Capacities of metabotropic glutamate modulators in counteracting soman-induced seizures in rats

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ABSTRACT

Current treatment of nerve agent poisoning with ionotropic drugs proves inadequate, and alternative treatment strategies are searched for. Based on positive findings with metabotropic glutamate modulators in microinfusion studies, the present study was initiated to examine anticonvulsant effects of MPEP (2-Methyl-6-(phenylethynyl)pyridine hydrochloride), a metabotropic glutamate receptor 5 antagonist, and DCG-IV ((2*S,*2'*R*,3'*R*)-2-(2',3'-dicarboxycyclopropyl)glycine), a metabotropic glutamate receptor 2/3 agonist, when administered systemically in combinations with HI-6 (1-[([4-(aminocarbonyl)pyridino] methoxy)methyl]-2-[(hydroxyimino)methyl]pyridinium) and procyclidine or HI-6 and levetiracetam relative to the combination of HI-6, procyclidine, and levetiracetam. The results showed that MPEP or DCG-IV combined with HI-6 and procyclidine resulted in substantial antidotal efficacy when administered 20 min after onset of seizures elicited by soman. MPEP or DCG-IV combined with HI-6 and levetiracetam did not terminate seizures and preserve lives. When given 20 min before challenge with soman, DCG-IV in combination with HI-6 and procyclidine provided protection, whereas MPEP combined with HI-6 and procyclidine did not. Combinations with metabotropic glutamate receptor modulators did not achieve the same high level of antidotal efficacy as the combination of HI-6, procyclidine, and levetiracetam. MPEP alone inhibited pseudocholinesterase activity in the brain markedly. A positive correlation was found between latency to seizure onset or full protection and level of pseudocholinesterase activity in brain. MPEP and DCG-IV can serve as effective anticonvulsants against nerve agent poisoning when combined with HI-6 and procyclidine. Metabotropic glutamate receptor modulators may represent an alternative or supplement to treatment with ionotropic drugs.

Keywords: DCG-IV, HI-6, Levetiracetam, MPEP, Procyclidine, Soman

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1. Introduction

Organophosphorus nerve agents are highly potent irreversible inhibitors of the enzyme acetylcholinesterase that hydrolyses acetylcholine. Accumulation of acetylcholine in the synaptic cleft results in over-stimulation of muscarinic and nicotinic receptors. It has been hypothesized that several neurotransmitter systems become involved sequentially in the initiation and maintenance of seizures elicited by nerve agents (McDonough and Shih, 1997). The progression of events can conceptually be divided into 3 phases. An early cholinergic phase lasting from the time of exposure to about 5 minutes after onset of seizures is dominated by high cholinergic activity followed by a transitional phase of cholinergic and glutamatergic hyperactivity and finally a predominantly glutamatergic phase after about 40 minutes (McDonough and Shih, 1997).

Exposure to nerve agent requires immediate medical treatment. For this purpose, a number of armed forces have based their therapy against nerve agent intoxication on an oxime (obidoxime (1,1'-(oxydimethylene)bis(4-formylpyridinium) dioxime), 2-PAM (2-[(hydroxyimino)methyl]-1-methylpyridinium chloride), HI-6 (1-[([4-(aminocarbonyl) pyridino]methoxy)methyl]-2-[(hydroxyimino)methyl]pyridinium)), an anticholinergic (atropine), and a $GABA_A$ (γ-amminobutyric acid A receptors) agent (diazepam, avizafone) combined with carbamate (pyridostigmine) pretreatment (Aas, 2003). However, such treatment regimens can 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). Such seizures rapidly progress to status epilepticus, a condition that is strongly associated with mortality and brain damage in experimental animals (Shih *et al.*, 2003). In the search for novel strategies able to prevent or terminate nerve agent-evoked seizures, soman has been used in animal models because it takes a higher dose of anticonvulsants to stop seizures triggered by soman than other classical

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nerve agents (tabun, sarin, cyclosarin, VX). This finding suggests that drugs effective against soman will also be effective against other nerve agents (Shih and McDonough, 2000).

Seizures that have lasted more than 40 minutes are gradually more difficult to terminate (Carpentier et al., 2001; Lallement et al., 1999). The refractory nature of sustained seizures represents a great challenge in treatment of nerve agent poisoned victims long time after exposure. The increased glutamatergic activity induced by soman causes excitotoxic damage and cell death (Munirathinam and Bahr, 2004), and the initial signs of injury are seen about 20 minutes after seizure onset (Lallement et al., 1994; McDonough et al., 1995). Seizure activity lasting 30 minutes or more (status epilepticus) has been shown to cause up-regulation of NMDA (N-methyl-D-aspartic acid) and AMPA (α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptors along with internalization of $GABA_A$ receptors in hippocampal slices (Chen and Wasterlain, 2006; Wasterlain and Chen, 2008). This outcome means that $GABA_A$ receptors are inactivated, because they are no longer within reach of the neurotransmitter. Furthermore, NMDA and AMPA receptor subunits move to the synaptic membrane where they form additional excitatory receptors (Chen and Wasterlain, 2006; Wasterlain and Chen, 2008). These configurations may in part explain why glutamatergic antagonists and GABAergic agonists become gradually ineffective during the development of status epilepticus.

Given the implication of glutamatergic neurotransmission in epileptiform discharges, alternative targets for control of glutamatergic activity are of great interest. Through a large number of studies, metabotropic glutamate receptors have been shown to fulfil unique presynaptic and postsynaptic roles (Alexander and Godwin, 2006). In contrast to ionotropic glutamate receptors, which mediate fast synaptic transmission, metabotropic glutamate receptors often modulate on-going activity. When located postsynaptically, metabotropic glutamate receptors may modulate membrane properties by second messenger interactions,

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whereas presynaptic metabotropic glutamate receptors have been shown to control neurotransmitter release (Alexander and Godwin, 2006). These modulatory aspects appear to have attracted attention in experimental epilepsy. Group I metabotropic glutamate receptor antagonists and Group II metabotropic glutamate receptor agonists have been demonstrated to exert anticonvulsant efficacy, whereas Group III metabotropic glutamate receptor agonists show mixed responses in animal models of epilepsy (Alexander and Godwin, 2006).

We have previously examined the anticonvulsant potency of metabotropic glutamate receptor modulators microinfused into the perirhinal cortex of rats. The results show that the metabotropic glutamate receptor 5 antagonist MPEP hydrochloride (2-Methyl-6-(phenylethynyl)pyridine hydrochloride) and the metabotropic glutamate 2/3 receptor agonist DCG-IV ((2*S*,2'*R*,3'*R*)-2-(2',3'-dicarboxycyclopropyl)glycine) cause full protection against seizures or increased latency to onset of seizures, whereas the metabotropic glutamate receptor 1 antagonist LY367385 ((S)-(+)-α-Amino-4-carboxy-2-methylbenzeneacetic acid) does not produce anticonvulsant efficacy in response to systemically administered soman (Myhrer et al., 2010). The present study was designed to investigate the anticonvulsant capacities of MPEP and DCG-IV during systemic administration. However, in contrast to microinfusion studies in which anticonvulsant action of a single drug can be measured, combinations of drugs are necessary for achieving antiseizure efficacy when systemic administration is used (Myhrer et al., 2011). Both MPEP and DCG-IV pass the blood-brain barrier (Jesse et al., 2008; Tomita et al., 2000).

Procyclidine has been shown to be the most potent anticonvulsant tested in our microinfusion studies, and its potency can be further enhanced when combined with the antiepileptic drug levetiracetam (Myhrer et al., 2011). Inclusion of the oxime HI-6 serves as an important factor in optimizing the antidotal efficacy of procyclidine and levetiracetam (Myhrer et al., 2013a). The purpose of the present study was to examine antidotal effects of

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MPEP or DCG-IV combined with either HI-6 and procyclidine or HI-6 and levetiracetam relative to the combination of HI-6, procyclidine, and levetiracetam 20 minutes after onset of seizures elicited by soman in rats pretreated with pyridostigmine. Treatment later than 20 minutes after seizure onset requires pretreatment with HI-6 in order to have a reasonable number of surviving rats, but this oxime may mask genuine anticonvulsant effects because of several pharmacological actions (Myhrer et al., 2011). The most potent combinations were also tested as prophylactic therapies and were for that purpose given 20 minutes before soman poisoning. Because MPEP has been reported to protect acetylcholinesterase activity against inhibition caused by the cholinergic agonist pilocarpine (Jesse et al., 2008), it was investigated whether MPEP could prevent inhibition of acetylcholinesterase activity by soman.

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 minutes a day. The climatized vivarium (21ºC) was illuminated from 0700 to 1900 hours.

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

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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 and nerve agent

Some of the drug doses chosen were derived from previous studies of anticonvulsant effects against soman-evoked seizures in rats; HI-6 dimethanesulphonate 125 mg/kg, procyclidine hydrochloride 6 or 20 mg/kg, levetiracetam (Keppra®) 50 mg/kg, pyridostigmine bromide 0.1 mg/kg (Myhrer et al. 2011, 2013a). MPEP doses (15, 30, or 60 mg/kg) were derived from a study in which seizures were induced by electrical stimulation (Lojková and Mareš, 2005). The dose of 4 mg/kg DCG-IV was established from our own pilot experimentation of anticonvulsant effect against soman-evoked seizures. The drugs were dissolved in 0.9% saline, except MPEP that was dissolved in 10% dimethyl sulfoxide in distilled water (33% 2-hydroxy propyl-β-cyclodextrin) (Ali, 2001). All drugs were administered intraperitoneally. The soman dose was 1.3 x LD_{50} (100 μg/kg) resulting in convulsions and death in all rats of our strain (Sterri et al. 1980). Soman was given subcutaneously. Procyclidine and pyridostigmine were purchased from Sigma (St Louis, Missouri, USA), and MPEP and DCG-IV were purchased from Tocris Cookson Ltd (Bristol, United Kingdom). HI-6 dimethanesulphonate was a gift from Defence Research and Development (Suffield, Medicine Hat, Canada).

2.4. 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

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thick and dried in an incubator (37ºC) for 12 h before they were stained with hematoxylin and eosin or Fluoro-Jade B (Schmued and Hopkins 2000). Since Fluoro-Jade staining 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 hematoxylin and eosin. Therefore, hematoxylin and eosin staining had to be used to determine neuropathology in the animals. Otherwise Fluoro-Jade staining was used for evaluation of neuronal degeneration. 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. Determination of neuropathology versus intact neurons is often facilitated by the use of DAPI staining. However for illustration, the photographs of neuropathology become clearer without DAPI staining, in particular in the paper version of photographs.

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2.5. 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 hippocampal CA1 region, the basolateral amygdala, and the piriform cortex in both hemispheres, 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%. Each animal was given a total neuropathology score based on the mean score from left and right hemispheres in the 3 individual brain areas chosen. The criterion used to characterize the pathology was neuronal degeneration.

2.6. 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 (ElectroEnchephaloGraphy) recording was made while the animals were situated in their home cages (50 x 30 x 15 cm). Measures were made 24 hours prior to drug treatment, immediately after, and 24, 48, or 144 hours after treatment.

2.7. Observation of animals

The rats were observed for convulsions and visible signs of intoxication continuously for the first 2-3 hours and then for 10 minutes at 24 and 48 hours after soman injection. Rats that displayed aphagia/adipsia following termination of convulsions were given 5 ml of 0.9% saline (intraperitoneally) twice a day for 1-3 days until they started to eat.

2.8. Determination of acetylcholinesterase and pseudocholinesterase activity

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The activity of acetylcholinesterase and pseudocholinesterase was determined in plasma, red blood cells, and brain according to the method published by Molecular Probe (Amplex™ Red Acetylcholine/Acetylcholinesterase Assay Kit (A-12217)) based on measurement of H_2O_2 with the use of Amplex Red reagent (Zhou et al., 1997). The acetylcholinesterase and pseudocholinesterase activities were expressed as pmol min^{-1} mg $protein^{-1}$. Acetylcholinesterase activity was measured after inhibition of pseudocholinesterase with ethopropazine (Todrik, 1954). The rats were divided into 7 groups with 6-8 rats in each. They were given various doses of MPEP 20 min before they received either 0.9% saline or soman (1.3 x LD_{50}). One control group received 2 doses of saline, and 1 control group received saline and soman. Survival time after soman exposure or saline was 1 hour, or dying rats were decapitated just before natural death.

2.9. Statistics

Group comparisons were made with two-sided Fisher's exact test, two-tailed Mann-Whitney *U* test, or two-tailed Spearman correlation analysis. When treatment is given before exposure to soman, anticonvulsant effects of the drugs would appear as prolonged latency from injection of soman to onset of seizures/convulsions or prevention of seizures. In order to combine the data from these 2 reaction categories a latency criterion was used for the nonconvulsing rats. One minute more than the longest latency among the convulsing rats was set as criterion for full anticonvulsant effect. The use of this criterion prevented normal distribution of data, and nonparametric statistics were required. For this reason, the scores had to be expressed in median and range. Overall analyses were carried out with one-way Kruskal-Wallis or Friedman analysis of variance (ANOVA). Group comparisons were made with Dunn's multiple comparison test. Use of the grading system of neuropathology resulted

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in nonparametric data. Computations were made with the Prism statistical software program (GraphPad Software, Sand Diego, California, USA).

3. Results

3.1. Soman and treatment

Large variations in antidotal efficacy were seen for the various treatment categories (Table 1). One-way ANOVA did not reveal significant differences in mean latency to seizure onset (P>0.05). Fisher's exact test showed that both the antiseizure response ratio and lethality response rate were reliably higher for the HI-6, procyclidine, levetiracetam regimen compared with the regimens consisting of HI-6, levetiracetam, MPEP or HI-6, levetiracetam, DCG-IV (P=0.0022). ANOVA did not show significant differences in latency to seizure termination among the groups (P>0.05). All 6 rats from the HI-6, procyclidine, levetiracetam group survived 7 days (time for harvesting brains) and were in a good condition, whereas only 3 rats from the HI-6, procyclidine, MPEP group and 2 rats from the HI-6, procyclidine, DCG-IV group survived 7 days. Rats that did not recover satisfactorily were euthanized before 7 days of survival.

When the therapies were administered 20 minutes before challenge with soman, no reliable differences among the groups were found in terms of antiseizure response and lethality response ratio (Table 2). However, the difference in percentage of lethality was relatively high between the HI-6, procyclidine (20 mg/kg), levetiracetam group and the HI-6, procyclidine, MPEP group. Only 1 rat from the latter group survived 7 days, whereas 4 rats from the HI-6, procyclidine, DCG-IV group, 6 rats from the HI-6, procyclidine (20 mg/kg), levetiracetam group, and 5 rats from the HI-6, procyclidine (6 mg/kg), levetiracetam group survived 7 days.

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The highest dose of MPEP (60 mg/kg) evidently caused anticonvulsant effect when administered 20 minutes before soman intoxication (Table 3). Friedman one-way of ANOVA revealed reliable differences among the groups in median latency to seizure onset (H (3)=9.478, P=0.0017). Dunn's multiple comparison test showed that the MPEP (60 mg/kg) group had significantly longer latency to seizure onset than both the saline and MPEP (30 mg/kg) groups (P<0.05). Two-tailed Spearman correlation analysis showed a significant positive correlation between latencies to seizure onset or full protection and activity levels of acetylcholinesterase and pseudocholinesterase in brain homogenate (Table 4) for the groups that received MPEP in Table 3 (N=12) (r=0.7030, P=0.0108 and r=0.6658, P=0.0181, respectively).

3.2. Soman, MPEP, and acetylcholinesterase/pseudocholinesterase activity measures

Table 4 shows the acetylcholinesterase and pseudocholinesterase activities in blood and brain of rats treated with MPEP and injected with saline or soman. When the 2 groups with 30 and 60 mg/kg of MPEP were combined in 1 MPEP group, evident differences were seen in comparison with the control groups. Mann-Whitney *U* test showed that the combined MPEP group that was treated with saline had significantly lower levels of acetylcholinesterase activity in brain homogenate than the control group that only received saline (P=0.0492). The combined MPEP group that was treated with saline also had reliably lower values of pseudocholinesterase activity in brain homogenate than the saline-treated control group (P=0.0151). The levels of pseudocholinesterase activity in brain homogenate was significantly higher in the combined MPEP group that received soman than the control group that received saline and soman (P=0.0323). The latter MPEP group displayed a brain pseudocholinesterase activity level that was about 7% of that seen for the control group that only was given saline.

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3.3. Histology

When treatment was given 20 minutes after seizure onset, neuropathology in terms of fluorescent staining was observed for all treatment categories (Table 5). One-way Kruskal-Wallis ANOVA revealed no significant differences in neuropathology scores among the groups ($P>0.05$). When treatment was given 20 minutes before exposure to soman, clear differences in neuropathology were seen between the groups (Table 6; Fig. 1). ANOVA revealed a reliable overall effect between the groups ($H(4)=11.08$, $P=0.0113$). The severity of total neuropathology scores was significantly smaller in the HI-6, procyclidine (20 mg/kg), levetiracetam group relative to the HI-6, procyclidine, DCG-IV, and HI-6, procyclidine (6 mg/kg), levetiracetam groups ($P<0.05$).

4. Discussion

The results from the present study showed that MPEP or DCG-IV combined with HI-6 and procyclidine produced robust antidotal efficacy when administered 20 minutes after onset of seizures evoked by soman. On the other hand, MPEP or DCG-IV combined with HI-6 and levetiracetam did not terminate seizures and preserve lives. When the treatments were given 20 minutes before soman exposure, DCG-IV in combination with HI-6 and procyclidine protected well, whereas MPEP combined with HI-6 and procyclidine did not. No treatment combinations with metabotropic glutamate receptor modulators were able to achieve similar high level of antidotal performance as HI-6, procyclidine, and levetiracetam, both pre- and post-poisoning. MPEP (30 and 60 mg/kg) reduced brain acetylcholinesterase and pseudocholinesterase activities considerably in rats treated with saline. In animals intoxicated with soman (1.3 x LD_{50}), acetylcholinesterase and pseudocholinesterase activities were almost completely inhibited.

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The antidotal efficacy of MPEP or DCG-IV combined with HI-6 and procyclidine was weaker than that obtained by HI-6, procyclidine, and levetiracetam. Procyclidine administered alone 20 minutes after onset of soman-induced seizures in rats pretreated with pyridostigmine has no anticonvulsant effect (Myhrer et al., 2011). The antiglutamatergic property of procyclidine was probably enhanced by the action of the metabotropic glutamate receptor modulators, whereas both the antiglutamatergic and anticholinergic properties of procyclidine were probably increased by levetiracetam. Lack of anticonvulsant reinforcement is seen when levetiracetam is used together with other drugs with a single function such as muscimol, NBQX (2,3-Dihydro-6-nitro-7-sulphamoyl-benzo(f)quinoxaline), and ketamine, whereas robust anticonvulsant effects are achieved when levetiracetam is administered along with the multifunctional drugs procyclidine or caramiphen (Myhrer et al., 2011).

During prophylactic treatment, the regimen consisting of HI-6, procyclidine, and MPEP caused low anticonvulsant potency as well as high lethality response. When HI-6 was not included in the latter regimen during a pilot experiment, seizures were prevented, although the rats were still incapacitated 24 hours after soman exposure (Myhrer and Enger, unpublished data). In rats not exposed to soman, approximately 18% of HI-6 enters the brain and binds reversibly to acetylcholinesterase (Cassel et al., 1997; Sket and Brzin, 1986; Svensson et al., 2005). The half-life of HI-6 in plasma of rats is 24 minutes (Garrigue et al., 1990), and the MPEP receptors occupancy in the brain of rats is 100% for at least 1 hour (Anderson et al., 2003). An adverse interaction between HI-6 and MPEP appears to result in decreased antidotal efficacy. This finding is not readily accounted for.

When procyclidine is used in prophylactic treatment, effective doses can be lower than those required for post-poisoning treatment (Myhrer et al., 2004, 2011). A dose of 1 mg/kg of procyclidine is sufficient in combination with physostigmine (0.1 mg/kg) to protect fully against a soman dose of 1.3 x LD_{50} (Myhrer et al., 2004). The reduction of procyclidine to 6

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mg/kg in combination with HI-6 and levetiracetam did not yield optimal protection, indicating that the chosen dose of 20 mg/kg of procyclidine should also be used for pre-poisoning treatment.

During administration of MPEP alone only the highest dose (60 mg/kg) caused anticonvulsant efficacy against soman-induced seizure activity. However, positive correlations were found between latency to seizure onset or full protection and acetylcholinesterase and pseudocholinesterase activities in brain homogenate for the MPEP-treated groups encompassed in Table 3 and lower part of Table 4. This finding emerges as comprehensible; the lower cholinergic activity the more efficient the anticonvulsant properties of MPEP. Both 30 and 60 mg/kg reduced the levels of acetylcholinesterase and pseudocholinesterase activities in brain homogenate of rats treated with saline. However, in experiments with soman, acetylcholinesterase and pseudocholinesterase activities were inhibited to about 7% of the level seen in control rats treated with saline alone. Both 30 and 60 mg/kg of MPEP inhibited the activity levels of brain pseudocholinesterase, but 60 mg/kg had to be applied for the anticonvulsant impact of MPEP to become effective against soman-evoked seizures.

When treatment was administered 20 minutes after seizure onset, convulsions were terminated after about 15 minutes. The interval of seizing (35 minutes) gives room for development of neuropathology, because the first signs of morphological changes can be observed about 20 minutes following seizure onset (Lallement et al., 1994; McDonough et al., 1995). No differences in neuropathology were seen for the treatment categories when used post-poisoning. When used pre-poisoning, clear differences occurred. The degree of protection in combinations with metabotropic glutamate receptor modulators was low compared with that of the HI-6, procyclidine (20 mg/kg), levetiracetam regimen. It was also seen that the use of 6 mg/kg procyclidine was insufficient in providing neuroprotection

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compared with 20 mg/kg procyclidine in combination with HI-6 and levetiracetam. The latter finding implies that procyclidine has crucial neuroprotective properties, most likely exerted by the drug's powerful NMDA antagonism (Raveh et al., 1999).

Both MPEP and DCG-IV were shown to cause substantial anticonvulsant impact when combined with HI-6 and procyclidine. In a recent microinfusion study, double infusions of MPEP or DCG-IV into the perirhinal cortex (to reach a larger area) prevented seizure onset in up to 83% of rats exposed to soman (Myhrer et al., 2013b). This percentage is higher than ever obtained before in our microinfusion studies (Table 3 in Myhrer, 2010). An increase in glutamatergic activity most likely occurs already during the cholinergic phase of the 3-phase model (McDonough and Shih, 1997), because focal administration of metabotropic glutamate receptor modulators can protect against evolvement of seizures in response to soman. During pretreatment, the impact of drugs can be more potent when applied directly into seizure controlling forebrain structures than by systemic administration. However, the relation between focal and global administration is reversed when treatment is carried out after seizure onset (Myhrer, 2010).

MPEP administered alone at high dose has evident anticonvulsant efficacy against soman-induced seizures, but MPEP may lose efficacy if it is combined with another agent binding to acetylcholinesterase, like HI-6. DCG-IV also possesses NMDA agonism and can at high doses cause proconvulsant effect (Folbergrová et al., 2001). The metabotropic glutamate receptor Group I modulator MPEP and the metabotropic glutamate receptor Group II modulator DCG-IV may have relevant action as adjuvant drugs in treatment of sustained seizures, when ionotropic agents gradually become ineffective (Wasterlain and Chen, 2008). Group I of metabotropic glutamate receptor modulators (MPEP, LY367385) reduces persistent epileptiform bursts more than 5 hours after onset in hippocampal slices (Merlin, 2002), and down-regulation of Group II of metabotropic glutamate receptors starts as late as

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24 hours following onset of status epilepticus (Garrido-Sanabria et al., 2008). Clinical studies of metabotropic glutamate receptor modulators for treatment of epilepsy are expected to begin in the near future (Ngomba et al., 2011).

Levetiracetam is an antiepileptic that increases the anticonvulsant actions of drugs affecting glutamatergic, GABAergic, or cholinergic neurotransmission, and the specific binding site is believed to be the synaptic vesicle protein 2A (Kaminski et al., 2009; Lynch et al., 2004; Meehan et al., 2012; Vogl et al., 2012). Although the exact mechanisms are not well known, levetiracetam probably reduces release of glutamate by which the efficacy of glutamatergic antagonists are highly enhanced (Kaminski et al., 2009). MPEP has antagonistic effect postsynaptically on metabotropic glutamate receptors, and DCG-IV has agonistic effect presynaptically on metabotropic glutamate 2/3 receptors, by which spillover of glutamate is diminished. Levetiracetam does not seem to enhance effects already obtained by the metabotropic glutamate receptor modulators as reflected in the present results of anticonvulsant efficacy, although additive effects may well have occurred. Lack of multifunction in the combination of levetiracetam and metabotropic glutamate receptor modulators probably puts limits for the results to be achieved.

In conclusion, MPEP or DCG-IV combined with HI-6 and procyclidine can as post-poisoning therapies terminate seizures and preserve lives of rats exposed to soman. As pre-poisoning therapy, only the combination of HI-6, procyclidine, and DCG-IV appears to act adequately. Metabotropic glutamate modulators may have potency as adjuvant drugs in treatment of sustained seizures, because they can maintain anticonvulsant efficacy for a considerably longer time than ionotropic drugs.

The authors declare that there are no conflicts of interest.

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Figure legend

Fig. 1. Sections stained with Fluoro-Jade B 7 days following soman exposure (1.3 x LD_{50}). From rats that received HI-6, procyclidine (20 mg/kg), and levetiracetam (A) or HI-6, procyclidine, and DCG-IV (B) before soman intoxication. Photographs with DAPI staining were not used for illustrations (see section 2.4 Histology in Materials and Methods). The magnification was x200.

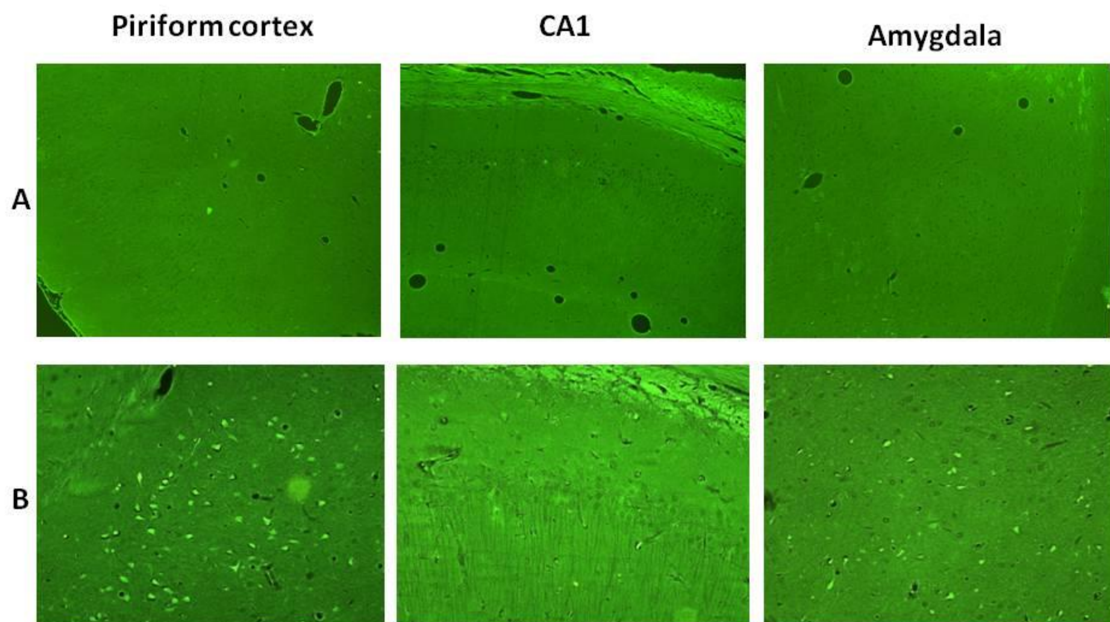

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Table 1

Anticonvulsant effects of 5 treatment categories on soman-induced (1.3 x LD_{50}) seizures 20 minutes after onset. The rats were pretreated with pyridostigmine (0.1 mg/kg). Mean (±SEM) latencies in minutes.

Drug	Dose mg/kg	N	Latency to seizure onset	Antiseizure response ratio	Latency to seizure termination	Lethality response (24 hours)	
						Ratio	Percent
HI-6 Procyclidine MPEP	125 20 30	7	8.7±1.3	5/7	15.6±2.3	2/7	29
HI-6 Levetiracetam MPEP	125 50 30	6	7.9±1.6	0/6	-	6/6	100
HI-6 Procyclidine DCG-IV	125 20 4	8	8.0±1.4	6/8	17.0±3.2	3/8	38
HI-6 Levetiracetam DCG-IV	125 50 4	6	7.5±1.7	0/6	-	6/6	100
HI-6 Procyclidine Levetiracetam	125 20 50	6	7.3±1.6	6/6*	12.7±1.8	0/6[a]	0

[a]Significantly different from the HI-6, levetiracetam, DCG-IV and HI-6, levetiracetam, MPEP groups with Fisher's exact test (*P*=0.0022).

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Table 2

Prophylactic effects of 4 therapies given 20 minutes before soman intoxication ($1.3 \times LD_{50}$).

Group	Dose mg/kg	N	Antiseizure response ratio	Lethality response (24 hours)	
				Ratio	Percent
HI-6 Procyclidine MPEP	125 20 30	6	3/6	4/6	67
HI-6 Procyclidine DCG-IV	125 20 4	6	5/6	1/6	17
HI-6 Procyclidine Levetiracetam	125 20 50	6	6/6	0/6	0
HI-6 Procyclidine Levetiracetam	125 6 50	6	5/6	1/6	17

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Table 3

Anticonvulsant effects of MPEP in rats when administered 20 minutes before exposure to soman (1.3 x LD_{50}).

Drugs	Dose mg/kg	N	Latency to seizure/ nonseizure		Noncon-vulsing rats
			Median	Range	
Saline	-	8	5.7	3.3-7.0	0
MPEP	30	6	6.2	3.0-14.0	0
MPEP	60	6	13.5[a]	6.0-24.0	1

[a]Significantly different from the saline-treated control group with Friedman's one-way ANOVA and Dunn's multiple comparison test (*P*<0.05).

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Table 4

Acetylcholinesterase and/or pseudocholinesterase activity determination in red blood cells, plasma, and brain homogenate from rats exposed to 1.3 x LD_{50} of soman or saline 1 hour before euthanasia. MPEP or saline was administered 20 minutes before soman. The values are pmol hydrolized acetylcholinesterase or pseudocholinesterase min^{-1} mg $protein^{-1}$.

Groups	N	AChE red blood cells	ChE plasma	AChE brain homogenate	ChE brain homogenate
		Median (range)			
Saline		5.2	6.4	138.7	106.2
+ saline	8	(2.6-7.0)	(4.6-7.3)	(58.3-235.1)	(73.4-186.9)
MPEP 15 mg/kg		7.3	6.7	138.6	101.8
+ saline	6	(2.6-16.7)	(3.9-10.6)	(59.1-275.7)	(58.1-178.3)
MPEP 30 mg/kg		6.1	7.1	106.1	64.0
+ saline	6	(3.8-18.5)	(5.8-10.8)	(75.0-127.8)	(12.8-160.0)
MPEP 60 mg/kg		4.8	5.8	97.0[a]	83.5[b]
+ saline	6	(2.7-12.7)	(5.3-11.0)	(50.3-104.5)	(34.3-87.1)
Saline		0.1	0.0	4.9	0.0
+ soman	8	(0.0-0.4)	(0.0-0.0)	(3.2-6.5)	(0.0-3.0)
MPEP 30 mg/kg		0.3	0.0	5.7	9.2
+ soman	6	(0.0-0.4)	(0.0-0.0)	(0.0-9.2)	(0.0-15.0)
MPEP 60 mg/kg		0.3	0.0	7.6	6.1[c]
+ soman	6	(0.0-0.5)	(0.0-0.0)	(4.6-33.1)	(0.0-33.3)

Significantly different from the saline-treated control group for the combined 30 and 60 mg/kg of MPEP (marked for the 60 mg/kg group) that received saline for acetylcholinesterase (AChE) activity ([a]$P<0.05$) and pseudocholinesterase (ChE) activity ([b]$P<0.05$) in brain homogenate. Significantly different from the control group that received saline and soman for the combined 30 and 60 mg/kg of MPEP (marked for the 60 mg/kg group) that received soman ([c]$P<0.05$). The statistical analysis was performed with Mann-Whitney *U*-test.

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Table 5

Median (range) neuropathology scores of rats that lived for 2-7 days after exposure to a soman dose of 1.3 x LD_{50}. The rats were treated with 3 medical therapies 20 minutes after seizure onset.

Group	Dose mg/kg	N	Neuropathology score			
			Piriform cortex	Hippocampal CA1	Amygdala	Total
HI-6 Procyclidine MPEP	125 20 30	3	2.0 (0.5-2)	0.0 (0-3)	2.0 (0-2)	2.0 (1-7)
HI-6 Procyclidine DCG-IV	125 20 4	3	3.0 (0.5-3)	0.5 (0-0.5)	1.0 (1-2)	4.0 (1.5-4.5)
HI-6 Procyclidine Levetiracetam	125 20 50	6	1.8 (0-2)	0.0 (0-0)	0.3 (0-2)	2.0 (0-4)

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Table 6

Median (range) neuropathology scores of rats that lived for 2-7 days after exposure to a soman dose of 1.3 x LD_{50}. The rats were treated with 4 medical therapies 20 minutes before challenge with soman.

Group	Dose mg/kg	N	Neuropathology score			
			Piriform cortex	Hippocampal CA1	Amygdala	Total
HI-6 Procyclidine MPEP	125 20 30	2	1.0 (0-2)	0.5 (0-1)	0.8 (0-1.5)	2.3 (1.5-3)
HI-6 Procyclidine DCG-IV	125 20 4	5	1.0 (0-4)	0.0 (0-1)	1.5 (0-3)	3.0 (0.5-7)
HI-6 Procyclidine Levetiracetam	125 20 50	6	0.0 (0-1)	0.0 (0-0)	0.0 (0-0.5)	0.0[a] (0-1)
HI-6 Procyclidine Levetiracetam	125 6 50	5	1.5 (0-4)	0.5 (0-3.5)	1.0 (0-2)	3.0 (0.5-7)

[a] Significantly different from the HI-6, procyclidine, DCG-IV and HI-6, procyclidine (6 mg/kg), levetiracetam groups with one-way Kruskal-Wallis ANOVA and Dunn's multiple comparison test ($P<0.05$).

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