Prophylaxis Against Nerve Agent Toxicity: Physiological, Behavioral, and Neuroprotection of Current and Novel Treatments

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ABSTRACT

Compounds with another mechanism of action than enzyme inhibition alone were tested in their capability to counteract or prevent incapacitation and lethality after 2xLD_{50} soman when used as a pretreatment or as a post-intoxication therapy in guinea pigs. To increase the validity of extrapolating data to man, crucial experiments were performed in marmoset monkeys. The efficacy was evaluated in terms of survival, intoxication symptomatology, hypotermia, cortical electro-encephalogram (EEG) and behavioral incapacitation. The treatment scenarios in the guinea pigs were pyridostigmine (PYR), physostigmine (PHY), procyclidine (PC), PHY/scopolamine (SCO), PHY/PC, and (+)-PHY/PC. These treatments were given 30 minutes prior to soman intoxication. To test the efficacy of these compounds as a post-intoxication therapy the treatments were administered on indication after the first sign appeared in a parallell group of guinea pigs The subacute pretreatment scenarios in marmosets were PYR, PHY/SCO, PHY/PC. In the marmoset the treatments were administered by using Alzet osmotic mini-pumps inserted twelve days prior to soman intoxication. All animals received atropine sulphate after soman.

Pretreatments with PYR (0.05 mg/kg), PHY (0.3 mg/kg), and PC (3 mg/kg) did not protect sufficiently against lethality, intoxication symptoms, EEG and incapacitation. All animals pretreated with PYR deceased within 24 hrs. The most effective pretreatments in guinea pigs regarding survival and behavioral performance showed to be the combinations of PHY/PC and PHY/SCO(0.1 mg/kg) with a survival of 100%. These scenarios did also offer the best protection against hypothermia, seizures and convulsions. The PHY/PC combination showed to be even more protective, since none of these animals showed EEG seizure activity, in contrast to some PHY/SCO pretreated guinea pigs. Used as a therapy after soman intoxication, the PHY/PC combination was also the best treatment option, although not fully protective. (+)PHY (0.36 mg/kg), which does not bind to cholinersterase, in combination with PC, was the second best pretreatment group on survival. This means that also direct effects of PHY is responsible for survival.

In marmoset monkeys, the pretreatment combinations PHY(0.0125 mg/kg/hr)/PC(0.125 mg/kg/hr) and PHY/SCO(0.01 mg/kg/hr) showed minor improvements compared to PYR (0.02 mg/kg/hr).

In conclusion, the protective effects are based on the combination of 1) temporary cholinesterase inhibition, to prevent incapacitation and peripheral effects, and 2) direct effects on the cholinergic receptor, to prevent convulsive behavior, and 3) NMDA receptor antagonism, to prevent seizure activity. The sign free combinations of PHY/PC or PHY/SCO are promising alternatives for the PYR pretreatment. Furthermore, PHY/PC used as therapy increases chances of survival compared to PHY/SCO. Therefore, the combination of PHY/PC seems to be very promising and should be further investigated.

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1.0 INTRODUCTION

Pretreatment is an essential element in the passive defense against nerve agents which may reduce the extent of the intoxication and thereby improve the pathological outcome of the exposed subject. This was already stated by Wolthuis *et al*. [1] and is still valid. The pathological result is based on a cascade of reactions starting with the inhibition of the enzyme acetylcholinesterase (AChE) [2]. Current pretreatment against organophosphate (OP) intoxication aims at the first step in the cascade of reactions, the protection of a fraction of the available AChE from irreversible binding by the OP, thereby allowing sufficient residual AChE activity to prevent lethality [3,4].
Currently pyridostigmine bromide (PYR) is used in most NATO countries as a pretreatment against OP intoxication. PYR binds covalently to AChE after which this bond is hydrolyzed gradually with time, thus liberating active AChE. However, PYR alone fails to protect against OPs [5]. The limited protective capacity of PYR is probably due to its molecular structure: a quaternary nitrogen atom prohibits PYR to enter the brain, and will thus not protect against central effects of OPs. Failure to conserve brain AChE activity may lead to brain damage and post-intoxication incapacitation.
Incapacitation is a huge problem from a military operational point of view, as the medical system will be overloaded with the care for the incapacitated soldiers. Moreover, over-stimulation of central ACh receptors may lead to convulsions, which in turn may induce irreversible brain damage in cholinergic areas. Clearly, (pre)treatment strategies should aim at prevention of permanent brain damage as well as post-intoxication incapacitation. Consequently, a need for a better treatment drug in terms of protection against lethality, post-exposure symptomatology and incapacitation still exists.

Pretreatment with a more centrally active compound or compounds with a different mode of action is considered. The centrally effective carbamate PHY has proven to be effective against lethality and post-intoxication incapacitation against soman in guinea pigs and marmoset monkeys [6,7,8].
Because the central effects of PHY, unwanted side-effects can be induced that can be reduced by co-administration of scopolamine (SCO). Guinea pigs pretreated with PHY and SCO all survived 3xLD_{50} soman, whereas 43% survived with PYR and SCO [7]. These findings suggest that PHY is more effective compared to PYR. This is in accordance with findings of others [9,10]. The addition of SCO to PHY also results in a faster recovery in guinea pigs [11]. In most of the published studies a cholinolytic compound was added to the pretreatment to acquire a better protection against OP intoxication [6,12,13]. Subchronic PHY pretreatment offered a higher survival rate when combined with SCO [14]. Lallement *et al.*[15] also found a better protection against early mortality after soman intoxication with PHY and SCO pretreatment compared with PYR or PHY alone.
The mechanism of action of the carbamates, such as PYR and PHY, is assumed to be the temporary inhibition of a fraction of the available AChE, thus shielding this fraction from irreversible inhibition by a nerve agent. Consequently, research on pretreatment compounds has focused on reversible inhibitors of AChE. However, it is suggested that other processes than enzyme inhibition contribute to the protection against nerve agents.
PHY can also affect cholinergic receptors directly [16,17,18,19]. Nerve agents such as VX and soman also directly affect ACh receptors. Bakry *et al*.[20] reported that soman can act as a partial agonist of the nicotinergic ACh receptor. VX and soman may also affect a sub-population of muscarinergic receptors that have similar affinity for OP compounds as AChE. This observation suggests that toxicity of soman may involve combined effects on nicotinergic and muscarinergic receptors. A direct effect of PHY on nicotinergic receptors can therefore be viewed as a potential mechanism in its protecting efficacy. Recently, the optical isomer of PHY, (+)PHY that does not inhibit AChE, appeared to offer nearly the same protection against symptomatology and post-intoxication incapacitation after 2x LD_{50} soman as the

isomer that does inhibit this enzyme [21]. This finding suggests that, indeed, other processes, like other factors or even other transmitter systems, contribute to the protection against nerve agents.

Besides the cholinergic system, the NMDA receptor complex appears to play an important role in the protective effect of anticholinergic drugs against soman poisoning [22]. Uncontrolled and progressive seizure activity after soman intoxication recruits the NMDA system. This was confirmed at our institute in a study with the NMDA antagonist piperidine (TCP) against soman [23]. This study underlined the current hypothesis that cholinergic mechanisms are responsible for eliciting seizure activity after OP intoxication and that the subsequent recruitment of other excitatory neurotransmitters and loss of inhibitory control are responsible for the maintenance of seizures and the development of subsequent brain damage.
Thus, drugs with anticholinergic and anti-NMDA activity would be beneficial against the neurotoxic effects and brain damage after OP intoxication. In that respect, the carbamate PHY, its enantiomer that does not inhibit AChE, and/or the cholinergic and NMDA antagonist procyclidine (PC) will be very interesting. PC exerts antagonism on both muscarinic and nicotinic receptors and has antagonistic effects on NMDA receptors. It aims for reduction of cholinergic and glutamatergic excitatory action during intoxication, in both peripheral and central nervous system. PC in combination with PHY and a post-intoxication therapy with AS and HI-6 is reported to offer a high protective ratio of 21.5 in guinea pigs against soman [24]. We found in guinea pigs a 100% survival using PHY and PC pretreatment against 2xLD_{50} soman (Philippens, unpublished results, 2002), whereas animals pretreated with PYR all died. Therefore, PC is an interesting compound to consider as an alternative for the current pretreatment with PYR against OP-intoxication as a longer term solution.

In this study compounds with another mechanism of action than enzyme inhibition alone will be tested and compared with PYR in their ability to counteract or prevent post-intoxication incapacitation and lethality after 2xLD_{50} soman. The different treatment scenarios are: PYR, PHY, PC, PHY/SCO, PHY/PC, and (+)PHY/PC. For practical experimental reasons the efficacy againt soman poisoning of subacute pretreatment of PYR and the combination of PHY/SCO and PHY/PC was tested by using osmotic mini-pumps placed subcutaneous in marmoset monkeys.
If the hypothesis, that other factors than enzyme inhibition play a major role in the protection against nerve agents, proves to be correct, then such pretreatment drugs are likely to be also effective when administered post-exposure, i.e. as therapeutics. From a military operational point of view it would be highly attractive to have a drug that can be used both as a pretreatment and as a therapeutic. Therefore, the ability of these compounds to reduce post-intoxication incapacitation and lethality after the first symptoms of nerve agent intoxication appear will be evaluated.

2.0 METHODS

2.1 Animals

Male Dunkin-Hartley albino guinea pigs (Harlan BV, The Netherlands) with an initial body weight of 400-450 g were used. The animals are kept individual (Makrolon type IV) in agreement with our Standard Operating Procedures for housing and care of experimental animals for neurobehavioral studies. The ambient temperature is regulated between 20-22^{o}C. Relative humidity is monitored but not regulated and is kept over 50%. Food and water is available.
Adult Marmoset monkeys (*Callithrix jacchus*) of both sexes bred and raised at the Biomedical Primate Research Centre (BPRC, Rijswijk, The Netherlands) were used. During the study, the animals were housed separately in cages (61 x 61 x 41 cm) in a room kept at 23-25^{o}C and at a relative humidity >60%. In this room a 12-hour day and night cycle was maintained. Daily they were fed with pellet chow, peanuts, boiled egg, fruit, baby biscuits, sunflower seeds, and beans after training or testing. Water was available *ad libitum*.

All aspects of animal care are in agreement with current guidelines of the European Community. The experiments described received prior approval from an undependent Ethical Committee on Animal Experimentation (DEC) (DEC no. 1704, 1796 and 1869).

2.2 Drugs

Physostigmine (eserine) hemisulphate salt, pyridostigmine bromide, and procyclidine hydrochlorid were obtained from Sigma (St.Louis, U.S.A.) and scopolamine hydrobromid from Merck (Darmstadt, Germany). (+)-Physostigmine was a gift from Dr McDonough supplied from the USAMRICD, USA (article number ICD#1071, batch no. BL50263). Identity and purity was tested at TNO which was found to be 100% (+)PHY and 79% respectively. Atropine Sulphate was obtained from ACF (Amsterdam, The Netherlands). Soman (O-pinacolyl methylphosphonofluoridate) was synthesized at TNO Defence, Security and Safety. The doses were collected and selected from other guinea pig and marmoset studies in our institute and from literature.

2.3 Study design

Compounds were tested in their capability to counteract or prevent post-intoxication incapacitation and lethality after soman intoxication when used as a pretreatment or as a post-intoxication therapy in guinea pigs. The efficacy of two promising alternatives for the current pretreatment was evaluated in the marmoset monkey. The efficacy was evaluated in terms of survival, post-intoxication symptomatology and incapacitation on behavior and cortical electro-encephalogram (EEG). The different treatment scenarios in the guinea pigs are: PYR (0.05 mg/kg s.c.), PHY (0.3 mg/kg s.c.), PC (3 mg/kg s.c.), PHY/SCO (resp. 0.3 and 0.1 mg/kg s.c.), PHY/PC (resp. 0.3 and 3 mg/kg s.c.), and (+)-PHY/PC (resp. 0.36 and 3 mg/kg s.c.) against 2x LD_{50} soman (49 µg/kg s.c., LD_{50} was adapted from DSTL, Porton Down). The different subacute pretreatment scenarios in marmoset monkeys are: PYR (0.02 mg/kg/hr), PHY/SCO (resp. 0.0125 and 0.01 mg/kg /hr.), PHY/PC (resp. 0.0125 and 0.125 mg/kg/hr) against 2x LD_{50} soman (18 µg/kg s.c., LD_{50} was adapted from DSTL, Porton Down). The doses for PYR and PHY lead to a blood AChE inhibition of 30-40% [7]. The dose of PC was adapted from Kim *et al.* [24].
In order to obtain baseline values the body weight, shuttle box (guinea pigs), bungalow and hand-eye coordination (marmoset monkey), and EEG were registered after training of the behavioral tasks and placement of the EEG-electrodes. Thereafter, 30 minutes prior to OP-intoxication the pretreatment was injected subcutaneously in guinea pigs. In the marmoset monkeys the pretreatments were administered by using an Alzet osmotic mini-pump inserted twelve days prior to soman intoxication.
To test the efficacy of these compounds when given as a post-intoxication therapy, in a parallell group of guinea pigs, the treatment was given after the first sign appeared. All animals received an injection with AS (guinea pigs: 3.6 mg/kg i.m., marmosets: 5 mg/kg i.m.) after soman intoxication. From one hour before until three hours after soman intoxication, EEG was recorded continuously. In the meantime the observations for the intoxication signs took place and the body temperature was monitored. Three hours after intoxication the behavioral tests were performed and repeated one day later. In the marmoset monkeys the behavioral tests were also repeated one and two weeks after intoxication.

2.4 Shuttle box (guinea pigs)

The performance of the active avoidance is measured in a computerized two-way shuttle box, consisting of two connected equal compartments. Guinea pigs have to learn how to avoid a stream of air (about 6 l/s, air tube diameter 1 cm) aimed at their fur within 10 s after presentation of a sound stimulus (the conditioned stimulus) with a random inter-trial interval. After reaching correct responses of 70% or more, the animals are ready to be tested. The number of correct avoidance responses is used to express the retrieval of learned behavior (for more details see [25]).

2.5 Loco-motor and exploratory activity (marmoset monkeys)

Explorative loco-motor activity was measured in a computerized setup, the so-called “bungalow” test [26,8]. Marmoset monkeys were allowed to move freely for 20 min in an apparatus consisting of four compartments interconnected by tubes. A video tracking system records measured for movement pattern and speed (e.g. time spent in each compartment, number of changes between compartments). The motor activity was expressed as the number of compartment changes in this time period.

2.6 Hand-eye coordination (marmoset monkeys)

Hand-eye coordination was tested in a robot-guided computerized test-system [27]. This system uses marshmallow-like rewards as a motivating stimulus. The small sweets were presented by a robot and moved in front of the marmoset monkey at different speeds (non-moving, slow moving and fast moving), challenging the animal to follow and reach out. This system tests for aspects of alertness, vigilance, reaction time, motor speed and accuracy [8,28]. The percentage of correct hits (successful trial) was used as criterion to judge the performance of the animal.

2.7 EEG registration

Under isoflurane/O_2 anesthesia combined with the local anesthetic lidocaine two stainless electrodes were placed into a small hole in the skull both 3 mm lateral to the *sutura sagitalis* and 5 mm caudal from *intra-aural* or 2 mm anterior from *intra-aural* leaving the *dura mater* intact. Both electrodes were connected by a plug for telemetric registration of the EEG (Data Sciences) and fixed on the skull with dental cement. EEG signals were amplified, filtered and fed into an AD converter of a PC; sampling frequency was 50 Hz. The appearance and duration of seizures is recorded. Furthermore, Fast Fourier Transformation (FFT), to obtain power spectra, was performed from EEG epochs of 10.24 s. The obtained power spectra was averaged per treatment group and subdivided into 8 frequency classes (delta1: 0.8-2, delta2: 2-3.5, theta1: 3.5-5.5, theta2: 5.5-7.5, alpha: 7.5-12.5, beta1: 12.5-18, beta2:18-25 Hz). The difference in power of the different frequency classes and the presence of seizures were used for the evaluation of the brain activity.

2.8 Observations

The post-intoxication symptomatology was observed by experienced personnel unaware of the treatment. The registration of signs started immediately after soman intoxication. The following symptoms were scored: 1) chewing: clear chewing-like movement in which the entire head is involved; 2) hyper salivation: extensive drooling; 3) mild tremor: slight shivering; 4) severe tremor: intense shivering, the entire body is involved; 5) convulsions: involuntary tensed movement in which the entire body is involved, during which the animal is refractory to stimulatory impulses and 6) dyspnoea: low respiratory rate and heavy breathing, in guinea pigs often accompanied with dark eyes. The severity of a symptom was calculated as the % of total scoring hits of the animals in which the symptom was observed. Also the number of animals suffering from each symptom was used to indicate the severity of the post-intoxication symptoms.

2.9 Statistical analysis of the results

For statistical analysis of the behavioral tests And EEG an analysis of variance (ANOVA) was used followed by a Newmann Keuls post-hoc test. Paired T-tests were used to investigate differences between vehicle and specific treatments. For the symptomatology after soman intoxication a Fisher exact probability test or an unpaired t-test with Welch's correction was used. In all tests p-values < 0.05 was considered significant.

3.0 RESULTS

3.1 Survival and symptomatology

In Table 1 and 2 the survival and symptoms tremors, convulsions and dyspnoea in resp. guinea pigs and marmoset monkeys are shown. In guinea pigs, the currently used PYR pretreatment increases the time spent having convulsions (compared to control) which are due to the longer time till death of the animals. Convulsion activity was found in almost all groups but was absent in the group pretreated with PHY/PC. Also the lack of dyspnoea after the PHY/PC pretreatment indicates that this pretreatment prevents the more severe intoxication symptoms in guinea pigs and marmoset monkeys.

Table 1: 24 hrs survival and symptomatology of different pretreatment and therapy scenarios in guinea pigs.

Prophylaxis	Therapy	n=	Survival 24h (n=)	T (n=)	C (n=)	D (n=)
Saline	Saline	8	0	6	8	8
PYR	Saline	8	0	6	8	5
PHY	Saline	8	3	8	8	4
PC	Saline	8	3	5	5	4
PHY/PC	Saline	8	8	1	0	0
(+)PHY/PC	Saline	8	4	8	8	8
PHY/SCO	Saline	8	8	5	2	2
Saline	PHY	8	1	8	7	7
Saline	PC	8	1	7	7	5
Saline	PHY/PC	8	4	5	6	5
Saline	(+)PHY/PC	8	1	8	8	8
Saline	PHY/SCO	8	1	8	8	8

T: tremors; C: convulsions; D: dyspnoea

PHY/PC and PHY/SCO pretreatment lead to a 100% 24 hours survival, whereas both the control as the de-centrally effective compound PYR did not protect against the lethal effects of soman in guinea pigs. Nevertheless, only inhibiting central AChE activity is neither enough for protection against intoxication; the PHY treatment only protected less than half of the animals against lethality.

Table 2: 24-hrs and 1-week survival and symptomatology of different pretreatment scenarios in marmoset monkeys.

Pretreatment	n=	Survival 24h (n=)	Survival 1wk (n=)	T (n=)	C (n=)	D (n=)
PYR	6	6	6	6*	1	1
PHY/SCO	6	6	5	2	1	1
PHY/PC	6	6	6	1	0	0

*significantly different compared to PHY/SCO or PHY/PC pretreatment (Fisher exact probability test: $p<0.05$)

Only in the PHY/SCO pretreated group one monkey died after 24-hours after intoxication. In the PHY/PC pretreated group the monkeys showed less symptoms and less monkeys were affected. Other not scoreable behavioral aspects were observed. The PYR pretreated monkeys exposed an anxiety-like behavior. The PHY/SCO pretreated monkeys showed scratching activity on the legs arms and head. The PHY/PC pretreated animals showed a slow lateral movement of the head. All these signs were only found after intoxication with soman in all monkeys of each group.

3.2 Body temperature

The body temperature after soman intoxication is shown in Figure 1. It was found that the body temperature of the non-surviving animals is increased for several hours before the guinea pigs deceased in the groups of the PHY or PYR pretreatment and therapy. In the PYR pretreatment group all guinea pigs died. In the pretreatment groups PHY/PC and PHY/SCO all guinea pigs survived with relatively stable body temperature. The body temperature stayed stable in the guinea pigs treated with PC alone or in combination with PHY.

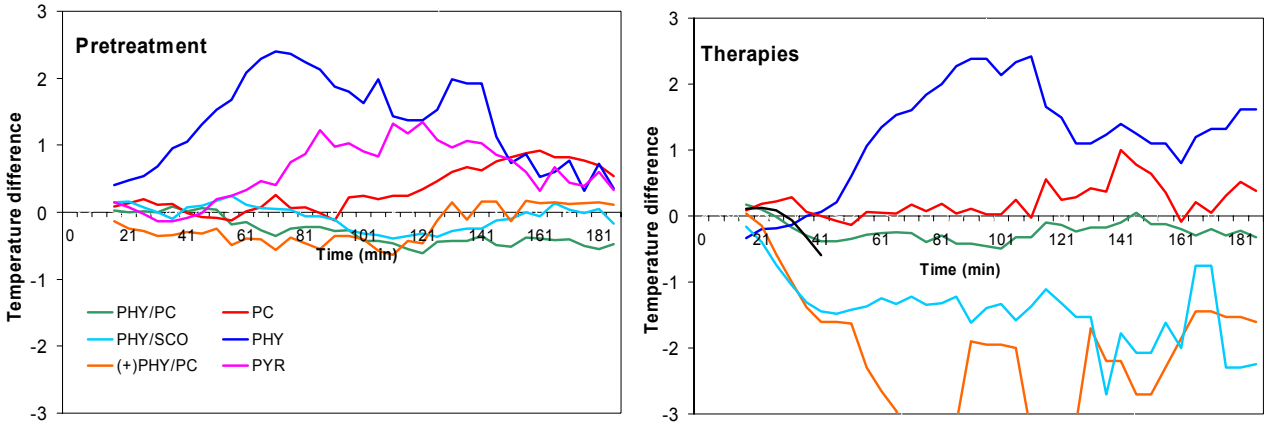

Figure 1: Mean body temperature of pretreated guinea pigs (left figure) or guinea pigs receiving a therapy after intoxication (right figure) at several time points during a 3-hour period after intoxication with 2xLD$_{50}$ soman.

3.3 EEG

The EEG of the PHY/PC and PHY/SCO pretreated groups after soman intoxication was not different from normal compared with their baseline values (see Figure 2). All other groups showed an increase of the delta power and a decrease of the theta power. PYR pretreated guinea pigs showed also a clear increase of the slow alpha activity like the untreated guinea pigs. In case only a post-intoxication therapy was used the EEG of all groups was affected.

Visual inspection of the EEG signals indicates that the brain activity of the PHY/C and PHY/CO pretreatment groups are least affected. The PHY/C pretreatment group showed neither seizures nor any behavioral convulsions and had a 100% 24h survival (see Figure 3).

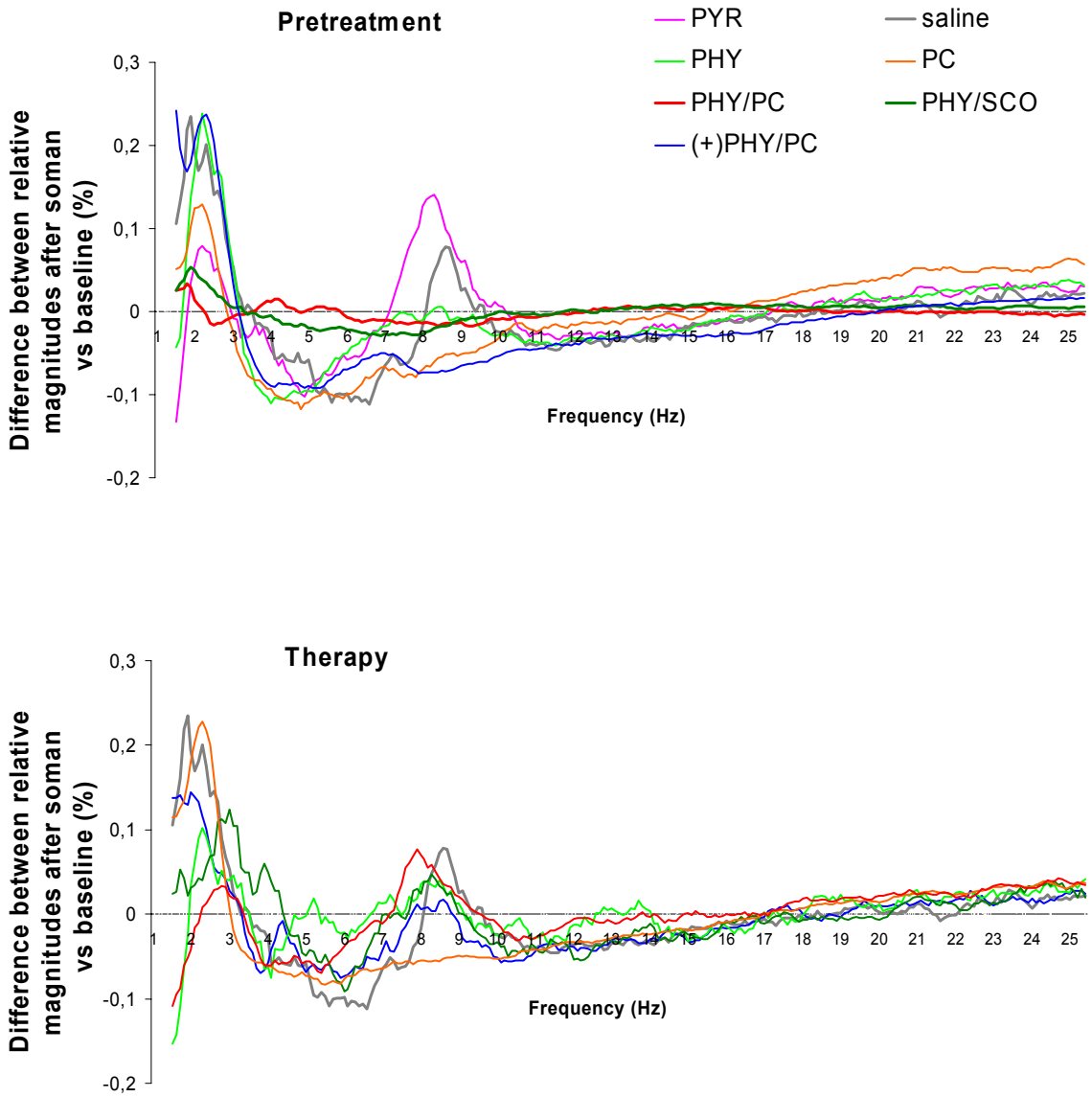

Figure 2: Mean difference between relative EEG Power magnitudes after post-intoxication vs baseline EEG of pretreated guinea (upper curve) or guinea pigs using a post-intoxication therapy (lower curve). The FFT was only performed before the start of seizure activity.

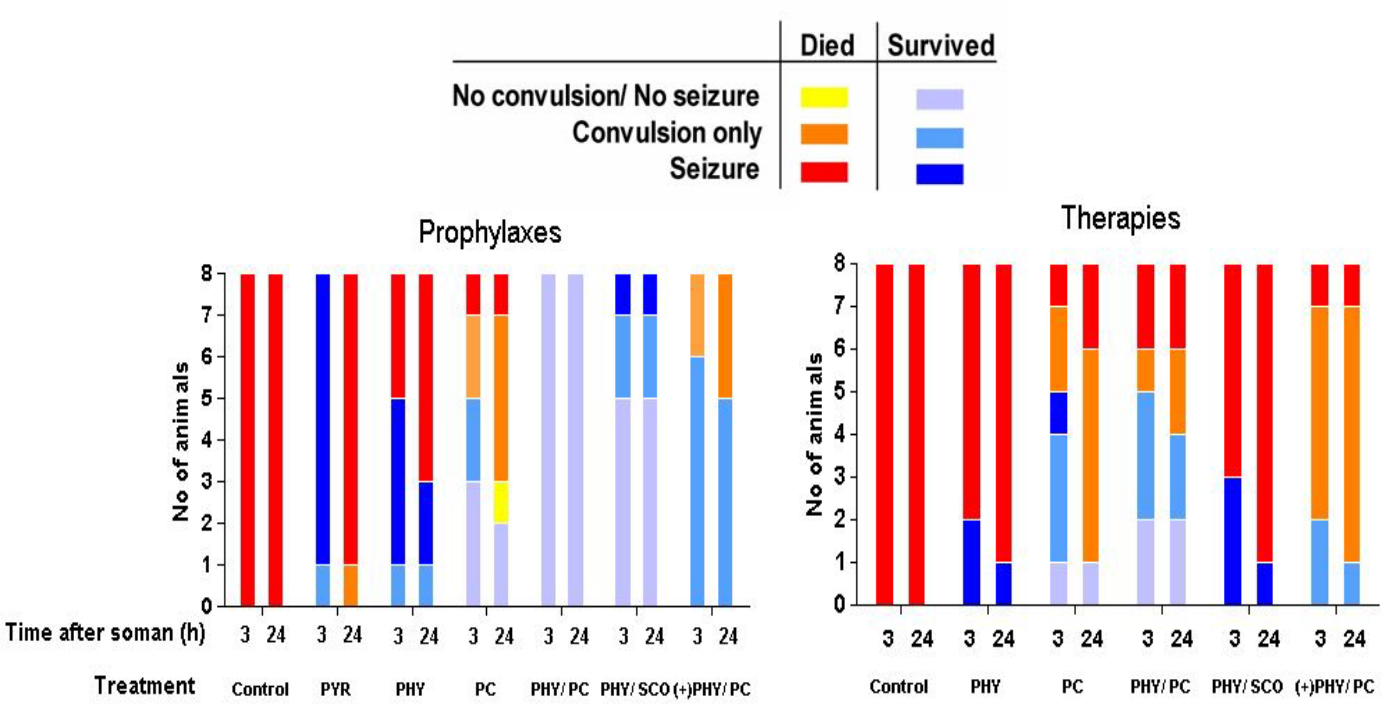

Figure 3: Number of guinea pigs, treated with pretreatment (prophylaxes) or therapy, with or without seizure activity and behavioral convulsions related to survival.

3.4 Post-intoxication incapacitation

3.4.1 Shuttle box performance (guinea pigs)

All treatments show a decline in active avoidance performance 3 hrs after intoxication. The PYR pretreatment and (+)PHY/PC therapy groups did not respond at all (see Figure 4). Central AChE inhibition plays an important role in preventing incapacitation; PHY alone, PHY/PC and PHY/SCO prevent a prolonged performance decline.

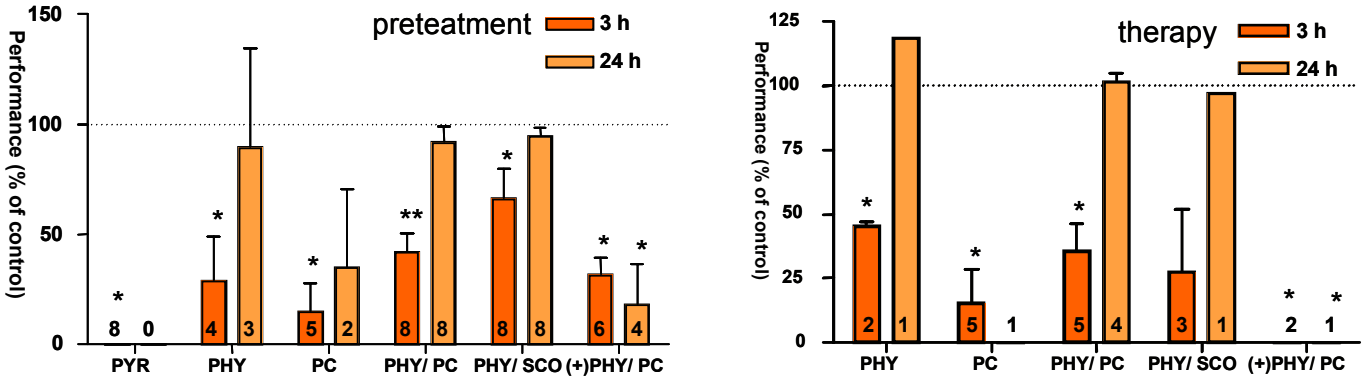

Figure 4: Mean (+SEM) of the percentage of correct avoidance responses (CARs), 3 and 24 hrs after intoxication.* indicate statistical significances compared to baseline. Numbers in bars indicate the number of animals measured (survivors only), PZ is saline.

3.4.2 Loco-motor activity (marmoset monkeys)

The loco-motor activity measured with the so-called Bungalow showed a decline in activity shortly after soman intoxication in the PYR pretreated monkeys. The activity of the monkeys from the other two groups was not affected (see Figure 5). 24 Hrs after soman a decline of the activity was found in all groups. Only in the PYR and PHY/PC pretreated animals this effect was found to be significant ($P<0.05$).

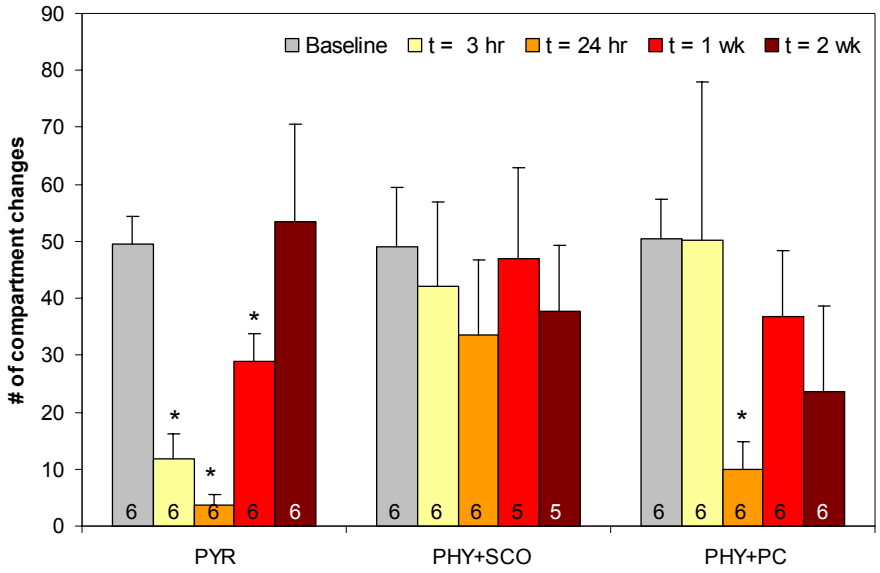

Figure 5: Mean (+SEM) number of compartment changes at 3, 24 hrs, 1 and 2 weeks after 2xLD_{50} soman in PYR, PHY/SCO, and PHY/PC pretreated marmosets. *Statistical significant compared to baseline.

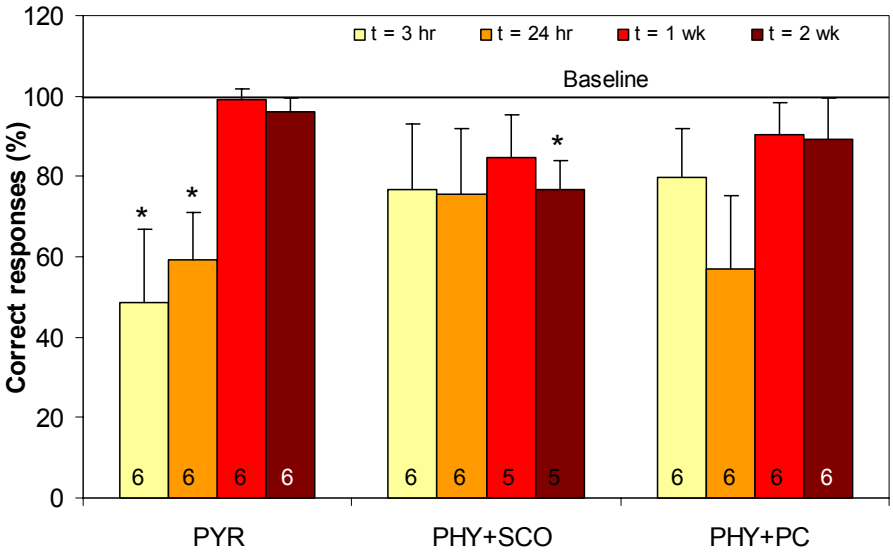

Figure 6: Mean (+SEM) percentage of baseline (100%) correct responses at 3, 24 hrs, 1 and 2 weeks after 2xLD_{50} soman in PYR, PHY/SCO, and PHY/PC pretreated marmosets. The numbers in the bars represents the number of monkeys tested. *Statistical significances compared to baseline.

3.4.3 Hand-eye coordination (HEC) (marmoset monkey)

In Figure 6 the effects on the HEC is shown. Similar to the effects on the activity the performance on this learned task was significantly declined shortly after soman intoxication in animals pretreated with PYR. Animals from the two other groups (PHY/SCO, PHY/PC) still performed at 80% of their baseline level.

4.0 DISCUSSION

The efficacy of new and better medical countermeasures against chemical threat exposures in a guinea pig and marmoset monkey model was investigated. Alternative compounds with another or additional mechanisms of action than AChE inhibition alone were investigated. Sign-free doses were used. PHY and SCO were already tested for side-effects in guinea pigs and marmoset monkeys [29,30,31,7,8,11]. The dose levels of PHY/PC used in this study were also sign free on EEG, behavior and cognition in guinea pigs and marmoset monkeys (not shown), as was also described by Myhrer *et al.* [32] in a study using therapeutic doses of PHY and PC in rats.

In this study the combination of PHY/SCO and the combination of PHY/PC did offer the best protection against seizure activity in the brain and the development of motor convulsive activity. From PC and SCO it is known that they can terminate seizure activity after 2xLD_{50} soman intoxication in PYR pretreated guinea pigs [33]. The combination of PHY/PC as a post-intoxication therapy after 2xLD_{50} soman without any pretreatment was also effective in preventing the development of convulsions and hippocampally monitored seizures in rats [34]. The occurrence of convulsions and/or seizures after intoxication is a good predictor for neuronal damage [35]. From our results we can also conclude that the absence of convulsions and/or seizures is a good predictor for survival. Indeed, animals with no seizures and/or convulsions (in particular animals from the PHY/SCO and PHY/PC groups) survived the intoxicant. This was the case for guinea pigs as for marmoset monkeys. The presence of convulsion and seizure activity did not necessarily lead to death within 24 hrs. However, these surviving animals only showed convulsions or seizures for a very short period of time (< 10 min). Animals from these groups also showed an increase in delta EEG activity. An increase of the relative power in the delta band might be a real-time marker of the ongoing development of soman-induced, seizure-related cerebral lesions and a reliable predictor for the final neuronal losses to come [36]. Unfortunately, no histological data of the PYR pretreated guinea pigs is available, but based on the seizure activity and on the increase of the power on the delta frequency band it is likely that brain damage was apparent.

Notably, convulsions without seizures were observed in a considerable amount of non-survivors. It seems that despite the fact that no seizure activity was observed on the EEG, indicating some protection against the effects of intoxication on the brain, the peripheral effects of the intoxication such as convulsions and heart failure may remain and can result in mortality. This was clearly the case in the PC and (+)PHY/PC (both pretreatment and therapy) groups. Remarkably, only in these two test groups the AChE was not protected against binding to soman. Therefore, the protection of AChE may prevent peripheral intoxication effects. This was also the case with PYR pretreatment which only inhibits AChE de-centrally and did not protect against the intoxication. From the PYR pretreatment group, none of the guinea pigs survived. The only observed improval was that less guinea pigs pretreated with PYR suffered from dyspnoea. This was presumably due to the peripheral protection of the respiration. This indicates that the AChE inhibition must also affect the central compartment in order to be effective. However, the PHY pretreatment (3/8 24h survival) shows that central AChE inhibition only is insufficient to reach complete protection. This was also found by Lim *et al.* [12] and Gordon *et al.* [5]. On the other hand, despite the absence of seizure activity in the brain, the post-intoxication incapacitation could not be prevented by direct effects on receptors alone as was the case of (+)PHY/PC pretreatment. Regarding the performance on the active avoidance task only animals in which the enzyme AChE was protected by the carbamate PHY, recovered within 24 hours.

As already indicated survival rate was highest in the pretreatment groups PHY/PC and PHY/SCO (both 100 %). It was shown earlier that pretreatment with the combination of PHY/PC was effective on survival and post-intoxication incapacitation against 1.3xLD_{50} in rats [37] and against 2xLD_{50} in guinea pigs [38] and together with HI-6 against soman intoxication in dogs [39]. Strikingly, the pretreatment with (+)PHY/PC was the third best group on survival. In a previous study we found that the symptomatology and the post-intoxication incapacitation after 2xLD_{50} soman were similar for (+)PHY and PHY pretreated guinea pigs [21]. This means that not only the enzyme inhibition of PHY is responsible for survival but

also direct effects of PHY on presumably nicotinergic receptors. It is known that PHY can also affect cholinergic receptors directly [16,17,18,19]. The ED_{50} of PHY agonism at the nicotinergic receptor even appears to be lower than its IC_{50} of AChE inhibition [17]. In a previous study with guinea pigs (not published) we found that the addition of mecamylamine to the pretreatment prevent the animals completely from convulsions and dyspnoea which led to less post-intoxication incapacitation. Because mecamylamine augments the pretreatment with PHY the involvement of the nicotinic receptor could be more prominent than indicated before. Indeed, release of glutamate, leading to excitotoxicity and subsequently to an increase of Ca^{2+} in the neurons resulting in cell death, can be stimulated by nicotine in brain tissue [40].

The interaction between the cholinergic and glutaminergic system was also found in the effects on the body temperature. In the combination pretreatment groups the body temperature remained relatively stable in all guinea pigs. These three pretreatment groups were the groups with the best survival rates. In general we found that the body temperature of the non-surviving animals is increased several hours before the animals deceased. Normally the animals that died very shortly after intoxication which was the case in the untreated animals after soman intoxication, showed a decline in the body temperature. In this study only the guinea pigs from the PHY/SCO and (+)PHY/PC post-intoxication groups showed a decline in the body temperature which was not found when these compounds were used as a pretreatment before soman intoxication. It is known that soman intoxication leads to a drop in body temperature which is a muscarinic receptor related event [41]. Anti-muscarinics may prevent this temperature effect. As the pretreatment of PHY/SCO is indeed able to prevent this event which was also found by Wetherell *et al*. [42], this treatment given after soman intoxication may presumably too late to stop this muscarinic effect which was also found by Wetherell *et al.* [43]. Remarkably the post-intoxication therapy of the combination of PHY/PC was still able to block the temperature drop after soman. Presumably the cholinergic system is only responsible for the eliciting of the temperature drop and the activation of the NMDA receptor are responsible for the persistance of the body temperature loss. Nevertheless, both PHY and NMDA antagonists like PC seems to be able to prevent hypotermia [44].

The added NMDA agonistic activity of PC might have also some added value over SCO in the efficacy of the PHY/PC pretreatment because the acute PHY/SCO pretreatment group did show some behavioral motor convulsions, while the acute PHY/PC group did not. Since pretreatment of either the PHY or PC alone are clearly less effective, the efficacy of the PHY/PC pretreatment lies within a synergistic effect, an interaction or accumulatory effect, of these different mechanisms of action.

Notably, PHY/PC pretreatment as well as PHY/PC therapy resulted in higher survival rates and lower incapacitation in guinea pigs than PYR pretreatment which is the currently used method of protection against intoxication. On the other hand, in the monkey study comparable efficacy was found between the different treatments. None of the animals showed any signs of seizures on the EEG and in only two animals (PYR and PHY/SCO group) short lasting convulsions were observed. The protective effects found in these monkey experiments results fully on the pretreatments. In a similar study with the same dose of soman, all marmosets treated with AS (5 mg/kg im) one minute after soman died within two hours [7].

On post-intoxication incapacitation small differences were found in the marmoset monkey. At 3 and 24 hours after intoxication the hand-eye coordination performance and the loco-motor activity was significantly reduced in the monkeys pretreated with PYR. Also the post-intoxication symptoms were slightly worse in this group: all animals were suffering from tremors ($p<0.05$). In the PHY/SCO pretreated monkeys only the performance of the hand-eye coordination was decreased two weeks after intoxication. This was mainly due to the decrease in variance. The behavior in the PHY/PC pretreated animals was only affected 24 hours after intoxication on the loco-motor activity. Based on the post-intoxication signs and incapacitation the PYR pretreated monkeys were less protected. This is in accordance with the finding that central protection of the AChE is important to prevent post-intoxication incapacitation in guinea pigs. This was not reflected in the survival; no differences were found between the three test groups.

In spite of the small differences of the pretreatment scenarios in the monkey, the therapeutic approach in the guinea pig indicates that the addition of anti-NMDA effects in the combination of PHY and PC did also offer some protection when given after the first intoxication signs have appeared and increases chances of survival compared to PHY/SCO treatment. The protective effects are based on the combination of enzyme inhibition and direct effects on the cholinergic and NMDA receptors. This underlines the hypothesis that cholinergic mechanisms are responsible for eliciting seizure activity after OP intoxication and that the subsequent recruitment of other excitatory neurotransmitters and loss of inhibitory control are responsible for the maintenance of seizures and the development of subsequent brain damage [2]. The direct effects on the cholinergic system are partly because of the anti-muscarinic and partly because of the anti-nicotinic actions of the treatment. Because PHY and PC both have a high potency for the nicotinic receptor and mecamylamine augments the pretreatment with PHY (not published data) the involvement of the nicotinic receptor could be more prominent than indicated before. Therefore, treatment may be improved by the addition of a nicotinergic antagonist. The role of the nicotinic receptor in the therapeutic approach should be further examined. Besides the anti-cholinergic effects, the central AChE inhibition by PHY is important to prevent convulsive behavior, incapacitation and peripheral effects. It was shown that convulsive behavior without seizure activity on the EEG can also lead to death, presumably by peripheral effects. Because PHY/PC also showed some protective effects when given after the first intoxication signs appeared, the addition of NMDA antagonising effects helps to prevent seizure activity that may lead to death. It was shown that soman-induced neuropathology was not observed in non-seizing animals in which the delta activity was not increased above the pre-soman baseline [our results and 36].

In conclusion, the combination strategies showed to offer the best potency in the efficacy against soman intoxication compared with the single treatments of the compounds. The PHY/PC treatment shows the most potential and should preferably be used as a pretreatment and in case this is not possible, using PHY/PC as a therapy, along with AS, still increases chances of survival compared to PHY/SCO treatment. It is recommended to explore the potential of the addition of PC to the treatment with PHY as a post-intoxication therapy and the optimal treatment protocol.

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