Pretreatment and prophylaxis against nerve agent poisoning: Are undesirable behavioral side effects unavoidable?

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Running title: Pretreatment and side effects

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Abstract

The threat of chemical warfare agents like nerve agents requires life saving measures of medical pretreatment combined with treatment after exposure. Pretreatment (pyridostigmine) may cause some side effects in a small number of individuals. A comprehensive research on animals has been performed to clarify effects on behavior. The results from these studies are far from unambiguous, since pyridostigmine may produce adverse effects on behavior in animals in relatively high doses, but not in a consistent way. Other animal studies have examined the potential of drugs like physostigmine, galantamine, benactyzine, trihexyphenidyl, and procyclidine, but they all produce marked behavioral impairment at doses sufficient to contribute to protection against a convulsant dose of soman. Attempts have also been made to develop a combination of drugs capable of assuring full protection (prophylaxis) against nerve agents. However, common to all combinations is that they at anticonvulsant doses cause behavioral deficits. Therefore, the use of limited pretreatment doses may be performed without marked side effects followed by post-exposure therapy with a combination of drugs.

Keywords: Nerve agents; Pharmacological protection; Enzymatic protection; Behavioral side effects

1. Introduction

Organophosphates called nerve agents are considered to be the most toxic among all chemical weapons. Nerve agents can create a substantial threat on the battlefield, and in the hands of terrorist groups they will represent a threat to civilians. The nerve agents were originally synthesized during the 1930s in Germany in order to obtain more effective pesticides based on organophosphorus compounds. Some of these agents, however, turned out to be too toxic for their original purpose. The organophosphorus nerve agents are highly potent inhibitors of the enzyme acetylcholinesterase (AChE) that hydrolyzes acetylcholine (ACh). Accumulation of ACh in the synaptic cleft results in over-stimulation of muscarinic and nicotinic receptors. This increased cholinergic activity can affect all organ systems. The toxic signs include miosis, hypersalivation, respiratory distress, tremor, seizures/convulsions, coma, and death (Taylor, 2001).

Acute exposure to nerve agent, particularly by inhalation, requires immediate medical treatment. Compared with other agents, the time window of opportunity for therapeutic intervention is very limited following nerve agent intoxication, in particular after exposure to soman vapor. In the case of military deployment, medical pretreatment represents an option to be considered, but might be of minor relevance for civilian populations. Pretreatment drugs are administered prior to nerve agent and are part of a continuum requiring post-exposure treatment (partial protection). The term prophylaxis denotes drugs applied before exposure to intoxication, but are not supposed to be followed by adjuvant therapy (full protection). The purpose of a pretreatment is to provide a more efficacious impact of post-poisoning therapy. Prophylactic treatment is intended to ensure anticonvulsant and life preserving effects when/if no post-exposure therapy is available. It may, however, occur unsafe to give medical pretreatment to healthy persons. It is therefore crucial that the countermeasures administered do not by themselves impair normal functions.

During the cold war and prior to entry into force of The Chemical Weapons Convention in 1997, use of large quantities of chemical warfare agents, in particular soman with its brief onset to the aging process, was a real threat (Aas, 2003). Pretreatment against nerve agents was introduced in most armies to be combined with a post-poisoning treatment to increase survival. Since the threat of large scale use of chemical warfare weapons has decreased, but potential for use against civilians has increased, the prevalent opinion has been to reduce reliance on pretreatment against nerve agent. Pretreatment against nerve agents can be obtained by the use of a reversible AChE inhibitor (pyridostigmine) 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, pralidoxime-2-chloride (2-PAM), 1-[([4-aminocarbonyl)pyridino]methoxy)methyl]-2-[(hydroxyimino)methyl]pyridinium (HI-6)), an anticholinergic (atropine), and a benzodiazepine (diazepam, avizafone, midazolam) combined with carbamate (pyridostigmine) pretreatment (Aas, 2003). Atropine is, however, considered as the most important component of the therapy (Newmark, 2004). Such treatment regimens can reduce immediate lethality, but they do not attenuate the occurrence of nerve agent-induced seizure activity and concomitant convulsions if treatment is delayed (McDonough and Shih, 1997). Such seizures rapidly progress to status epilepticus, a condition that is strongly associated with brain damage and mortality in experimental animals (Shih et al., 2003).

Pretreatment with pyridostigmine was used on a large scale during the “Operation Desert Storm” in Kuwait in 1991. Neurocognitive deficits, neuroendocrine alterations as well as anxiety and mood alterations in Gulf War veterans have been attributed to the use of pyridostigmine and pesticides during deployment (Research Advisory Committee on Gulf

War Veterans' Illnesses, 2008). However, Institute of Medicine of the National Academies is of a different opinion. In its report of 2010 (Gulf War and Health), the Institute disagrees with the Research Advisory Committee's conclusion and maintains that current available evidence is not sufficient to establish a causative relationship between chronic multi symptom illness and any specific drug, toxin, plume, or other agent, either alone or in combination. The US Food and Drug Administration (FDA) has summarized the existing knowledge and concluded that despite a long history of pyridostigmine being used in the treatment of myasthenia gravis in humans, no evidence of long-term health effects has emerged to date (FDA, 2009).

The purpose of the present review was to examine whether pretreatment or prophylaxis against nerve agent intoxication can be administered without causing adverse effects on the recipients. This process was performed by reviewing animal studies of pretreatments and prophylaxes against nerve agent and their potential effects on cognitive behavior. A critical evaluation was made of the ability of various behavioral tests to reveal subtle cognitive deficits. The results from relevant studies presented in sections 2-6 are discussed in view of additional information in section 7. Comparisons of drug doses for animals and humans are made in the discussion section (7).

2. Pyridostigmine

Pretreatment with the carbamate pyridostigmine is a well-established method to enhance the efficacy of post-exposure therapy against nerve agent intoxication in the armed forces in a number of nations. A tablet (30 mg) of pyridostigmine bromide is supposed to be taken every 8 hour by the service personnel. The rationale behind this use is that carbamate occupies a portion of the available AChE (15-40 % of the erythrocyte AChE) and renders it inaccessible to nerve agents in the blood, since nerve agents only bind to unprotected enzyme (Dirnhuber et al., 1979; Leadbeater et al., 1985). The AChE that has been reversibly inhibited

by pyridostigmine spontaneously decarbamoylates, and the enzyme is again able to hydrolyze ACh. The quaternary carbamate pyridostigmine does not readily cross the blood-brain barrier (BBB), even at a dose that inhibits blood AChE, pyridostigmine does not substantially change brain AChE activity in rats (Amourette et al., 2009), guinea pigs (Lallement et al., 1998), or mice (Grauer et al., 2000). Hence, pyridostigmine only protects the peripheral nervous system, and alone it does not protect against nerve agent poisoning.

In a number of studies, pyridostigmine has been reported to have no detrimental physiological or psychological effects on military personnel or healthy volunteers when given 30 mg/8-h. Effects of pyridostigmine on aircrew performance has been examined in several studies. Twenty-one C-130 pilots flew 2 familiarization and 4 data flights in simulator. The results show that the aircrews successfully completed their assigned mission without being affected by pyridostigmine (Gawron et al., 1990). In a similar study, 10 pilots performed normally in flight simulator when the whole blood AChE level was reduced by 29% of control (Israeli et al., 1990). Selected visual functions were measured in 4 aviator candidates. Under the influence of pyridostigmine, the subjects` visual abilities were not compromised. Only refractive error and pupil diameter were significantly different (Wiley et al., 1992).

Increased arousal and attention have been demonstrated following administration of pyridostigmine in healthy volunteers. Results from recordings of psychomotor performance and visual function show that visual-motor coordination was not impaired for each session, but with pooling the data there was a drug effect. The observation suggests an increase in central arousal (Borland et al., 1985). Performances in a standardized test battery show improved reaction time on tests of memory and attention when AChE activity was reduced by 36% of control. Under non-stressful laboratory conditions pyridostigmine does not seem to cause adverse effects on physiological and psychological performance (Cook et al., 2002).

Effects of pyridostigmine on physiological responses to heat and exercise have been examined in several studies. In one study, 5 men underwent heat and exercise stress tests at 35º C by 25 min treadmill walks under various conditions of hypohydration. The results show that pyridostigmine has little effect on physiological responses to moderate exercise-heat stress (Wenger and Latzka, 1992). Side effects of chronic pyridostigmine administration were studied in 7 male soldiers performing moderate-intensity exercise in a desert environment during 7 consecutive days. It was concluded that pyridostigmine does not negatively impact soldiers' ability to perform physical work in a desert environment (Cook et al., 1992). Effects of pyridostigmine, protective gear, and heat-exercise exposure on psychomotor performance and subjective sensations were investigated in 8 healthy male volunteers. Multiple complaints of subjective discomfort arose from wearing the protective garment, but no major cognitive decrements in a multiple-stress state of chemical alertness were measured (Arad et al., 1992). Most post-1991 studies were carried out to explain what has been described as the Gulf War Syndrome.

In one study, a brief questionnaire covering broad topics of questions including chemical defense and antidotes was given to 148 soldiers, over two-thirds of whom were aviators, at the conclusion of "Operation Desert Storm". Questions were asked about chemical defense, work/rest schedules, an aspect of pharmacological support, and heat stress/physical training during the operations. Some of the most noteworthy findings concerned side effects related to pyridostigmine. Of the 89% of the sample that used pyridostigmine one third reported nausea, diarrhea, abdominal cramps, muscle cramps, and muscle weakness (Caldwell, 1992). In 2 other studies, pyridostigmine has also been administered to soldiers under a chemical warfare threat during the Gulf War. A total of 41,650 US soldiers received 30 mg orally every 8 h for 1 to 7 days. About half of the population noted physiological changes that were not incapacitating, but it turned out that they had a higher incidence of

minor intestinal and urinary symptoms than expected (Keeler et al., 1991). A study comprising 213 Israeli soldiers used the standard regimen for pyridostigmine during 24 h before completing a questionnaire. The most frequent symptoms were nonspecific and included dry mouth, general malaise, fatigue, and weakness. The symptoms appeared around 1.6 h after the medication and recurred after each intake. In the situation of combat stress, the frequency and severity of subjective symptoms following administration of pyridostigmine were increased compared with previous studies under peacetime (Sharabi et al., 1991).

In the results from animal studies, behavioral effects of pyridostigmine seem to be more pronounced than in data from human studies. Although it has been stated that pyridostigmine does not pass the BBB, repeated stress (avoidance conditioning) in combination with pyridostigmine (1.5 mg/kg/day peroral for 12 days) (blood AChE activity 54.6% of control) has been demonstrated to change expression of genes involved in learning and memory in the hippocampus of rats. No effects of stress or pyridostigmine alone (blood AChE activity 53.1% of control) were seen on the gene expression (Barbier et al., 2009). Furthermore, evidence has been presented that pyridostigmine can modulate brain activity in mice. Activation of c-fos in the hippocampus, thalamus, and piriform cortex was seen to follow exposure to a stress situation (electric foot shock) combined with administration of 2 doses a day (0.2 mg/kg subcutaneously) for 12 days. Such activation of c-fos was seen in a lower degree in mice that received pyridostigmine alone (Taysse et al., 2005).

Different opinions have been presented whether the BBB increases permeability during stress (Amourette et al., 2009). In the latter study, ^{3}H-pyridostigmine (i.v. 1.22 nmole/rat, injection volume 100 µl) was used as a tracer in rats to evaluate BBB breakdown. Because of the heterogeneity of BBB permeability, the passage of pyridostigmine was determined in a number of brain areas. The results show that brain micro-punches and coronal cryosections do not reveal any radioactivity in rats chronically stressed and treated with

pyridostigmine (1.22 nmole/rat intravenously/day for 12 days). Accordingly, no changes in AChE activity was noted in any of the regions examined in the forebrain and cerebellum. The AChE levels in whole blood and red blood cells were reduced by 45% and 35% of control, respectively (Amourette et al., 2009).

Administration of pyridostigmine alone can cause behavioral changes in rodents. In an early investigation (Wolthuis and Vanwersch, 1984), it came as a surprise that pyridostigmine, which hardly passes the BBB, can cause behavioral disturbances at relatively low doses. Pyridostigmine (<10% LD_{50} (2.6 mg/kg) intraperitoneally) impairs shuttle-box learning, open field behavior, and hurdle-stepping task in rats. It has been examined in a simple operant visual discrimination task whether deficits produced by pyridostigmine alone are mediated by central and/or peripheral cholinergic mechanisms. Use of atropine (central effect) or methylatropine (peripheral effect) together with pyridostigmine (12 mg/kg perorally) in rats show that the debilitating effects of pyridostigmine on the operant behavior are primarily antagonized by the stimulation of peripheral muscarinic receptors by methylatropine (Liu, 1991). In the 2 studies cited above, no AChE measures were presented.

In a subsequent study (Servatius et al., 1998), it was examined whether pyridostigmine (1.3, 2.6, or 7.2 mg/kg perorally) may affect central or peripheral mechanisms of acoustic startle responses in Wistar-Kyoto (WKY; the normotensive control strain) rats with inherently low butyrylcholinesterase (BuChE) activity compared with Sprague-Dawley (SD) rats with normal BuChE activity. The results show that the WKY, but not the SD rats, display a delayed-onset, persistently exaggerated startle response after 7 consecutive days with pyridostigmine. Inhibition of plasma AChE activity was reduced by about 20% at day 7 in both SD and WKY rats. The startle response was still evident 22 days after the end of pyridostigmine treatment in the WKY rats. Both the duration and magnitude of the enhanced startle responses were related to the dosage of pyridostigmine. Treatment of the rats for the

second time with pyridostigmine, 7 weeks after the end of the first treatment, resulted in elevated startle response that appeared sooner and dissipated faster than was evident after the first pyridostigmine treatment. Because BuChE works as a scavenger for pyridostigmine, it is suggested that pyridostigmine may influence Central Nervous System (CNS) function in rats with low BuChE activity (Servatius et al., 1998).

Administration of pyridostigmine (1.3 mg/kg perorally for 15 days, BuChE activity was 96% of control) was assessed in rats by a battery of sensorimotor ability tests. Animals treated with pyridostigmine alone show deficits in beam-walk score as well as beam-walk time compared with controls. Pyridostigmine causes inhibition of midbrain AChE activity (40%), but no change in enzyme activity in the brainstem, cortex, or cerebellum. Increased ligand binding for M2 muscarinic ACh receptors was seen in the cortex. Plasma BuChE activity was reduced by only 4%. It is concluded that pyridostigmine leads to neurobehavioral deficits and region-specific alterations in AChE activity and ACh receptors (Abou-Donia et al., 2001). The findings in the latter study imply that pyridostigmine may affect the AChE activity in the midbrain, an area not examined in the study of Amourette et al. (2009).

Response acquisition in an operant task has been tested in rats exposed to pyridostigmine (1.5 mg/kg perorally) for 7 consecutive days. Pyridostigmine delays response acquisition in male and female rats, and results in higher response rates on the inactive lever in female rats (van Haaren et al., 2000). Impairment of operant performance was not observed at a pyridostigmine dose of 3 mg/kg, but was observed in a dose-dependent manner at 10 and 30 mg/kg perorally in a fixed-ratio and fixed-interval task (van Haaren et al., 2001). No measures of AChE activity were presented in the 2 latter studies.

3. Physostigmine and related AChE inhibitors (Alzheimer drugs)

Because pyridostigmine poorly penetrates into the brain, physostigmine which inhibits central AChE activity has been suggested as a replacement. Physostigmine effectively protects against neurological symptoms and severe behavioral incapacitation in animals which are often seen to follow organophosphate intoxication (Leadbeater et al., 1985). The problem, however, with AChE inhibitors that readily cross the BBB, is that they can cause undesirable behavioral side effects at high doses. Administration of physostigmine (0.6 mg/kg subcutaneously) to guinea pigs results in impaired shuttle-box performance and increased acoustic startle response. The shuttle-box impairment is antagonized by scopolamine (0.1 mg/kg subcutaneously), but the startle response deficit is enhanced by the combination with scopolamine (Philippens et al., 1996). Behavioral side effects of physostigmine and scopolamine have been demonstrated to offset each other when dogs are protected against 2.5 x LD_{50} of sarin and full recovery is seen after a brief period of incapacitation (Meshulam et al., 2001), but exceptions are observed in addition to the one cited above. For instance, scopolamine (0.15 mg/kg) given alone causes reduced preference for novelty in rats, but the reduction is further increased when scopolamine is combined with physostigmine (0.1 mg/kg), which alone does not affect the preference response (Myhrer et al., 2004a).

Alternatives to physostigmine have been investigated. The half-life of physostigmine is relatively short; 17 min in plasma of rats (Somani and Khalique, 1986) and 30 min in plasma of humans (Walter et al., 1995). For this reason, the Alzheimer drugs donepezil, galantamine, and huperzine with relatively long half-lives are drugs that could be studied as possible alternative prophylactic AChE inhibitors against nerve agent intoxication (Aas, 2003). Several of the Alzheimer drugs have, moreover, too high affinity to the AChE enzyme. The enzyme regeneration is therefore too slow. Donepezil is a partial reversible centrally acting and highly selective inhibitor of the AChE (Sugimoto et al., 2002). Galantamine is another drug approved for treatment of mild to moderate Alzheimer's disease. The drug is a

reversible AChE inhibitor that crosses the BBB (Corey-Bloom, 2003). Huperzine is a slow, reversible inhibitor of the AChE at both peripheral and central levels (Ashani et al., 1992). This drug is used for treatment of Alzheimer's disease in China (Wang et al., 2000).

In a study of rats, physostigmine (0.1 mg/kg), galantamine (3 mg/kg), huperzine (0.5 mg/kg), or donepezil (2.5 mg/kg) were given intraperitoneally, and the effects were tested in the novelty task (Fig. 1). The results show that only galantamine produces a mild cognitive deficit in terms of reduced preference for novelty. However, for all groups of rats a tremendous depression of locomotor activity and rearing was seen. Even if the decline in activity was rather uniform for all groups, galantamine reduces locomotion and rearing even more than the other AChE inhibitors (Myhrer et al., 2010). Effects of AChE inhibitors were found to be rather modest on cognition, but motor functions are severely impaired by this class of drugs.

4. Antiparkinson drugs and cognitive impact

The group of antiparkinson drugs including benactyzine, biperiden, caramiphen, procyclidine, and trihexyphenidyl (Gao et al., 1998; Vargas et al., 1998) possesses potent anticonvulsant properties against nerve agent-induced seizures, since these drugs exert both cholinergic and glutamatergic antagonism in mice and rats (Gao et al., 1998; McDonough and Shih, 1995; Raveh et al., 2002). Antiparkinson agents are therefore well suited as anticonvulsants against soman-evoked seizures and have been used in animal studies on pretreatment or prophylaxis against nerve agent poisoning.

It is important to use particularly sensitive tests in revealing cognitive dysfunctions to ensure that drugs used as pretreatments or prophylactics do not by themselves cause impairment of cognitive capability. Exploration of a discrete novel object is one form of inquisitive activity frequently seen among rats. This activity appears as a strong preference for

novelty, the recognition of which is probably based on polymodal information (Berlyne, 1960). The novelty test has proven very sensitive in uncovering cognitive deficits following selective disruption of neuronal connections in the temporal region of rats (Myhrer, 1988, 1989). A brief description of the test is given in the legend of Fig. 1. The novelty test was used to make a comparative assessment of potential cognitive effects of fixed doses of benactyzine (0.3 mg/kg), biperiden (0.11 mg/kg), caramiphen (10 mg/kg), procyclidine (3 mg/kg), and trihexyphenidyl (0.12 mg/kg) separately and each in combination with physostigmine (0.1 mg/kg) (Myhrer et al., 2008). The results show that benactyzine, caramiphen, and trihexyphenidyl reduce rats` innate preference for novelty, whereas biperiden and procyclidine do not. When benactyzine, caramiphen, and trihexyphenidyl were combined with physostigmine the cognitive impairment disappeared. This counteracting effect, however, causes changes in locomotor and rearing activity not seen by each drug alone. AChE inhibitors and anticholinergics used as prophylactics can offset each other, but as previously shown in rats a very potent anticholinergic (scopolamine 0.15 mg/kg) results in cognitive deficits that become even worse by coadministration with physostigmine of 0.1 mg/kg (Myhrer et al., 2004a). Among the drugs tested in the study presented above, procyclidine (3 mg/kg) appears to be a robust anticonvulsant with few cognitive side effects (Myhrer et al., 2008).

Both cholinergic and glutamatergic antagonists produce cognitive malfunction in a number of behavioral tasks (Myhrer, 2003). Hence, it appears somewhat intriguing that coadministration with physostigmine completely compensated for the cognitive deficits caused by some antiparkinson drugs when administered alone (Myhrer et al., 2008). This finding might suggest that the deleterious impact on behavior was most prominently exerted by cholinergic antagonism. Rats injected with the glutamatergic N-methyl-D-aspartic acid (NMDA) antagonist 3-amino-1-hydroxy-2-pyrrolidinone (HA-966) (30 mg/kg) display

reduced preference for novelty in the novelty task (Myhrer, 1999). However, impairment of preference for novelty that has spontaneously recovered 2-3 weeks after a combination of 2 denervations in the temporal region (fiber connections between temporal and entorhinal cortices plus hippocampal perforant path) is more effectively reactivated by atropine than HA-966 (Myhrer, 1999). Thus, normal performance in the novelty task might potentially be more vulnerable to cholinergic than glutamatergic antagonism.

5. Prophylactic regimens and behavioral side effects

The inclusion criteria for this section have been prophylactic regimens demonstrated to protect effectively against nerve agent intoxication and additionally have been tested for behavioral side effects. In our laboratory, a number of potential prophylactic regimens against soman-induced seizures and lethality have been examined. In order to determine whether they possess ability to act as acceptable prophylactic measures they have systematically been tested for possible side effects in the novelty test (Table 1).

Four prophylactic therapies previously shown to exert varying degrees of protection against a convulsant dose of soman in rats (Myhrer et al., 2013a, 2013b) have been assessed for potential behavioral side effects in our novelty test (Myhrer et al., 2014). In one experiment, the combination of HI-6 (125 mg/kg), scopolamine (1 mg/kg), and physostigmine (0.1 mg/kg) (termed the physostigmine regimen) or HI-6 (125 mg/kg), levetiracetam (50 mg/kg), and procyclidine (20 mg/kg) (termed the procyclidine regimen) were tested. In another experiment, the metabotropic glutamate modulators 2S,2'R,3'R-2-(2',3'-dicarboxycyclopropyl)glycine (DCG-IV) (4 mg/kg) and 2-methyl-6-(phenylethynyl)pyridine (MPEP) (30 mg/kg) were each tested in combination with HI-6 (125 mg/kg) and procyclidine (20 mg/kg) (termed the DCG-IV regimen and the MPEP regimen, respectively). The results show that the physostigmine and procyclidine regimens both produce severe cognitive

impairment (lack of preference for novelty) and reduce locomotor and rearing activities. The DCG-IV and MPEP regimens cause milder deficits on the same behavioral measures. Some relations are seen between prophylactic capacity and degree of behavioral side effects. The relatively high dose of procyclidine (anticholinergic and antiglutamatergic) required for prophylactic efficacy may have played a major role for the side effects of the regimens in which the drug was used (Myhrer et al., 2014).

The dose of procyclidine can be radically lowered if the drug is used with physostigmine as an additional treatment. Physostigmine (0.1 mg/kg) in combination with procyclidine doses of 1, 3, or 6 mg/kg can effectively prevent the development of seizure when the soman doses are 1.3, 1.6, or 2 x LD_{50}, respectively. Physostigmine (0.1 mg/kg) and procyclidine in a dose of 1 mg/kg do not prevent onset of seizures when the soman dose is 1.6 x LD_{50} (Myhrer et al., 2004a). Behavioral side effects of the above combinations with physostigmine and procyclidine have been tested in our novelty test. Physostigmine (0.1 mg/kg) combined with procyclidine (6 mg/kg) causes a marked deficit in preference for novelty. A much milder deficit is seen when physostigmine is combined with lower doses (1 or 3 mg/kg) of procyclidine. The latter combinations also have milder adverse impact on the rats' interest in the test environment and activity measures than physostigmine combined with 6 mg/kg of procyclidine (Myhrer et al., 2004a). As seen from the studies cited, the options can, in extreme cases, either be heavy medication on the cost of cognitive capability or lighter medication and subsequent potential problems when intoxication occurs. In the latter case, post-poisoning treatment was given if the prophylaxis turned out to be insufficient. The combination of physostigmine (0.1 mg/kg) and procyclidine (1 mg/kg) does not protect against a soman dose of 1.6 x LD_{50}. However, subsequent treatment with scopolamine (0.5 or 1 mg/kg) immediately after (3 min) seizure onset shows that only the highest dose of scopolamine produces a reliable termination. If scopolamine (1 mg/kg) is given later (10 min)

after onset of seizures, no effects are achieved. The sustained seizures can subsequently be treated with diazepam (10 mg/kg) and pentobarbital (30 mg/kg) and finally be terminated 25 min after onset (Myhrer et al., 2004b).

Donepezil (2.5 mg/kg) in combination with procyclidine (3 mg/kg) protects effectively against seizures and death when rats are exposed to a soman dose of 1.6 x LD_{50} (Haug et al., 2007). Rats treated with the same combination of drugs at the same doses display a mild cognitive deficit, but locomotor and rearing activities are radically depressed in the novelty test (Myhrer et al., 2010).

6. Pretreatment and prophylaxis for humans in use or proposed

Pretreatment with pyridostigmine and follow-up treatment consisting of atropine, an oxime, and a benzodiazepine (for some nations) after exposure is a standardized regimen for protection against nerve agent poisoning. Most nations use pyridostigmine and atropine, but they differ in their choice of oxime and benzodiazepine. Of oximes, obidoxime is in use by Finland, Germany, Norway, and the Netherlands. Canada and Sweden introduced HI-6 several years ago, United Kingdom uses pralidoxime mesylas (P2S), and USA uses 2-PAM. Of benzodiazepines, some countries use diazepam. United Kingdom and The Netherlands use avizafone, which is rapidly converted to diazepam in the body (Aas, 2003). It is proposed by several NATO nations to choose a regimen consisting of pyridostigmine, atropine, HI-6, and diazepam or avizafone.

A prophylactic treatment with pyridostigmine, benactyzine, and trihexyphenidyl in tablets has been designated PANPAL (Prophylactic Antidote against Nerve Paralytic Agent) and introduced into the Czech Army (Fusek et al., 2000). The combination with anticholinergics allows a higher dose of pyridostigmine to be applied, and effects of the

mixture have been examined in a number of animal studies, some of which will be presented in the following.

Rats pretreated intramuscularly with PANPAL consisting of pyridostigmine (0.75 mg/kg), benactyzine (16 mg/kg), and trihexyphenidyl (6.3 mg/kg) were exposed to a sublethal dose of soman (60 µg/kg) 30 min later. The treatment was effective in alleviating the disturbed respiratory and circulatory function without increasing soman-inhibited AChE activity in several brain areas (Kassa and Fusek, 1997). In a subsequent study, however, when the rats were challenged with a supralethal dose of soman (2 x LD_{50}) after the same treatment as used above (PANPAL), they all died within 30 min. On the other hand, when the rats were pretreated with PANPAL and posttreated with the antidotes HI-6 (13,8 mg/kg) and benactyzine (3.7 mg/kg) 30 sec after soman poisoning (2 x LD_{50}), respiration as well as circulation were completely restored and the rats survived for at least 120 min after intoxication (Kassa and Fusek, 1998).

A comparison has been made between the efficacies of PANPAL and a Bulgarian prophylaxis of pyridostigmine (0.75 mg/kg) and biperiden (2.5 mg/kg) when both prophylaxes were combined with antidotal posttreatment by HI-6 (15.6 mg/kg) and atropine (10 mg/kg) in rats exposed to a sublethal dose of soman (54 µg/kg). The combination of PANPAL or the Bulgarian mixture and antidotal treatment was more effective in elimination of soman-induced neurotoxicity at 24 h than pretreatment or antidotal treatment alone. However, the Bulgarian mixture turned out to be more efficacious than PANPAL (Kassa et al., 2003). PANPAL administered perorally before intoxication by tabun is much more effective than pyridostigmine alone in rats and mice (Kassa and Vachek, 2002). The combination of PANPAL pretreatment and antidotal posttreatment (HI-6, atropine) appears to be slightly more effective in eliminating tabun-induced neurotoxicity in rats after 24 h than PANPAL or antidotal treatment alone (Krejčová and Kassa, 2003). The prophylactic ability of

PANPAL is well documented, but potential cognitive side effects of the regimen do not seem to have been examined in animals and published.

PANPAL has been taken into use in the Czech Army and also the Slovak Army (Bajgar et al., 2009). The prophylactic use of antidote tablets (benactyzine total 8 mg, trihexyphenidyl total 6 mg, pyridostigmine total 35 mg) has been tested on healthy volunteers and no significant side effects have been reported (Fusek et al., 2000). Transdermal prophylactic antidote against nerve agents (TRANSANT) containing a patch impregnated with HI-6 has been clinically tested and found to have no harmful effects and has been introduced into the same armies (Bajgar et al., 2009).

A pretreatment consisting of physostigmine and hyoscine (scopolamine) able to reduce the initial effects of intoxication and extend the time available for post-poisoning intervention has been suggested by British investigators. Continuous pretreatment of guinea pigs by using subcutaneously implanted mini-osmotic pumps delivering physostigmine and hyoscine up to 13 days provides complete protection against lethal effects of soman (equivalent to LD99) and minimizes incapacitation and weight loss (Wetherell, 1994). In an extension of the latter study, physostigmine and hyoscine were administered for 6 days before exposure to nerve agent. The pretreatment was shown to protect well against subcutaneously injected lethal dose (1.05-1.25 x LD_{50}) of tabun, sarin, soman, cyclosarin, or VX (Wetherell et al., 2002).

In a study of marmosets, the animals were pretreated with either pyridostigmine or physostigmine and hyoscine by way of implanted mini-osmotic pumps for 13 days before they were challenged with a lethal dose of either sarin or soman. All monkeys pretreated with pyridostigmine needed adjuvant treatment with atropine, the oxime P2S, and avizafone to survive. In the group treated with physostigmine and hyoscine, only 2 animals of those exposed to sarin (N=8) or soman (N=8) needed adjuvant treatment. No adverse effects were

seen on performance of a visually guided reaching test by either pretreatment during the period before nerve agent exposure (Scott, 2007).

Two-way active avoidance (shuttle-box) has been used to test the performance of guinea pigs affected by physostigmine and/or scopolamine. Physostigmine injected subcutaneously at doses of 0.3, 0.6, or 1.2 mg/kg causes dose-dependent impairment of shuttle-box performance. However, scopolamine at a dose (100 µg/kg) that by itself does not affect the avoidance behavior counteracts the physostigmine impairment (Philippens et al., 1992). Behavioral side effects of the combination of physostigmine and hyoscine in guinea pigs have not been examined by the British group, but they have studied behavioral performance in marmosets treated with the combination of physostigmine and hyoscine (Muggleton et al., 2003). Common marmosets were trained to perform a two-choice discrimination serial reversal task. The subjects received a sublethal dose of either soman or sarin after 2 weeks of pretreatment with physostigmine and scopolamine via osmotic pumps. The results show no effects of the drugs on task accuracy or response rates, not even when poisoned with nerve agent. However, the dose of scopolamine used is equivalent to an acute dose of 0.009 mg/kg and the doses of soman and sarin induced only minor transient signs of intoxication. According to Muggleton et al (2003), a likely explanation of their negative behavioral results is attributable to the low dose of scopolamine that is lower than doses shown to affect cognitive performance in marmosets (e.g ., 0.06 mg/kg; Harder et al., 1998).

Based on animals studies referred to above the pretreatment with physostigmine and hyoscine, which improve the efficacy of subsequent therapy, has been brought to the level of clinical trials in the United Kingdom (Scott, 2007). The need for continuous delivery of both physostigmine and hyoscine because of their relatively short half-lives is intended to be solved by the use of transdermal patch. When co-administered, pharmacological antagonism between the 2 drugs mitigates the potentially adverse effects of each and thus, selection and

optimization of dosage regimens continues to represent both an opportunity and a technical challenge (Scott, 2007). A key challenge for this approach is to deliver controlled doses of the 2 drugs which provide effective protection, but are below the levels which lead to adverse effects (Tattersall, personal communication, April 2015).

Rivastigmine is a reversible AChE inhibitor that crosses the BBB and is approved for the treatment of Alzheimer's disease and dementia related to Parkinson's disease (cf., Lavon et al., 2015). This drug has recently been tested for safety as pretreatment against nerve agent poisoning in healthy volunteers in Israel. Three groups completed 3 treatment periods: 0, 1.5, and 3 mg twice a day, for a total of 5 intakes of rivastigmine. Mean maximal AChE inhibition after the 1st and 5th 1.5 mg dose intake was 6.9%±2.7 and 10.7%±2.6, respectively. Mean maximal enzyme inhibition after 1st and 5th 3 mg dose intake was 20.2%±3 and 27.9%±2.4, respectively. From this comparison study, it emerged that the unpredictable nature of the adverse effects, the non-linear pharmacological property, wide variety between subjects, and the negative influence on some cognitive functions limit the potential use of rivastigmine as pretreatment against nerve agent poisoning in high-performance operational population (Lavon et al., 2015).

As an alternative approach to protect against nerve agent intoxication, attempts have been made to identify human proteins that can remain stable in circulation for long periods of time and exert detoxification by acting as biological scavengers for nerve agents. Two different ways have been used: specific enzymes binding nerve agent (stoichiometric scavengers) and enzymes hydrolyzing nerve agent (catalytic scavengers).

The use of plasma-derived human butyrylcholinesterase (HuBuChE) alone has been shown not only to increase survivability after exposure (i.v.) to multiple lethal doses of tabun, VX, sarin, or soman, but also to alleviate manifestation of toxic symptoms in mice and rats without the need for additional post-exposure therapy (Raveh et al., 1993). In a subsequent

study, the same group reported similar protective action against a soman dose of 3.3 x LD_{50} or a VX dose of 2.1 x LD_{50} i.v. in rhesus monkeys. A marked protection was also seen against soman-induced behavioral deficits in a spatial discrimination task (Raveh et al., 1997). In another study, guinea pigs were protected against a cumulative 5.5 x LD_{50} dose s.c. of either soman or VX. At 7 or 14 days after nerve agent challenge, histopathology studies were made and no signs of abnormal tissue were found. Also cynomolgus monkeys were protected in a similar way against 5.5 x LD_{50} of soman (Lenz et al., 2005). Inhalation toxicity makes up a more realistic simulation exposure to violate nerve agent than systemic administration. HuBuChE-treated guinea pigs are very well protected against poisoning by high dose of soman vapor (Allon et al., 1998). Similar results have recently been shown with HuBuChE against high doses of sarin vapor in minipigs (Saxena et al., 2015).

Because plasma derived HuBuChE is based on outdated human blood and the supply can variably be scarce, an alternative has been the use of goat milk derived recombinant HuBuChE. Experiments with recombinant HuBuChE from transgenic goat milk have yielded results in guinea pigs similar to those described for plasma derived material (Cerasoli et al., 2005). HuBuChE produced by Baxter Pharmaceuticals, using outdated human blood has been a source of material. It has also been shown that polyethylene-glycol conjugated recombinant human AChE serves as an effective bioscavenger against a soman dose of 2.5 x LD_{50} (i.v.) in mice (Kronman et al., 2007). Experiments with guinea pigs have shown that HuBuChE can be an effective therapy following percutaneous exposure to VX (Lenz et al., 2010). Such HuBuChE scavengers can be used by military personnel as well as civilian first responders, if this protection becomes available in the future.

Stoichiometric scavengers require relatively large quantity to neutralize nerve agent, whereas catalytic scavengers would in smaller quantities produce the same or even greater extent of protection against nerve agent poisoning (cf. Masson, 2015). The naturally occurring

human serum enzyme, paraoxonase-1, has the capacity to catalyze the hydrolysis of nerve agents, but the protective efficacy is modest (Lenz et al., 2007). A number of catalytic enzymes have been examined, and they are indicative of therapies for use in animals and eventually in humans. There is, however, limited information on the efficacy of catalytic bioscavengers in non-anesthetized animals. Treatment of mice with parathion hydrolase purified from *Pseudomonas sp.* (15 or 22 µg/animal) ensures a protective ratio of 3.94 and 5.65, respectively against tabun poisoning without any need for post-exposure treatment (Raveh et al., 1992). Guinea pigs given (i.v.) 5 units (~ 600 µg of protein) of recombinant wild-type paraoxonase1 (PON1) 30 min before inhalation exposure to 1.2 x LCt_{50} of sarin or soman displayed reliably higher survival rate than control animals and minimal signs of toxicity (Valiyaveettil et al., 2011). Human paraoxonase-1 (HuPON1) has been proposed as a catalytic bioscavenger of nerve agents. *Trichoplusia ni* larvae expressed recombinant PON1 fails to protect guinea pigs against 2 x LD_{50} of tabun, sarin, soman, or cyclosarin. The results suggest that wild-type HuPON1 does not have sufficient catalytic activity to provide *in vivo* protection against nerve agents (Hodgins et al., 2013). Worek et al. (2014a) has studies the efficacy of the recombinant PON1 (rePON1) mutant llG1 (chimeric PON1 mutant) to prevent cyclosarin toxicity *in vivo*. Results in a guinea-pig model demonstrated that llG1 had a high catalytic efficiency. In another recent study, the small molecule β-cyclodextrin derivative bearing a pyridinium oximate in 6-position of one glucose (6-OxP-CD; 100 mg/kg) was injected in guinea pigs and is reported to prevent toxicity of cyclosarin in a dose of 2 x LD_{50}. The brain AChE activity is preserved, whereas erythrocyte AChE is not. A lower dose of 6-OxP-CD (50 mg/kg) reduces systemic toxicity of cyclosarin and prevents death in all animals. Hence, 6-OxP-CD may be considered as a potential small molecule scavenger to protect against nerve agents (Worek et al., 2014b). In addition, one more recent study by Worek et al. (2014c) has demonstrated the ability of a catalytic bioscavenger (engineered

phosphotriesterase mutant C23) to prevent systemic VX toxicity when given alone as single post-exposure medical treatment in guinea-pigs.

7. General discussion

7. 1. Cholinergic functions

According to the results presented in Section 2, administration of pyridostigmine to military or healthy volunteers under laboratory conditions has not been shown to impair physiological or psychological functions. Studies based on the reactions of soldiers on the battlefield show that under a chemical warfare threat administration of pyridostigmine can increase the frequency and severity of subjective symptoms (Caldwell, 1992; Keeler et al., 1991; Sharabi et al., 1991).

Pyridostigmine administered to experimental animals during diverse test conditions has apparently given inconsistent results. In rats, pyridostigmine alone impairs learning/memory and spontaneous performance in a number of behavioral tasks. In some of these studies, the detrimental effects of pyridostigmine were shown to be antagonized by the stimulation of peripheral muscarinic receptors by methylatropine (Liu, 1991), whereas CNS function was probably affected by pyridostigmine in rats with low BuChE activity (Servatius et al., 1998). When pyridostigmine is combined with stress, gene expression involved in learning and memory in the hippocampus is changed (Barbier et al., 2009), and activation of c-fos in several brain areas is seen, also with pyridostigmine alone, although in a more moderate extent (Taysse et al., 2005). However, the BBB appears to remain intact for pyridostigmine, even under sustained stress (Amourette et al., 2009).

Even if pyridostigmine is not centrally active, the increased cholinergic activity caused by pyridostigmine would be expected to improve or enhance functions, not impair as seen

from the above reports. However, enhancement of behavioral functions has been seen to follow administration of physostigmine. Physostigmine at a low dose (0.03 mg/kg) has been reported to improve passive avoidance performance in rats (Santucci et al., 1989). A physostigmine dose of 0.1 mg/kg improves radial maze performance (Ennaceur, 1998). Physostigmine at 0.125 mg/kg does not affect working memory in operant continuous delayed response, whereas higher doses decrease responding indiscriminately (Heise and Hudson, 1985). The impairing effect obtained with higher doses of physostigmine may be due to an excessive presence of acetylcholine leading to a blockade rather than a facilitation of neurotransmission (Ennaceur, 1998). Studies reporting improved performance following administration of pyridostigmine at low doses are hard to find. On the other hand, it has been reported that a peroral pyridostigmine dose of 3 mg/kg before each test session has no adverse effect on operant behavior, whereas higher doses (6-40 mg/kg) impair performance in a dose-dependent manner (Liu, 1992; Shih et al., 1991; van Haaren et al., 2001). There is, however, a big difference between the sites of action of physostigmine and pyridostigmine, since physostigmine passes the BBB and pyridostigmine does not and has only direct influence on peripheral processes. Pyridostigmine can act on a number of cholinergic synapses in the peripheral nervous system. It is possible for pyridostigmine to affect cholinergic transmission at autonomic ganglia and postganglionic parasympathetic terminals. In the lateroventral sympathetic ganglions, terminals of the preganglionic sympathetic fibers are cholinergic. Drinking and eating motivation are reduced by pyridostigmine (Liu, 1992; Van Haaren et al., 2001), and it has been suggested that such reactions are associated with cholinergic activation of the gastrointestinal tract (Liu, 1992).

Cholinergic terminals in the adrenal medulla stimulate adrenal chromaffin cells to release catecholamines that can indirectly affect the brain. It has been demonstrated significant potentiation of acute lethality in mice when pyridostigmine is combined with

selected fixed doses of drugs that operate within the sympathetic nervous system to stimulate β-adrenoceptors, antagonize α-adrenoceptors or, in the case of caffeine, cause release of catecholamines. Pretreatment with atropine or methylatropine diminishes or abolishes the lethal effect of these drug combinations. The ability of methylatropine to protect against these lethal effects suggests that a peripherally mediated toxic interaction occurs following simultaneous exogenous activation of adrenergic and cholinergic receptor systems (Chaney et al., 1997).

The vagal nerve is provided with cholinergic afferents and efferents. A growing body of evidence from anatomical, electrophysical, and neurochemical studies indicate that ascending fibers of the vagus nerve play a crucial role in transmitting the memory enhancing action of peripheral epinephrine to limbic structures by activating central norepinephrine release during memory consolidation (Hassert et al., 2004). By these different pathways, pyridostigmine may interact at various levels on peripheral cholinergic neurotransmission related to arousal and memory.

The discrepant findings from administration of pyridostigmine in humans and animals may be associated with the use of incompatible doses of pyridostigmine for the species. In a study of rats (Scremin et al., 2003), a pyridostigmine concentration of 80 mg/l in the drinking water that corresponds to an estimated dose about 10 mg/kg body mass/day inhibits 20-30% plasma BuChE activity. This is close to the rat equivalent (9 mg/kg body mass/day) of the dose used in humans for pretreatment of nerve agent poisoning (1.29 mg/kg body mass/day x 70 = 90.3 mg/kg), based on the surface area dosage conversion (Freireich et al., 1966). The degree of plasma BuChE inhibition obtained with this dose was within the range reported for humans taking 90 mg of pyridostigmine perorally per 24 h, divided in 3 doses (Keeler et al., 1991). In the study of Scremin et al. (2003), the rats were tested for passive avoidance,

nociceptive threshold, acoustic startle, and open field activity 2, 4, or 16 weeks after treatment with pyridostigmine (10 mg/kg perorally a day for 3 weeks) and no effects were recorded.

In humans, systemic administration of 2.5 mg of pyridostigmine corresponds to an oral dose of 120 mg (Aquilonius et al., 1980). If this conversion factor of about 50 times is transformed to rats, a peroral dose of 10 mg/kg corresponds to 0.2 mg/kg by systemic administration. In a recent study, we have shown that 2 groups of rats given either 0.1 or 0.2 mg/kg of pyridostigmine systemically both display a small cognitive impairment in the novelty test, whereas locomotor and rearing activities are unaffected. The dose of 0.2 mg/kg of pyridostigmine reduced plasma AChE by 20-30% (Mariussen, Enger, Myhrer, unpublished data). In comparison, rats given a physostigmine dose of 0.1 mg/kg and tested in the same behavioral task exhibit unimpaired cognitive performance, but radically reduced locomotor and rearing activities (Myhrer et al., 2004a).

If 10 mg/kg perorally a day of pyridostigmine is used as a benchmark for the rat equivalent to the human dose of pyridostigmine (90 mg a day), behavioral effects of lower doses of pyridostigmine have been found in several studies. Peroral pyridostigmine doses of 1.3 or 1.5 mg/kg have been shown to impair behavioral responses in rats (Abou-Donia et al., 2001; Servatius et al., 1998; van Harren et al., 2000). In other studies, the doses producing adverse effects on behavior have been 6 mg/kg or higher (Liu, 1991, 1992; Shih et al., 1991; van Harren et al., 2001). This diversity of results may be attributed to the specific nature of the behavioral test situation used or subtle differences in responding to pyridostigmine across the rat strains used. With regards to the latter matter, Servatius et al (1998) report enhanced acoustic startle for a long period of time with a pyridostigmine dose of 1.3 mg/kg in WKY rats, but not in SD rats. The WKY strain differs from other commonly used strains, and there are behavioral and neurobiological differences among the various WKY strains (Sagvolden and Johansen, 2012). Furthermore, behavioral testing has been performed within minutes of

dosing and with no long-term follow up in some studies in contrast to the procedures followed in other studies. Thus, peroral administration of pyridostigmine to rats can cause measurable behavioral changes or not, depending on differences in responding between rat strains and/or differences in the experimental procedures applied.

Collectively, the results from animal studies imply that pyridostigmine can, even at comparatively low doses, cause adverse effects on behavior, but not invariably or necessarily. Similarly, humans may differ in their reactions to pyridostigmine depending on the circumstances under which the drug is administered. In a meta-analysis, it is concluded that that carbamate can cause side effects in predisposed individuals (Golomb, 2008).

7. 2. Pretreatment

Many investigators have found physostigmine to be superior to pyridostigmine in protecting against organophosphate poisoning (Harris et al., 1984; Leadbeater et al., 1985; Miller et al., 1993). It is generally accepted that this result is associated with physostigmine's capability to cross the BBB. The drugs also differ in other ways, inasmuch as the half-life for pyridostigmine in plasma of rats is 1.9 h and 16-17 min for physostigmine (Miller et al., 1993). However, relatively modest doses of physostigmine can have detrimental impact on behavior, probably due to blockade of cholinergic neurotransmission (Ennaceur, 1998). It will, therefore, be important to assess adverse effects of physostigmine in clinical studies, also in combination with the anticholinergic agent scopolamine (half-life 17 min) (Lyeth et al., 1992).

Pretreatment with galantamine and posttreatment with atropine have been advanced as an effective countermeasure against poisoning by organophosphorus insecticides and nerve agents in guinea pigs (Albuquerque et al., 2006). In the latter study, it is asserted that galantamine is likely to maintain normal cognitive performance of organophosphate-exposed

subjects. Results from testing rats in the novelty test show that galantamine causes cognitive impairment as well as reduced locomotor and rearing activities; the latter even more pronounced than by using physostigmine, huperzine, or donepezil (Myhrer et al., 2010).

PANPAL (pyridostigmine, benactyzine, trihexyphenidyl) was originally developed as a prophylactic treatment. However, to manage a supralethal dose of soman (2 x LD_{50}) it appeared necessary to combine PANPAL with post-exposure treatment in terms of HI-6 and benactyzine in rats (Kassa and Fusek, 1998). Pretreatment of rats with PANPAL or the Bulgarian regimen pyridostigmine (0.75 mg/kg) and biperiden (2.5 mg/kg) has been followed by HI-6 and atropine to become more effective (Kassa et al., 2003). Behavioral effects of the doses used in the above studies (benactyzine 16 mg/kg, biperiden 2.5 mg/kg, trihexyphenidyl 6.3 mg/kg) do not seem to have been tested in rats. Results from the novelty test show that benactyzine (0.3 mg/kg) and trihexyphenidyl (0.12 mg/kg) impair preference for novelty, whereas biperiden (0.11 mg/kg) does not (Myhrer et al., 2010). Since the doses used for pretreatment against nerve agent intoxication are 20-50 times higher than those used for the novelty testing, PANPAL doses will hardly leave cognitive performance unaffected in rats. The doses chosen for novelty test have previously been reported to assure anticonvulsant effects against soman when administered 20 or 30 min before the nerve agent (Myhrer et al., 2010).

The combination of physostigmine and procyclidine as pretreatment has been examined in several studies. A transdermal patch containing 1.5% (blood concentration of approximately 1 ng/ml after 1 day and 18% enzyme inhibition) of physostigmine and 6% (blood concentration of approximately 8 ng/ml after 1 day) of procyclidine has been administered to beagle dogs for 2 days before they were challenged with subcutaneous injection of serial doses (2-10 x LD_{50}) of soman. One min after exposure to soman the dogs received atropine (2 mg) and 2-PAM (600 mg) or atropine (2 mg) and HI-6 (500 mg)

intramuscularly. The patch exerts a high protective ratio (4.7 x LD_{50}) in comparison with the relatively low effects of traditional antidotes (atropine plus 2-PAM, atropine plus HI-6) without pretreatment. However, a synergistic increase in the protection ratio is achieved by the combination of the patch with atropine and HI-6 (9 x LD_{50}), but not with atropine and 2-PAM (5 x LD_{50}) (Kim et al., 2005). In a subsequent study by the same group, rhesus monkeys issued with a patch containing physostigmine (0.54 ng/ml in blood) and procyclidine (10.8 ng/ml in blood) are protected against a soman dose of 2 x $LD_{50.}$ If the patch is combined with atropine (0.5 mg/kg) and HI-6 (50 mg/kg) after soman exposure, the monkeys are protected against a soman dose of 5 x LD_{50} (Cho et al., 2012). In the studies of dogs and monkeys, potential behavioral side effects of the regimens used do not appear to have been investigated. In the study of Kim et al (2005), the authors asserted that: ”It was expected that the possible side effects of carbamates and anticholinergics in combinational pretreatment might be offset by each other, although somewhat different results were reported (Myhrer et al., 2004a)”.

The combination of physostigmine and procyclidine as pretreatment (30 min before soman) has also been examined in rats. Physostigmine (0.1 mg/kg) and procyclidine in a dose of 1 mg/kg do not prevent seizures when the soman dose is 1.6 x LD_{50} (only if it is 1.3 x LD_{50}). Subsequent treatment with scopolamine (1 mg/kg) within 10 min after seizure onset produces a reliable termination (Myhrer et al., 2004b). Even such a low procyclidine dose causes a slight cognitive deficit in the novelty test when combined with physostigmine (Myhrer et al., 2004a). Physostigmine (0.1 mg/kg) in combination with procyclidine doses of 1, 3, or 6 mg/kg effectively prevent the development of seizures when the doses of soman are 1.3, 1.6, or 2 x LD_{50}, respectively (Myhrer et al., 2004b). The combination of physostigmine and procyclidine (6 mg/kg) results in a marked deficit in preference for novelty. A much milder deficit is observed when physostigmine is combined with a lower dose (1 or 3mg/kg) of procyclidine (Myhrer et al., 2004a). Hence, heavy pretreatment with physostigmine and

procyclidine can give efficacious protection against nerve agent poisoning, but will most likely produce severe cognitive side effects. On the other hand, light premedication with slight or moderate side effects can effectively be supplemented by adjunct therapy.

In order to ensure complete protection against a convulsant dose of soman, powerful pharmacological interference with neuronal activity is required. Thus, from a theoretical point of view, behavioral side effects will likely occur. If pretreatment followed by adjunct treatment after exposure to nerve agent is used, pretreatment with HI-6 and levetiracetam accompanied by procyclidine might be an option. HI-6 (125 mg/kg) and levetiracetam (50 mg/kg) are without behavioral side effects in the novelty test (Myhrer et al., 2014). The potent anticonvulsant procyclidine (anticholinergic and antiglutamatergic) is more appropriately used as a post-poisoning drug than as a pretreatment agent. The potency of HI-6 and levetiracetam used as prophylactics followed by procyclidine as adjunct has partly been tested before. Rats pretreated with HI-6 (125 mg/kg) that were about to die (very close to respiratory arrest) 5-10 min after onset of soman-induced seizures survived and recovered well when they were treated with levetiracetam (50 mg/kg) and procyclidine (20 mg/kg) (Myhrer et al., 2011).

Pretreatment with the carbamate pyridostigmine is a well established method to protect against nerve agent intoxication in the armed forces in a number of nations (Aas, 2003). A tablet (30 mg) of pyridostigmine bromide is supposed to be taken every 8 hour by the service personnel. The rationale behind this use is that carbamate occupies a portion of the available AChE (15-40 % of the erythrocyte AChE) and renders it inaccessible to nerve agents in the blood, since nerve agents only bind to unprotected enzyme (Leadbeater et al., 1985). The AChE that has been reversibly inhibited by pyridostigmine spontaneously decarbamoylates, and the enzyme is again able to hydrolyze ACh. Because pyridostigmine does not penetrate the blood-brain barrier to any extent, it only protects the peripheral nervous

system. In most military protocols, the antidotal combinations consist of an oxime, anticholinergics, and benzodiazepine. The results from animal research suggest that pyridostigmine may and may not produce adverse effects on behavior depending on various variables discussed in Section 7.1.

As mentioned on page 18, pretreatment with a patch impregnated with HI-6 (TRANSANT) has been taken into use by the Czech and Slovak Army. No harmful effects have been observed in clinical tests (Bajgar et al., 2009). In animal studies, HI-6 (125 mg/kg) does not produce any adverse effects in the novelty test (Myhrer et al., 2014).

7.3. Prophylaxis

A number of attempts have been made in animals to develop a combination of antidotes capable of yielding prophylaxis against nerve agent intoxication without requiring any adjunct treatment. A marked protective efficacy is obtained by donepezil (2.5 mg/kg) combined with procyclidine (3 or 6 mg/kg) when given prophylactically against a lethal dose of soman (1.6 x LD_{50}) in rats (Haug et al., 2007). The combination of HI-6 (125 mg/kg) and huperzine (0.5 mg/kg) also provides an effective protection against soman (1 x LD_{50}) in rats when given 30 min before exposure (Tonduli et al., 2001). However, the combination of donepezil (2.5 mg/kg) and procyclidine (3 mg/kg) results in a cognitive deficit and radically depressed locomotor and rearing activities in the novelty test. Huperzine (0.5 mg/kg) depresses motor activity as well, whereas HI-6 (125 mg/kg) does not affect behavior in the same test (Myhrer et al., 2010, 2014). AChE inhibitors like physostigmine, galantamine, huperzine, and donepezil seem to have in common that they can cause fear and freezing in rats (Plotnik et al., 1974). Administration of various doses of physostigmine (0.025 – 0.2 mg/kg) results in a dose-related increase of freezing, suppression of feeding, and suppression of time near aversive stimulus (Mollenauer et al., 1979). In the latter study, it was suggested

that the effect of physostigmine is not to depress behavior in general, but rather to increase or potentiate the innate defensive response of freezing. Thus, the freezing effect is central to other changes. In correspondence with the latter view, reduced freezing induced systemically by scopolamine can be reinstated by donepezil (Lindner et al., 2006). Electrophysiological experiments with amygdaloid slices from young rats exposed to a convulsant dose of soman (brains harvested 24 h or 14 days after exposure) reveal reduced GABAergic (y-amino butyric acid) inhibition in the basolateral amygdala which may relate to increased anxiety in *in vivo* studies (Prager et al., 2014). A plausible explanation of the remarkable decline in locomotor and rearing activities in the novelty test may be that the anticholinesterases activate the freezing response. This interpretation receives support from the data in the novelty test, inasmuch as both locomotion and rearing in rats that received physostigmine, donepezil, or galantamine were normal only during the last phase in Session III when the adaptation to the test situation was optimal (Myhrer et al., 2010).

It has been well documented that the anticholinesterases can mitigate symptoms of Alzheimer's disease. However, cholinesterase inhibitors used in healthy persons can have perturbing effects. The influence of physostigmine on stimulus-selectivity and/or task-related responses is often opposite between Alzheimer patients and healthy controls. In control subjects, excessive cortical activation (functional magnetic resonance imaging-scanning) during task-irrelevant conditions occurs in addition to enhanced cholinergic activation in the frontoparietal and sensory cortex during low-attention conditions that do not normally engage such brain areas (Bentley et al., 2008). These results support a model of anxiety in which increased release of cortical acetylcholine augments the expression of fear and anxiety (Berntson et al., 1998). Commercial pesticide (organophosphate compounds) sprayers show elevated anxiety and lower plasma cholinesterase activity than control subjects (Levin et al., 1976). The most prevalent neuropsychiatric symptoms seen in victims of the sarin attacks in

Japan are anxiety disorders, including posttraumatic stress disorder (Yanagisawa et al., 2006). Hence, there is an apparent correspondence between the findings of increased fear/freezing in animals and elevated anxiety in humans following exposure to AChE inhibitors. The behavioral inhibition obtained in animals suggests that centrally active AChE inhibitors may not be suitable as prophylactics against nerve agent intoxication.

The combination of physostigmine and procyclidine has been demonstrated to exert very effective protection against soman intoxication in both rats and guinea pigs. In rats, physostigmine (0.1 mg/kg) given together with procyclidine in various doses (0.3 - 6 mg/kg) 30 min prior to soman (1.3 x LD_{50}) results in 1.92 - 5.07 folds of protection ratio (Kim et al., 2002). In the novelty test, physostigmine (0.1 mg/kg) combined with procyclidine (1, 3, or 6 mg) cause adverse behavioral effects in a dose-related manner. Even in the combination with the lowest dose of procyclidine impaired behavior is salient (Myhrer et al., 2004a).

Prophylactic treatment with pyridostigmine (0.1 mg/kg) combined with either caramiphen (10 mg/kg) or scopolamine (0.1 mg/kg) provide survival of soman exposure (1 x LD_{50}) and no convulsions were observed (Raveh et al., 2002). However, both scopolamine (0.15 mg/kg) and caramiphen (10 mg/kg) have been shown to cause marked cognitive deficits in rats in the novelty test (Myhrer et al., 2004a, 2008).

Both procyclidine and caramiphen belong to the same group of antiparkison drugs exerting cholinergic and glutamatergic antagonism. Through a series of microinfusion studies, it turned out that the excellent anticonvulsant properties of procyclidine are superior to those of caramiphen (Myhrer, 2010). The NMDA antagonism of procyclidine is far more potent than that seen for caramiphen which must be given in higher doses than procyclidine to achieve anticonvulsant capability (Raveh et al., 2014). On this background, procyclidine has been combined with other relevant drugs to make up several treatment regimens against soman intoxication. Table 1 summarizes the behavioral side effects of these regimens in the

novelty task. The therapies containing HI-6 and procyclidine are supposed to be used as universal treatment regardless of the time of application, whereas the regimen containing HI-6, scopolamine, and physostigmine was suggested for prophylactic use. The latter regimen was included in the table as an example of a very successful prophylactic treatment (without HI-6) against all the classical nerve agents in guinea pigs (Wetherell et al., 2002). However, prophylaxis consisting of HI-6, scopolamine, and physostigmine does not protect adequately against neuropathology in rats exposed to a convulsant dose of soman (Myhrer et al., 2013a).

From the results presented in Table 1, a dose-response relation can be seen for procyclidine. When it is combined with physostigmine, which shields a fraction of AChE, the dose levels can be relatively moderate. When procyclidine is combined with HI-6 and levetiracetam, the dose has to be enhanced to obtain effective protection against soman poisoning. The latter regimen exerts the most devastating impact on normal behavior in the novelty test, whereas the regimens in which procyclidine is combined with HI-6 and DCG-IV or HI-6 and MPEP produce a slightly lower level of adverse effects. However, also the regimen specially designed for prophylactic use (HI-6, scopolamine, physostigmine) causes marked behavioral impairments. In order to assure complete protection against a convulsant dose of soman, powerful pharmacological interference with the activity in the CNS is required. A radical impact on normal actions in cholinergic, glutamatergic, or GABAergic systems has to be made. Hence, from a theoretical point of view, behavioral side effects will most likely occur.

Full protection against lethal doses of soman or VX in monkeys has been achieved by using HuBuChE as biological scavenger for nerve agents. In these studies, no observable behavioral changes of HuBuChE administration have been reported for the period of 1.5-11 h prior to soman or VX (Lenz et al., 2005; Raveh et al., 1997). However, a number of studies have been carried out to measure potential cognitive impairment of the enzyme itself in

standardized behavioral tasks. In mice, HuBuChE alone at a dose 30-fold higher than estimated to be necessary for protection against 2 x LD_{50} of soman in humans, does not impair acoustic startle reflex or prepulse inhibition (Clark et al., 2005). Similar negative effects have been found in rats. Administration of horse serum BuChE resulting in increased blood enzyme activity for up to 72 h has no effect on acquisition and retention of passive avoidance, operant performance, total daily motor activity, or circadian pattern of activity (Genovese and Doctor, 1995). In 2 studies of rhesus monkeys, a serial probe recognition task has been used. Repeated administration of commercially prepared equine BuChE has no systematic influence on the recognition task, despite 7- to 18-fold increases in baseline BuChE levels in blood (Matzke et al., 1999). In a subsequent study of monkeys, intravenous injection of 150 mg of HuBuChE 1 h prior to testing does not result in cognitive decrements of any kind in the recognition task and no toxic signs in clinical pathology were detected in blood assays during the 5 weeks of observation (Myers et al., 2012). Collectively, the results demonstrate the behavioral and physiological safety of HuBuChE in animals and support its development as a bioscavenger for the prophylaxis of chemical warfare agent toxicity in humans. There are, however, safety concerns due to contaminants such as viruses, bacterial endotoxins and various serum proteins that may cause intravascular clots etc. Such concerns must be clarified in future studies.

The extrapolation from animal data to therapeutic effects in humans is especially critical in studies intended to estimate the protective impact of pharmacological agents against nerve agent intoxication. In protection therapies, it is crucial that excessive doses may lead to adverse effects on mental capacities. The common method of comparing dose per body weight may result in erroneous extrapolation. An alternative approach is based on the comparison of plasma concentrations at steady state required to obtain a given pharmacodynamic endpoint. When similar dose response curves are obtained in at least 2

animal models, the extrapolation to expected therapeutic effects in humans might be considered more reliable (Levy et al., 2007).

PANPAL seems to be the only prophylactic therapy that has been taken into use for humans. According to the results from animal studies, components of PANPAL (benactyzine, trihexyphenidyl) cause cognitive deficits (Myhrer et al., 2008).

8. Concluding comments

In the late 1980s, several nations introduced pyridostigmine as an effective pretreatment against nerve agent and in particular against soman that is considered to be the most problematic to manage because of the very short time to onset of the aging process. Pyridostigmine also improves the effectiveness of supporting therapy against cyclosarin (Koplovitz et al., 1992a) and tabun (Koplovitz et al., 1992b). During the cold war with large stockpiles of nerve agents, it was necessary as a precaution to take into use pyridostigmine to serve as a life saving measure. Since no stand-alone post-poisoning therapy currently exists, which is effective against all nerve agents, it is still reassuring to have an adequate pretreatment in place. After the end of the Gulf War, a large number of animal studies have been performed to clarify whether use of pyridostigmine may lead to adverse effects on cognition and other aspects of behavior. The results from experiments on animals presented in this study are not unambiguous, because pyridostigmine may and may not impair behavioral functions depending on the animal strain, the doses, and the test procedures used.

Pretreatment with drugs other than pyridostigmine has been examined in a number of animal studies. Procyclidine has been combined with physostigmine as pretreatment prior to soman exposure and supplemented by atropine and HI-6 post-exposure in dogs and monkeys. The same pretreatment has also been used in rats followed up by scopolamine after exposure. However, the problem with this pretreatment is that both procyclidine and physostigmine

alone produce behavioral deficits that can be further enhanced when the drugs are combined (Myhrer et al., 2004a). A potential combination may be HI-6 and levetiracetam that each has no behavioral effects (Myhrer et al., 2014) and procyclidine given as adjuvant therapy after challenge with nerve agent. In humans, TRANSANT in terms of a patch with HI-6 is used as pretreatment in the Czech and Slovak armies.

For prophylaxis against nerve agent, the anticonvulsant efficacy of countermeasures has to be even more powerful than for pretreatment agents, because no additional treatment is supposed to be given following exposure. The prophylactic treatments listed in Table 1 can protect against a convulsant dose of soman, in particular 2 of them (HI-6, levetiracetam, procyclidine and HI-6, scopolamine, physostigmine), but they both exert pronounced impairment of behavior. On the other hand, stoichiometric scavengers have been shown to ensure adequate protection against high doses of soman without causing recordable side effects in animals. However, prophylactic use of HuBuChE is probably not suited for use on a large scale in the field, because the cost of production under Good Manufacturing Practice (GMP) will be high, and the compound will need storing at low temperature. Therefore, overall, catalytic bioscavengers will be more suitable than stoichiometric bioscavengers as medical countermeasure against nerve agent intoxications. The use of any prophylactic bioscavenger treatment would most likely require additional therapy available, because instances of very severe intoxication or long-lasting exposure may occur.

Effective pharmacological prophylaxes will most likely cause undesirable behavioral side effects. Actually, such action may be inevitable because of the need for marked interference with central nervous activity to achieve sufficient antidotal efficacy. Pretreatments like pyridostigmine and TRANSANT may be used with only small behavioral side effects, and it should be emphasized that pyridostigmine is important for its life saving

properties. Application of enzymatic prophylaxis, catalytic scavengers in particular, may perhaps solve the problem with side effects in the future.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgment

The authors would like to thank Dr. Lily Raveh (Israel Institute for Biological Research) and Dr. John Tattersall (Dstl, UK) for constructive comments on an earlier version of the manuscript.

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Figure legend

Fig. 1. Novelty test measuring rats` innate preference for novelty, locomotor activity, rearing, and grooming. Testing is carried out in 3 sessions (1 each day). Each session consists of 5 min exploring with neutral cubes present (Phase 1) and Phase 2 (5 min) after 10 min in the home cage during which the central cube has been replaced by a novel one. The novelty is represented by uneven top (Session I), spot of cheese on 1 side (Session II), or smaller cube (Session III).

Table 1

Preference for novelty, locomotor activity, and rearing in rats treated with 1 of 5 prophylactic regimens (1 with 2 different doses of procyclidine) ensuring protection against a convulsant dose of soman (1.3 x LD_{50}). The results are relative to the behavior of saline-treated rats.

Group/Drugs	Dose	Preference for novelty			Locomotion			Rearing		
		Session			Session			Session		
	mg/kg	I	II	III	I	II	III	I	II	III
Physostigmine Procyclidine	0.1 3	↓	─	─	─	↓1	↓1	↓1	↓1	─
Physostigmine Procyclidine	0.1 6	↓	↓	↓	↓1	↓1	↓2	↓1	↓1	↓2
HI-6 Levetiracetam Procyclidine	125 50 20	↓	↓	↓	↓2	↓2	↓2	↓2	↓2	↓2
HI-6 DCG-IV Procyclidine	125 4 20	─	↓	↓	↓1	↓2	↓2	─	↓2	↓2
HI-6 MPEP Procyclidine	125 30 20	─	↓	↓	↓1	↓2	↓2	─	↓2	↓2
HI-6 Scopolamine Physostigmine	125 1 0.1	↓	↓	↓	↓2	↓2	↓2	─	↓1	↓2

↓, decreased; ─, unchanged; ↓1, 1 phase; ↓2, 2 phases. The data are from Myhrer et al. (2004a, 2014).