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Evaluating the Anti-Seizure Efficacy of Novel Adenosine Treatment Regimens in a Soman Rat Model

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14. ABSTRACT

The severe brain-damaging effects of organophosphorus nerve agents are difficult to treat with current medical countermeasures. Diazepam and midazolam have been shown to have anti-seizure capabilities but are limited by sensitivities to dosing and timing parameters. We recently reported that central adenosine receptor (AR) stimulation with the adenosine A1 agonist (6)-cyclopentyladenosine (CPA) improved survivability and minimized neuropathology after soman intoxication. The goal for this study was to further explore adenosine's therapeutic applications and obtain a deeper understanding of the neuroprotective mechanism. We first investigated the neuroprotective efficacy of intracerebroventricularly delivered CPA (700 µg) when given 20 minutes after the onset of soman-induced seizure. To further promote survival, we then tested the therapeutic benefit of incorporating monoisonitrosoacetone, a centrally active cholinesterase-reactivating oxime. Since it is important to return the warfighter to combat after treatment, our last objective was to investigate if the centrally acting AR antagonist 8-cyclopentyl-1,3-dipropylxanthine could reverse CPA-induced sedation. Although further study is needed to validate efficacy and safety, the results from this study demonstrated that both peripherally and centrally delivered adenosine agonists have significant therapeutic benefits for acute and delayed treatment of nerve agent poisoning

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Abstract

The severe brain-damaging effects of organophosphorus (OP) nerve agents are difficult to treat with current medical countermeasures. Diazepam and midazolam have been shown to have anti-seizure capabilities but are limited by sensitivities to dosing and timing parameters. We recently reported that central adenosine receptor (AR) stimulation with the adenosine A1 agonist (6)-cyclopentyladenosine (CPA) improved survivability and minimized neuropathology after soman intoxication. The goal for this study was to further explore adenosine's therapeutic applications and obtain a deeper understanding of the neuroprotective mechanism. We first investigated the neuroprotective efficacy of intracerebroventricularly delivered CPA (700 μg) when given 20 minutes after the onset of soman-induced seizure. Delayed CPA treatment terminated seizure and protected against neuropathology with statistical significance. Next, we tested the efficacy of systemic adenosine treatment, which is a more clinically relevant route of administration. Results showed that systemic CPA co-administered with the peripherally acting AR antagonist 8-(p-sulfophenyl)theophylline (8-SPT) prevented soman-induced seizure while minimizing CPA's cardiovascular side-effects. To further promote survival, we then tested the therapeutic benefit of incorporating monoisonitrosoacetone (MINA), a centrally active cholinesterase-reactivating oxime. MINA at 60 mg/kg provided little neuroprotection, while at 120 mg/kg it was highly toxic. Since it is important to return the warfighter to combat after treatment, our last objective was to investigate if the centrally acting AR antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) could reverse CPA-induced sedation. DPCPX given 3 hours after CPA induced severe seizure activity and neuropathology in animals not exposed to soman. Caution should be used when reversing central AR stimulation; sudden antagonism may induce neuropathology similar to a nerve agent. Although further study is needed to validate efficacy and safety, the results from this study demonstrated that both peripherally and centrally delivered adenosine agonists have significant therapeutic benefits for acute and delayed treatment of nerve agent poisoning.

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; AR, adenosine receptor; AMN, atropine methylnitrate; BBB, blood brain barrier; CWNA, chemical warfare nerve agent; CNS, central nervous system; GABA, ɣ-aminobutyric acid; CPA, (6)-cyclopentyladenosine; ICV, intracerebroventricular; IM, intramuscular; IP, intraperitoneal; LV, lateral ventricle; OP, organophosphorus compound; SC, subcutaneous; 8-SPT, 8-(p-sulfophenyl)theophylline; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; MINA, monoisonitrosoacetone.

Introduction

Chemical warfare nerve agents (CWNAs), such as sarin and soman, are deadly organophosphorus (OP) compounds. They inhibit the enzyme acetylcholinesterase (AChE) that hydrolyzes the neurotransmitter acetylcholine (ACh) in the cholinergic synapses and neuromuscular junctions (Goodman 2001). The resulting accumulation of ACh causes a cholinergic crisis that affects the peripheral and central nervous systems. An especially devastating effect is the generation of excitotoxic activity in the brain that leads to permanent brain damage (el-Etri et al. 1992; Fosbraey et al. 1990; Lallement et al. 1991; Lallement et al. 1992; O'Donnell et al. 2010; O'Donnell et al. 2011; Shih 1982; Wade et al. 1987). Excitotoxicity due to extended periods of elevated extracellular excitatory neurotransmitters ACh and glutamate causes cell death and brain damage. While many potential inhibitory compounds have been developed to combat these events, they have limited neuroprotective efficacy in protecting the central nervous system (CNS), particularly in cases of prolonged seizure activity (Shih et al. 1997). Diazepam is one such drug used to terminate seizure activity; unfortunately, it loses efficacy as a result of endocytosis of the γ-amino-butyric acid ($GABA_A$) receptors (Wei et al. 2011). Furthermore, diazepam's respiratory depressant effects may enhance soman's lethality (Shiomi et al. 2000). Therefore, investigation and exploration of new/novel therapeutic targets for CWNA countermeasures are needed.

Adenosine is an endogenous compound whose primary CNS effect is the inhibition of neuronal activity and neurotransmitter release (Cunha 2005; Lynge et al. 2000; Svenningsson et al. 1997). Normal metabolic activity produces the accumulation of adenosine into the extracellular space where it modulates cell function by operating on G-protein-coupled receptors (Ribeiro et al. 2002). Adenosine receptor (AR) subtypes are classified according to their effect on adenylyl cyclase: A1 subtype inhibits via $G\alpha_i$ proteins, whereas A2A subtype enhances via $G\alpha_s$ proteins (St. Hilaire et al. 2009). A1 receptors are widely distributed throughout the CNS. They are located in the cortex and thalamus, and have the highest densities in critical cholinergic centers, the hippocampus and striatum (Bjorness et al. 2009; Svenningsson et al. 1997). Activation of A1 receptor subtypes and GABA receptors has similar effects; both decrease neuronal excitability (Ribeiro et al. 2002). These attributes have been exploited by scientists to protect neurons from various trauma including epilepsy, hypoxia and ischemia (Basheer et al. 2004; Schubert et al. 1997; Wardas 2002). Data suggest that adenosine's protective mechanism involves the partial neutralization of neuronal Ca^{++} overload that causes cell death (Schubert et al. 1997). Adenosine agonists have also been shown to be effective anticonvulsants for treating drug-resistant epilepsy (Gouder et al. 2003; Huber et al. 2002). Unfortunately, clinical applications have not been accomplished on account of the profound reduction in heart rate and blood pressure that peripheral AR stimulation causes (Biaggioni 1992; Dunwiddie et al. 2001).

Despite such cardiovascular effects, van Helden et al. (1998) recognized adenosine's potential as a CWNA countermeasure. In their early study, the A1 adenosine agonist (6)-cyclopentyladenosine (CPA) was shown to reduce nerve agent lethality; intramuscular (IM) injections of CPA decreased extracellular ACh levels, diminished seizure activity, and improved survivability in rats challenged with soman. Other researchers, many of whom are affiliated with van Helden, pursued adenosine in nerve agent models and identified its neuroprotective properties (Bueters et al. 2002; Bueters et al. 2003; Compton 2004; Joosen et al. 2004). However, the mechanism of protection has yet to be agreed upon. Much of the contention can be attributed to the systemic administration method. After recognizing the significant therapeutic potential of AR stimulation in treating nerve agent exposure, we performed a series of experiments that explored the neuroprotective benefits of CPA in a soman-induced seizure rat model (Thomas et al. 2014). To avoid adenosine's confounding cardio-respiratory effects, CPA was microinjected directly into the brain's lateral ventricles in those experiments. While the primary effect of CPA was a deep sleep, unexpectedly there was evidence that peripheral adenosine receptors were also stimulated. Rats receiving CPA without nerve agent experienced a dose-related decrease in heart and respiration rates, but made a full recovery by the next day. Since CPA is permeable to the blood brain barrier (BBB), a fraction of the CPA likely escaped the CNS and entered peripheral circulation. Despite the peripheral effects, preliminary findings demonstrate that central AR stimulation is potentially a very effective neuroprotective treatment. In an experiment where rats received CPA one minute after a 1.6 x LD_{50} dose of soman, all convulsive and seizure activity was blocked. The animals simply slept through the nerve agent exposure. Eleven of the twelve animals continued to display no central or peripheral cholinergic symptoms 24 hours after soman exposure. One of the animals that received CPA treatment died late overnight but never experienced symptoms of a cholinergic crisis. That animal likely succumbed to the combined cardio-respiratory depression of peripheral adenosine receptor stimulation and nerve agent exposure.

Our long-term goal is to develop a nerve agent medical countermeasure that prevents neuropathology, promotes survival, is clinically relevant, and allows the warfighter to remain combat-ready. To achieve that goal, modifications to adenosine treatment are needed. Since direct brain injections are not practical in the field, new systemic administration strategies need to be explored. Peripheral injection of an adenosine agonist may be lethal for a patient exposed to nerve agent. Given that cardiovascular function is already compromised by AChE inhibition, any additional depression of cardiac output via peripheral AR stimulation may result in death. We hypothesize that systemic CPA treatment is a viable strategy if a BBB impermeable AR antagonist was co-administered. Peripheral AR antagonism would minimize CPA's cardiac effects while maintaining its positive central effects. One such AR antagonist is 8-(p-sulfophenyl)theophylline (8-SPT). It has been shown to counteract AR-induced

cardiovascular effects at 50 mg/kg in a rat model (Evoniuk et al. 1987). Reactivation of inhibited AChE may also promote survival in CPA-treated nerve agent-exposed patients. Monoisonitrosoacetone (MINA) is a tertiary oxime that has been shown to be centrally active and effective in countering nerve agent-induced AChE inhibition (Skovira et al. 2010). It is believed that combining adenosine's pre- and post-synaptic inhibitory effects with MINA's ability to reactivate AChE would produce a more effective treatment regimen. In addition to being neuroprotective, an optimal treatment regimen would allow the soldier to maintain combat readiness. While CPA-induced inhibition of neurotransmission and neuronal excitability prevents seizure activity, that inhibition also leads to loss of consciousness. A method to reverse that sedation so that the warfighter could return to combat or extract himself from harm would be helpful. Administration of a BBB permeable AR antagonist such as 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) is a possible approach.

Given the promising data from our previous research, we believe adenosine may be an important component to future nerve agent countermeasures. This study aimed to develop new strategies that would minimize adenosine's limitations while optimizing treatment outcomes. The first objective was to assess adenosine's efficacy when treatment was delayed and seizure developed. Since immediate medical care may not be available after a CWNA attack, it is essential that treatment can both prevent and terminate seizure activity. To test the efficacy of delayed stimulation of central ARs, CPA was injected intracerebroventricularly (ICV) 20 minutes after seizure onset. The second objective was to evaluate whether systemic CPA administration could produce the same neuroprotective benefits as ICV treatment. To exclusively stimulate central receptors and avoid adverse side-effects, 8-SPT was co-injected with CPA intraperitoneally (IP). The third objective was to test a multifaceted treatment approach that combined CPA and MINA. It was hypothesized that a positive synergistic effect would occur that would enhance neuroprotection and survival. The fourth objective examined a strategy for reversing CPA-induced sedation such that the patient would regain neuromuscular control. In this experiment, rats were first treated with CPA and then 3 hours later received the centrally active antagonist DPCPX. We hypothesized that AR antagonism would restore cognitive and motor control.

Methods

Subjects/Animals

Male Sprague-Dawley rats weighing 250 – 350 g were purchased from Charles River Labs (Kingston, NY) and were individually housed at 21±2 °C and 50±10% humidity with a 12-hour light – dark schedule (with lights on at 0600 h). Laboratory rodent chow and filtered tap water were freely available whenever the animals were in home cages.

Surgical Procedure

Using aseptic surgical techniques, animals were prepared first with the insertion of an electronic temperature ID transponder between the shoulder blades subcutaneously (SC) (Bio Medic Data Systems Inc., Seaford, DE) and then had wire-electrodes implanted into the skull for recording brain electroencephalographic (EEG) activity and for detection of seizure onset and termination. A stereotaxic frame with computer-assisted guidance (Leica Microsystems Inc., Buffalo Grove, IL) was then used to drill two holes into the skull and insert 26 gauge cannulae bilaterally toward the lateral ventricles [Atlas Coordinates mm (AP, DV, L) (0.0, -4.5, ±1.5)] (Paxinos et al. 2009) for drug administration. The rats were allowed to recover for 7 days before experimentation.

Animal Model

A soman-induced seizure rat model developed at the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) for nerve agent-related neuroprotection studies was used for this study (Shih 1990; Shih et al. 1991). Animals were placed in an EEG recording chamber (43 x 30 x 25 cm), and baseline brain activity data were collected for 30 minutes. Then animals were treated with HI-6 (125 mg/kg) intraperitoneally (IP). Thirty minutes following HI-6 treatment, rats were challenged with a subcutaneous (SC) injection of 1.6 x LD_{50} (180 µg/kg) soman. After exposure, animals were treated with an intramuscular (IM) injection of 2 mg/kg atropine methylnitrate (AMN) and one of the adenosine treatment regimens. The HI-6 and AMN injections were incorporated into this model to mitigate soman's peripheral effects and promote 24-hour survivability; seizure activity and neuropathology are not affected. The EEG data were collected from the CDE 1902 amplifiers and analyzed using Spike2 software (Cambridge Electronic Design, Ltd., Cambridge, England) and custom written MATLAB code. EEG data were continuously assessed by a trained technician who rated the seizure activity as absent or present. Non-pharmaceutical grade CPA and 8-SPT were purchased from Sigma-Aldrich. No equivalent veterinary or human drug was available for experimental use. All drugs were prepared in sterile containers and solutions (i.e., saline, DMSO, and multisol) on the day of experimentation. Soman was obtained from the U.S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). HI-6 was purchased from Phoenix Chemical Inc. (Bromborough, England), and AMN was purchased from Wedgewood Pharmacy (Swedesboro, NJ).

Animals continued to have EEG recorded for 5 hours after exposure, during which physiological responses including heart rate, respirations and toxic signs were measured. In addition, during the first 5 hours after exposure, behavioral assessment tests were performed to quantify and describe the animals' response to treatment. These noninvasive procedures for testing gross functional behaviors, i.e., functional observation batteries (FOB), have been widely used within the USAMRICD and by other

researchers to assess pharmacologic reactions (Bowen et al. 1997; Shih et al. 2006; Youssef et al. 1997). Qualitative measures of righting reflex, approach response, gait description, ease of handling and level of arousal were scored using the FOB (Appendix) and toxic sign scores (Table 1). Assessments were made at 0, 4, 8, 15, 30, 45, and 60 min, and thereafter at 30-minute increments for 5 hours after exposure. At 24 and 48 hours after nerve agent exposure, a gross behavioral assessment was made, and EEG data were recorded for 30 minutes.

Assessment of Neuropathology

Once the *in vivo* segment of the experiment was completed, the rats were anesthetized with sodium pentobarbital based euthanasia solution and perfused transcardially with saline followed by 4% paraformaldehyde in phosphate-buffered saline. The brain was then extracted and stored in paraformaldehyde. The brains were serial sectioned at 5 μm, stained with hematoxylin and eosin (H&E), and evaluated for neuropathology using established methodology (McDonough et al. 1995). A trained pathologist, who was unaware of the treatment paradigm, analyzed and scored four brain regions (the piriform cortex, the thalamus, the dorsal and ventral hippocampus) using the standard rubric: 0= No lesion; 1= Minimal (1-10%); 2= Mild (11-25%); 3= Moderate (26-45%); 4= Severe (>45%). To further stratify the data and obtain a more comprehensive measure of brain damage, a total score was calculated by summing the 4 regional scores. A total score of 16 indicates widespread severe damage.

Effects of Delayed Central CPA Treatment

Animals for this experiment were surgically prepared with the bilateral implantation of 26 gauge guide cannulae directed toward the lateral ventricles. Direct ICV injections of saline or CPA via the cannulae were performed using a micro syringe pump. Each group contained 12 rats. A control group was used to compare differences in soman-induced seizure characteristics for animals with and without adenosine treatment. This group received 10 μl multisol (a vehicle containing 48.5% H_2O, 40% propylene glycol, 10% ethanol, and 1.5% benzyl alcohol) over 3 minutes via ICV injection into the cannulae 1 minute after soman exposure. A baseline treatment group of rats received CPA at a dose previously determined to be efficacious (700 μg in 10 μl multisol via ICV injection over 3 minutes) 1 minute after exposure to a 1.6 x LD_{50} dose of soman (Thomas and Shih 2014). The delayed treatment group received the CPA via ICV injection 20 minutes after seizure. After soman exposure and CPA treatment, EEG was continuously recorded, and physiologic and behavioral assessments were made at regular intervals. After those 5 hours, the animals were returned to husbandry. Twenty-four hours after exposure, EEG data were collected for 30 minutes to assess seizure activity. Forty-eight hours after exposure, EEG was collected for another 30 minutes, and the animals were euthanized and fixed for histology. An experimental timeline is

depicted in Figure 1. Neuropathology and assessment scores for the baseline and delayed treatment groups were compared to the control group.

Effects of Co-Administration of Adenosine Peripheral Antagonist and CPA

Part A of this experiment tested the neuroprotective efficacy of CPA (700 µg) in 10 µl multisol delivered directly to the brain (ICV) with an accompanying systemic injection of the BBB impermeable adenosine antagonist 8-SPT (50 mg/kg) to prevent the side-effects of CPA leaking into peripheral circulation. 8-SPT was dissolved in 0.4 ml of dimethyl sulfoxide (DMSO). All animals were surgically prepared (bilateral cannulae implantation) for ICV CPA injections. A control group that receives no nerve agent was used to serve as a reference for responses to CPA (central + peripheral agonist) and 8-SPT (peripheral antagonist). Thirty minutes after HI-6 pretreatment, animals received saline (control group) or soman (exposure group) and then were treated with 8-SPT IP and CPA via ICV 1 minute later.

Part B of this experiment tested the neuroprotective efficacy of systemically delivered CPA (in 0.2 ml multisol). These rats did not require surgery since there were no ICV injections. CPA (50-55 mg/kg, IP) and 8-SPT (50 mg/kg, IP) were both delivered to the rats 1 minute after saline or soman (1.6 x LD_{50}) exposure. EEG, behavioral and toxic signs were recorded, and then the animals were returned to husbandry. At 24 and 48 hours, EEG was collected for 30 minutes and assessed for seizure activity. Animals in Parts A and B were euthanized and prepared for histology immediately following the 48-hour EEG recording. The protection offered by central and systemic CPA delivery with 8-SPT antagonism was evaluated and compared.

Effect of Combined Adenosine Treatment with Oxime

With the aim of developing a comprehensive therapeutic strategy, the BBB permeable oxime MINA was administered along with a centrally delivered adenosine agonist treatment. Animals were surgically prepared with the implantation of cortical screws for EEG and bilateral cannulae directed toward the lateral ventricles. First, the neuroprotective benefits of MINA alone were investigated. Rats received HI-6 pretreatment 30 minutes prior to soman exposure. One minute after soman, a dose of MINA was injected intramuscularly. The combined treatment group (MINA + CPA) received an ICV microinjection of CPA at the dose of 700 µg in addition to the MINA. The initial dose of MINA (120 mg/kg) that was used in this experiment was taken from previous work done at USAMRICD (unpublished) that demonstrated efficacy in a guinea pig model. Unlike the guinea pig where no lethality was observed, the 120 mg/kg dose proved to be toxic to the rat; all 12 rats died within 2 hours after treatment. Since that rate of lethality was significantly higher than in any previous experiment, that group of animals was excluded from the study, and the experiment was repeated using a reduced dose of MINA at 60 mg/kg. The animals in all groups had EEG, behavioral and

toxic signs recorded for 5 hours after saline/soman exposure, after which they were returned to husbandry. At 24 hours, a 30-minute EEG was recorded. Forty-eight hours after exposure, a final 30-minute EEG recording was made, and the animals were euthanized and prepared for histology. The neuropathology of this combined exposure group was compared to an exposure group that received only MINA as treatment, and to the control group that received no CPA treatment after soman exposure.

Functional Restoration with Peripheral and Central AR Antagonism Treatment

To reverse the sedative effects of CPA, the peripherally and centrally acting adenosine antagonist DPCPX was administered. Animals in this experiment were surgically prepared with bilateral cannulae implantation toward the lateral ventricles. The control group that did not receive nerve agent was used to measure DPCPX's ability to reverse CPA's effects. This group received CPA via ICV and MINA IM 1 minute after a saline SC injection (in lieu of soman). Three hours after CPA, the animals received an IP injection of DPCPX (5 mg/kg). The exposure group received the same treatments as the control except that soman was injected SC instead of saline. Behavioral, toxic signs and seizure activity were recorded for 5 hours post-exposure. At 24 and 48 hours, the animals had 30 minutes of EEG recorded. After the 48-hour measurement, the rats were euthanized for histological analysis, and differences between control and exposure were determined.

Data Analysis

The Anderson-Darling normality test was used to determine whether the dataset would be analyzed with a parametric or nonparametric test. The total neuropathology scores (0=normal, 16=severe) for each treatment group were compared to their controls using the, Kruskal-Wallis test and compared between treatment groups using the Mann-Whitney test if significant. Rates of seizure prevention and survival were compared using the Fisher's exact test. Estimation and comparison of the mean time to seizure and death were done using the Kaplan Meier survival analysis and log rank test. Statistical differences in the severity of toxic motor signs (fasciculation, tremor and convulsion) and FOB scores between treatment and control groups were detected using a generalized linear model. Differences in body temperatures after GD exposure between the group receiving CPA+8-SPT treatment to the groups receiving only saline (control) or just CPA (ICV) were analyzed using the unpaired two-tailed t-test. Since multiple statistical tests are being performed on a single data set, a Bonferroni correction was made to the level of significance, $p<0.025$.

Results

Effects of Delayed Central CPA Treatment

We tested the efficacy of treating soman exposure with 700 µg of CPA via ICV either one minute after soman exposure or 20 minutes after seizure onset in groups of 12 rats.

Similar to what was previously observed in a 24-hour study (Thomas and Shih 2014), acute CPA ICV provided complete protection from seizure for the total observation period of 48 hours. Whereas all 12 rats exposed to soman and treated with saline seized, none of the 12 rats acutely treated with CPA ICV seized ($p<0.0001$). Only one animal who received treatment 1 minute after soman exposure experienced a brief period (45 minutes) of tremors but did not go on to develop any EEG spiking or seizure activity. None of the other acutely treated rats showed peripheral signs of OP intoxication. Although 100% seizure prevention was obtained, acute treatment did not significantly improve survival. Four control and seven treated animals survived up to the 48-hour endpoint. The median time to death for control was 5 hours and for CPA-treated rats it was 48 hours (the study endpoint). That difference was not significant according to the Kaplan Meir test ($p=0.15$). For those animals that did survive to the end, damage to neuronal tissue was minimized by acute CPA treatment. Animals receiving CPA scored an average total neuropathology score of 2.1±2 [1.0, 3.3] (± std, [95% CI]), which was a significant reduction from the control group that averaged 11.3±1.0 [10.0,12.6] ($p<0.01$) (Figure 2).

Delaying the treatment of CPA ICV for 20 minutes after the detection of EEG seizure provided protective benefits to the majority of animals. Whereas control animals continually seized until their death or until the study's endpoint, seizure in 8 of the 12 animals was terminated by CPA within the 48-hour endpoint. Seizure was terminated in 6 of the rats within 5 hours after treatment; the other two rats stopped seizing sometime overnight between day one and day two. The remaining four animals that received delayed treatment seized until their time of death, two of which died approximately 10 minutes after treatment administration. Of the eight animals in which seizures terminated, 5 survived until the study endpoint. Similar to the acute treatment group, delayed treatment did not significantly improve survival. Neuropathology was significantly reduced for the delayed treatment group compared to the control according to the Mann-Whitney test ($p=0.014$); the average pathology score for those 5 surviving animals was 6.2±10 [5.0, 7.3]. Three of the delayed treatment rats that survived experienced severe damage to the piriform cortex. That region received a score of 4 (out of 4); the other two surviving treatment animals received scores of 1 and 2. The average time of seizure for those 3 animals was 13.9 hours; the other 2 surviving rats in the delayed treatment group seized 3.5 hours on average.

Effects of Co-Administration of Adenosine Peripheral Antagonist and CPA

Since nerve agent medical countermeasures will not be centrally delivered in practice, this experiment aimed to (1) determine the efficacy of intraperitoneally delivered CPA treatment and (2) compare peripheral CPA treatment to centrally delivered treatment. Although peripheral administration is a more clinically relevant approach, the stimulation of peripheral adenosine receptors has been shown to reduce cardiac output. To suppress such negative side-effects and promote survival, the BBB

impermeable adenosine antagonist 8-SPT was co-administered with CPA. The results from this experiment demonstrated that peripherally delivered CPA in conjunction with 8-SPT was as effective as centrally delivered CPA for preventing seizure after soman exposure (Figure 3). None of the rats that received CPA via IP or ICV with 8-SPT (IP) after soman exposure produced seizure activity at any time in the 48-hour study. While seizure was prevented, neither IP nor ICV delivered CPA with 8-SPT produced statistically significant survival rates when compared to the control (soman with saline treatment). Whereas 33% of the control group survived until 48 hours, only 50% from the CPA (IP) + 8-SPT group and 70% from the CPA (ICV) + 8-SPT group survived ($p>0.20$). Two of the 12 rats in the group that received CPA ICV and 8-SPT after soman exposure were excluded from the study because of cannula blockages and the consequential inability to administer all treatment. One of those rats died within 2 hours after partial treatment and displayed signs of severe peripheral cholinergic symptoms such as convulsions and hyper secretions. While peripheral signs were evident, that rat did not develop seizure; CPA at <700 µg was centrally protective. The other rat that received partial treatment also displayed some evidence of neuroprotection. Seizure onset for that animal was delayed for 188 minutes after soman exposure, and peripheral cholinergic signs were suppressed.

The rats that were administered CPA (ICV) + 8-SPT after exposure to a 1.6 x LD_{50} dose of soman displayed minimal pathology (group average 2.8±2 [2, 4.1]), a statistically significant reduction from the control (group average 11.3±1.5 [10.0, 12.6]) ($p<0.01$). Animals receiving CPA (IP) + 8-SPT displayed relatively minor signs of neuropathology (group average 3.3±2.8 [1, 5.6]). The origin of that pathology may not be entirely due to the soman exposure. Control animals that were not exposed to soman but were injected with CPA (IP) and 8-SPT displayed some neuropathology as well (group average 3.8±0.8 [3.0, 4.3]). Since CPA injected directly into the brain did not produce neuropathology in previous studies (Thomas and Shih 2014), the cause for that minor damage may be the vehicle that was used for 8-SPT, DMSO. Among the treatment groups (1) CPA (ICV) group, (2) the CPA (ICV) and 8-SPT group, and (3) the CPA (IP) and 8-SPT group, there were no statistically significant differences in neuropathology score.

Body temperature data from this experiment suggest that the peripherally acting antagonist 8-SPT counteracted some of CPA's side-effects. Animals receiving just CPA via ICV experienced mild hypothermia and reached a body temperature of 29.9±1.9°C within five hours after soman exposure. In contrast, the animals that received the co-injection of 8-SPT maintained a significantly higher temperature of 32.0±1.8°C ($p=0.016$). While 8-SPT suppressed some of the peripheral temperature effects of adenosine receptor stimulation, not all of CPA's side-effects were mitigated. After soman exposure, the CPA (ICV) with 8-SPT group experienced a reduction in body

temperature (32.0±1.8°C) compared to the group that received soman without treatment (36.9±1.2°C) ($p<0.01$).

Effects of Combined Adenosine Treatment with Oxime

This experiment aimed to further improve treatment efficacy of AR stimulation with an adjunct centrally active AChE reactivator, MINA. The results from this experiment suggest that MINA at 60 mg/kg (IM) did not significantly improve survival or neuroprotection by itself, or in combination with 700 µg CPA (ICV). Only 4 of the 12 rats survived until the endpoint in the group that received CPA + MINA treatment, and only 6 of the 12 that received just MINA after exposure survived. Those results are not significantly different from the soman control group where 4 of the 12 survived. Similar to the soman-exposed group of rats that received acute CPA (ICV) treatment, none of the rats that were treated with CPA + MINA seized after exposure to soman. However, 7 of the 12 that received just MINA developed seizure. Since 5 of those rats from the MINA group were in fact protected from seizure, the data suggest that both CPA and MINA individually contributed to seizure prevention when combined. Although the rate of seizure prevention in the MINA group (42%) was significantly different from the soman control group (100%) ($p<0.01$), all rats in the MINA group displayed peripheral signs of a cholinergic crisis (e.g., tremors and seizure). Typical of previous seizure research, the animals that developed seizure in the MINA treatment group developed severe neuropathology. The MINA group average neuropathology scores for the 6 surviving animals was 9.0±7.8. In contrast, the four rats that survived in the CPA + MINA group had no detectable neuronal damage (score was 0.0).

Functional Restoration with Peripheral and Central AR Antagonism Treatment

The saline exposure control group was first tested in this experiment. It aimed to collect baseline physiologic responses to reversing the effects of the adenosine agonist CPA at 700 µg by administering a BBB permeable adenosine antagonist, DPCPX. That experiment yielded unanticipated results; after injecting CPA into the lateral ventricles, the rats displayed the expected behavior of reduced locomotion and brain activity. After three hours, 5 mg of DPCPX was administered IP to reverse the widespread inhibitory effects of the agonist. As predicted, the rats displayed increased levels of brain activity following DPCPX treatment. However, 9 of the 12 rats proceeded to develop seizures and entered *status epilepticus* (Figure 4). This reaction was unexpected since soman was not administered. The seizures were as severe as those seen in animals exposed to soman. Two of the 12 rats died within 48 hours. Those rats that survived developed moderate brain damage; the average total neuropathology score for this group was 9.2±4.9 [6, 12.2]. As a result of the seizure-inducing properties of CPA + DPCPX in saline-exposed animals, we did not conduct the experiment that was planned to test efficacy in a soman-exposed group of rats.

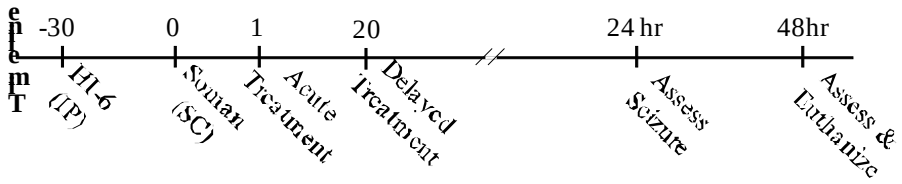

Figure 1. Timeline of experiment. Neurobehavioral and toxicity assessments were made at 0, 4, 8, 15, 30, 45, and 60 min, and thereafter at 30-minute increments for 5 hours after exposure. At 24 and 48 hours after nerve agent exposure, a gross behavioral assessment was made, and EEG data were recorded for 30 minutes.

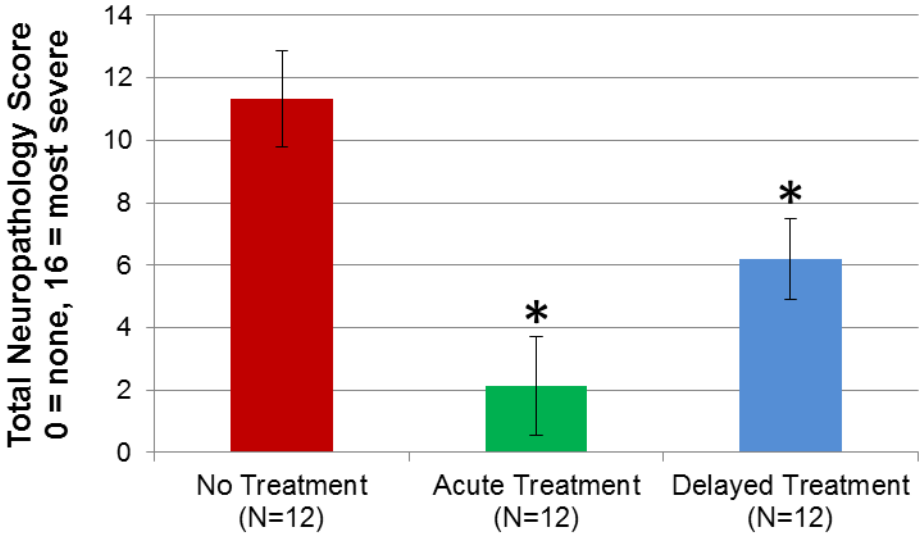

Figure 2. The average total neuropathology scores for the control, acute treatment (1 minute after soman) and the delayed treatment (20 minutes after seizure) groups are shown. The results demonstrate that acute or delayed treatment of 700 µg CPA produces a statistically significant reduction in neuropathology according to the Mann-Whitney test (* $p<0.05$).

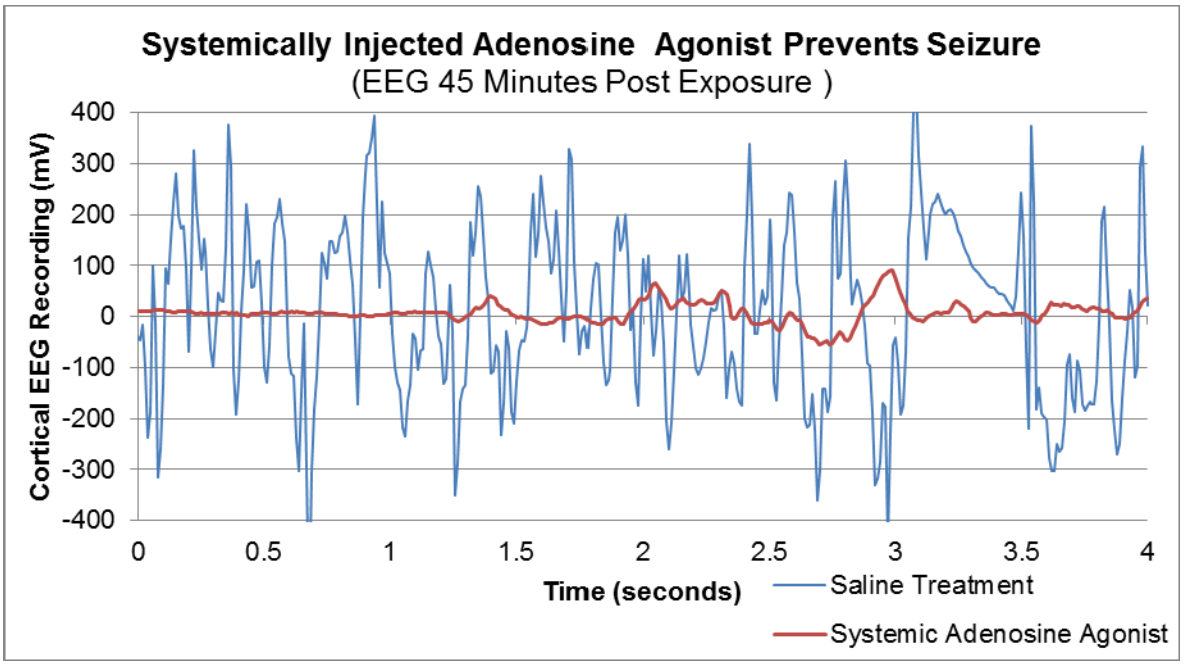

Figure 3. The systemic administration of the adenosine agonist CPA in combination with a peripherally acting adenosine antagonist 8-SPT (red) was able to inhibit soman-induced excitotoxic neuronal activity (blue). This representative EEG window illustrates the reduction of high frequency-high amplitude brain activity in a seizing rat to low frequency-low amplitude activity in a rat receiving treatment (CPA at 50-55 mg/kg, and 8-SPT 50 mg/kg, IP).

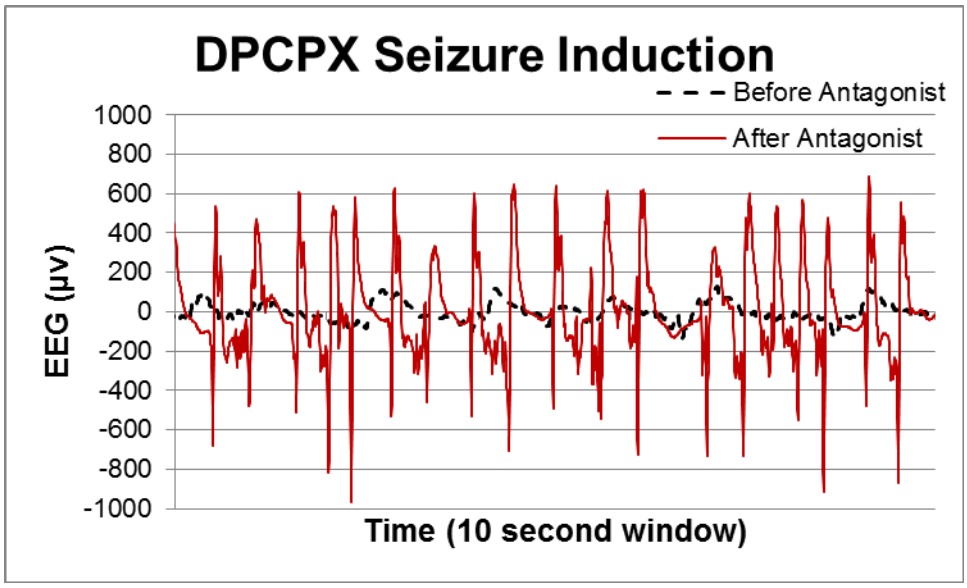

Figure 4. Example EEG data illustrating the seizure inducing effect of adenosine agonism and subsequent antagonism without nerve agent. After the ICV injection of CPA (700 µg), EEG power was reduced (dashed line). Three hours later, administration (IP) of DPCPX (5 mg/kg) induced spiking activity within approximately 30 minutes (solid line). These seizures resulted in the development of neuropathology.

Table 1. Scoring system for toxic signs assessment

Toxic Signs Scores	
Motor	0 = Normal 1 = Fasciculation's 2 = Tremors 3 = Convulsions
General	0 = Normal 1 = Mildly Uncoordinated 2 = Impaired Movement 3 = Prostrated
Salivation	0 = Normal 1 = Salivation
Lacrimation	0 = Normal 1 = Lacrimation
Eye	0 = Normal 1 = Nystagmus

Toxic signs were continuously scored following drug administration during the 5-hour observation period after exposure on the day of the experiment and also scored again at the 24- and 48-hour time points.

Table 2. Summary of treatment efficacy in rats after exposure to a 1.6 x LD_{50} dose of soman.

Treatment after Soman	Survival Rate	Seizure Prevention / Termination Rate	Total Neuropathology (avg±std)
Saline (Control)	33%	0%	11.3 ± 1.5
Acute CPA (ICV)	58%	100%	2.1 ± 1.6
Delayed CPA (ICV)	42%	67%	6.2 ± 1.3
Peripheral CPA (IP)	50%	100%	3.3 ± 2.8
CPA + MINA	33%	100%	0.0 ± 0.0

Discussion

The overall goal for this study was to further investigate central adenosine receptor stimulation and gain a better understanding of potential therapeutic strategies. It is essential that nerve agent countermeasures are easily administered and effective even if administration is delayed minutes after nerve agent exposure. Therefore, the first objective for this research was to determine if administration of CPA 20 minutes after seizure onset could terminate excitotoxic brain activity and provide neuroprotection. The results from that experiment suggest that CPA is indeed capable of suppressing seizure activity well after exposure and seizure onset. Our next objective was to establish efficacy for peripherally administered CPA treatment. While our previous research demonstrated that direct brain injections of CPA provided significant neuroprotection, application of that strategy to the warfighter would be very limited. Our research initially applied central delivery to avoid the cardiac effects of peripheral adenosine receptor stimulation. Specifically, stimulation of the peripheral ARA1 is believed to slow the heart rate and consequently decrease cardiac output. If soman's cardiovascular depression were to be compounded by that side-effect, the patient would not be expected to survive. Therefore, we incorporated a BBB impermeable adenosine antagonist to counteract CPA's peripheral side-effects. The results from these experiments demonstrated that CPA delivered both centrally and systemically offers significant neuroprotective benefits (Table 2).

In addition to preventing seizure generation, a key objective for medical countermeasures is to promote survival. The survival rates for adenosine-treated animals in these experiments are similar to previously obtained results (Thomas et al. 2014). In the previous research, 10 of the 12 rats exposed to 1.6 x LD_{50} soman and treated with 700 µg CPA (ICV) survived to the endpoint of 24 hours. In this experiment, 11 of the 12 rats that received CPA ICV were alive at the 24-hour time point, and 4 died the second night after exposure. Such lethality in treated animals is a likely consequence of soman's peripheral effects, e.g., hyper secretions and cardio-respiratory distress. Lethality could also be due to other factors related to CPA treatment including a prolonged period of sedation where access to food/water was limited or the reduction in body temperature (from 37° to 30°C). The fact that CPA did not mitigate the nerve agent's peripheral cholinergic symptoms does not necessarily discount its potential use as a medical countermeasure. CPA was originally pursued for its therapeutic actions within the CNS, something that current countermeasures lack. Since the optimal countermeasure needs to suppress soman's peripheral and central effects, the best therapy will likely involve a multi-pronged approach. Such a strategy could include therapeutics focused on mitigating peripheral cholinergic accumulation (e.g., AMN) and others that suppress the cholinergic and glutamergic hyperexcitatory neuronal activity (e.g., CPA).

The peripherally acting antagonist 8-SPT was incorporated to minimize the risk of CPA-induced bradycardia. The responses to the co-administration of 8-SPT and CPA indicated that peripheral ARs were partially antagonized. Body temperature can be used as an indicator for CPA's peripheral effects as it is directly related to peripheral metabolic and cardiovascular activity. The group of rats receiving 8-SPT maintained a significantly higher core body temperature than those receiving just CPA. However, it is likely that all peripheral ARs were not antagonized; the 8-SPT and CPA group dropped to significantly lower temperatures than the control group. Perhaps the optimal dose of 8-SPT was not used. Future experiments aim to address this concern and perform an 8-SPT dose escalation experiment to maximize cardiac function.

The combination of an oxime with CPA was expected to have a positive synergistic effect in soman-exposed rats. Although ageing renders most AChE reactivators ineffective within several minutes after soman-AChE docking, we hypothesized that immediate treatment of MINA would be beneficial. However, the results from the experiment did not support that hypothesis. While MINA has proven to be an effective AChE reactivator in a guinea pig model (Skovira et al. 2010), our data suggest that a rat reacts differently to MINA treatment. Doses comparable to previously reported guinea pig data (60 and 120 mg/kg) did not provide significant neuroprotection or proved to be toxic in rats. Perhaps MINA at a dose between the ineffective 60 mg/kg and toxic 120 mg/kg would offer some protection for AChE. Since reactivation of OP-inhibited AChE improves patient outcomes, future testing aims to further evaluate MINA and other oximes as adjuncts to adenosine treatment strategies.

To maximize therapeutic potential, nerve agent countermeasures should be given immediately after exposure is detected. However, acute treatment may not always be possible on the battlefield. Whether because the soldier does not realize that an exposure occurred or the countermeasure is not readily available, sustained seizure activity and subsequent brain damage may develop. In that event, it is critical that the countermeasure is able to terminate the seizure well after its onset. However, stopping status epilepticus once it develops is much more difficult than preventing its onset. The mechanism for terminating seizure may be different from the mechanism that prevents seizure generation and propagation. A mechanism that terminates seizure after onset would suggest post-synaptic neuronal effects and the inhibition of calcium influx. If a therapeutic were able to prevent but not terminate seizure, its mechanism may only involve the pre-synaptic inhibition of neurotransmitter release. To better understand CPA's mechanism of action, we tested its anti-seizure efficacy in a delayed treatment scenario, i.e., CPA administered 20 minutes after seizure onset. The results from that experiment showed that adenosine could terminate seizure well after an extended period of time for 67% of the animals. At 3 - 3.5 hours after treatment, the spiking EEG signal dissipated and took on an amplitude and frequency similar to the EEGs of rats

treated acutely with CPA. In contrast, the animals that did not receive treatment displayed EEG spiking throughout the observation period. While CPA ICV treatment did not terminate seizure in all animals, it is important to note that two of the four animals that were not responsive to treatment died within 10 minutes after treatment administration. Since the time to AR-induced pre- and post-synaptic effects is likely longer than that time period, those data points should not be considered evidence for lack of efficacy. The ability of CPA to terminate sustained seizure activity suggests that treatment suppressed neuronal excitability via post-synaptic effects. Perhaps better termination potential will be achieved with greater CPA doses, either administered peripherally or centrally.

Conclusion

While many therapeutic compounds have been developed to combat the deadly effects of nerve agent exposure, they have limited neuroprotective efficacy in the CNS. Previous research demonstrated that central AR stimulation via direct brain injection of CPA was an effective medical countermeasure to the nerve agent soman. This study continued to explore that therapeutic mechanism and demonstrated that peripherally delivered CPA also has significant neuroprotective capabilities. Furthermore, data suggests that CPA is able to terminate seizure long after its onset. Efficacy in seizure prevention and termination demonstrates that CPA or another adenosine ARA1 agonist may have significant applications to the clinic and chemical warfare battlefield. Future studies will further investigate alternative strategies that exploit adenosine's positive central effects while minimizing undesirable side-effects.

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Appendix

U-1009 Functional Observation Battery Score Sheet

Paw/Ear Color:
0 Pink, normal
1 Pale
2 Blue

Startle Reflex:
Stimulus: Snap your fingers close to and above the rodent's head
0 jumps, seems startled
1 more energetic response than (2) – may include vocalization
2 slight reaction, ear flick or some evidence that a sound was heard
3 no reaction

Righting reflex:
0 normal (immediately rights itself)
1 slightly impared (>1 sec)
2 impaired (>2 sec)
3 totally impaired (remains on back)

Animal Handling (Ease of Handling Rodent in Hand):
0 Difficult, squirming, twisting, attempting to bite, with or without vocalizations
1 Moderately easy; vocalizations, little or no squirming
2 Easy, but alert, limbs may be pulled against body
3 Easy, but lethargic

Arousal:
0 Normal (alert, exploratory movements)
1 Somewhat low (some exploratory movements with periods of immobility)
2 Low (some head or body movement)
3 Very low (little or absent)

Gait description:
0 Normal
1 Impairment
  Uncoordinated movement (ataxia)
  Walking on toes
  Splayed hind limbs
  Exaggerated hind limb flexion
  Staggered gait
  Dragging hind limbs
  Unable to walk
2 No movement

Approach Response:
Stimulus: Slowly use pen to approach the rodent from the front and make sure they are aware of the approach.
0 more energetic response than (2), possible vocalizations
1 slow approach, sniffing or turning away
2 no reaction