Anticonvulsant efficacy of antihistamine cyproheptadine in rats exposed to the chemical warfare nerve agent soman.

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Abstract

Organophosphate compounds, such as soman and sarin, are highly toxic chemical warfare nerve agents that cause a build-up of acetylcholine in synapses and neuromuscular junctions. Current therapies aim to prevent seizures and protect against brain injury following exposure. The present study was designed to evaluate the effectiveness of the antihistamine cyproheptadine in improving survival and controlling seizures in rats exposed to soman. Rats were pretreated with the oxime reactivator HI-6 (125 mg/kg, ip) 30 min prior to soman exposure (225 µg/kg, sc) and then treated with atropine methylnitrate (AMN, 2.0 mg/kg, im) 1 min after soman.

Cyproheptadine (10, 13, 16 or 20 mg/kg, ip) was given at one of three time points: 1 min after soman intoxication, at the onset of soman-induced seizures or 5 min after seizure onset. Control animals were exposed to soman and given an equivalent volume of sterile water instead of cyproheptadine. The incidence of seizures, mortality, neuron counts, neuropathology and apoptosis in specific regions of the brain were evaluated. In animals given HI-6 and AMN the incidence of soman-induced seizure and mortality rate within the first 24 hours were 100%.

When cyproheptadine was given at a dose of 13 or 20 mg/kg 1 min after soman exposure, the incidence of seizures was reduced from 100% to 13% and 30%, respectively. In addition, cyproheptadine given at 1 min after soman exposure increased the survival rate to 100% regardless of dose. When cyproheptadine was administered at seizure onset, seizures were terminated in 100% of the animals at doses above 10 mg/kg. The survival rate with cyproheptadine treatment at the onset of seizure was $\geq 83\%$. Seizures terminated in $\geq 75\%$ of the animals that received cyproheptadine 5 min after soman-induced seizure onset. When given at 5 min after seizure onset the survival rate was 100% at all tested doses of cyproheptadine. The neuropathology scores and the number of TUNEL positive cells in the brain regions examined

decreased at all time points and cyproheptadine doses tested. These observations indicate that cyproheptadine treatment can effectively control seizures, improve survival, reduce seizure duration and reduce the number of dying cells in the brain following soman exposure.

Keywords: Nerve agent, Soman, Seizure, Anticholinergic, Cyproheptadine, Neuropathology.

1. Introduction

Chemical warfare nerve agents (CWNAs), such as tabun, sarin, cyclosarin, soman and VX, are a class of phosphorus-containing organophosphates (OP). CWNAs are a realistic threat to military personnel on the battlefield and to civilian populations through a terrorist attack. Organophosphates irreversibly bind to acetylcholinesterase (AChE), an enzyme responsible for the degradation of acetylcholine (ACh), resulting in an accumulation of ACh in the synaptic terminals of the peripheral and central nervous systems (CNS). The accumulation of ACh results in overstimulation of cholinergic receptors, which produces a variety of toxic effects. Symptoms of cholinergic toxicity include excess salivation, bronchial constriction, muscle fasciculations, seizures, respiratory failure, neuronal damage and possibly death (Taylor, 2001, Wiener and Hoffman, 2004).

Nerve agent countermeasures emphasize the reduction of post-exposure brain pathologies and prevention of death. This is especially difficult with soman exposure because it permanently and irreversibly inhibits AChE within minutes after intoxication (a result of rapid aging) (Romano, 2007, Bateman, 2014). Pharmacological control of nerve agent-induced seizures is critical for survival following nerve agent exposure (Shih et al., 2003). In animal models of acute exposure to OPs, such as soman, current treatment include; oximes, such as HI-6 or 2-PAM, which reactivate CWNA-inhibited AChE, a muscarinic antagonist, such as atropine, to block peripheral and CNS receptors and benzodiazepines, such as diazepam, to treat or prevent convulsions (Shih et al., 1991, Shih and McDonough, 1999). Once the nerve agent AChE complex has aged, oximes are no longer effective in reactivating AChE. Development of new or improved therapies for OP poisoning has generally focused on terminating seizures and providing neuroprotection.

Limited studies have been conducted to evaluate the efficacy of antihistamines as a therapy for nerve agent exposure. Two studies have shown the protective effects of the antihistamine diphenhydramine against organophosphate poisoning (Faris and Mohammad, 1997, Bird et al., 2002). More recently the antihistamine promethazine has been shown to be effective at reducing paraoxon, dicotophos, and soman toxicity (Kan et al., 2008, Nurulain et al., 2015). These studies show the potential of antihistamines as a medical countermeasure following nerve agent intoxication. In this study, cyproheptadine, a first generation antihistamine with anticholinergic effects (Simons, 1989) was evaluated as an adjunct therapy to the standard countermeasures which include an oxime and muscarinic agonist in soman-intoxicated animals. This study examined the effect cyproheptadine had on soman-induced seizure incidence, seizure duration, and 24 hour survivability. In addition, since soman-induced seizures have been shown to cause neuronal damage in the cortices, amygdala and thalamus (Kim et al., 1999, Baille et al., 2005) and cyproheptadine has been shown to have neuroprotective properties through inhibition of activated caspase-3, resulting in a reduction of apoptosis in models of Huntington's disease (Sarantos et al., 2012), neuropathology, neuronal counts and apoptosis in the piriform cortex, dorsal cortex, lateral cortex, laterodorsal thalamus and amygdala regions of the brain were evaluated.

2. Materials and Methods

Animal model: Male Sprague-Dawley rats (Charles River Laboratories, Kingston, NY) weighing 250-350 g were used for these studies. The animals were acclimated for at least one week prior to experiments. Animals were individually housed in a controlled room environment (temperature 21±2 °C, 12-hour light/dark cycle) and supplied with food and water ad libitum. Experiments were conducted in compliance with the regulations and standards of the Animal

Welfare Act and adhered to the principles of the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where the research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

Cortical electroencephalographic electrode implantation: A small subset of cyproheptadine-treated animals (N=8) were implanted with cortical electroencephalographic (EEG) electrodes to monitor seizure activity. EEG electrodes were implanted as previously described (Skovira et al., 2012). In brief, rats were anesthetized with isoflurane (3% induction, 2-3% maintenance, with oxygen) and placed into a stereotaxic frame. Two stainless steel cortical screws were placed equidistant apart between bregma and lambda and 2-3 mm lateral from the midline in each hemisphere, with a third screw was placed over the cerebellum, as a reference. The electrodes were held in place and electrically isolated using dental acrylic cement. Buprenorphine HCl (0.05 mg/kg, sc) was administered for pain upon recovery from anesthesia. Electrode-implanted rats were allowed to rest at least 7 days prior to experimentation.

Soman exposure: On the day of exposure rats were first treated with the oxime reactivator HI-6 (1-(((4-(aminocarbonyl)pyridinio)methoxy)methyl)-2-((hydroxyimino)methyl) pyridinium chloride) (125 mg/kg, ip; Kalexsyn, Kalamazoo, MI) 30 minutes prior to soman exposure. Animals were then given an injection of the nerve agent soman (225 µg/kg, sc; US Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD). One minute after soman intoxication animals were treated with the muscarinic receptor antagonist atropine methyl nitrate (AMN, 2.0 mg/kg, im; Wedgewood Pharmacy, Swedesboro, NJ). HI-6 and atropine were used to enhance survivability of soman-exposed animals (Shih and McDonough, 1999, Shih et

al., 2010). After soman intoxication, animals were randomly divided into groups and treated with cyproheptadine (10, 13, 16 or 20 mg/kg, ip; Tocris Biologicals, Bristol, UK) at 1 min after soman exposure, at seizure onset or at 5 min after seizure onset. The doses of treatment were calculated by incrementally decreasing or increasing the effective dose of cyproheptadine (16mg/kg) by 0.1 log unit intervals. Untreated animals were given the same soman, HI-6 and AMN regimen. Untreated animals (HI-6, AMN and soman, 225 µg/kg) did not survive 24 hours. Therefore, in order to study 24-hour neuropathology in untreated, soman exposed animals a dose of 180 µg/kg was used. This dose of soman has been previously described to produce seizures in 100% of exposed animals and a survival rate of approximately 60% 24 hours after exposure (Shih et al., 1991).

Seizure monitoring: Animals were monitored for behavioral seizures for 7 hours post exposure. Seizure activity was determined using the Racine scale: stage 1, mouth and facial movements; stage 2, head nodding; stage 3, forelimb clonus; stage 4, rearing; and stage 5, rearing and falling (Racine, 1972). EEG recordings were done on a subset of animals, in parallel with the Racine scale of seizure determination to ensure accuracy of determining cortical seizures using behavioral seizure signs. To monitor EEG, animals were connected to recording leads and placed in individual plastic recording chambers. EEG recordings were done using CDE Model 1902 amplifiers and displayed on a computer running Spike2 Software (Cambridge Electronic Design, Ltd., Cambridge, UK). To establish a baseline recording, animals were monitored for at least 30 minutes prior to any treatments. Seizure durations were calculated by using the seizure times in both EEG observed and behaviorally observed seizures. Animals in which seizures did not terminate during the 7 hours of observation were not used in seizure duration calculations.

Twenty-four hours post exposure animals were deeply anesthetized with pentobarbital (65 mg/kg, ip; Vortech Pharmaceuticals, Dearborn, MI) and transcardially perfused with 0.9% Saline (Sigma-Aldrich, St Louis, MO), followed by 10% formalin (Fisher Scientific, Waltham, MA). Brains were extracted after perfusion and post fixed in 10% formalin. Following fixation brains were processed and embedded in paraffin. Five micron coronal plane sections were made using a rotary microtome and adhered to positively charged slides (Fisher Scientific, Waltham, MA).

Histopathological evaluation: Five-micron sections were stained with Mayer's hematoxylin and eosin (H&E) using a conventional method (Mikel, 1994). H&E stained brains were blindly evaluated both quantitatively and qualitatively. Quantitative analysis was performed by counting the hematoxylin-positive neurons in brain regions of interest and dividing the hematoxyline-positive neurons by the area of the region used to count (cells/mm²). A qualitative neuropathology analysis was performed on the following brain regions of interest: piriform cortex (Pir), laterodorsal thalamus (LD), amygdala (AM), lateral cortex (LCx) and dorsal cortex (DCx). Pathology was scored using a previously described scale for damage: 0=no sign of damage, 1=minimal damage (1-10%), 2=mild damage (11-25%), 3=moderate damage (25-45%), and 4=severe damage (>45%) (McDonough et al., 1995, Shih et al., 2003, Shih et al., 2011).

Cell death: Terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate-biotin nick-end labeling (TUNEL) staining was carried out using the Apop Tag Plus Peroxidase In Situ Apoptosis Kit (EMD Millipore, Billerica, MA) as directed by the manufacturer's instructions.

Microscopy: Slides were scanned using a NanoZoomer 2.0-RS slide scanner (Hamamatsu Photonics, Hamamatsu City, Japan). Scans were viewed using the NDP software (Hamamatsu).

The drawing tool on the software was used to outline an area in the brain regions of interest. The drawing tool gives the area within the outlined region. The numbers of cells and area of the brain regions of interest were recorded. H&E and TUNEL positive cells were blindly counted in the desired brain regions and the total was divided by the area of the region used to determine the number of TUNEL positive cells per area. Cell counts are the number of cells per mm² of tissue.

2.1 Statistics

A Fisher's exact test was utilized to determine statistical significance of seizure incidence between groups. A one-way ANOVA followed by a Dunnett's test was used to determine the significance between no cyproheptadine and cyproheptadine group's seizure duration. A mixed-methods ANOVA followed by a Dunnett's test was used to determine significance between no cyproheptadine and cyproheptadine group's H&E counts.

3. Results

Incidence of seizure and 24-hour survivability

Animals given HI-6 and AMN only showed signs of intoxication after nerve agent exposure. All untreated (no cyproheptadine) rats dosed with 225µg/kg of soman seized and died within the first 24 hours after intoxication. The number of animals that experienced seizure, seizure termination and 24-hour survival rates of cyproheptadine-treated, soman-intoxicated animals can be found in Table 1. When given at 1 min after soman exposure cyproheptadine at 10, 13, 16 or 20 mg/kg reduced the incidence of seizure from 100% in the untreated animals to 60%, 12.5%, 80% and 30%, respectively.

Cyproheptadine treatment increased 24-hour survivability at all doses and time points tested (Table 1). Twenty-four-hour survivability when 10, 13, 16 or 20 mg/kg cyproheptadine was given at 1 minute after soman intoxication went from 0% to 60%, 100%, 100% and 100%,

respectively. When 10, 13, 16 or 20 mg/kg cyproheptadine was given at seizure onset survivability increased from 0% to 80%, 83%, 100% and 100%, respectively. Survivability increased to 100% at all cyproheptadine doses when given 5 minutes after seizure onset. These data indicate that cyproheptadine treatment for soman exposure decreases both seizure incidence and mortality.

Seizure duration and termination

Cyproheptadine significantly reduced seizure duration at all tested doses and time points (Figure 1). Soman-intoxicated animals receiving only HI-6 and AMN continuously seized for the 7 hours (420 minutes) that they were observed. When cyproheptadine was given at 1 minute after soman intoxication seizures were all terminated except in one animal receiving 10 mg/kg. The average seizure durations for animals in this group were 14.8 (10 mg/kg), 0.6 (13 mg/kg), 1.8 (16 mg/kg) and 1.4 (20 mg/kg) minutes. Seizures terminated in all animals when cyproheptadine was given at the onset of seizure, and the average seizure duration was 54.8 (10 mg/kg), 4.0 (13 mg/kg), 4.0 (16 mg/kg) and 3.0 (20 mg/kg) minutes. When cyproheptadine was given at 5 minutes after seizure onset, seizures terminated in 100% (10 mg/kg), 75% (13 mg/kg), 88% (16 mg/kg) and 100% (20 mg/kg) of the animals, soman-induced seizure duration was decreased to 13.3, 8.3, 8.8 and 9.5 minutes, respectively. Seizure duration and termination were similar in the EEG-monitored group compared to the non-EEG animals (data not shown).

Figure 1. *Cyproheptadine reduces soman-induced seizure (sz) duration.* Cyproheptadine reduced sz duration at all tested doses and time-points of treatment. Results are the mean $\pm$ standard deviation (SD). *** $p < 0.001$.

Regions of neuronal damage

Neuropathology scores of untreated, soman-exposed rats (no cyproheptadine) showed damage in the Pir, DCx, LCx, LD and the AM regions of the brain (Table 2). Neuropathology scores for the untreated animals were 4.0 ± 0 in the Pir, 2.3 ± 0.1 in the DCx, 2.2 ± 0.1 in the LCx, 4.0 ± 0 in the LD and 3.1 ± 0.1 in the AM (values are $\pm$ the SD scores). There were no signs of neuronal damage in animals treated with cyproheptadine at 1 minute after soman intoxication and at seizure onset in animals that stopped seizing (Table 2). Soman-intoxicated animals that did not stop seizing after treatment with cyproheptadine at seizure onset had neuronal damage in the Pir, LD and AM regions of the brain (Table 2). When cyproheptadine was given 5 minutes after seizure onset, the neuropathology scores were greatly reduced compared to the scores of

untreated animals. Neuronal damage was observed in 2 out of 7, 1 out of 7 and 2 out of 4 in the 10 mg/kg, 16 mg/kg and 20 mg/kg cyproheptadine animals treated at 5 minutes after seizure onset, respectively. When 13 mg/kg cyproheptadine was given 5 minutes after seizure onset, no neuronal damage was observed in the brain regions of animals whose seizures were terminated (Table 2).

Figure 2. Representative micrographs of lateral dorsal thalamus in a soman exposed rat with and without cyproheptadine. Hematoxylin and eosin stained LD 24 hours after soman exposure in an untreated (A) and cyproheptadine treated (B) animal. TUNEL staining 24 hours after soman intoxication in an untreated (C) and a cyproheptadine-treated (D) rat. Arrow heads indicate blood vessels, asterisks are areas of edema and arrows are areas of hemorrhage and circles are TUNEL positive nuclei. Bar is 250 μ m.

Hematoxyline and eosin staining was used to compare the number of neurons in untreated and cyproheptadine-treated, soman-exposed rats. Representative micrographs can be found in Figure

2A and 2B. There was a significant reduction of neuronal loss in the Pir region of the brain at all time points and cyproheptadine doses tested, except 20mg/kg at 5 minutes after seizure onset (Figure 3A). There was approximately a 2-fold decrease in the number of neurons in the Pir region of untreated (no cyproheptadine) animals compared to the cyproheptadine-treated animals (Figure 3A). There wasn't a significant reduction of neuronal loss in the DCx region of the brain, except when cyproheptadine (13mg/kg) was given at 1 minute after soman (Figure 3B). No significant difference was found at any time point or dose in the neuron counts of LCx region of the brain (Figure 3C). In the LD there was a significant reduction of neuronal loss at all tested doses when cyproheptadine was given 1 minute after soman (Figure 3D). When cyproheptadine was given at seizure onset there was a significant decrease in the loss of neurons in the LD with the 10mg/kg and 20mg/kg doses (Figure 3D). When cyproheptadine (10, 13 and 16mg/kg) was given 5 minutes after seizure onset there was a significant reduction of neuron loss in the LD (Figure 3D). There was a significant reduction of neuron loss in the AM when cyproheptadine was administered one minute after soman cyproheptadine (13 and 16 mg/kg), at seizure onset (16 and 20mg/kg) and 5 minutes after seizure onset (10 and 13mg/kg) (Figure 3E).

Figure 3. *There was a reduction in neuron loss in the piriform cortex, dorsal cortex, lateral dorsal thalamus and amygdala with cyproheptadine treatment of soman exposed animals.* Hematoxyline and eosin (H&E) counts of neurons in the piriform cortex (Pir, 3A), dorsal cortex (DCx, 3B), lateral cortex (LCx, 3C), laterodorsal thalamus (LD, 3D) and amygdala (AM, 3E) regions of the brain show there was greater survival of neurons in all the examined regions except in the LCx of animals treated with cyproheptadine at various doses and time points tested compared to the no cyproheptadine group. Neuron counts were blindly scored. Results are the mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Cell death

The terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) technique was used to determine the regions of damage and quantify the number of dying cells in soman-exposed brains 24 hours after exposure. Representative micrographs for TUNEL staining of treated and untreated soman-exposed rats can be found in Figure 2C and 2D. Animals that were not exposed to soman had no TUNEL positive cells in the brain regions examined. There were TUNEL positive cells in the Pir, DCx, LCx, LD and AM regions of untreated, soman-intoxicated animals 24 hours after exposure (Table 3). In contrast, animals given cyproheptadine (10, 13, 16

or 20 mg/kg) at one minute after soman intoxication had no TUNEL positive cells in the brain regions examined (Table 3). When cyproheptadine was given at seizure onset, the number of TUNEL positive cells was greatly reduced in animals that had their seizures terminated (Table 3). Eight out of nine animals that were given 10 mg/kg at seizure onset had their seizures terminated. These animals had no TUNEL positive cells in any of the regions of the brain that were examined. The animal that did not stop seizing had no TUNEL positive cells in the DCx, LCx and AM, but did have TUNEL positive cells in the Pir and LD regions of the brain, similar in number to the untreated animals. There were no TUNEL positive cells in the brain regions examined from animals dosed with 13 or 20 mg/kg at seizure onset. The animals dosed with 16 mg/kg of cyproheptadine at seizure onset (N=9) all stopped seizing and had no TUNEL positive cells in the DCx and LCx, but did show a substantial reduction in the number of TUNEL positive cells in the Pir, AM and LD. The majority of animals in the 16 mg/kg group (N=7) had no TUNEL positive cells in the brain regions examined, but two of the nine animals in this group had TUNEL positive cells in the Pir and LD regions, and one of these animals had TUNEL positive cells in the AM.

The results in animals dosed with cyproheptadine five minutes after seizure onset were similar to those of the animals that were treated at seizure onset (Table 3). The 10 mg/kg group (N=7) all stopped seizing, had no TUNEL positive cells were identified in the DCx, and the number of apoptotic cells in the Pir, LCx, LD and AM regions were largely reduced when compared to the untreated animals. Five out of the seven animals in the 10 mg/kg 5 minutes after seizure onset group had no TUNEL positive cells in the brain regions examined, but two had TUNEL positive cells in the Pir and LD regions, and one of these two animals also had TUNEL positive cells in the LCx and AM regions. In the 13 and 16 mg/kg groups there were no TUNEL positive cells in

any of the regions examined for animals that had their seizures terminated. The animals that did not stop seizing in these groups had TUNEL positive cells in the same brain regions and similar in numbers as that of the untreated animals. The animals treated with 20 mg/kg at 5 minutes after seizure onset (N=4) all stopped seizing and had no TUNEL positive cells in the Pir, DCx, LCx and AM regions. There were greatly reduced numbers of TUNEL positive cells in the LD, with just one of those four animals having TUNEL positive cells in the LD region of the brain.

4. Discussion

Two of the major challenges in the medical treatment of nerve agent poisoning are the prevention of death and a reduction in nerve agent induced brain pathologies. In the case of soman exposure, these challenges are especially difficult to overcome because of soman's ability to rapidly age (1-3 minutes) and irreversibly bind AChE (Romano, 2007, Bateman, 2014). Once this aging process has occurred, reactivation of CWNA-inhibited AChE through oxime therapy is no longer effective. Current treatments for organophosphate poisoning are inadequate in their ability to fully prevent death and brain pathologies. Current therapies include oximes to reactivate CWNA-inhibited AChE, anticholinergics to block cholinergic effects and benzodiazepines to control the seizures (Marrs et al., 2006). Although current therapies increase survivability, they do not secure a full recovery for exposed victims (Svensson et al., 2005). The current study was undertaken to examine the possible therapeutic effectiveness of cyproheptadine in conjunction with the oxime HI-6 and the muscarinic receptor antagonist atropine in soman intoxicated animals.

Cyproheptadine is a first generation antihistamine with anticholinergic properties (Young et al., 2005). Cyproheptadine given in combination with HI-6 and atropine resulted in an overall reduction in the number of animals that seized, when given prior to seizure onset, greatly

reduced the duration of soman-induced seizures, when given after seizure onset, and increased survivability at all doses and treatment times tested.

Previous studies have shown that organophosphate poisoning results in apoptosis in the hippocampus, piriform cortex, amygdala, and cortices (Kim et al., 1999, Price, 2003). Price et al. (2003) determined that there was a time-related increase in caspase-3, a pro-apoptotic protein, 24-48 hours after soman exposure (Price, 2003). While we did see a reduction in the number of dying cells in the treated animals that did not seize or had seizures terminated, there was no apparent reduction in the number of TUNEL positive cells in the animals that did not have their seizures terminated. This led to the conclusion that cyproheptadine treatment in soman-intoxicated rats did not result in neuroprotection for the animals that did not stop seizing, since the number of apoptotic cells was similar to that in untreated animals. If the seizures were terminated there was overwhelmingly no damage in the brain, and any damage that did occur was greatly reduced and in a very small number of animals. In conclusion, seizure termination is likely the reason for protection against neuronal damage and unlikely the result of cyproheptadine having any direct neuroprotective properties in this model of soman-induced seizures.

Neuron loss, in multiple regions of the brain, is a common histopathological observance in temporal lobe epilepsy, a severe type of epilepsy (Curia et al., 2014). Similar results would be expected in this model of soman-induced seizures. Cell viability is difficult to fully distinguish using hematoxylin and eosin staining. Therefore, the difference between neuropathology scores and the neuron counts for the regions of the brain examined is likely due to the early time point at which these were investigated (24 hours); it may take longer to clear or remove dying or dead

cells in these regions of the brain. Later time-point studies of untreated and cyproheptadine-treated animals are necessary to test this hypothesis.

The greatest effect of cyproheptadine on soman-induced seizures is likely due to its anticholinergic properties. Anticholinergic properties are well-studied and therapeutically used properties of first generation antihistamines (Niemegeers et al., 1982, Gaudins et al., 1998, Kulkarni et al., 2006). A previous study compared anticholinergic properties of 10 different antihistamines and found that cyproheptadine had the most potent anticholinergic properties (Kubo et al., 1987, Orzechowski et al., 2005). Anticholinergics have been shown to effectively attenuate nerve agent-induced seizures and protect against neuronal damage resulting from nerve-agent intoxication (Green et al., 1977, Capacio and Shih, 1991, McDonough and Shih, 1993, Sparenborg et al., 1993). Compared to diazepam, when given shortly after seizure onset, anticholinergics stopped nerve agent-induced seizures more rapidly and reduced seizure reoccurrence (Capacio and Shih, 1991, Anderson et al., 1997).

In conclusion, the present study provides strong evidence that cyproheptadine can be used synergistically with HI-6 and atropine to protect against soman-induced seizures and neuronal damage. It is likely that cyproheptadine was effective due to its anticholinergic properties. Future studies examining the effect cyproheptadine against other organophosphate toxins, such as other nerve agents or pesticides as a monotherapy are warranted.

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	<u>Cypro dose</u>	<u># of animals</u>	<u>Sz</u>	<u>Sz Term</u>	<u>24hr survival</u>
Control	No Cypro	9	9/9	0/9	0/9
1 min after soman	10 mg/kg	9	6/9	5/6	6/9
	13 mg/kg	8	1/8***	1/1	8/8
	16 mg/kg	10	8/10	8/8	10/10
	20 mg/kg	10	3/10**	3/3	10/10
@sz onset	10 mg/kg	10	10/10	8/9 [†]	8/10
	13 mg/kg	6	6/6	6/6	5/6
	16 mg/kg	9	9/9	8/9	9/9
	20 mg/kg	5	5/5	5/5	5/5
5 min after sz onset	10 mg/kg	7	7/7	7/7	7/7
	13 mg/kg	4	4/4	3/4	4/4
	16 mg/kg	8	8/8	7/8	8/8
	20 mg/kg	4	4/4	4/4	4/4

Table 1. Seizure incidence was reduced and 24-hour survivability was increased in animals treated with cyproheptadine after soman intoxication. Sz=seizure, Sz term=seizures terminated, Cypro=cyproheptadine. [†]=seizure duration was not recorded for one animal. **p≤0.01. ***p≤0.001. Fisher's exact test was used to obtain statistical significance for seizure incidence.

	<u>Cypro</u> <u>dose</u>	<u>Sz</u> <u>outcome</u>	<u>Pir</u>	<u>DCx</u>	<u>LCx</u>	<u>LD</u>	<u>AM</u>
Control	No Cypro	Sz not terminated (n=9)	4.0±0	2.3±0.1	2.2±0.1	4.0±0	3.1±0.1
1 min after soman	10 mg/kg	Sz terminated (n=5)	0±0	0±0	0±0	0±0	0±0
	13 mg/kg	Sz terminated (n=6)	0±0	0±0	0±0	0±0	0±0
	16 mg/kg	Sz terminated (n=6)	0±0	0±0	0±0	0±0	0±0
	20 mg/kg	Sz terminated (n=4)	0±0	0±0	0±0	0±0	0±0
@ sz onset	10 mg/kg	Sz terminated (n=4) Sz not terminated (n=1)	0±0 4.0	0±0 0	0±0 0	0±0 4.0	0±0 1.0
	13 mg/kg	Sz terminated (n=7)	0±0	0±0	0±0	0±0	0±0
	16 mg/kg	Sz terminated (n=3) Sz not terminated (n=1)	0±0 2.0	0±0 0	0±0 0	0±0 4.0	0±0 1.0
	20 mg/kg	Sz terminated (n=5)	0±0	0±0	0±0	0±0	0±0
	10 mg/kg	Sz terminated (n=7)	0.4±0.3	0.1±0.1	0.1±0.1	0.7±0.6	0.3±0.2
5 min after sz onset	13 mg/kg	Sz terminated (N=3) Sz not terminated (N=1)	0±0 4.0	0±0 4.0	0±0 4.0	0±0 4.0	0±0 4.0
	16 mg/kg	Sz terminated (N=7) Sz not terminated (N=1)	0.6±0.5 4.0	0±0 4.0	0±0 4.0	0.6±0.5 4.0	0±0 4.0
	20 mg/kg	Sz terminated (N=4)	0.8±0.5	0.8±0.5	0.8±0.5	2.0±1.2	0±0

Table 2. Neuropathology scores 24 hours post soman exposure in untreated and cyproheptadine treated animals at 1 minute after soman intoxication, at seizure onset and 5 minutes after seizure onset. Animals that stopped seizing after being treated with cyproheptadine at 1 minute after soman and at seizure onset had no pathologies (0) in the brain regions examined: piriform cortex (Pir), dorsal cortex (DCx), lateral cortex (LCx), lateral dorsal thalamus (LD) and amygdala (AM). Animals that were treated with cyproheptadine 5 minutes after soman-induced seizure onset and that stopped seizing had greatly reduced neuropathology scores. Animals in which seizure was not terminated by treatment showed higher neuropathology scores. Values are averages $\pm$ SEM.

	<u>Cypro</u> <u>dose</u>	<u>Sz</u> <u>outcome</u>	<u>Pir</u>	<u>DCx</u>	<u>LCx</u>	<u>LD</u>	<u>AM</u>
Control	No Cypro	Sz not terminated N=7	340±26	108±43	66±17	197±26	143±27
1 min after soman	10 mg/kg	Sz terminated N=6	0±0	0±0	0±0	0±0	0±0
	13 mg/kg	Sz terminated N=8	0±0	0±0	0±0	0±0	0±0
	16 mg/kg	Sz terminated N=10	0±0	0±0	0±0	0±0	0±0
	20mg/kg	Sz terminated N=10	0±0	0±0	0±0	0±0	0±0
@ sz onset	10 mg/kg	Sz terminated (N=6)	0±0	0±0	0±0	0±0	0±0
		Sz not terminated (N=1)	88	0	0	202	0
	13 mg/kg	Sz terminated (N=4)	0±0	0±0	0±0	0±0	0±0
	16 mg/kg	Sz terminated (N=8)	9±9	0±0	0±0	42±40	16±15
		SZ not terminated (N=1)	129	0	0	182	0
	20 mg/kg	Sz terminated (N=5)	0±0	0±0	0±0	0±0	0±0
5 min after sz onset	10 mg/kg	Sz terminated (N=7)	21±14	0±0	17±17	23±17	19±19
	13 mg/kg	Sz terminated (N=3)	0±0	0±0	0±0	0±0	0±0
		Sz not terminated (N=1)	577	98	143	154	197
	16 mg/kg	Sz terminated (N=7)	0±0	0±0	0±0	0±0	0±0
		Sz not terminated (N=1)	299	122	74	230	267
	20 mg/kg	Sz terminated (N=4)	0±0	0±0	0±0	44±44	0±0

Table 3. *The number of TUNEL positive cells in specific brain regions decreased in animals treated with cyproheptadine after soman intoxication.* Numbers represent the average of the number of TUNEL positive cells per mm² in the following brain regions: piriform cortex (Pir), dorsal cortex (DCx), lateral cortex (LCx), lateral dorsal thalamus (LD) and amygdala (AM). Seizures were terminated in all animals. Counts are ±SEM. N=number of animals.