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Pharmacological Evaluation of Candidate
Adenosine Agonists as Novel Anticonvulsant
Medical Countermeasures to Soman Nerve
Agent Intoxication

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14. ABSTRACT Toward developing a more effective anti-seizure treatment for nerve agent (NA) intoxication, this study evaluated the efficacy of A1 adenosine (ADO) receptor (A1AR) agonists in a rat soman (GD) seizure model. One minute after exposure to GD (1.6 x LD50, SC) or control vehicle, rats were treated intraperitoneally with one of the following agonists at increasing dose levels until anti-seizure efficacy was achieved: N6-cyclopentadenosine (CPA), 2-Chloro-N6-cyclopentyladenosine (CCPA), and (±)-5'-Chloro-5'-deoxy-ENBA (ENBA). All A1AR agonists were efficacious in preventing seizure and promoting survival. The effective doses for the A1AR agonists were 60 mg/kg CPA, 36 mg/kg CCPA, and 62 mg/kg ENBA. Whereas saline-treated rats experienced 100% seizure and 21% survival (N=28), ADO treatments reduced seizure occurrence and improved survival rates: 8% seizure and 83% survival with CPA (60 mg/kg, N=12), 17% seizure and 75% survival with CCPA (36 mg/kg, N=12), and 8% seizure, 83% survival with ENBA (62 mg/kg, N=12). The brains of ADO-treated rats were also protected from neuropathology. While all ADO agonists provided neuroprotection, rats receiving CCPA and ENBA experienced less severe ADO-induced side effects (e.g., sedation, hypothermia, bradycardia) than with CPA. The data from this study suggest that the ADO signaling pathway is a promising mechanism for countering NA seizure.					
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Abstract

Organophosphorus nerve agents (NAs) irreversibly inhibit acetylcholinesterase, which results in the accumulation of acetylcholine and widespread excitotoxic seizure activity. To minimize neuropathology and death, suppression of seizure activity is critical. Current medical countermeasures, however, have limited efficacy in this respect. In previous studies, we demonstrated that stimulation of the A1 adenosine (ADO) receptor (A1AR) is a promising neuroprotective mechanism since treatment with the A1AR agonist N6-cyclopentyladenosine (CPA) effectively prevented the onset of seizure after exposure to NA. While CPA was effective at the high dose of 60 mg/kg, unwanted side effects, such as prolonged sedation, were observed. Toward further developing ADO treatment as a novel NA countermeasure, this study aimed to determine if CPA's neuroprotective efficacy could be achieved at lower doses and also to investigate additional A1AR agonists with different chemical structures and receptor affinities that could potentially provide neuroprotection with few side effects. To do so, Sprague Dawley rats were surgically implanted with a body temperature transponder and cortical electrodes for recording brain electroencephalographic (EEG) activity and detecting seizure onset. One week after surgery, the rats received a combination of HI-6 (125 mg/kg, IP) and atropine methyl nitrate (2 mg/kg, IM) to prevent peripheral cholinergic symptoms and, thus, promote survival without affecting central activity. Thirty minutes later, rats were exposed to soman (1.6 x LD50, SC) or control vehicle. One minute after exposure, rats were treated IP with one of the following A1AR agonists at increasing dose levels until anti-seizure efficacy was achieved: CPA, 2-Chloro-N6-cyclopentyladenosine (CCPA), and (\pm)-5'-Chloro-5'-deoxy-ENBA (ENBA). Twenty-four hours after exposure, the surviving rats were euthanized, prepared for histology, and scored for neuropathology (0=no damage, 16=most severe damage). All A1AR agonists proved to be highly efficacious in preventing seizure and promoting survival. The effective doses for the A1AR agonists were determined as follows: 60 mg/kg CPA, 36 mg/kg CCPA, and 62 mg/kg ENBA. Whereas soman-exposed saline-treated rats experienced 100% seizure and 21% survival (N=28), ADO treatments reduced seizure occurrence and improved survival rates. CPA-treated (60 mg/kg) rats experienced 8% seizure and 83% survival (N=12), CCPA-treated (36 mg/kg) rats experienced 17% seizure and 75% survival (N=12), and ENBA-treated (62 mg/kg) rats experienced 8% seizure, 83% survival (N=12). ADO's seizure prevention translated to protection from neuropathology. Saline-treated animals displayed severe brain damage and received an average pathology score of 14.8 ± 1.8 . Rats treated with ADO had significantly reduced pathology scores: 0.2 ± 0.6 for CPA, 0 ± 0 for CCPA, and 1.4 ± 4.4 for ENBA. In addition to suppressing cortical activity in the brain, A1AR agonists had significant physiologic effects; bradycardia and hypothermia were induced at all dose levels. CPA 60 mg/kg had the most significant effect on heart rate and body temperature which persisted to the 24 hr endpoint. The recovery from CCPA and ENBA treatment was much faster than from CPA. Whereas CPA 60 mg/kg rats were sedated with hypothermia at 24 hr, rats treated with CCPA 36 mg/kg and ENBA 62 mg/kg had recovered much of their body temperatures and heart rates. Moreover, most CCPA- and ENBA-treated rats had recovered their ability to ambulate (0% CPA, 89% CCPA, and 100% ENBA). The data from this study suggest that CCPA and ENBA are superior candidate ADO agonists for countering NA seizure with significant efficacy in preventing neuropathology and promoting survival.

Introduction

Exposure to an organophosphorus nerve agent (NA), such as soman, initiates a cascade of biochemical events that eventually lead to severe brain damage or death. The primary mechanism for this toxicity involves the irreversible inhibition of cholinesterase (ChE) enzymes, especially acetylcholinesterase (AChE), the enzyme responsible for clearing the excitatory neurotransmitter acetylcholine (ACh). Without adequate AChE activity, there is an excess amount of ACh at synapses, which leads to overstimulation of the downstream cells and eventually to cell death. This process leads to headache, confusion, tremors, convulsions, depression of neural respiratory centers, and seizure. Peripherally, NAs induce respiratory dysfunction via muscarinic-induced bronchorrhea and bronchoconstriction, and nicotinic-mediated respiratory paralysis. To preserve brain function and life, it is critical that both peripheral and central cholinergic symptoms are alleviated.

Many inhibitory compounds with different pharmacologic mechanisms have been investigated to combat the toxic sequelae after NA exposure. Drugs that block ACh receptors, increase the effect of γ -amino-butyric acid (GABA_A), and antagonize N-methyl-D-aspartic acid (NMDA) glutamatergic receptors are efficacious when administered quickly after NA exposure [1, 2]. The currently approved NA treatment system uses atropine to block ACh receptors and 2-pyridine aldoxime methyl chloride (2-PAM) to reactivate NA-inhibited AChE. However, after a period of sustained seizure activity, the central nervous system often becomes refractory to these treatment strategies, and seizures cannot be terminated [1, 3]. Because current inhibitory- and reactivator-based strategies are unable to sufficiently control seizure activity when treatment is delayed, it is essential that victims are treated quickly so that *status epilepticus* and neuropathology are prevented [4]. However, in the event of an unexpected or mass casualty NA event, acute treatment may not be feasible. New medical countermeasures capable of preventing and terminating seizure activity need to be developed. Toward developing more effective anticonvulsant treatments, our team has been investigating the adenosine (ADO) signaling pathway as a more effective mechanism to counter NA-induced seizure.

ADO is an endogenous purine nucleoside that has a variety of physiological effects throughout the periphery and central nervous systems. Activation of A1 adenosine receptors (A1ARs) in the brain elicits a profound inhibitory effect on neuronal excitability via presynaptic inhibition of neurotransmitter release and post-synaptic hyperpolarization [5, 6]. Therapeutic exploitation of this inhibitory effect on neuronal excitability has been investigated for neuroprotection against a variety of pathologies including drug-resistant epilepsy [7-10]. Although ADO has demonstrated neuroprotective efficacy, clinical adoption of historical ADO agonists has been impeded by their modulation of cardiovascular function. Peripherally administered ADO agonists reduce cardiac output via A2AAR-induced hypotension and A1AR-induced bradycardia [11]. Despite that phenomenon, our team has demonstrated that acute intraperitoneal

(IP) treatment with the A1AR agonist N6-cyclopentyladenosine (CPA) promotes survival and prevents neuropathology after exposure to 1.6xLD50 doses of soman [12]. While those results are encouraging, the previously tested dose of CPA at 60 mg/kg was perhaps too high, considering the prolonged sedation, profound hypothermia, and sustained neuronal inhibition [12]. For this study, we hypothesized that lower doses of CPA could be equally neuroprotective with diminished severity of side effects. Additionally, we theorized that new agonists with greater affinity for the A1AR would increase therapeutic efficiency and minimize off target effects. These selective A1AR agonists included 2-Chloro-N6-cyclopentyladenosine (CCPA) and (±)-5'-Chloro-5'-deoxy-ENBA (ENBA) (**FIGURES**

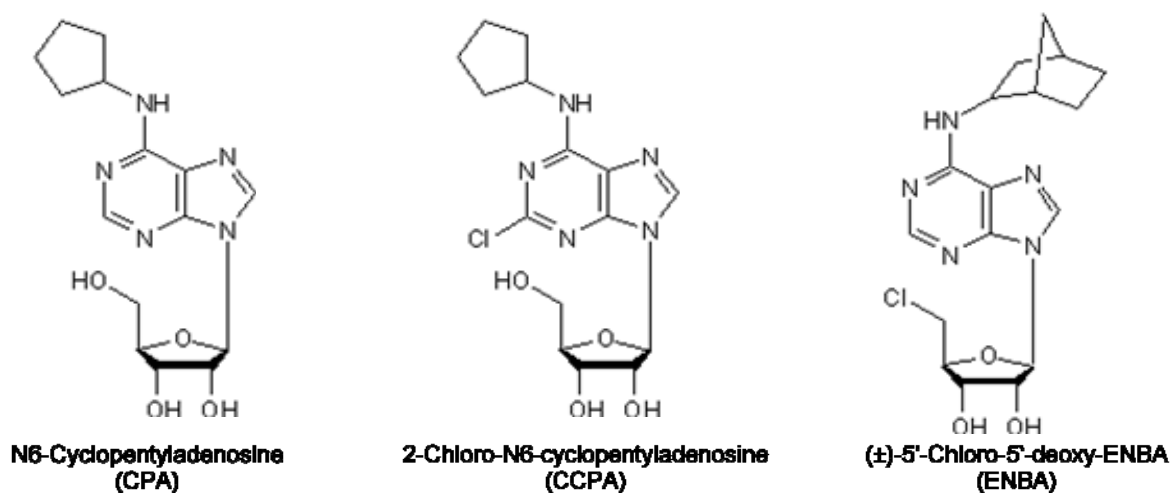


Figure 1). CCPA has been shown to be an effective anticonvulsant in pentylenetetrazol-induced seizure [13]. Compared to CPA's K_i of 2.3 nM, CCPA is more selective for the A1 agonist with a $K_i = 0.8$ nM. Furthermore, CCPA has improved selectivity for the A1 versus the A2A ($K_i = 2300$ nM) or A3 (42 nM) receptors. Another potent A1 agonist is ENBA. It is more selective for the A1 ($K_i = 0.51$ nM) than is CPA and has been shown to have powerful antinociceptive properties [14].

While the ultimate goal is to develop a countermeasure that is capable of terminating sustained seizure activity after delayed treatment, safety and efficacy in preventing seizure with acute treatment have to be first established. Therefore, the objective of this study was to 1) determine and compare the efficacy of CPA, CCPA and ENBA in preventing seizure and acute neurohistopathology in a rat soman seizure model, and 2) evaluate and compare the physiological and functional responses between these ADO agonists.

Material and Methods

Subjects:

Male Sprague Dawley rats weighing 275-300 grams were purchased from Charles River Labs (Kingston, NY). They were housed in individual cages in temperature ($21 \pm 2^\circ\text{C}$) and humidity ($50 \pm 20\%$) controlled quarters that were maintained on a 12-hour

light – dark schedule (with lights on at 0600 h). Laboratory chow and tap water were freely available whenever the animals were in home cages. Animals were quarantined for at least one week prior to experimentation.

Materials:

Saline (0.9% NaCl) injection, USP, was purchased from Cutter Labs, Inc. (Berkeley, CA). Atropine methyl nitrate (AMN) was purchased from Wedgewood Pharmacy (Swedesboro, NJ). Asoxime chloride (HI-6) was synthesized by Kalexyn Medicinal Chemistry (Kalamazoo, MI). N6-cyclopentaladenosine (CPA), 2-Chloro-N6-cyclopentyladenosine (CCPA) and (±)-5'-Chloro-5'-deoxy-ENBA (ENBA) were purchased from R&D Systems. Soman (pinacolyl methylphosphonofluoridate) was obtained from the US Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). Nerve agent was diluted in ice-cold saline prior to subcutaneous (SC) injection. AMN was injected IM, while HI-6, CPA, CCPA, and ENBA were injected IP. Injection volumes were 0.5 ml/kg for nerve agent and all treatment drugs.

Experimental procedures:

Approximately one week before experimentation, rats were surgically prepared with the implantation of a temperature transponder for recording body temperature and cortical electrodes for recording brain electroencephalographic (EEG) activity and detecting seizure onset. After collecting baseline brain activity on the day of experimentation, the standard soman-induced seizure rat model that is used for neuroprotection studies was implemented [1, 15]. Briefly, animals were pre-treated with HI-6 (125 mg/kg, IP) 30 minutes before a subcutaneous (SC) injection of $1.6 \times LD_{50}$ (180 µg/kg) soman. One minute later, animals were treated with an intramuscular (IM) injection of 2 mg/kg atropine methylnitrate (AMN). The administration of HI-6 and AMN, both with quaternary ammonium structures, does not affect EEG seizure onset or severity; they are used to minimize peripheral cholinergic symptoms and, thus, promote survival for adequate statistical comparisons of neuroprotection outcomes.

Since the objective of this study was to determine the anti-seizure dose efficacy of acute treatment, CPA, CCPA, or ENBA was given IP one minute after soman exposure at increasing dose levels. The following dose ranges were administered: CPA, 0 (for saline control), 25, 30, 40, 50, and 60 mg/kg; CCPA, 0, 15, 18, 21, 24, 30, and 36 mg/kg; and ENBA, 0, 10, 16, 20, 28, 34, 46, 54, and 62 mg/kg. All ADO agonists were prepared in multisol (48.5% distilled H₂O, 40% propylene glycol, 10% ethanol, 1.5% benzyl alcohol) vehicle on the day of treatment. Diluted preparations were sonicated until the drug was completely dissolved. During sonication, bath temperature was monitored for elevations above average ambient temperature to diminish the chance of structure degradation. CCPA readily dissolved in the solvent at all concentrations and stayed in solution until the time of treatment. CPA required additional time sonicating, but stayed in solution once dissolved. At the elevated concentrations (108 mg/ml)

required for the optimal dose, ENBA did not readily dissolve and required constant sonication until time of treatment delivery.

For each agonist dose level, up to 8 rats were exposed to multisol vehicle to measure the effects of the A1AR agonist without soman, and 12 rats were exposed to soman to determine the dose level's anti-seizure efficacy and rate of survival. The initial dose level for each agonist was determined from past experience and the pharmacological profiles of the new ADO agonists. To minimize the number of animals used, specific ADO dose levels were first tested in groups of 3-4 rats after soman exposure. If no seizure was detected, additional sets of 3-4 rats were tested until N=12 for the dose level. If seizure was detected in any rat, the dose level was escalated.

Brain activity of each rat was monitored continuously via EEG recording for 5 hr on the experimental day. The neurobehavioral responses were recorded using the functional observational battery (FOB) and toxic signs at 0, 4, 8, 15, 30, 45, and 60 min, and thereafter at 30-minute increments for 5 hours after soman or vehicle. Briefly, the FOB scored the rat's righting reflex, startle reflex, arousal, and ambulation/gait, and the presence of toxic signs, such as chewing, excessive salivation, lacrimation, head nodding, clonus, and convulsions, was recorded. Twenty-four hours after exposure, a final EEG recording (30-minute duration) and neurobehavioral assessment were made. The rats were then deeply sedated and perfused for histological analysis.

Data analysis:

After perfusion, the brains were extracted and prepared for histology with hematoxylin and eosin (H&E) staining. The brain tissue was evaluated and scored by a neuropathologist who was unaware of treatment, using established methodology [4]. Four regions associated with severe neurological deficits after NA exposure (i.e., piriform cortex, dorsal and ventral hippocampus, and thalamus) were each analyzed and scored from 0 (no damage) to 4 (severe damage). A total neuropathology score was then calculated for each rat from 0 (normal) to 16 (severe damage), and experimental groups were then compared to their respective controls using the Kruskal-Wallis test, followed by a Dunn's multiple comparison test if the treatment effect was significant. Differences in body temperature were also compared using the Kruskal-Wallis followed by a Dunn's multiple comparison test. Rates of seizure and survival for soman-exposed rats that received treatment and those that did not 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 significance was defined as $p < 0.05$. Because multiple comparisons were made to the control groups with the seizure and survival Fisher's tests, a Bonferroni correction was implemented ($p < 0.006 - p < 0.01$).

Results

Anticonvulsive effects:

The efficacy of each A1AR agonist was compared against the control group, which received saline in lieu of agonist. Seizure was readily detected from the EEG tracing; the signal associated with normal/baseline brain activity (i.e., low amplitude [$\leq \pm 100 \mu\text{V}$], high frequency) would quickly transition to severe spiking activity with high amplitude ($> \pm 1000 \mu\text{V}$) and high frequency. Behavioral signs such as convulsions and Straub tail coincided with such EEG power changes. Due to a high rate of mortality in our rat soman seizure model, a total of 28 control rats were required to generate a sufficient sample size for statistical analysis of neuropathology. Without ADO treatment, the rate of seizure prevention/absence was 0% (i.e., 100% seizure rate); the average (\pm standard deviation) latency to seizure was 5.28 ± 2.2 min. Of the 28 control rats, 6 survived to the 24-hour endpoint (21%). For those rats that did survive, signs of severe neuropathology were observed with an average total score of 14.8 ± 1.8 .

Similar to our previous experiments involving CPA treatment via ICV or IP [12], CPA proved to be a highly effective countermeasure for preventing NA-induced seizure and subsequent neuropathology. The anti-seizure effectiveness of CPA increased with escalating dose levels at 25, 30, 40, 50, and 60 mg/kg (Figure 2). A moderate level of protection was achieved even at the lowest dose of 25 mg/kg: 60% seizure prevention and 80% survival with average seizure onset of 3.7 min. Rats receiving 30 mg/kg experienced a greater rate of seizure (33% seizure prevention, 67% survival); however, the onset of seizure was slightly delayed to 8.9 ± 4.7 min. The 40 mg/kg and 50 mg/kg groups had 100% survival but only 50% and 60% seizure prevention, respectively. For those rats that seized, the average onset was much more delayed: average seizure onset was 19.4 and 22.7 min, respectively. The largest dose tested (60 mg/kg) provided the greatest protection. Only one of the 12 rats (8.3%) developed minimal EEG spiking/seizure activity (lower amplitude and frequency vs. control seizures) 36 mins after exposure. The neural and physiological effect of 60 mg/kg CPA on that rat was markedly diminished compared to the other CPA-treated rats. Whereas almost all rats, even those treated with the smallest dose of CPA (25 mg/kg), experienced rapid and deep sedation, this one rat maintained consciousness and a righting reflex. Because of that aberrant response, the actual dose of CPA delivered to the IP cavity was hypothesized to be less than prescribed. The most probable cause for this observation is an error in the execution of the IP injection, not with the compound or dose. Consequently, dose escalation was not performed further, and 60 mg/kg was accepted as the minimum effective dose.

CCPA was tested against soman at 15, 18, 21, 24, 30, and 36 mg/kg. CCPA at all dose levels improved survival. From 15 to 30 mg/kg, all rats survived to the 24-hour endpoint. Similar to CPA, some degree of protection from seizure was achieved at all dose levels (Figure 3). All rats receiving 21 mg/kg were protected from *status epilepticus*; however, there were signs of toxicity. Approximately two hours after soman exposure, rats treated with CCPA 21 mg/kg displayed brief episodes (< 3 min) of

tremors and minimal EEG spiking sporadically. Even though seizure/*status epilepticus* was not detected, we determined that the minimum effective dose was not achieved, and therefore, the dose level was increased. At the highest dose level of 36 mg/kg, 2 rats seized (average onset of 13 min) and later died. The remaining 10 rats were completely protected from seizure. Similar to the rationale for not escalating CPA beyond 60 mg/kg, CCPA was not escalated above 36 mg/kg because of a suspected error in the IP injection technique. Whereas all other rats treated with CCPA (same vial, same day) experienced rapid unconsciousness and bradycardia, the two rats that seized maintained consciousness, reflexes, and cardiovascular function. Because that response was extremely aberrant, we concluded that CCPA did not reach circulation and the lack of seizure prevention was due to human/injection error.

The third A1AR agonist evaluated in this study was ENBA at 10, 16, 20, 28, 34, 46, 54, and 62 mg/kg. ENBA at 10, 16, and 20 mg/kg was largely ineffective at preventing seizure onset ($\leq 25\%$) after soman exposure (Figure 4). ENBA at 28 and 34 mg/kg provided statistically significant seizure protection (75% and 50%, respectively); however, survival was low with only 50% and 38% respectively. While ENBA at 46 and 54 mg/kg significantly delayed the onset of seizure to more than two hours after soman exposure, most rats eventually seized. Significant anti-seizure and survival-promoting efficacy was only achieved with ENBA at 62 mg/kg. At that dose level, one of the 12 rats seized 155 mins after exposure. That rat survived to the 24 hr time point. In general, ENBA had similar physiological and neurological effects as the other A1AR agonists; i.e., reduction of brain activity, bradycardia, hypothermia, and sedation; however, the duration of effect was significantly shorter. Whereas rats receiving half the effective dose of CPA and CCPA remained sedated throughout the day of treatment, all rats receiving ENBA 34 mg/kg recovered consciousness after a period of deep sedation and appeared normal by the day's end.

Neuroprotective effects:

For all dose levels of CPA, CCPA, and ENBA, prevention of seizure translated to the absence of neuronal injury as assessed by neurohistopathology (i.e., regional scores ≤ 1). According to the Kruskal-Wallis test followed by a Dunn's multiple comparison test, CPA at 25 and 60 mg/kg, CCPA at 21 and 36 mg/kg, and ENBA at 62 mg/kg were statistically different from the control soman/saline group (Figure 5). Whereas saline-treated rats experienced severe neuropathology (average score = 14.8 ± 1.8), rats treated with ADO received significantly lower scores: CPA (60 mg/kg) = 0.2 ± 0.6 , CCPA (36 mg/kg) = 0 ± 0 , and ENBA (62 mg/kg) = 1.4 ± 4.4 . Animals that experienced seizure after saline or ENBA at any dose level developed severe neuropathology with widespread neuronal injury and death. In contrast, the severity of neuropathology was suppressed after CPA or CCPA treatment at suboptimal dose levels, even if seizure occurred. The average neuropathology scores for seizing rats with suboptimal ADO treatments are as follows: CPA (25-50 mg/kg) = 7.5 ± 3.7 , CCPA (15-30 mg/kg) = $6.2 \pm$

2.3, and ENBA (10-54 mg/kg) = 11.7 ± 2.8 . Suppression of neuropathology with seizure activity could not be assessed for the 3 rats that seized after CPA at 60 mg/kg (N=1) and CCPA at 36 mg/kg (N=2) because those rats died before the study endpoint. All other rats in the soman/CPA (60 mg/kg) and soman/CCPA (36 mg/kg) groups received a neuropathology score of 0 except for one soman/CPA (60 mg/kg) rat. That rat's dorsal and ventral hippocampus received scores of 1, which is considered normal, minor, injury. The single rat that seized after ENBA (62 mg/kg) developed severe neuropathology and received a score of 14. All other ENBA-treated rats did not seize and received a score of 0.

Cholinergic toxic signs and behavioral effects:

While CPA, CCPA, and ENBA all stimulate the A1AR with a high degree of specificity, the effects on EEG and brain activity after saline or soman exposure varied between agonists (Figure 6). After soman exposure and no ADO treatment, the rats displayed cholinergic symptoms (chewing, tremors) in less than 3 minutes. Within 5 minutes, most rats were convulsing with EEG seizure activity. That high frequency, high amplitude EEG signal continued throughout the day of experimental observation (5 hours). At 24 hours, the spiking activity was observed, albeit at a lower frequency (Figure 5, top row). Drastic behavioral changes correlated with CPA-, CCPA-, or ENBA-induced suppression of EEG. Animals first became lethargic and then quickly lost consciousness and a righting reflex as the EEG changed from normal (desynchronized) activity to slow-wave and flat-line. Conversely, the resumption of desynchronized EEG indicated behavioral recovery with ambulation, reflexes, and consciousness. CPA, CCPA, and ENBA effectively suppressed all cortical activity after GD exposure and thereby inhibited neuronal excitotoxicity. While CPA and CCPA had similar effects on EEG during the day of experimentation, rats receiving CCPA recovered more brain activity at 24 hours than did animals receiving CPA. Not only had they recovered their righting reflexes and arousal, their EEG was also of higher frequency and more similar to baseline. ENBA had the most rapid and pronounced effect on EEG of all agonists tested. In rats exposed to either vehicle or soman, ENBA suppressed all cortical activity in approximately 2 minutes after injection. Remarkably, most rats regained all brain activity at the 24-hour time point without any cholinergic symptoms. In addition to central inhibitory effects, ADO agonists also showed some inhibition of peripheral neural pathways. ADO treatments suppressed all peripheral cholinergic symptoms after soman exposure. Neither excessive salivation nor lacrimation was detected in animals receiving ADO at anti-seizure levels.

EEG power analysis:

The effect of soman or ADO on brain activity is further exemplified by an EEG power analysis (custom MATLAB Fast Fourier Transform script applied). As shown in Figure 7,

the power relative to baseline increased 10-fold within 30 minutes of soman exposure without ADO treatment, a time point that corresponded to maximum EEG spiking and seizure activity. In contrast, ADO treatment rapidly suppressed EEG power and respectively all reflexes and consciousness. ENBA's effects on brain activity were the most rapid and greatest in magnitude; EEG power was reduced to near zero in less than ten minutes of treatment. EEG power then slowly increased and eventually surpassed baseline power approximately 3 hours after treatment. The resumption of EEG power corresponded to an increase in body temperature from a hypothermic state, but not consciousness nor a return of a righting reflex. While EEG power was 1.5x baseline 5 hours post-treatment, that power resided within low frequency bands (i.e., very low frequency, delta, and theta) that are associated with sleep. CPA and CCPA also reduced total EEG power but at a slower rate than ENBA. The effects of CPA and CCPA on the day of treatment were similar; both reduced power to approximately half of baseline within 30 minutes of treatment, and suppressed EEG power at that level for the remainder of the experimental day.

Lethality:

Lethality in the control group for this rat soman seizure model was relatively high compared to previously published data [17]. After saline treatment, only 6 of the 28 rats survived to the 24-hour time point. Death occurred for the other 82% within 6.9 ± 9.3 hours after exposure on average. The lethal effects of soman were significantly minimized with all ADO treatment at the highest dose levels (*Figure 8*). The ability to promote survival was similar for CPA, CCPA, and ENBA; differences between agonists were not statistically different according to the Kaplan-Meier analysis with log rank (Mantel-Cox). Two deaths occurred after CPA, one within 1 hour of exposure and one overnight. The rat that died during the day was the only rat that seized with CPA at 60 mg/kg. Two of the 12 rats receiving CCPA at 36 mg/kg died at 0.8 and 1.0 hour post-exposure with seizure. The other CCPA death occurred overnight without seizing. ENBA protected most rats at 62 mg/kg; however, two rats died at 0.8 and 0.6 hour after exposure without seizure or cholinergic symptoms. While 100% survival was achieved with CPA (40-50 mg/kg), CCPA (15-30 mg/kg), and ENBA (46 mg/kg) at sub-anti-seizure dose levels, those groups had small sample sizes, and therefore significant differences among dose level groups could not be determined.

Effects of body temperature and heart rate:

Beyond suppression of neuronal activity in the central nervous system, A1AR agonists had significant peripheral effects. All ADO treatments with and without soman exposure induced some degree of hypothermia and bradycardia (i.e., reduction of heart rate). As seen in *Figure 9*, CPA, CCPA, and ENBA by themselves (i.e., no soman exposure) significantly reduced body temperatures with increasing magnitude at 0.5x and 1.0x their respective minimum effect doses. CCPA produced the greatest reduction in body temperature on the day of exposure; rats receiving 36 mg/kg decreased from a

baseline of $38.1 \pm 0.2^\circ\text{C}$ to $28.6 \pm 1.6^\circ\text{C}$ at 5 hours (300 min) after treatment, a reduction of 24.9%. That group continued to experience a decrease in body temperature overnight; the 24-hour average temperature was $27.8 \pm 7.7^\circ\text{C}$. Rats receiving 18 mg/kg of CCPA experienced a similar decline in temperature on the day of treatment ($29.1 \pm 0.8^\circ\text{C}$); however, their body temperatures increased to $37.1 \pm 0.0^\circ\text{C}$ at 24 hours (97.6% of baseline). CPA at 30 mg/kg and 60 mg/kg had similar effects on body temperature on the day of treatment ($84.0 \pm 4.9\%$ and $86.8 \pm 4.6\%$ of baseline, respectively). As with CCPA, body temperatures in rats receiving the high dose of CPA further decreased to $77.1 \pm 13.3\%$ of baseline at 24 hours. Only CPA and CCPA effects were statistically different at 24 hours in vehicle-exposed animals (*Figure 10, left*). ENBA also decreased body temperature but with less severity and for a shorter period of time. Rats receiving 34 mg/kg experienced maximum hypothermic effect 3 hours after treatment and slightly recovered to $88.4 \pm 1.7\%$ of baseline at 24 hours. The magnitude of reduction with ENBA at 62 mg/kg was comparable to CPA on the day of treatment; however, rats receiving ENBA demonstrated improved recovery with temperatures rebounding to $95.3 \pm 4.2\%$ of baseline at 24 hours.

For rats exposed to soman, ADO treatment reduced the body temperature in rats if seizure was prevented; otherwise, seizing rats did not develop hypothermia and maintained a temperature comparable to baseline. At the anti-seizure dose levels, CPA had the greatest effect on 24-hour body temperature (*Figure 10, right*). The body temperature of rats receiving CPA (60 mg/kg) after soman exposure decreased to $67.7 \pm 10.0\%$ of baseline. Soman-exposed rats that received CCPA (36 mg/kg) and ENBA (62 mg/kg) also experienced hypothermia on the day of exposure but had recovered to $89.2 \pm 7.6\%$ and $97.2 \pm 3.1\%$, respectively, of their baseline body temperatures at the 24-hour time point. The improvement in 24-hour body temperature after soman and ENBA treatment was statistically significant from soman-exposed CPA-treated animals. As evidenced by high rates of survival and seizure prevention in both hypothermic (CPA and CCPA) and normothermic (ENBA) rats, body temperature does not appear to be beneficial or deleterious to their outcomes at 24 hours.

In addition to lowering body temperature, A1AR agonists rapidly reduced heart rate at all dose levels. This phenomenon was reliably observed within 60 seconds of treatment, quickly accompanied by lethargy and pallor. Unlike the severity of hypothermia, the magnitude and rate of heart rate reduction were not dependent on the ADO dose level. That is, the lowest dose of each agonist had the same acute effect on the heart rate as the highest dose tested. However, the duration of heart rate depression was dose dependent; the recovery of baseline physiological parameters was quicker in rats receiving lower ADO doses. At the optimal/anti-seizure dose levels, depression of heart rate relative to baseline persisted until the 24-hour endpoint for all treatments after saline exposure with statistical significance. The effect was, however, smallest with ENBA (*Figure 11, left*). For soman-exposed animals (*Figure 11, right*),

CPA at 60 mg/kg induced the greatest bradycardia of all ADO treatments: heart rates at 24 hours averaged $33.6 \pm 20.4\%$ of baseline. CCPA and ENBA at anti-seizure dose levels also depressed heart rate but to a significantly less degree than CPA at 24 hours. Heart rates for CCPA-treated (36 mg/kg) rats were $67.9 \pm 17.0\%$ and ENBA-treated (62 mg/kg) rats had $78.7 \pm 13.4\%$ relative to baseline.

Effects on ambulation:

A functional observational battery (FOB), including assessments for startle reflex, righting reflex, arousal, and gait, was performed on each animal at regular intervals after soman exposure and treatment. Of the various functional assessments, ambulation was the most representative measure of function or incapacitation for these rats. That is, the ability to ambulate even slowly indicated the return of a righting reflex and at least a moderate degree of arousal or cognitive function. Rats receiving CPA at 60 mg/kg experienced the greatest immobility and depth of sedation; only 1 of the 11 surviving rats displayed some ability to walk at 24 hours, and the remaining animals were unconscious. The functional recovery after CCPA (36 mg/kg) and ENBA (62 mg/kg) was markedly better than after CPA; all rats except for one CCPA-treated rat were able to ambulate at 24 hr (Figure 12). Overall, approximately 33% of soman/saline, 90% of soman/CPA, 11% of soman/CCPA, and 0% of soman/ENBA rats were unable to ambulate.

FIGURES



Figure 1. Chemical structures of N6-Cyclopentyladenosine (CPA), 2-Chloro-N6-cyclopentyladenosine (CCPA), and (±)-5'-Chloro-5'-deoxy-ENBA (ENBA).

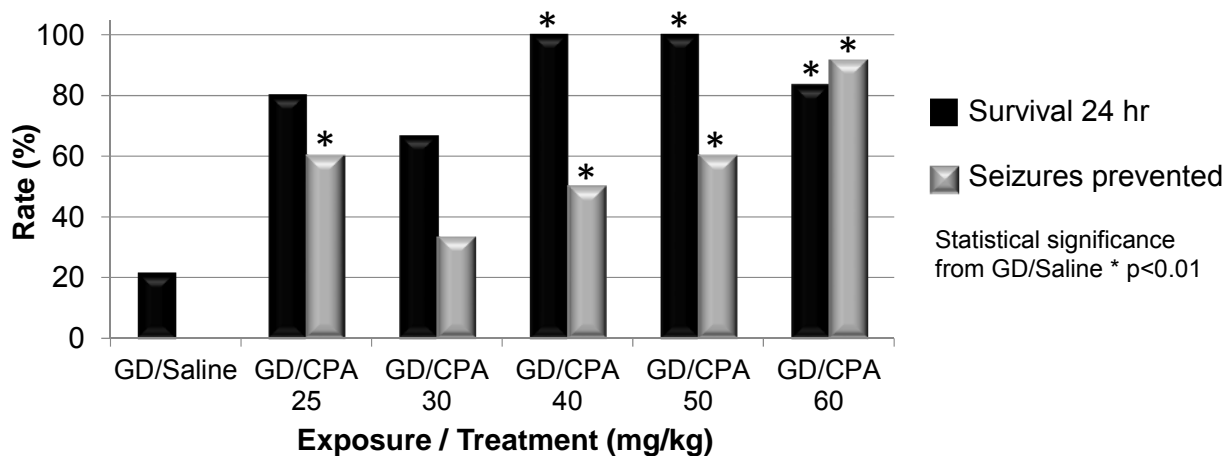


Figure 2. Anti-seizure and survival-promoting efficacy of CPA at escalating doses in a rat soman (GD) seizure model. Statistically significant differences from control (GD/Saline) indicated by * (Bonferroni corrected $p < 0.01$).

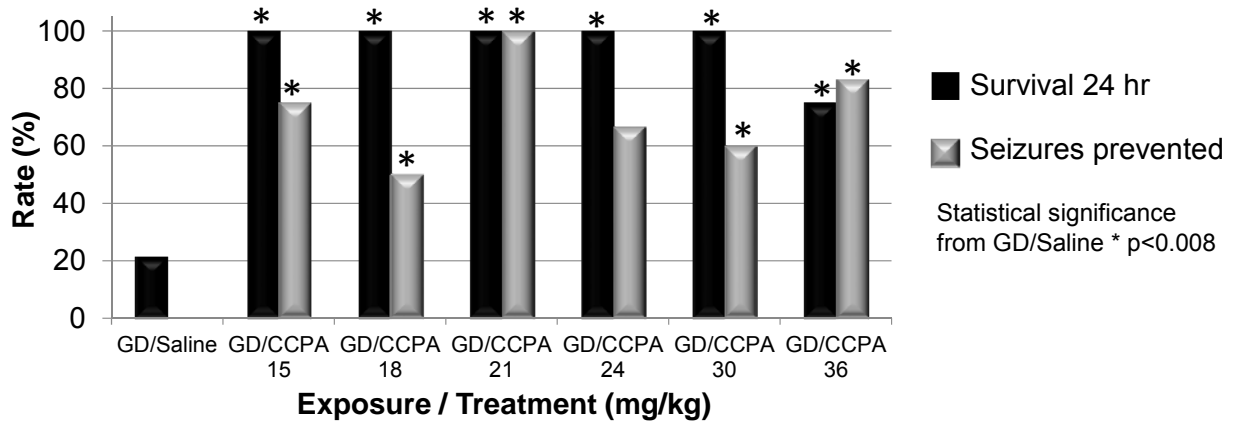


Figure 3. Anti-seizure and survival-promoting efficacy of CCPA at escalating doses in a rat soman (GD) seizure model. Statistically significant differences from control (GD/Saline) indicated by * (Bonferroni corrected $p < 0.008$).

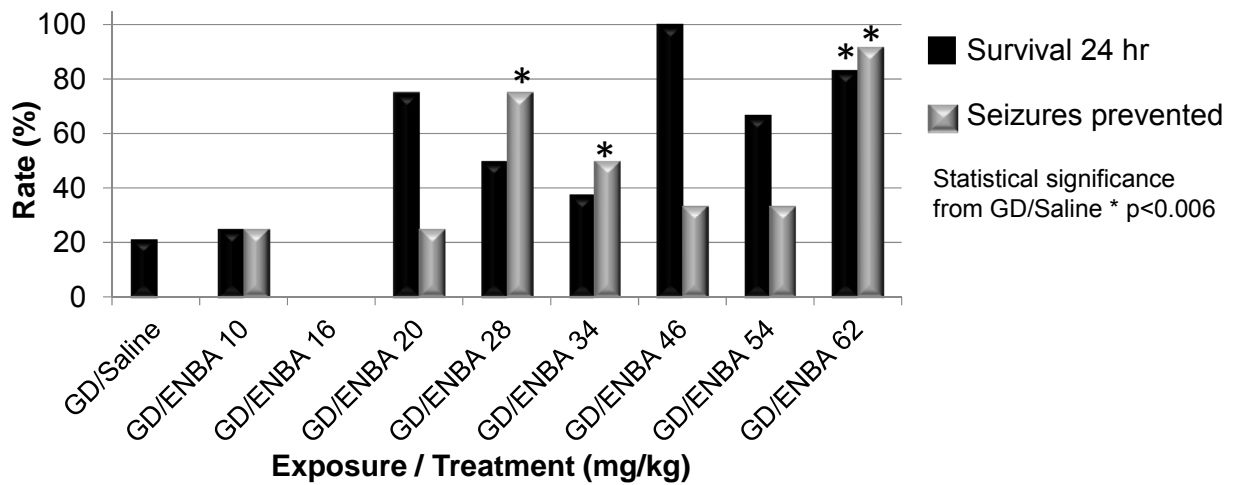


Figure 4. Anti-seizure and survival-promoting efficacy of ENBA at escalating doses in a rat soman (GD) seizure model. Statistically significant differences from control (GD/Saline) indicated by * (Bonferroni corrected $p < 0.006$).

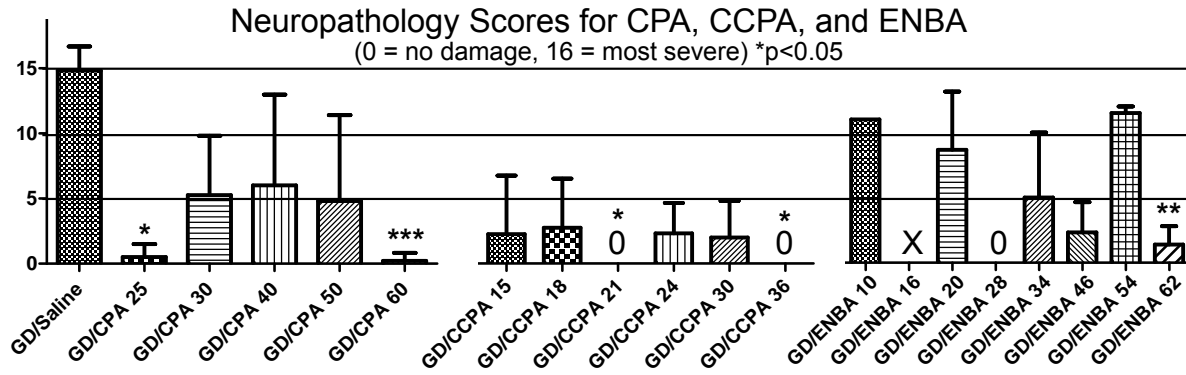


Figure 5. Neuropathology scores for groups exposed to soman (GD) and treated with saline, CPA, CCPA, or ENBA at escalating levels. Level of statistically significant differences from control (GD/Saline) is indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Example EEG Brain Activity after Soman Exposure and ADO Treatment

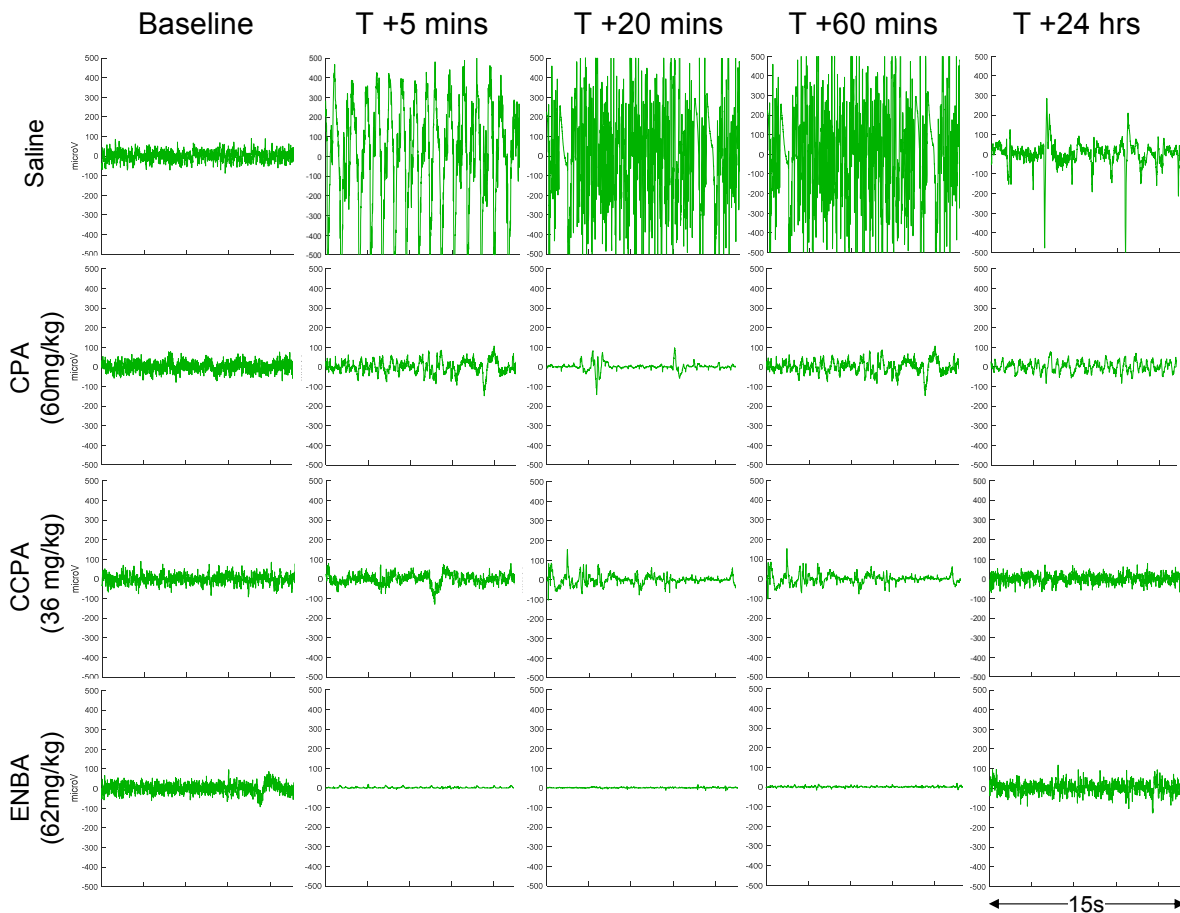


Figure 6. Exemplar EEG tracing for rats exposed to soman and treated with saline, CPA, CCPA, or ENBA. Whereas control animals (saline treatment) displayed severe spiking/seizure activity within 5 minutes of exposure (top row), the ADO agonists suppressed cortical activity and prevented the onset of seizure.

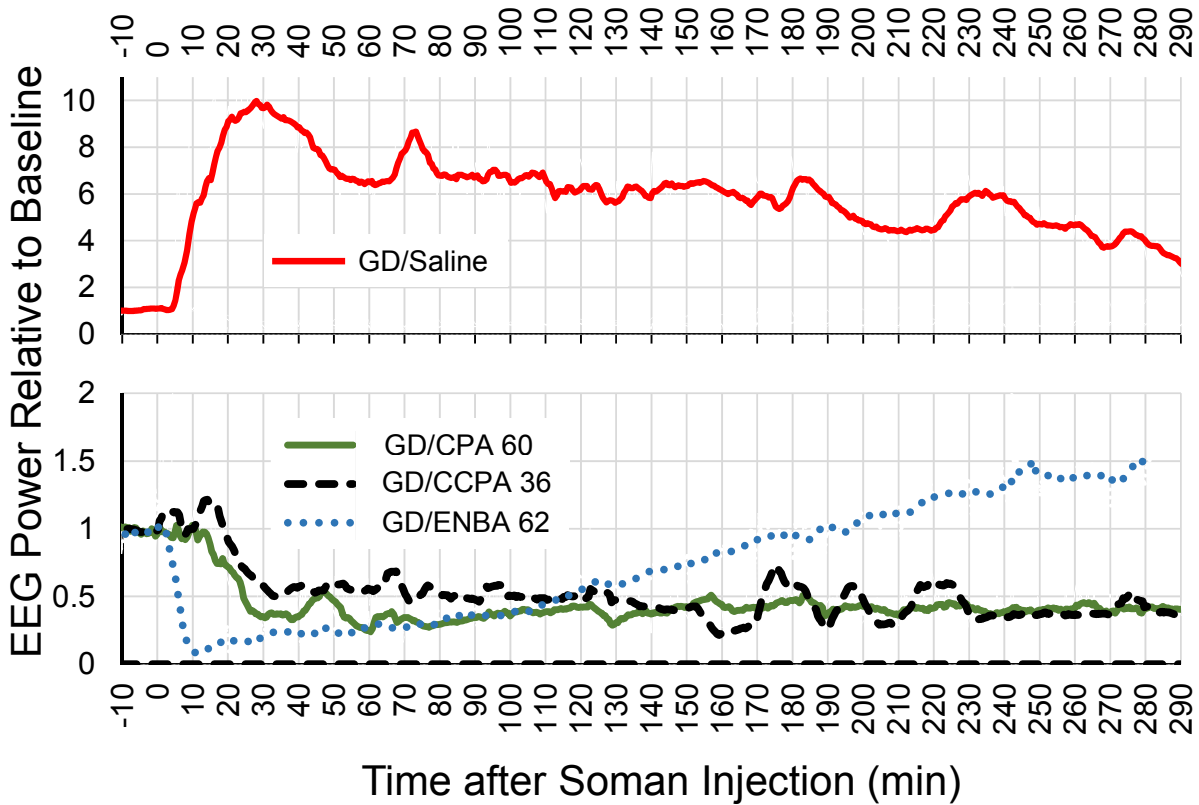


Figure 7. A power analysis of the EEG brain activity after soman (GD) exposure and treatment with saline, CPA, CCPA, or ENBA is shown. Whereas there is a dramatic increase in EEG power 7 minutes after GD exposure and 6 minutes after saline treatment, ADO treatments decreased EEG power and suppressed cortical brain activity.

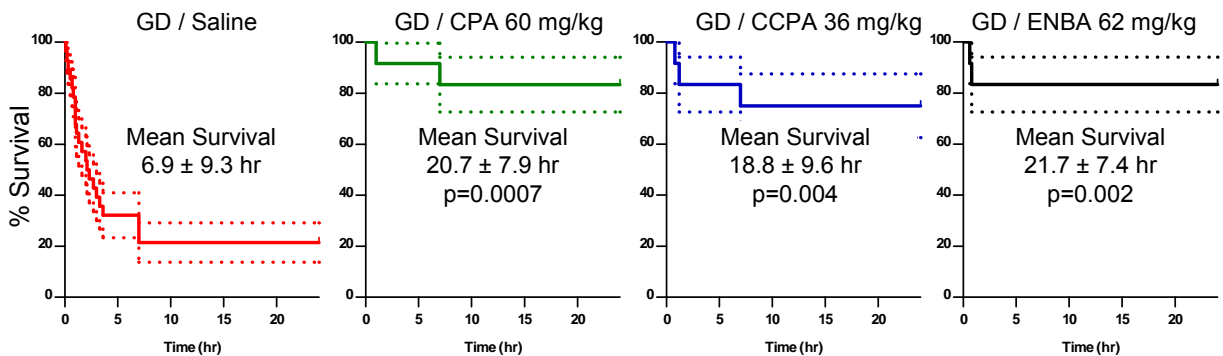


Figure 8. Kaplan-Meier survival analysis of groups exposed to soman (GD) and treated with saline, CPA (60 mg/kg), CCPA (36 mg/kg), or ENBA (62 mg/kg). All treatments were statistically significant from control (GD/Saline): $p < 0.05$.

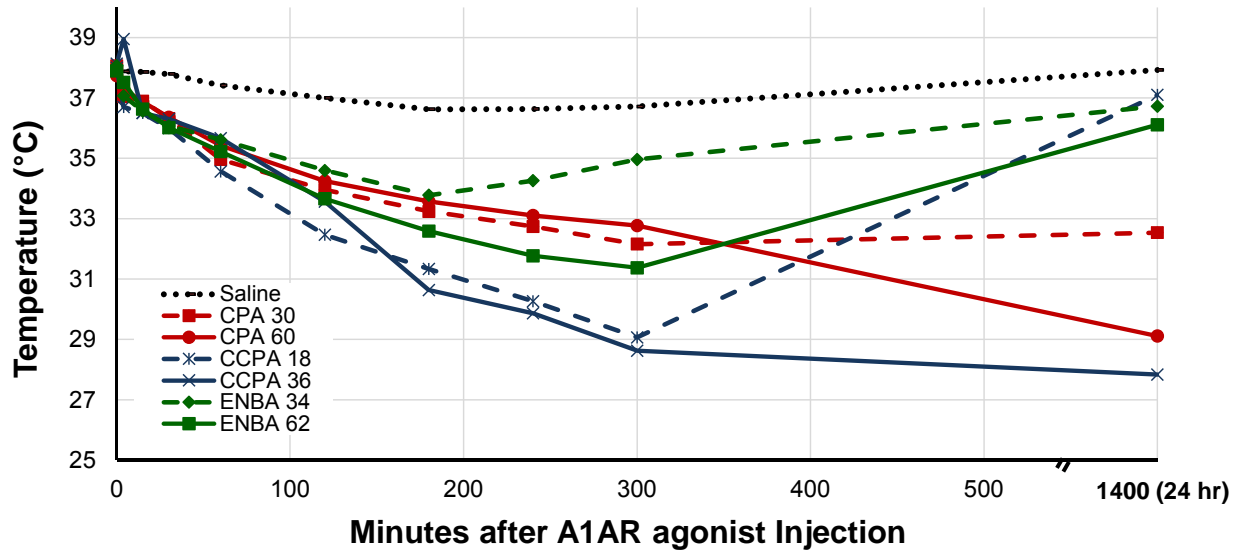


Figure 9. A1AR agonist effect on average body temperature without soman exposure.

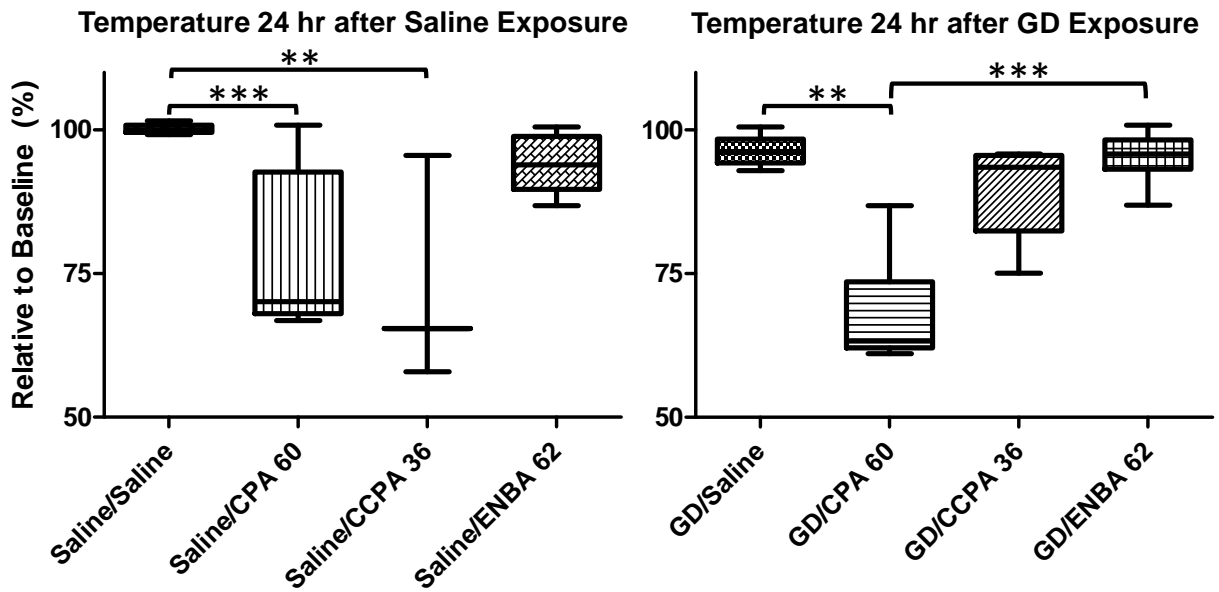


Figure 10. Box and whisker plot of median, min, and max body temperatures relative to baseline after vehicle (left) or soman (GD) exposure (right). Statistically significant differences are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

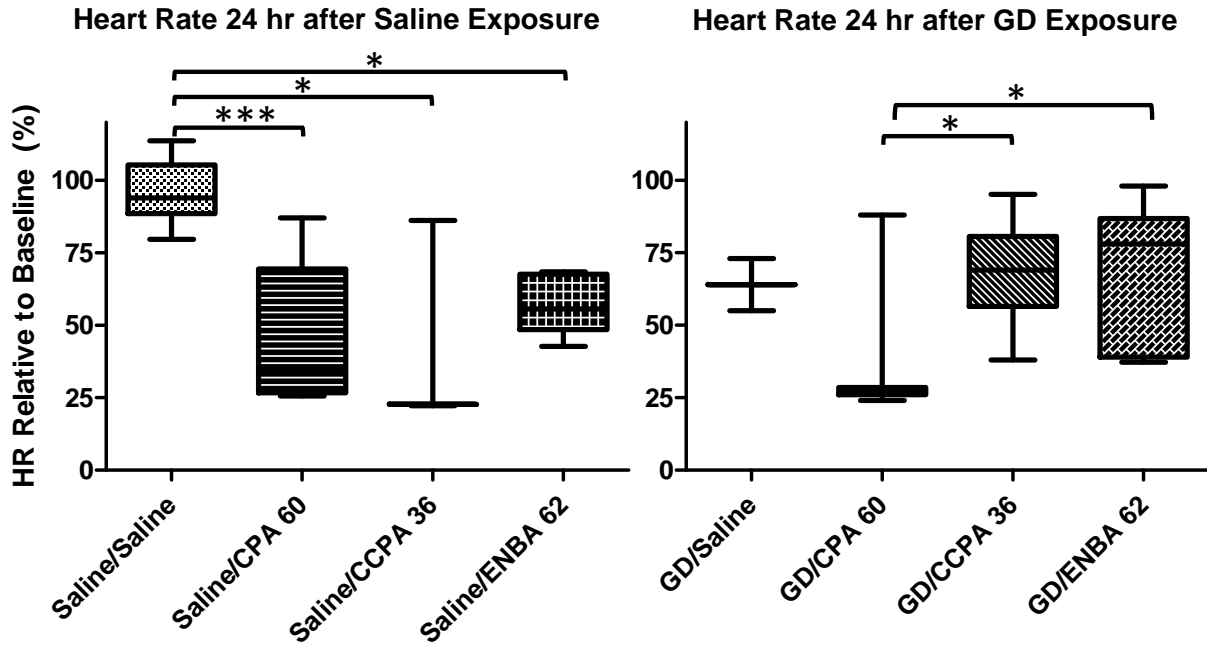


Figure 11. Box and whisker plot of median, min, and max heart rates at 24 hr after vehicle (left) or soman (GD) exposure (right) and treatment with CPA, CCPA, and CDENBA at anti-seizure dose levels. * indicates statistically significant differences * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

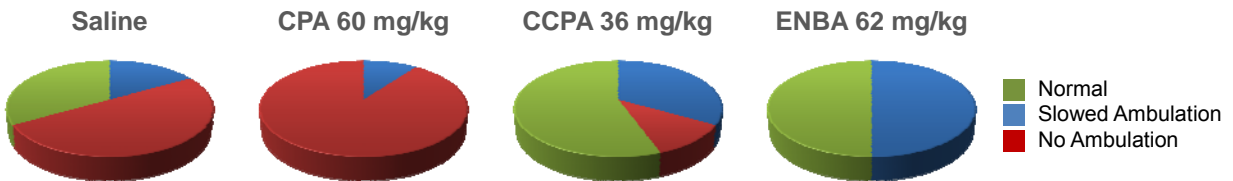


Figure 12. Ability to ambulate 24 hours after exposure to soman (GD) and treatment with saline, CPA, CCPA or ENBA at effective dose levels. Approximately 33% of GD/Saline, 90% of GD/CPA, 11% of GD/CCPA, and 0% of GD/ENBA rats were unable to ambulate.

Discussion

The use of NAs poses a severe threat to civilian and military personnel. Because current medical countermeasures have limited efficacy in preventing or terminating NA-induced seizure, we are investigating novel treatment strategies that utilize the ADO signaling pathway to suppress excitotoxic neuronal activity. In this study, we evaluated the efficacy of three candidate A1AR selective agonists for preventing seizure and neurohistopathology in a rat soman seizure model. We demonstrated that acute ADO treatment effectively prevented the onset of seizure and promoted survival with statistical significance. CPA at 60 mg/kg, CCPA at 36 mg/kg, and ENBA at 62 mg/kg were determined to be effective anti-seizure dose levels.

Of the three A1AR agonists tested in this study, ENBA at 62 mg/kg was the most efficacious treatment in that it was anti-seizure, was survival promoting, and allowed for ambulation at the study endpoint of 24 hours. CPA and CCPA also promoted survival and prevented seizure; however, those animals had not recovered consciousness nor a righting reflex at 24 hours. While protective sedation is a much better outcome than *status epilepticus* after NA exposure, the ideal countermeasure would allow the treated victim to return to combat or at least ambulate so that medical resources could be directed elsewhere. In addition to enabling a faster recovery, ENBA's suppression of neuronal activity was more rapid and pronounced than that of CPA and CCPA. Consequently, ENBA may offer better protection since cholinergic and glutamatergic excitotoxicity would have less opportunity to develop. Furthermore, ENBA's pharmacological profile would allow the first responder or clinician to better control the course of treatment and maintain seizure prevention with additional doses while minimizing side effects.

While the primary motivation for investigating ADO was to leverage the A1AR's profound inhibitory effect on cortical brain activity, data from this study suggest that ADO's protective efficacy can also be attributed to inhibition of peripheral neural pathways. Soman-induced hypersecretions often lead to high rates of mortality. In this study, ADO treatments suppressed all peripheral cholinergic symptoms after soman exposure. Neither excessive salivation nor lacrimation was detected in animals receiving ADO at anti-seizure levels. This peripheral phenomenon in addition to reduction in seizure activity is likely the driving mechanisms of ADO's promotion of survival after soman exposure.

ADO treatments had significant cognitive and physiological effects at all dose levels. While others have reported that ADO's peripheral effects (i.e., cardiac depression) limit clinical utility, the data from the present study clearly demonstrated that acute ADO-induced bradycardia is not lethal regardless of whether the animal was exposed to soman or not. There was zero lethality for all vehicle-exposed (no soman) ADO-treated rats at any dose level. Moreover, all ADO treatments promoted survival after soman exposure with great statistical significance concomitant to bradycardia and hypothermia.

Although these results suggest that A1AR stimulation is a promising mechanism for countering NA intoxication, further study is needed to establish the long-term safety and efficacy of relatively high ADO doses. Since ADO receptors are expressed in many different types of tissues (e.g., liver, heart, spleen, and kidney) and modulate a variety of physiologic functions [16], robust long-term behavioral and histological analyses are required to verify that effective dose levels do not induce deleterious side effects.

The ultimate goal for any NA medical countermeasure is to provide neuroprotection. However, neuroprotection is a difficult phenomenon to define and quantify. A common and relatively straightforward approach is to associate histologically quantified neuronal health or injury with neuroprotection. That is, the presence of spongiform changes, rarefaction, and aberrant neuronal staining/morphology indicates severe damage and likely poor functional outcomes. Conversely, brain sections that lack such indicators typically correlate to normal behavior and positive outcomes. From this definition, CPA (60 mg/kg), CCPA (36 mg/kg), and ENBA (62 mg/kg) provided significant neuroprotection against a high-dose soman exposure. In general, that neuroprotection closely correlated with seizure prevention. However, CPA and CCPA surprisingly provided moderate, yet not significant, neuroprotection even after soman-induced seizure. CPA-treated rats that seized received an average neuropathology score of 7.5 ± 3.7 (maximum score of 12), and CCPA-treated rats that seized received an average score of 6.2 ± 2.3 . In contrast, all ENBA-treated rats that seized displayed greater neuropathology, with an average score of 11.7 ± 2.8 . CPA's and CCPA's greater duration of effect may explain this partial protection. Whereas ENBA at a suboptimal dose level may have suppressed excitotoxic activity for a short period of time, that effect likely subsided overnight. However, CPA's and CCPA's effects were longer lasting than were ENBA's, and their suppression of the excitotoxic magnitude may have persisted overnight and consequently minimized neuronal damage. Further study into the mechanism of partial neuroprotection is required.

In this study, there were several aberrant cases of subdued or absent responses to ADO injection. At the optimal dose levels, CPA, CCPA, and ENBA produced rapid sedation, inhibition of EEG, and bradycardia after vehicle or soman exposure in the majority of rats. However, the responses of 1 CPA rat, 2 CCPA rats, and 1 ENBA rat were fundamentally different. Those rats displayed minimal reaction to ADO, i.e., lack of sedation, minimal bradycardia, and insignificant EEG power effects. While biological variability and sensitivity to A1AR stimulation may lead to slight differences in the duration of neuronal inhibition, the pronounced absence of central effects indicates other causative factors when strain, weight, age, and sex are controlled. One plausible explanation is variability in the exact location of the treatment injection. Whether it was from deformation of the IP cavity from improper animal holding or a misplacement in the injection site, clearly the amount of ADO agonist reaching the circulation was less. Consequently, the lack of anti-seizure and neuroprotection in the rats receiving ADO

agonists at the high dose levels could be attributed to human error and not the countermeasure. In follow-up experiments, alternative routes of administration such as IM should be evaluated not only to minimize injection error, but also to assess the efficacy of a more clinically relevant delivery technique.

Summary

Three candidate A1AR agonists were evaluated for anti-seizure and neuroprotective efficacy in a rat soman seizure model. After completing a dose-escalation study, CPA at 60 mg/kg, CCPA at 36 mg/kg, and ENBA at 62 mg/kg were determined to be effective anti-seizure doses. Suppression of soman-induced seizure activity translated to protection of brain tissue from neuropathology. Moreover, all ADO treatments promoted survival with great statistical significance. While CPA, CCPA, and ENBA concomitantly induced bradycardia and hypothermia in soman-exposed rats, those peripheral side effects did not negatively affect outcomes. Of the three agonists tested, ENBA displayed the most beneficial pharmacological profile. Not only did that agonist produce the most rapid and profound suppression of neuronal activity, but rats treated with ENBA after soman were fully functional 24 hours later. These data strongly suggest that A1AR agonists are promising NA medical countermeasures. Toward further developing this novel treatment strategy, future studies will comprehensively evaluate CPA, CCPA, and ENBA against a broad set of threat agents including sarin, cyclosarin, and VX, and in combination with currently approved oximes and benzodiazepines. Additionally, longer-term studies will be conducted to better assess physiological and functional recovery after ADO treatment with and without preceding NA exposure.

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