Two medical therapies very effective shortly after high levels of soman poisoning in rats, but only one with universal utility

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Dette er en postprint-versjon / This is a postprint version.
DOI til publisert versjon / DOI to published version: 10.1016/j.tox.2013.08.005

Abstract

A treatment regimen consisting of HI-6, scopolamine, and physostigmine (termed the physostigmine regimen) has been based on the serendipitous discovery that it exerts powerful antidotal effects against high levels of soman poisoning if it is administered 1 min after exposure. A medical therapy with corresponding efficacy, but without the time limitation of the latter regimen, has been developed through studies of microinfusions of anticonvulsants into seizure controlling sites in the forebrain of rats. From these studies procyclidine emerged as the most potent anticonvulsant, and its potency was further enhanced when being combined with the antiepileptic levetiracetam during systemic administration. In the present study, the capacity of HI-6, levetiracetam, and procyclidine (termed the procyclidine regimen) was tested against that of the physostigmine regimen. The results showed that both regimens were very effective against supralethal doses of soman (3, 4, 5 x LD_{50}) when given 1 and 5 min after intoxication. When the treatments were administered 10 and 14 or 20 and 24 min after soman exposure, only the procyclidine regimen was able to terminate seizures and preserve lives. When used as prophylactic therapies, both regimens protected equally well against seizures, but only the procyclidine regimen provided neuroprotection. The procyclidine regimen has apparently capacities to serve as a universal therapy against soman intoxication in rats.

Keywords: Soman intoxication; Seizures; HI-6; Scopolamine; Physostigmine; Levetiracetam; Procyclidine

1. Introduction

Intoxication by the organophosphorus nerve agent soman, an irreversible acetylcholinesterase (AChE) inhibitor, causes a progression of toxic signs including miosis, hypersalivation, respiratory distress, tremor, seizures/convulsions, coma, and death. In survivors, soman can evoke sustained seizure activity resulting in neuropathology in vulnerable brain areas like the piriform cortex, amygdala, and hippocampus. A large body of evidence relates brain damage to secondary massive release of glutamate, a neurotransmitter with excitotoxic potential (Carpentier et al., 2010).

Exposure to nerve agents requires immediate medical treatment. For this purpose, military personnel are issued with autoinjectors containing countermeasures for self-administration or "buddy aid". Antidotes against nerve agents are based on drugs acting at the muscarinic receptors and $GABA_A$ receptors (McDonough and Shih, 1997). In addition, partial protection against nerve agents can be obtained by the use of reversible (carbamate) AChE inhibitors shielding a portion of AChE from irreversible inhibition by nerve agents prior to nerve agent exposure. Furthermore, reactivation of any unaged AChE by an oxime is regarded as important immediate treatment after nerve agent exposure.

A number of armed forces have based their therapy against nerve agent intoxication on an oxime (obidoxime, 2-PAM, HI-6), an anticholinergic agent (atropine) combined with carbamate (pyridostigmine) pretreatment (Aas, 2003). Such treatment regimens can, however, reduce immediate lethality, but they do not attenuate the occurrence of nerve agent-induced seizure activity and concomitant convulsions, unless atropine is given early and at a high dose (McDonough and Shih, 1997). To overcome this shortcoming, several nations have provided their personnel with autoinjectors containing diazepam or avizafon (both benzodiazepine analogues with similar anticonvulsant action).

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The choice of oxime in autoinjector therapy differs among countries. Currently, there is no universal oxime that effectively can reactivate AChE inhibited by any known nerve agent. This oxime-nerve agent specificity makes the choice of a single oxime difficult; alternatively plural oximes have to be considered. Most studies indicate that obidoxime is more efficient than HI-6 (1-[([4-(aminocarbonyl)pyridinio]methoxy)methyl]-2-[(hydroxyimino)methyl]pyridinium) against tabun, whereas HI-6 is a better drug than obidoxime against soman. However, HI-6 is assessed to be a promising broad spectrum oxime against nerve intoxication (Aas, 2003; Kassa, 2002). Some countries use atropine along with obidoxime (e.g., Germany, Norway, The Netherlands, Finland). A future autoinjector regimen containing atropine, HI-6, and diazepam (presently used by Canada) is proposed by nations within NATO (Aas, 2003).

In a recent study (Myhrer et al., 2013), a comparative assessment was made of the antidotal capabilities of atropine/obidoxime/diazepam (termed the obidoxime regimen), atropine/HI-6/avizafone (termed the HI-6 regimen), and a recently recommended regimen consisting of scopolamine/HI-6/physostigmine (termed the physostigmine regimen). The latter therapy is supposed to be applied just 1 min after nerve agent exposure and has been suggested to represent next generation of countermeasures, because it will reduce reliance on pretreatment and requirement for 3 autoinjectors (Wetherell et al., 2007). The results from our study show that each regimen administered 2 times (1 and 5 min after exposure to soman doses of 2, 3, or 4 x LD_{50}) can effectively prevent or terminate epileptiform activity within 10 min (Myhrer et al., 2013). However, the regimens differ markedly in life saving properties with the physostigmine regimen ranking highest followed by the HI-6 and obidoxime regimens. Pretreatment with pyridostigmine increases the potency of the HI-6 regimen, but not the obidoxime regimen. The physostigmine regimen has outstanding antidotal capacity, but the very narrow time window (< 10 min) makes it unsuitable for use in the field (Myhrer

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et al., 2013). Conceptually, delayed administration of physostigmine will aggravate actions of soman by inhibiting residual active AChE. It has, however, been shown that physostigmine administered along with obidoxime 10 min after soman has some anticonvulsant effect in guinea pigs (Joosen et al., 2011), but determination of time limits of physostigmine in rats has not been carried out. The ultimate aim would be to develop a regimen effective shortly after high levels of soman poisoning that also can be effective at late points of time after exposure.

It has previously been suggested to work out specifications for what subreceptors in what sites of the forebrain that preferentially should be affected by countermeasures to obtain optimal efficacy (Myhrer, 2007). Without such specifications pharmacological research on anticonvulsants against nerve agents can take the form of a seemingly endless number of trial and error. In epilepsy research, more than 30000 compounds have been screened for antiepileptic properties (Crepeau and Treiman, 2010). In a series of studies, we have mapped critical pharmacological receptors in specified brain areas. Through this process, procyclidine turned out to have the highest impact of the drugs tested in seizure controlling sites in the forebrain of rats (Table 4 in Myhrer, 2010). Enhancement of procyclidine`s excellent antidotal properties (anticholinergic and antiglutamatergic) would make up a novel and interesting approach. Levetiracetam with a unique profile in preclinical models of epilepsy has been shown to increase the potency of other antiepileptic drugs up to 19-fold (Kaminski et al., 2009). In a recent study, we demonstrated that the combination of levetiracetam and procyclidine can effectively terminate soman-induced seizures 20 or 40 min after onset in rats pretreated with pyridostigmine or HI-6, respectively. This therapy could also save the lives of rats that were about to die 5-10 min after seizure onset (Myhrer el al., 2011).

The purpose of the present study was to determine the antidotal capacities of the physostigmine regimen (HI-6/ scopolamine/physostigmine) and a procyclidine regimen (HI-6/levetiracetam/procyclidine) against moderate or supralethal levels of soman poisoning (1.3,

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1.6, 3, 4, or 5 x LD_{50}) when given twice with 4 min interval at different points of time (1, 10, or 20 min) after exposure. Additionally, the importance of including HI-6 in the combination of levetiracetam and procyclidine was investigated. The prophylactic potency of the regimens was also examined.

2. Materials and methods

2.1. Animals

Male Wistar rats from a commercial supplier (Taconic Breeding Laboratories, Denmark) weighing 300-330 g served as subjects. The experiments were approved by the National Animal Research Authority. The animals were housed individually and had free access to commercial rat pellets and water. The rats were handled individually 3 days preoperatively and 3 days postoperatively, being allowed to explore a table top (80 x 60 cm) for 3 min a day. The climatized vivarium (21ºC) was illuminated from 0700 to 1900 h.

2.2. Surgery

The rats were anesthetized ip with diazepam (4.5 mg/kg) and fentanyl fluanisone (2 mg/kg). Of 2 stainless screws, one was lowered 1 mm into the parietal cortex (1 mm behind bregma, 3 mm lateral to midline), and the contralateral one served as ground. The screws were fixed with dental cement (Durelon; ESPE, Seefeldt, Germany). The rats were given a recovery period of 7 days.

2.3. Drugs

The drug doses chosen were derived from previous studies of anticonvulsant effects against soman-evoked seizures in rats; HI-6 dimethanesulphonate 125 mg/kg, scopolamine hydrobromide 1 mg/kg, physostigmine salicylate 0.1 mg/kg, procyclidine hydrochloride 20

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mg/kg, levetiracetam 50 mg/kg, pyridostigmine bromide 0.1 mg/kg (McDonough and Shih, 1993; Myhrer et al., 2011, 2013; Shih et al., 1999). The drugs were dissolved in 0.9% saline and were administered intramuscularly. The injection site alternated between the left and right muscle in the hind leg. The soman doses were 1.3 x LD_{50} (100 µg/kg), 1.6 x LD_{50} (128 µg/kg), 3 x LD_{50} (240 µg/kg), 4 x LD_{50} (320 µg/kg), or 5 x LD_{50} (400 µg/kg) resulting in convulsions and death in all rats of our strain (Sterri et al., 1980). Soman was given subcutaneously. All drugs were purchased from Sigma (St Louis, MO, USA), except HI-6 dimethanesulphonate that was a gift from Defence Research and Development (Suffield, Medicine Hat, Canada). Soman was purchased from TNO (Netherlands Organisation for Applied Scientific Research), The Netherlands.

2.4. Experimental design

For overview, see Table1. When the physostigmine regimen and the procyclidine regimen were used as prophylactic treatment, they were administered 20 min before a soman dose of 1.3 x LD_{50}. When used as postexposure treatment, the regimens were given 1 and 5 min after a soman dose of 3, 4, or 5 x $LD_{50,}$ respectively, 10 and 14 min after a soman dose 1.6 x LD_{50}, or 20 and 24 min after a soman dose of 1.3 x LD_{50} in rats pretreated with pyridostigmine.

2.5. Histology

The rats were anesthetized as described for surgery, perfused intracardially with 10% formalin, and the brains were post-fixed in 10% formalin for at least 24 h. The brains were dehydrated and embedded in paraffin (Schmued et al., 1997). The sections were cut 5 µm thick and dried in an incubator (37ºC) for 12 h before they were stained with hematoxylin and eosin (HE) or Fluoro-Jade B (Schmued and Hopkins, 2000). Since Fluoro-Jade staining

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requires perfusion of the brain, only live rats could be used for this purpose. Rats that recently died or rats about to die were decapitated, and the brain sections were stained with HE. Because Fluoro-Jade has been considered to be the compound most suitable for the detection of neuronal degeneration (Schmued et al., 1997), this fluorescent staining technique was used to supplement the more conventional HE staining technique. A degenerating neuron presumably expresses a strong basic molecule, since it has an affinity for the strongly acidic Fluoro-Jade (Schmued et al., 1997). The Fluoro-Jade method has previously been described in detail (Schmued et al., 1997; Schmued and Hopkins, 2000). In order to make a distinct contrast between degenerated neurons and intact ones the sections were co-stained with 4´, 6-diamidino-2-phenylindole dihydrochloride (DAPI) resulting in a blue fluorescence of cellular nuclei. Such nuclear staining is seen in all viable cells. A 0.01% stock solution of DAPI (10 mg/100 ml distilled water) was prepared and 2 ml of this stock solution was added to 98 ml of the Fluoro-Jade B staining solution. Blue counterstained normal cell nuclei can be visualized when excited by ultraviolet (330-380 nm) light (Schmued and Hopkins, 2000). Fluoro-Jade B staining is seen with blue excitation filter, whereas DAPI is visualized by filter set 49 from Zeiss with excitation at 365 nm and emission at 445/50 nm. One picture was superimposed on the other in order to see both simultaneously. A digital microscope camera (AxioCam, Zeiss, Jena, Germany) was used to make photomicrographs. This technique allows processing of the photographs so that elements of particular interest can be made clearer by adjusting contrasts.

2.6. Evaluation of neuropathology

A grading system of 0-4 previously described (McDonough et al., 1995), was used to determine severity of neuronal damage in the piriform cortex, the hippocampal CA1 region, and the basolateral amygdala based on the approximate percentage of tissue involvement: 0 – no lesion; 1 – minimal, 1-10%; 2 – mild, 11-25%; 3 – moderate, 26-45%; 4 – severe > 45%.

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Each animal was given bilateral neuropathology scores for the 3 individual brain areas chosen. The criterion used to characterize the pathology was neuronal degeneration.

2.7. EEG

The electrodes were connected with the polygraph (Grass Model 79E) with alligator clips and leads. The use of a swivel allowed the rats to move freely. Seizure activity was defined as terminated when epileptiform waves had ceased (absence of continuous high amplitude rhythmic spike or sharp wave activity). EEG recording was made while the animals were situated in their home cages (50 x 30 x 15 cm). Measures were made 24 h prior to drug treatment, immediately after, and 24, 48, or 144 h after treatment.

2.8. Observation of animals

The rats were observed for convulsions and visible signs of intoxication continuously for the first 2-3 h and then for 10 min at 24 and 48 h after soman injection. Rats that displayed aphagia/adipsia following termination of convulsions were given 5 ml of saline (ip) twice a day for 1-3 days until they started to eat.

2.9. Statistics

Group comparisons were made with two-sided Fisher's exact test, two-tailed *t* test, or two-tailed Mann-Whitney *U* test. Use of the grading system of neuropathology resulted in nonparametric data. Computations were made with the Prism statistical software program (GraphPad Software, Sand Diego, CA, USA).

3. Results

3.1. Seizures and drugs

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When treatment was given 1 and 5 min after a soman dose of 3 x LD_{50}, some rats in each group responded with clonic or tonic convulsions after 1-5 min (Table 2). Both the physostigmine and procyclidine regimens prevented or stopped seizures, and none of the rats displayed epileptiform activity 10 min after soman exposure. This pattern was also seen for higher doses of soman (4 or 5 x LD_{50}). When HI-6 was not combined with levetiracetam and procyclidine, all rats died within 14 min. The difference in lethality rate between the latter group and the groups treated with adequate regimens was significant with Fisher`s exact test (P= 0.0022). When the soman doses were 4 or 5 x LD_{50}, the physostigmine and procyclidine regimens both exerted efficacious antidotal actions.

The mean latency to seizure onset was not significantly different among the groups (P>0.05 with t test) when the treatments were given 10 and 14 min after soman exposure (1.6 x LD_{50}) (Table 3). However, the antiseizure response ratio was significantly higher for the procyclidine group compared with the physostigmine group (P=0.0022). Also the lethality response was reliably lower for the procyclidine group (P=0097). Only 2 rats in the physostigmine group survived 24 h. In the latter rats, the seizure activity ceased 1.5 h after onset. In 2 rats of each treatment category, the seizures were induced between 10 and 13 min after soman poisoning. In these instances, the treatment started 1 min after seizure onset. In the physostigmine group, seizures or ictal epileptiform activity was observed during the postexposure period, whereas the EEG was normal in the procyclidine group (Fig. 1).

The mean latency to seizure onset was not significantly different (P>0.05) when the treatments were administered 20 and 24 min after soman intoxication (1.3 x LD_{50}) in rats pretreated with pyridostigmine 20 min prior to soman (Table 4). However, both the antiseizure and survival rates were reliably higher for the procyclidine regimen relative to the physostigmine regimen (P=0.0022).

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Prophylactic treatment with the physostigmine or procyclidine regimens produced complete antiseizure response to a soman dose of 1.3 x LD_{50} given 20 min after therapy (Table 5). When the HI-6 was deleted from the procyclidine regimen, the antiseizure response was incomplete and 2 rats did not survive 24 h, but the differences were not statistically reliable (P>0.05).

3.2. Recovery and body weight

The recovery course after high levels of soman poisoning (3, 4, 5 x LD_{50}) were reflected in the gain of body weight, and did not differ significantly (P>0.05) between the groups (Table 6). The rats in both treatment categories displayed a weight loss of about 10% during the 2 first days after exposure, but otherwise seemed almost unaffected by the supralethal intoxication. They were incapacitated for a brief period of time (about 10 min), but started to walk soon after. Following 3 h, they were able to rear and started to eat and drink. At 24 h, they were all in a good condition and displayed no overt signs of intoxication.

When treatment was given 10 and 14 min after challenge with soman (1.6 x LD_{50}), the rats in the procyclidine group recovered well. During the first and second day after exposure they displayed a weight loss of 6.2% and 3.9%, respectively. Two rats from the physostigmine group that survived had a similar loss of body weight (6.3% and 3.2%, respectively).

When the treatment was administered 20 an 24 min after soman exposure (1.3 x LD_{50}) in rats pretreated with pyridostigmine, the rats in the procyclidine group recovered well. The loss of body weight for the first and second day after soman intoxication was 7.8% and 4.9%, respectively. All rats in the physostigmine group died within 24 h.

Both the physostigmine and procyclidine regimens protected the rats effectively against soman intoxication (1.3 x LD_{50}). In the physostigmine group, no rats were

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incapacitated, whereas 2 rats in the procyclidine group were incapacitated for a brief period of time (2-3 min) 6-8 min after soman challenge. All rats moved normally about 10 min following poisoning. When HI-6 was deleted from the procyclidine regimen, the rats showed great problems in moving even 24 h after soman exposure. For this reason, the surviving rats were euthanized.

3.3. Histology

Even if pharmacological treatments given 1 and 5 min after soman poisoning prevented or terminated epileptiform activity within 10 min in all rats, some neuropathology was discovered in the index areas piriform cortex and amygdala, but not in the hippocampal CA1 field (Table 7). Fluorescent staining was totally seen in 58% of the animals in the piriform cortex and/or amygdala, but predominantly in the left hemisphere (Fig. 2). Only 14% of the rats had damage in the right hemisphere. The neuronal injury was unrelated to whether the rats convulsed or not. No statistically reliable treatment effects between the groups were found ($P>0.05$). However, differences in neuropathology were seen within the physostigmine groups. When the soman dose was 4 or 5 x LD_{50}, two-tailed *U* test showed that the pathology scores were significantly higher in the left piriform cortex than in the right side ($P<0.05$).

When the rats were treated 10 and 14 min after a soman dose of 1.6 x LD_{50}, they convulsed for about 10 min. In these instances, the neuropathology was bilateral, and the scores were rather moderate for the procyclidine group (piriform cortex 1; CA1 0; amygdala 0.5) and almost similar for the 2 survivors from the physostigmine group. When the pretreated rats were treated 20 and 24 min after a soman dose of 1.3 x LD_{50}, they convulsed for about 25 min. The neuropathology scores were: Piriform cortex 1.5; CA1 0; amygdala 0.5.

When given treatment 20 min before a soman dose of 1.3 x LD_{50}, the physostigmine regimen did not prevent neuropathology in the index areas piriform cortex and amygdala,

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whereas the procyclidine regimen provided neuroprotection (Table 8). Two-tailed *U* test showed that the pathology scores were significantly lower in the procyclidine group compared with the physostigmine group in the piriform cortex (*P*=0.0022) and the amygdala (*P*=0.0260). Four rats pretreated with levetiracetam and procyclidine (without HI-6) that were euthanized after 24 h displayed no neuronal damage.

4. Discussion

The present results showed that the physostigmine regimen (HI-6/scopolamine/ physostigmine) and the procyclidine regimen (HI-6/levetiracetam/procyclidine) yielded equal antidotal efficacy against high levels of soman poisoning (3, 4, 5 x LD_{50}) when administered 1 and 5 min after exposure. When the treatments were carried out 10 and 14 min or 20 and 24 min after soman intoxication, the procyclidine regimen terminated seizures and preserved lives, whereas the physostigmine regimen did not. When used as prophylactic therapies, both regimens protected equally well against overt toxic signs of soman poisoning, but only the procyclidine regimen provided neuroprotection. The very narrow time window (<10 min) of the physostigmine regimen makes it unsuitable for use in the field, whereas the procyclidine regimen has no time limits and may serve as a universal medical therapy against soman poisoning, as shown in rats.

During high levels of soman intoxication, some animals suffered from convulsions. When soman doses of 3, 4, and 5 x LD_{50} are applied, the mean latencies to seizure onset in rats are 1-3 min (Myhrer et al., 2006). Hence, prevention of seizures cannot be obtained in all rats during the comparatively short interval between soman exposure and optimal pharmacological action of the drugs for the initial injections (1 min after soman).

HI-6 has evidently a life preserving action, because treatment with levetiracetam and procyclidine alone following 3 x LD_{50} of soman resulted in death among all rats within 14

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min after exposure (Table 2). HI-6 has been reported to protect respiratory function, both centrally and peripherally during nerve agent poisoning. The oxime causes recovery of neuronal transmission in the respiratory center possibly by affecting GABAergic mechanisms and causes recovery of neuromuscular transmission in the diaphragm (van Helden et al., 1996). In addition, HI-6 appears to alleviate motor dysfunctions induced by soman. When HI-6 was not combined with levetiracetam and procyclidine in prophylactic treatment against soman poisoning, incapacitation was still present in the surviving rats 24 h after exposure. A statistically significant reactivation of inhibited AChE activity in the rat brain has been shown when HI-6 is applied up to 20 min after soman (Sket and Brzin, 1986). The half-time of aging of soman-inhibited erythrocyte AChE is 8.6 min in the rat (Talbot et al., 1988). About 18% of HI-6 enters the brain of control rats not exposed to soman (Cassel et al., 1997).

Maximal loss of body weight (14-15%) 2-4 days after soman-elicited seizures has been observed in rats that had convulsions terminated pharmacologically after 45 min (McDonough and Shih, 1993; Myhrer et al., 2005). Even if the present rats treated 1 and 5 min after soman exposure did not convulse or convulsed only for a brief period of time (5-8 min), they initially lost about 10% of their body weight during the first 2 days, but recovered well (Table 6). Rats treated with HI-6, atropine, and avizafone 1 and 5 min following a soman dose of 3 x LD_{50} still have significantly lower body weight 7 days after exposure than rats treated with the present physostigmine regimen at corresponding times (Myhrer et al., 2013). The aphagia/adipsia caused by soman intoxication may be related to dysfunction in cholinergic input to the hypothalamus (Myhrer, 2007). This dysfunction can apparently be initiated by high levels of soman poisoning or epileptiform activity per se, because microinfusion of soman into the perirhinal or posterior piriform cortices of rats can trigger seizure activity resulting in aphagia/adipsia (Myhrer et al., 2010).

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The physostigmine and procyclidine regimens differ in their neuroprotective capabilities. Rats treated with the physostigmine regimen following 4 or 5 x LD_{50} of soman displayed more extensive neurodegeneration in the left perirhinal cortex than in the right hemisphere (Table 7). A corresponding asymmetry was not seen for the procyclidine groups. This phenomenon has previously been encountered and has tentatively been related to local glutamatergic excitotoxic activity (Myhrer et al., 2013). Neuropathology was also detected in rats treated with the physostigmine regimen before exposure to soman. This finding was rather surprising, particularly because physostigmine along with scopolamine (hyoscine) has been advanced as a future prophylactic treatment (Scott, 2007; Wetherell, 1994). The occurrence of brain damage after prophylactic treatment with the physostigmine regimen is intriguing, because no overt signs of intoxication were observed, and the EEG activity was normal 1, 2, and 6 days after soman exposure. A potential cause might be that the AChE inhibition by physostigmine adds up to remnant action of soman rather than the inclusion of HI-6 in the present physostigmine regimen. It is also possible that lack of glutamatergic antagonism of the physostigmine regimen can result in neuronal damage, because the combination of physostigmine and procyclidine protects effectively against soman intoxication and no neuropathology is seen (Myhrer et al., 2004). Hence, the procyclidine regimen stands out as a more efficacious antidotal therapy than the physostigmine regimen.

The physostigmine and procyclidine regimens affect very different mechanisms of action. The only common component is HI-6 that has been discussed above. Scopolamine is a cholinergic antagonist binding to muscarinic receptors 1-5, and the potency of scopolamine is reported to be 170 times higher than for atropine against soman intoxication in rats (Capacio and Shih, 1991). Physostigmine is a reversible inhibitor of AChE that has a relatively short half-life in plasma of rats (17 min) (Somani and Khalique, 1986). The half-life in plasma of rats for HI-6 is 24 min (Garrigue et al., 1990) and for scopolamine 17 min (Lyeth et al.,

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1992). Levetiracetam is an antiepileptic drug that strongly enhances the anticonvulsant effects of compounds affecting either glutamatergic or GABAergic neurotransmission (Kaminski et al., 2009). The distinctive binding site of levetiracetam appears to be the synaptic vesicle protein 2A (SV2A) (Lynch et al., 2004). Although the exact mechanisms are not well known, levetiracetam probably reduces release of glutamate by which the effects of glutamatergic antagonists are highly increased (Kaminski et al., 2009). Levetiracetam has affinity to SV2A in GABA terminals and reduces release of transmitter. This apparently paradoxical finding for the antiepileptic effect of levetiracetam has partly been explained by subsequent more robust spontaneous inhibitory activity (Meehan et al., 2012). It has been reported that levetiracetam may both reduce release of acetylcholine and reduce postsynaptic responsiveness in cholinergic synapses (Oliveira et al., 2005). In vitro results show that levetiracetam binds to SV2A and contributes to reduced release of acetylcholine (Vogl et al., 2012). Procyclidine has a powerful capability to antagonize a lethal dose of NMDA in mice (Raveh et al., 1999). Procyclidine inhibits the phencyclidine site at the NMDA receptor very potently (Reynolds and Miller, 1988) in a concentration-dependent manner (Myhrer et al. 2004). In addition, procyclidine binds to the muscarinic receptors 1 and 2 (Waelbroeck et al., 1992). The half-life in plasma of rats for procyclidine is 120 min (Jang et al., 2001) and for levetiracetam 150 min (Löscher et al., 1998).

From the above overview of mechanisms of action, the procyclidine regimen seems to have qualifications above those of the physostigmine regimen in serving as a universal antidotal treatment for soman poisoning in rats. In a previous study, it was shown that levetiracetam combined with procyclidine terminates soman-evoked seizures when administered as late as 40 min after onset in rats pretreated with HI-6 (Myhrer et al., 2011). Theoretically, the physostigmine regimen should have advantages as a prophylactic therapy, but it appears to have shortcomings in neuroprotection, as shown in the present study. The

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brief half-life of the components in the physostigmine regimen will probably require continuous administration, whereas that is not the case for the procyclidine regimen.

In conclusion, the physostigmine and procyclidine regimens are very effective shortly after high levels of soman poisoning. They are also effective in preventing seizures and incapacitation when given as prophylactics. However, only the procyclidine regimen is capable of terminating seizure activity when administered at late times after soman exposure. Likewise, only the procyclidine regimen provides adequate neuroprotection when used as prophylactic as well as post-poisoning treatment. It will be of interest to examine whether the potency of the procyclidine regimen, as demonstrated in rats, can apply to other animal species and other classical nerve agents than soman.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Statement of funding

This work was not supported by any external funding.

References

Aas, P., 2003. Future considerations for the medical management of nerve agent intoxication. Prehosp. Disast. Med. 18, 208-216.

Carpentier, P., Testylier, G., Baille, V., Foquin, A., Delacour, C., Dorandeu, F., 2010. Cerebral edema in soman poisoning. In Weissman, B.A., Raveh, L. (Eds) The Neurochemical Consequences of Organophosphate Poisoning in the CNC. Pp 1-17 Transworld Research Network, Kerala.

Cassel, G., Karlsson, L., Waara, L., Ang, K.W., Göransson-Nyberg, A., 1997. Pharmacokinetics and effects of HI 6 in blood and brain of soman-intoxicated rats: A microdialysis study. Eur. J. Pharmacol. 332, 43-52.

Capacio, B.R., Shih, T.-M., 1991. Anticonvulsant actions of anticholinergic drugs in soman poisoning. Epilepsia 32, 604-615.

Crepeau, A.Z., Treiman, D.M., 2010. Levetiracetam: a comprehensive review. Expert. Rev. Neurotherap. 10, 159-171.

Garrigue, H., Maurizis, J.C., Nicolas, C., Madelmont, J.C., Godeneche, D., Hulot, T., Morge, X., Demerseman, P., Sentenac-Roumanou, H., Veyre, A., 1990. Disposition and metabolism of two acetylcholinesterase reactivators, pyrimidoxime and HI-6, in rats submitted to organophosphate poisoning. Xenobiotica 20, 699-709.

Jang, E.-J., Lee, Y.-J., Chung, S.-J., Shim, C.-K., 2001. Nasal absorption of procyclidine in rats and dogs. Arch. Pharm. Res. 24, 219-223.

Joosen, M.J.A., Smit, A.B., van Helden, H.P.M., 2011. Treatment efficacy in soman-poisoned guinea pig model: added value of physostigmine? Arch. Toxicol. 85, 227-237.

Kaminski, R.M., Matagne, A., Patsalos, P.N., Klitgaard, H., 2009. Benefit of combination therapy in epilepsy: A review of preclinical evidence with levetiracetam. Epilepsia 50, 387-397.

Dette er en postprint-versjon / This is a postprint version.
DOI til publisert versjon / DOI to published version: 10.1016/j.tox.2013.08.005

Kassa, J., 2002. Review of oximes in the antidotal treatment of poisoning by organophosphorus nerve agents. J. Toxicol. Clin. Toxicol. 40, 803-816.

Lyeth, B.G., Ray, M., Hamm, R.J., Schnabel, J., Saady, J.J., Poklis, A., Jenkins, L.W., Gudeman, S.K., Hayes, R.L., 1992. Postinjury scopolamine administration in experimental traumatic brain injury. Brain Res. 569, 281-286.

Lynch, B.A., Lambeng, N., Nocka, K., Kensel-Hammes, P., Bajjalieh, S.M., Matagne, A., Fuks, B., 2004. The synaptic vesicle protein SV2A is the biding site for the antiepileptic drug levetiracetam. Proc. Natl. Acad. Sci. U.S.A. 101, 9861-9866.

Löscher, W., Hönak, D., Rundfeldt, C., 1998. Antiepileptogenic effects of the novel anticonvulsant levetiracetam (ucb L059) in the kindling model of temporal lobe epilepsy. J. Pharamacol. Exp. Therap. 284, 474-479.

McDonough Jr., J.H., Dochterman, W., Smith, C.D., Shih, T.-M., 1995. Protection against nerve agent-induced neuropathology, but not cardiac pathology, is associated with the anticonvulsant action of drug treatment. Neurotoxicology 15, 123-132.

McDonough Jr., J.H., Shih, T.-M., 1993. Pharmacological modulation of soman-induced seizures. Neurosci. Biobehav. Rev. 17, 203-215.

McDonough Jr., J.H., Shih, T.-M., 1997. Neuropharmacological mechanisms of nerve agent-induced seizure and neuropathology. Neurosci. Biobehav. Rev. 21, 559-579.

Meehan, A.L., Yang, X., Yuan, L.-L., Rothman, S.M., 2012. Levetiracetam has an activity-dependent effect on inhibitory transmission. Epilepsia 53, 469-476.

Myhrer, T., 2007. Neuronal structures involved in the induction and propagation of seizures caused by nerve agents: Implications for medical treatment. Toxicology 239, 1-14.

Myhrer, T., 2010. Identification of neuronal target areas for nerve agents and specification of receptors for pharmacological treatment. Neurotoxicology 31, 629-638.

Dette er en postprint-versjon / This is a postprint version.
DOI til publisert versjon / DOI to published version: 10.1016/j.tox.2013.08.005

Myhrer, T., Andersen, J.M., Nguyen, N.H.T., Aas, P., 2000. Soman-induced convulsions in rats terminated with pharmacological agents after 45 min: Neuropathology and cognitive performance. Neurotoxicology 26, 39-48.

Myhrer, T., Enger, S., Aas, P., 2006. Efficacy of immediate and subsequent therapies against soman-induced seizures and lethality in rats. Basic. Clin. Pharmacol, Toxicol. 98, 184-191.

Myhrer, T., Enger, S., Aas, P., 2010. Roles of perirhinal and posterior piriform cortices in control and generation of seizures: A microinfusion study in rats exposed to soman. Neurotoxicology 31, 147-153.

Myhrer, T., Enger, S., Jonassen, M., Aas, P., 2011. Enhanced efficacy of anticonvulsants when combined with levetiracetam in soman-exposed rats. Neurotoxicology 32, 923-930.

Myhrer, T., Enger, S., Aas, P., 2013. Determination of anti-convulsant and life-preserving capacities of three types of auto-injector therapies against soman intoxication. Drug Test. Analysis (in press).

Myhrer, T., Nguyen, N.H.T., Andersen, J.M., Aas, P., 2004. Protection against soman-induced seizures: relationship among doses of prophylactics, soman, and adjuncts. Toxicol. Appl. Pharmacol. 196, 327-336.

Oliveira, A.A., Nogueira, C.R.A., Nascimento, V.S., Aguiar, L.M.V., Freitas, R.M., Sousa, F.C.F., Viana, G.S.B., Fonteles, M.M.F., 2005. Evaluation of levetiracetam effects on pilocarpine-induced seizures: Cholinergic muscarinic system involvement. Neurosci. Lett. 385, 184-188.

Raveh, L., Chapman, S., Cohen, G., Alkalay, D., Gilat, E., Rabinovitz, I., Weissman, B.A., 1999. The involvement of NMDA receptor complex in the protective effect of anticholinergic drugs against soman poisoning. Neurotoxicology 20, 551-560.

Dette er en postprint-versjon / This is a postprint version.
DOI til publisert versjon / DOI to published version: 10.1016/j.tox.2013.08.005

Reynolds, I.J., Miller, R.J., 1988. [^{3}H]MK-801 binding to the N-methyl-D-aspartate receptor reveals drug interaction with the zinc and magnesium binding sites. J. Pharmacol. Exp. Therap. 24, 1025-1031.

Schmued, L.C., Albertson, C., Slikker Jr., W., 1997. Fluoro-Jade: A novel fluorochrome for the sensitive and reliable histochemical localization of neuronal degeneration. Brain Res. 751, 37-46.

Schmued, L.C., Hopkins, K.J., 2000. Fluoro-Jade B: A high affinity fluorescent marker for the localization of neuronal degeneration. Brain Res. 874, 123-130.

Scott, L., 2007. Pretreatment for nerve agent poisoning. In Marrs, T.C., Maynard, R.L., Sidell, F.R. (Eds) Chemical Warfare Agents: Toxicology and Treatment (2nd Edition). John Wiley & Sons, Ltd, Chichester.

Shih, T.-M., McDonough Jr., J.H., Koplovitz, I., 1999. Anticonvulsants for soman-induced seizure activity. J. Biomed. Sci. 6, 86-96.

Sket, D., Brzin, M., 1986. Effects of HI-6, applied into the cerebral ventricles, on the inhibition of brain acetylcholinesterase by soman in rats. Neuropharmacology 25, 103-107.

Somani, S.M., Khalique, A., 1986. Distribution and pharmacokinetics of physostigmine in rat after intramuscular administration. Fundam. Appl. Toxicol. 6, 327-334.

Sterri, S.H., Lyngaas, S., Fonnum, F., 1980. Toxicity of soman after repetitive injection of sublethal doses in rat. Acta Pharmacol. Toxicol. 46, 1-7.

Talbot, B.G., Anderson, D.R., Harris, L.W., Yarbrough, L.W., Lennox, W.J., 1988. A comparison of in vivo and in vitro rates of aging of soman-inhibited erythrocyte acetylcholinesterase in different animal species. Drug Chem. Toxicol. 11, 289-305.

Van Helden, H.P.M., Busker, R.W., Melchers, B.P.C., Bruijnzeel, P.L.B., 1996. Pharmacological effects of oximes: how relevant are they. Arch. Toxicol. 70, 779-786.

Dette er en postprint-versjon / This is a postprint version.
DOI til publisert versjon / DOI to published version: 10.1016/j.tox.2013.08.005

Vogl, C., Mochida, S., Wolff, C., Whalley, B.J., Stephens, G.J., 2012. The synaptic vesicle glycoprotein 2A ligand levetiracetam inhibits presynaptic Ca^{2+} channels through an intracellular pathway. Molecul. Pharmacol. 82, 199-208.

Waelbroeck, M., Camus, J., Tastenoy, M., Mutschler, E., Strohmann, C., Tacke, R., Schjelderup, L., Aasen, A., Lambrecht, G., Christophe, J., 1992. Stereoselective interaction of procyclidine, hexahydro-difenidol, hexbutinol and oxyphencyclimine, and of related antagonists, with four muscarinic receptors. Eur. J. Pharmacol. 227, 33-42.

Wetherell, J.R., 1994. Continuous administration of low dose rates of physostigmine and hyoscine to guinea-pigs prevents the toxicity and reduces the incapacitation produced by soman poisoning. J. Pharm. Pharmacol. 46,1023-1028.

Wetherell, J., Price, M., Mumford, H., Armstrong, S., Scott, L., 2007. Development of next generation medical countermeasures to nerve agent poisoning. Toxicology 233, 120-127.

Dette er en postprint-versjon / This is a postprint version.
DOI til publisert versjon / DOI to published version: 10.1016/j.tox.2013.08.005

Figure legends

Fig. 1. EEG recordings from the parietal cortex in 2 rats treated with either HI-6, scopolamine, and physostigmine (A) or HI-6, levetiracetam, and procyclidine (B) 10 and 14 min after exposure to a soman dose of 1.6 x LD_{50}. The recordings show epileptiform activity (A) and normal EEG (B) 20 min after poisoning.

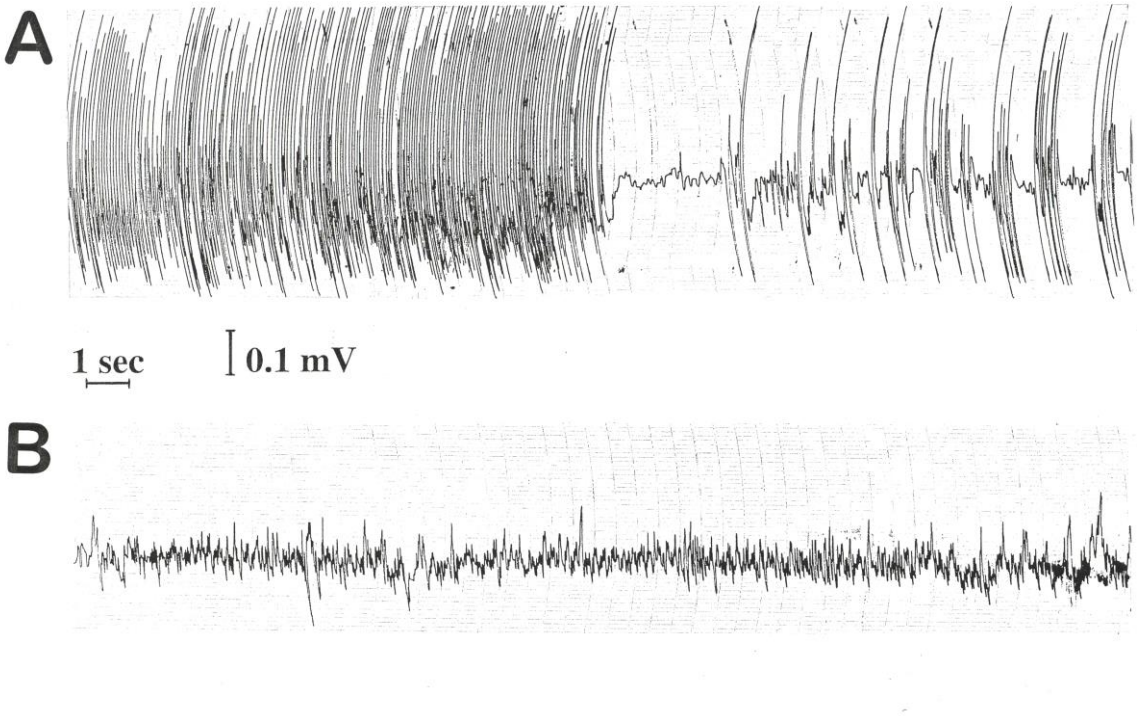

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Fig. 2. Sections stained with Fluoro-Jade B 7 days following soman exposure (5 x LD_{50}). All photos are from the same rat that was treated with HI-6, scopolamine, and physostigmine 1 and 5 min after poisoning. The neuronal damage is seen in the left hemisphere only in the piriform cortex and amygdala. The sections are viewed from the caudal end of the brain. The magnification was x 200.

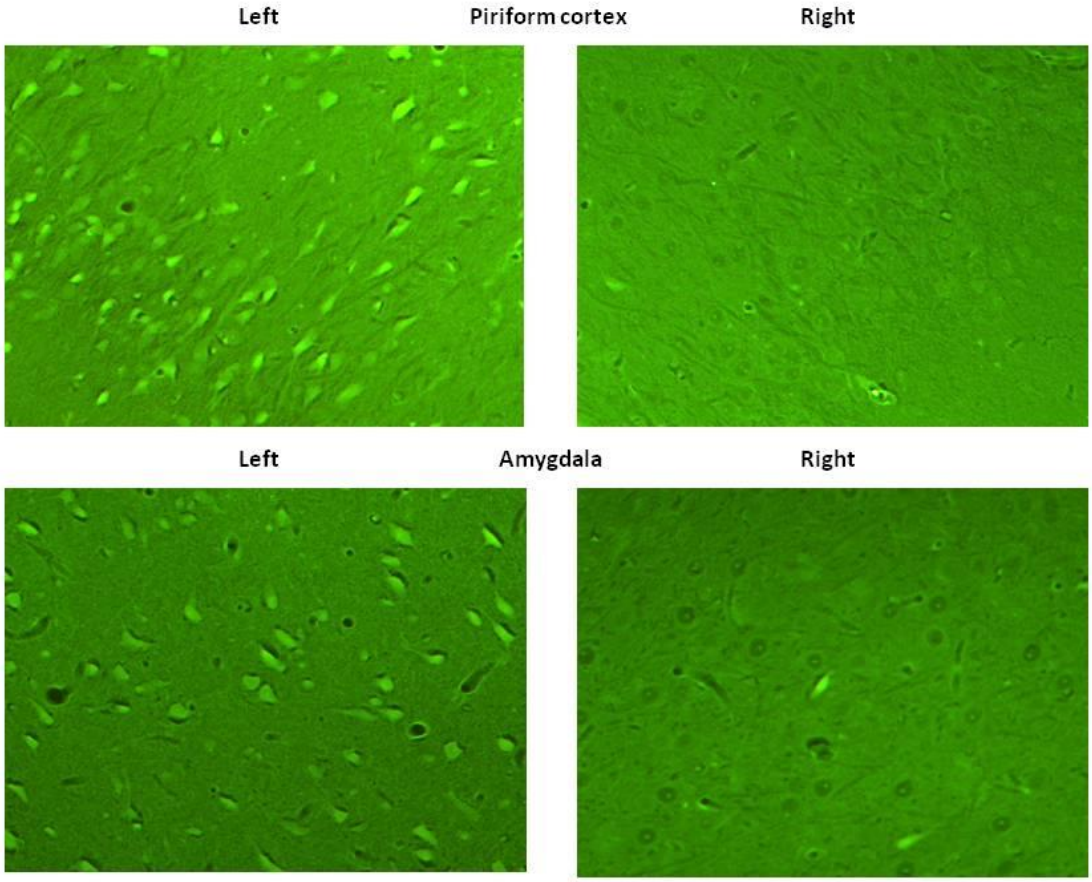

Table 1

Schematic overview of the experimental design and antidotal results achieved

Soman dose at 0 min:	1.3 x LD_{50}	3 x LD_{50} 4 x LD_{50} 5 x LD_{50}	1.6 x LD_{50}	1.3 x LD_{50} (with pyridostigmine pretreatment)
Time for treatment:	-20 min	1 and 5 min	10 and 14 min	20 and 24 min
HI-6 Scopolamine Physostigmine	↑	↑↑	0	0
HI-6 Levetiracetam Procyclidine	↑↑	↑↑	↑↑	↑↑
Levetiracetam Procyclidine	↑	0	--	--

↑↑ High protection (anticonvulsant and neuroprotective effects), ↑ Low protection (neuroprotection, but weak anticonvulsant effect and high lethality or anticonvulsant effect, but no neuroprotection), 0 No protection, -- Not tested. -20 min denote prophylactic treatment.

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Table 2

Anticonvulsant and life preserving effects of 2 or 3 medical therapies in rats challenged with 3, 4, or 5 x LD_{50} of soman

Soman	*N*	Treatment 1 and 5 min after soman	Clonic or tonic convulsions 1-5 min after soman	Epileptiform EEG 7-10 min after soman	Lethality rate 24 h after soman (Time of death before 24 h)
3 x LD_{50}	6	HI-6 (125 mg/kg) Scopolamine (1 mg/kg) Physostigmine (0.1 mg/kg)	2/6	0/6	0/6*
3 x LD_{50}	6	Levetiracetam (50 mg/kg) Procyclidine (20 mg/kg)	3/6	0/2	(6.3-14 min N=6) 6/6
3 x LD_{50}	6	HI-6 (125 mg/kg) Levetiracetam (50 mg/kg) Procyclidine (20 mg/kg)	3/6	0/6	0/6*
4x LD_{50}	6	HI-6 (125 mg/kg) Scopolamine (1 mg/kg) Physostigmine (0.1 mg/kg)	4/6	0/6	0/6
4 x LD_{50}	6	HI-6 (125 mg/kg) Levetiracetam (50 mg/kg) Procyclidine (20 mg/kg)	3/6	0/6	0/6
5 x LD_{50}	6	HI-6 (125 mg/kg) Scopolamine (1 mg/kg) Physostigmine (0.1 mg/kg)	2/6	0/6	0/6
5 x LD_{50}	6	HI-6 (125 mg/kg) Levetiracetam (50 mg/kg) Procyclidine (20 mg/kg)	3/6	0/6	0/6

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Significantly different from the levetiracetam, procyclidine group; *P= 0.0022. In the levetiracetam, procyclidine group, 4 of 6 rats seized and died before 7 min after exposure to soman.

Table 3

Anticonvulsant and life preserving effects of 2 medical therapies 10 and 14 min after exposure to 1.6 x LD_{50} of soman. Mean latencies in min (±SEM).

Drug	Dose (mg/kg)	*N*	Latency to seizure onset	Antiseizure response ratio	Latency to seizure termination	Lethality response ratio (24 h)
HI-6	125					
Scopolamine	1	8	7.3±1.1	0/8	No termination	6/8
Physostigmine	0.1					
HI-6	125					
Levetiracetam	50	6	8.3±1.2	6/6**	8.7±0.9	0/6*
Procyclidine	20					

Antiseizure response ratio 0/8 means that the therapy did not stop seizures in any of the 8 rats. Significantly different from the HI-6, scopolamine, physostigmine group; *P=0.0097, **P=0.0022.

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Table 4

Anticonvulsant and life preserving effects of 2 medical therapies 20 and 24 min after exposure to 1.3 x LD_{50} of soman in rats pretreated with pyridostigmine (0.1 mg/kg). Mean latencies in min (±SEM).

Drug	Dose (mg/kg)	*N*	Latency to seizure onset	Antiseizure response ratio	Latency to seizure termination	Lethality response ratio (24 h)
HI-6	125					
Scopolamine	1	6	8.6±1.5	0/6	No termination	6/6
Physostigmine	0.1					
HI-6	125					
Levetiracetam	50	6	7.5±1.7	6/6*	12.3±1.8	0/6*
Procyclidine	20					

Antiseizure response ratio 0/6 means that the therapy did not stop seizures in any of the 6 rats. Significantly different from the HI-6, scopolamine, physostigmine group; *P=0.0022.

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Table 5

Prophylactic effects of 3 medical therapies given 20 min before soman intoxication (1.3 x LD_{50})

Group	Dose (mg/kg)	*N*	Antiseizure response ratio	Lethality respone ratio (24 h)
HI-6	125			
Scopolamine	1	6	6/6	0/6
Physostigmine	0.1			
Levetiracetam	50			
Procyclidine	20	6	4/6	2/6
HI-6	125			
Levetiracetam	50	6	6/6	0/6
Procyclidine	20			

The antiseizure response ratio and lethality response ratio were not significantly lower in the levetiracetam, procyclidine group than in the other 2 groups (P>0.05).

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Table 6

Mean (±SEM) percent body weight 7 days after exposure relative to the day of soman intoxication

Treatment 1 and 5 min after soman	*N*	Dose of soman 3 x LD_{50}	*N*	Dose of soman 4 x LD_{50}	*N*	Dose of soman 5 x LD_{50}
HI-6 (125 mg/kg) Scopolamine (1 mg/kg) Physostigmine (0.1 mg/kg)	6	102±0.7	6	99±1.1	6	99±0.4
HI-6 (125 mg/kg) Levetiracetam (50 mg/kg) Procyclidine (20 mg/kg)	6	99±1.0	6	99±2.3	6	100±0.5

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Table 7

Median (range) neuropathology scores of rats that lived for 7 days after exposure to various levels of soman intoxication. The rats had been subjected to 2 medical therapies

Treatment 1 and 5 min after soman	*N*	3 x LD_{50} of soman				*N*	4 x LD_{50} of soman				*N*	5 x LD_{50} of soman			
		Piriform cortex		Amygdala			Piriform cortex		Amygdala			Piriform cortex		Amygdala	
		Left	Right	Left	Right		Left	Right	Left	Right		Left	Right	Left	Right
HI-6 (125 mg/kg) Scopolamine (1 mg/kg) Physostigmine (0.1 mg/kg)	6	0.5 (0-4)	0.0 (0-0)	1.5 (0-4)	0.0 (0-0)	6	1.5* (0-4)	0.0 (0-0)	0.0 (0-2)	0.0 (0-0)	6	1.5* (0-4)	0.0 (0.0)	1.0 (0-3)	0.0 (0-0)
HI-6 (125 mg/kg) Levetiracetam (50 mg/kg) Procyclidine (20 mg/kg)	6	0.0 (0-2)	0.0 (0-2)	0.0 (0-0)	0.0 (0-1)	6	0.0 (0-2)	0.0 (0-2)	0.0 (0-0)	0.0 (0-1)	6	1.5 (0-4)	0.0 (0-1)	0.5 (0-3)	0.0 (0-1)

Significantly different from the right side; *P<0.05.

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Table 8

Median (range) neuropathology scores of rats that lived for 7 days after exposure to a soman dose of 1.3 x LD_{50}. The rats were pretreated with 2 medical therapies 20 min before challenge with soman.

		Neuropathology score		
Group	*N*	Piriform cortex	Hippocampal CA1	Amygdala
HI-6 Scopolamine Physostigmine	6	2.8 (1 – 3)	0.0 (0 – 0)	1.0 (0 – 3)
HI-6 Levetiracetam Procyclidine	6	0.0** (0 – 1)	0.0 (0 – 0)	0.0* (0 – 0.5)

Significantly different from the HI-6, scopolamine, physostigmine group: ***P*=0.0022, **P*=0.026

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