Comparison of Pre-Treatment Regimens Against Soman: *In Vivo* and *in Vitro* Analysis of Treatment Tolerability and Efficacy

John R. Mikler, Christine S. Stewart, Sara T.C. Bohnert,
Kara N. Williamson, Robert Frew, and Thomas W. Sawyer
Medical Therapy Group, Casualty Management Section
Defence Research and Development Canada-Suffield
Box 4000, Medicine Hat, Alberta T1A 8K6, Canada

Phone 403-544-4421, Fax 403-544-4714

John.Mikler@drdc-rddc.gc.ca

ABSTRACT

Pyridostigmine bromide (PYR) continues to be the predominant pre-treatment for organophosphate nerve agent (NA) exposure. However, its effectiveness is limited by its non-selective inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), as well as its inability to enter the CNS. Physostigmine (PHY), which readily enters the brain and has been reported to be more effective than PYR, has not been widely used due to cholinergic toxicity and a short half life. Scopolamine (SCO) is commonly co-administered with PHY to attenuate cholinergic toxicity. Huperzine A (HUP) and Galantamine (GAL) are two possible pre-treatment candidates. Both enter the CNS and selectively inhibit AChE, which leaves BuChE free to scavenge NAs. The antimuscarinic, Caramiphen (CRM), has inherent anticonvulsant/neuroprotective properties, potentially making it a suitable replacement for SCO. This study compares the tolerability and efficacy of HUP (0.05 and 0.25 mg/kg) and GAL (8 mg/kg), alone and in combination with CRM (100 mg/kg), to PHY/SCO (0.5/5.0 mg/kg) as pre-treatments against soman exposure in guinea pigs. In the tolerability studies, animals received a pre-treatment regimen and were monitored for 7 days for signs of toxicity, body weight changes and blood and brain cholinesterase activity. CRM and 0.05 mg/kg HUP alone and in combination were the only pre-treatments which did not cause observable signs of toxicity. To assess efficacy, animals were pre-treated 10 min prior to a 1.5x LD50 soman (sc) challenge. The combination of CRM with either HUP (0.05 mg/kg) or GAL (8.0 mg/kg) protected animals almost sign free up to 4 hours after soman exposure. Based on tolerability and efficacy against 1.5x LD50 challenge, the HUP (0.05 mg/kg) and CRM combination was assessed as the most effective and was thus tested against a 3.0x LD50 challenge. Four of six animals survived with two animals eliciting only mild symptoms. In vitro studies using a phrenic nerve/diaphragm preparation suggest that HUP and CRM are more effective than GAL and CRM at restoring contractile function following soman exposure. These results suggest that the combination of HUP and CRM should be considered as a future pre-treatment regimen.

1.0 INTRODUCTION

The effectiveness of therapy against nerve agent (NA) exposure is limited by the ability of current oximes to regenerate phosphonylated (NA inhibited) cholinesterase. Enzyme regeneration can be completely prevented by specific nerve agent chemical characteristics and/or occurrence of dealkylation of enzyme-bound NA (aging); consequently the medical therapy of NA exposure can be severely compromised. To enhance the efficacy of post-exposure oximes, a pretreatment with the reversible cholinesterase inhibitor, pyridostigmine bromide (PYR) was implemented. PYR exists as an ionized quarternary ammonium

Mikler, J.R.; Stewart, C.S.; Bohnert, S.T.C.; Williamson, K.N.; Frew, R.; Sawyer, T.W. (2007) Comparison of Pre-Treatment Regimens Against Soman: *In Vivo* and *in Vitro* Analysis of Treatment Tolerability and Efficacy. In *Defence against the Effects of Chemical Hazards: Toxicology, Diagnosis and Medical Countermeasures* (pp. 15-1 – 15-16). Meeting Proceedings RTO-MP-HFM-149, Paper 15. Neuilly-sur-Seine, France: RTO. Available from: http://www.rto.nato.int.

compound that is unable to cross the blood brain barrier and is therefore confined to peripheral tissues (Gordon et al., 1978). PYR provides temporary protection of cholinesterase enzymes by carbmoylating the active-site serine in a reversible manner which prevents irreversible binding of NA's and increases the time for the removal of NA from the vital physiological site (Ellin and Kaminskis, 1989). Several insufficiencies exist with the current use of PYR. First, due to its short half-life PYR must be given three times a day, which may affect compliance. Secondly, PYR pretreatment failed to improve the protective ratio of animals challenged with sarin (GB) or VX (Koplovitz et al., 1992). Finally and maybe most importantly because PYR cannot enter the CNS, it fails to provide protection against OP induced seizures and subsequently seizure induced brain injuries (Leadbeater et al., 1985). Consequently, although PYR pretreatment does offer a substantial increase in protection against soman (GD) exposure compared to oxime and atropine (ATR) alone, significant improvements may be achieved by increasing the amount of drug reaching the CNS and improving the pharmacokinetic profile.

To achieve CNS protection a reversible inhibitor of cholinesterase which crosses the blood brain barrier would be preferred. Physostigmine (PHY), a non-ionized tertiary amine carbamate, which readily enters the CNS has been studied. Based on lethality and post exposure symptoms, PHY was more effective than PYR against soman (GD) and sarin (GB) poisoning. Unfortunately, due to PHY's higher toxicity liability and shorter half-life, it has not been favoured to replace PYR as the current pretreatment (Gordon et al, 1978).

HUP is an alkaloid isolated from the Chinese club moss, *Huperzia serrata* and has been demonstrated to be superior to PHY for its AChE potency and selectivity, increased biological half-life and reduced cholinergic toxicity (Laganiere et al, 1991; Lallement et al., 2002). These characteristics allow for selective inhibition of AChE without inhibiting BuChE which can act as an endogenous bioscavenger (Tang and Han, 1999); a longer half-life which allows for more convenient dosing and longer protection following OP exposure (Lallement et al., 2002); and decreased peripheral cholinergic symptoms while still providing CNS protection (Lallement et al., 1997). HUP has been shown to inhibit AChE activity throughout the CNS, and following oral administration, AChE inhibition peaked between 30-60 minutes (Tang and Han, 1999). Efficacy against lethal GD exposure has been shown in both rhesus monkeys and guinea pigs with doses ranging from 50 to 500 μg/kg (Lallement et al., 1997; Lallement et al., 2002). Compared to PYR a greater percentage of animals survived and a significant decrease in seizure incidence was observed. Survival and anti-convulsant effectiveness was shown to be dose dependent based on studies using doses of 100 and 500 μg/kg although transient adverse cholinergic effects were observed in animals receiving the higher dose (Tonduli et al., 2001). Consequently, HUP appears to be more effective than PYR and PHY based on efficacy and tolerability and the protective effect of HUP may be enhanced by increasing the dose with the worsening of adverse effects.

Several cholinolytic drugs have been used to limit the adverse cholinergic effects of PYR or PHY pretreatment. Although ATR has traditionally been used other compounds have increased efficacy against NA exposure. For example, cholinolytics with increased CNS penetration such as scopolamine (SCO), as well as other drugs that antagonize additional receptors such as benactyzine (BNZ), trihexyphenidyl (THP) and caramiphen (CRM) all show increased efficacy over ATR against NA exposure (Lennox et a., 1992). CRM (10 mg/kg) has been shown to prevent seizures and reduce brain damage and cognitive deficits in rats exposed to GD (Raveh et al., 2002) as well as double the protective ratio of PYR (Raveh et al., 1999). These anticonvulsant and protective effects appear to be related to anticholinergic and antiglutamatergic properties of CRM (Raveh et al., 2002). This makes CRM an ideal adjunct for pretreatment with a reversible cholinesterase inhibitor such as HUP.

The objectives of this study were to;

1) Establish the most effective pretreatment dose of HUP and CRM individually and in combination. The effectiveness being based on the tolerability of each pretreatment regimen and its efficacy against a 1.5x LD_{50} GD challenge (subcutaneous).

2) Determine the efficacy of the combined regimen and compare it against an established pretreatment of PHY and SCO. Protection efficacy will be assessed by survival numbers and health status of surviving animals.

2.0 MATERIALS AND METHODS

2.1 Animals: All experiments were carried out in accordance with the Canadian Council on Animal Care (CCAC) guidelines and the study protocol was approved by the DRDC Suffield Animal Care Committee. Male Hartley guinea pigs weighing 400 – 600 g and Sprague-Dawley rats weighing 200 – 300 g were obtained from Charles River (Saint-Constant, QC). Animals were acclimatized for at least 2 weeks prior to use and were housed in pairs in clear shoebox cages using a 12 hour light/dark cycle. Standard chow and tap water were provided *ad libitum* with 2 carrot slices (~10 g/slice) provided per guinea pig per day for enrichment.

2.2 Chemicals: (-) Huperzine A (HUP) was provided by Biomedisyn (Woodbridge, CT**).** Caramiphen edisylate was purchased from ADF Pharma (Georgetown, ON). Galantamine hydrobromide (GAL), physostigmine free base (PHY), and scopolamine hydrobromide (SCO) were purchased from Sigma-Aldrich Canada (Oakville, ON). Soman (GD, pinacolylmethlyphosphonofluoridate) was synthesized by the Single Small Scale Facility at DRDC Suffield, Alberta, Canada and was determined to be >98% pure by GC/MS and NMR. Euthansol (sodium pentobarbital) was purchased from Schering Canada Inc. (Point-Claire, QC). All other chemicals were purchased from Sigma-Aldrich Canada (Oakville, ON) or elsewhere as indicated.

All chemicals were diluted immediately prior to injection. Soman was diluted in anhydrous ethanol and then saline for a final injection solution of 10% ethanol saline. HUP, GAL, PHY and SCO were all dissolved in ethanol and then transferred to saline for a final injection solution of 10% ethanol saline. CRM was dissolved directly in saline.

2.3 Pretreatment Tolerability Studies: Animals were randomly assigned to 1 of 9 experimental groups with each group consisting of 6 animals. The groups were:

a) 10% Ethanol-Saline Control
b) 100 mg/kg CRM
c) 0.05 mg/kg HUP
d) 0.25 mg/kg HUP
e) 8.00 mg/kg GAL
f) 0.05 mg/kg HUP + 100 mg/kg CRM
g) 0.25 mg/kg HUP + 100 mg/kg CRM
h) 8.0 mg/kg GAL + 100 mg/kg CRM
i) Positive Control – 0.5 mg/kg PHY + 5 mg/kg SCO

All compounds except CRM were administered via an intraperitoneal (ip) injection; injection volumes were less than 2 mL. CRM was administered by intramuscular injection in a volume less than 0.5 mL. In studies using 2 compounds, CRM or SCO were administered 10 min prior to HUP, GAL or PHY.

The health status of the animals was assessed by changes in body weight (measured every morning) and visual assessment. Animals were observed every 5 min for the first hour following initial treatment, then every 30 minutes for the next 6 hours and once in the morning and afternoon for the rest of the treatment period. Qualitative health status was assessed using a 5 level scale. Scoring was based on the following criteria: 1 for animals appearing and behaving as normal, 2 for mild tremors or fasciculations present, 3 for severe tremors and fasciculations with the addition of any of the following; salivation, lacrimation, increased mouth movement, vocalizations, decreased mobility or paritial paralysis, 4 for full body

convulsions or paralysis with severely laboured breathing (gasping) and 5 for death. Animals that scored a 4 at anytime were monitored at 5 minute intervals. If the rating continued to be a 4 for 10 consecutive minutes, animals were considered terminal and euthanized using a 1.0 mL ip injection of pentobarbiotal.

Blood samples were taken immediately prior to test compound administration (baseline), 1 and 24 hours following the first injection and at the end of the experimental period. Blood samples of 0.05 to 0.10 mL were collected by pipette aspiration following laceration of an ear vein. Samples were immediately aliquoted into a 1.5 mL centrifuge tube containing 0.004 mL of a 15% K_3-EDTA solution (BD Biosciences, Oakville, ON) and stored at -80°C.

2.4 Efficacy Studies Against 1.5x LD_{50} Soman Challenge: The protocol followed that of the tolerability studies but following pre-treatment animals were subcutaneously (sc) challenged with a 1.5x LD50 of GD 10 min after the last pre-treatment dose. Health status assessment, euthanasia, blood sampling and tissue sampling were all conducted as in the previous tolerability studies.

2.5 Efficacy Studies Against 3.0x LD_{50} Soman Challenge: The protocol followed that of the efficacy animals. Animals were pretreated with 0.05 mg/kg of HUP and 100 mg/kg of CRM but then challenged with a 3.0x LD_{50} of GD. Only health status was monitored in these animals.

2.6 Modified Ellman Method for Cholinesterase Activity Determination: Blood cholinesterase activities were determined using a modified Ellman method (Wilson et al, 2002). Acetylthiocholine iodide (ATC) or Butyrylthiocholine iodide (BTC) were used as substrates for selective acetyl- and butyrylcholinesterase activity respectively. Thawed whole blood was diluted 1:20 in phosphate buffer (pH 7.3). To each well of a 96 well plate 10 µL of diluted blood sample (final concentration of 1:400) and 0.05 mL of 0.25mM DTNA was added. The plate was then incubated at room temperature for 5 minutes. Finally, 10 µL of ATC substrate (0.5 mM) or 20 µL of BTC substrate (1.0 mM) was added to each well to a final volume of 200 µL. The plate was incubated at 37ºC and absorbance readings were acquired at 340 nm every 2 minutes for 30 minutes with shaking prior to each reading. Thirty minute absorbance values (baseline corrected) were compared between treated and control samples and expressed as percent of control activity.

2.7 Hemi-Diaphragm Phrenic Nerve Functional Study: Male Sprague Dawley rats (~250 g) were euthanized prior to the removal of the diaphragm with the phrenic nerve intact. Removed tissue was placed in a tissue holder with an attached transducer to measure force of contraction. Diaphragms were pretreated with, saline (control), 10 mM CRM + 3 mM HUP or 10 mM CRM + 10 mM GAL. Diaphragms were challenged with 1 nM soman (30 min after pretreatment) for 15 min before wash out. Tetanic nerve stimulation was measured at 100 Hz for 3 seconds at various time points up to 240 min post-GD challenge. Contractions are presented as a percentage of control values.

3.0 RESULTS

3.1 Tolerability of Pretreatment Regimens: Animals were monitored for 7 days following pre-treatment administration. Three pre-treatment regimens, 100 mg/kg CRM, 0.05 mg/kg HUP and the combination of both did not produce any observable adverse effects in the animals tested (Figure 1). HUP pre-treatment of 0.25 mg/kg produced moderate adverse effects in 1 of the 6 animals tested during the first 2 hours following injection. GAL (8 mg/kg) produced adverse effects in all 4 animals tested with 3 animals exhibiting moderate signs. The combination of CRM with 0.25 mg/kg HUP did not lower the incidence of adverse effects as 2 of 6 animals exhibited adverse effects with 1 showing moderate signs. The combination of CRM did decrease the adverse effects associated with GAL treatment as 3 of 4 animals showed only transient mild signs. The combination of PHY and SCO produced mild signs in all 3 animals tested. No significant difference in body weight gain following pre-treatment was observed between groups (Figure 2).

Blood samples were collected from each animal 1, 24, and 96 or 168 hours after the initial pre-treatment administration. The acetylcholinesterase activity measured in three treatment groups, PHY/SCO, 0.25 mg/kg HUP and the combination of 100 mg/kg CRM and 0.05 mg/kg HUP were significantly lowered (30%-50%) from their control values at 1 hour (Figure 3). Butyrylcholinesterase activity was inhibited only after SCO/PHY pre-treatment. The inhibition was observed only at 1 hour following pre-treatment as activity in all treatments returned to control values by 24 hr. Low dose HUP treated animals appeared to have a significantly elevated AChE value at 7 days and was the only treatment to produce such a response.

3.2 Efficacy Studies Against 1.5x LD_{50} Soman Challenge: In untreated animals that received a subcutaneous injection of 1.5x LD_{50} of GD (37.5 µg/kg), toxic signs appeared within 5 to 10 min with all animals displaying severe signs by 30 to 35 min (Figure 4). All animals (10 of 10) had died or were euthanized due to severe signs 45 min following GD exposure. Pretreatment with CRM delayed the onset of signs until 35 to 55 min but prevented death in only 2 of 6 animals past 48 hours. Low dose HUP (0.05 mg/kg) delayed the onset of symptoms in 2 of 3 animals but did not prevent death in any animal past 5 hours following exposure. High dose HUP (0.25 mg/kg) did not appear to delay the onset of signs and prevented death in only 1 of 6 animals past 48 hours post exposure. GAL did not delay symptoms but if animals survived the first 2-3 hours (3 of 5) they were sign free and appeared normal for the rest of the experimental period. Combination of CRM with low dose HUP provided almost complete protection for 60 minutes following exposure. Five of 6 animals exhibited only transient mild signs but 1 animal did die at 4 hours. The combination of CRM and high dose HUP did not delay the appearance of signs and 2 of 6 animals died within the first 24 hours. Signs of toxicity were not observed in animals that survived past the first 24 hours. In animals pre-treated with CRM and GAL transient mild signs were exhibited in 4 of 5 animals within the first 30 min but all animals survived. All animals pretreated with PHY/SCO survived but 5 of 6 animals exhibited mild signs of toxicity immediately following exposure for up to 2 hours. No significant difference in body weight gain following pre-treatment was observed between groups except for animals treated with CRM alone (Figure 5). The body weight of CRM animals failed to return to initial values and appeared to be decreasing by the end of the 7 day test period.

At 1 hour post GD exposure all AChE activity was significantly decreased in all pre-treatment groups except high dose HUP (Figure 6). Twenty-four hours following exposure only high dose HUP in combination with CRM had significantly recovered to ~60% of initial activity. By 7 days all AChE in all pre-treatment groups returned to control values except for CRM pretreated animals which remained at ~20%. Similar inhibition was observed with BChE activity except CRM in combination with low dose HUP reached only ~40% of control activity.

3.3 Efficacy Studies Against 3.0x LD_{50} Soman Challenge: Based on the results from tolerability and efficacy against 1.5x LD_{50} GD studies, CRM in combination with low dose HUP was tested against a 3.0x LD_{50} GD (75 µg/kg) challenge. The pretreatment delayed the onset of symptoms for ~20 min post-exposure and 4 of 6 animals survived (Figure 7). Body weight and cholinesterase activity were not measured in these animals.

3.4 Hemi-Diaphragm Phrenic Nerve Functional Study: The combination of HUP and CRM appeared to provide better protection of contractile function than GAL and CRM following GD exposure (Figure 8).

4.0 DISCUSSION

The present study demonstrates the improved effectiveness of combining a reversible cholinesterase inhibitor, HUP or GAL with the anti-muscarinic compound, CRM. This combination can delay the onset of toxicity providing an increased window for post-exposure treatment and can protect animals up to 3 times the lethal dose of GD without the requirement of additional medical countermeasures. Maximum

efficacy however was not achieved with the highest dose of HUP which suggests dose selection should not be based solely on the level of cholinesterase inhibition or tolerability.

An improved pretreatment regimen should provide protection to a range of NA, delay the onset of NA toxicity and negate the requirement of additional medical countermeaures (Layish et al., 2005). The effectiveness of pretreatment must be achieved at a dose that is completely tolerated under all physiological conditions (Phillippens et al., 2005). Current pretreatment with PYR is only effective against GD exposure and must be used in conjunction with post-exposure medical countermeasures (Koplovitz et al., 1992). Based on the mechanism of action it can be hypothesized that increasing the dose of reversible cholinesterase inhibitor would increase pretreatment effectiveness (Harris et al., 1991). Furthermore increasing availability of the pretreatment compound into the CNS would increase efficacy against NA seizure induced brain damage (Albuquerque et al., 2006). However increased efficacy would also increase the liability of cholinergic toxicity. The addition of an antimuscarinic compound has been shown to decrease the cholinergic peripheral and neurophysiological adverse effects of reversible cholinesterase inhibitors (Harris et al., 1991; Albuquerque et al., 2006).

Huperzine doses used in this study had previously been shown to produce little or no toxicity (Lallement et al, 2002). In this study only 1 animal at 0.25 mg/kg exhibited mild cholinergic signs. The addition of CRM did not decrease the occurrence of toxicity as 1 animal also showed adverse signs when the combination was administered. The addition of CRM did appear to lessen the extent of blood AChE inhibition of high dose HUP. Other studies have used higher doses of HUP without reported toxicity but several of these used a racemic mixture of HUP where the percentage of the active isomer (-)HUP was not known (Lallement et al., 2001; Tonduli et al., 2001; Albuquerque et al., 2006). In this study only the active isomer of HUP, (-) HUP was used which may account for the mild toxicity observed at 0.25 mg/kg. The dose of GAL, 8 mg/kg, had been previously reported to be completely tolerated (Albuquerque et al., 2006) however in this study adverse effects were noted in all animals at this dose (Figure 1). These effects were observed despite very little inhibition of blood AChE (Figure 3). The addition of CRM significantly improved the tolerability of GAL suggesting these effects were mediated by activation of muscarinic receptors. The combination of PHY/SCO was used as a positive control but there is limited published data on the use of this combination as a one time pretreatment. Previous studies demonstrated that chronic administration of PHY/SCO was effective against GD exposure (Wetherell et al., 2002; Lallement et al., 2001). However these same doses administered as a single injection failed to protect any animals at 1.5x LD_{50} GD (not shown). Therefore, a higher dose of each compound was used, and despite the combination adverse signs were observed (Figure 1). As expected this combination was the only regimen to inhibit blood BuChE activity (Figure 3). This result supports previous reports of the selective AChE inhibition of HUP and GAL and the non-selective action of PHY (Albuguerque et al., 2006; Lallement et al., 2001). The low dose of HUP (0.05 mg/kg) and its combination with CRM were the only cholinesterase inhibitor regimens that were completely tolerated by all animals (Figure 1). This combination did not produce any adverse effects despite 40% inhibition of blood AChE (Figure 3). Consequently, based on the results from tolerability studies the combination of low dose HUP and CRM would be considered the most acceptable regimen.

As reported in previous studies the addition of an antimuscarinic compound also improves the pretreatment efficacy of cholinergic inhibitors (Lallement et al., 2001; Albuquerque et al., 2006). GAL was reported to be less effective against GD exposure in the absence of ATR (Albuquerque et al., 2006). Those results are supported in this study as the addition of CRM to GAL pretreatment prevented any lethality following GD exposure (Figure 4). Complete protection was also observed in animals pretreated with PHY/SCO but in both pretreatment regimens the onset of signs was not delayed. The effectiveness of high dose HUP was significantly improved with the addition of CRM however 2 animals still did not survive. Interestingly, CRM pretreatment on its own did delay signs until 35 to 40 min post-exposure which suggests the level of ChE inhibition with high dose HUP, GAL and PHY is near or at the level to produce cholinergic overstimulation. The use of low dose HUP and CRM prevented signs for up to 2 hrs

following GD which suggests a safer margin of ChE inhibition although 1 animal failed to survive the challenge (Figure 4).

Based on the tolerability and efficacy results, low dose HUP in combination with CRM was chosen to test against a 3x LD_{50} GD challenge. Four of six animals survived supporting the effectiveness of this pre-treatment however it remains to be determined if a HUP dose between 0.05 and 0.25 mg/kg would provide increased efficacy with continued tolerability.

In vitro diaphragm contraction studies also suggest that HUP/CRM is more effective than GAL/CRM at protecting muscle function following GD exposure. Although previous studies have shown that HUP is less effective than carbamate inhibitors at protecting muscle function (Eckert et al., 2006) the inhibition time of 15 min might favour HUP action as it has a relatively short time of cholinesterase inhibition.

These results support previous work demonstrating the effectiveness of centrally acting cholinesterase inhibitors, such as HUP, in combination with an antimuscarinic compound, such as CRM, against GD exposure. Further studies are required to determine the optimal dose regimen and the range of effectiveness when combined with post-exposure medical countermeasures.

5.0 FIGURES

(a) 100mg/kg CRM

		1hr																	
	CRM	5	10	15	20	25	30	35	40	45	50	55	60	2h	3h	4h	5h	6h	24h	48h	7days
06SEP001																					
06SEP002																					
06SEP003																					
06SEP004																					
06SEP005																					
06SEP006																					

(b) 0.05mg/kg HUP

		1hr																	
	HUP	5	10	15	20	25	30	35	40	45	50	55	60	2h	3h	4h	5h	6h	24h	48h	7days
06SEP001																					
06SEP002																					
06SEP003																					
06SEP004																					
06SEP005																					
06SEP006																					

(c) 0.25mg/kg HUP

		1hr																	
	HUP	5	10	15	20	25	30	35	40	45	50	55	60	2h	3h	4h	5h	6h	24h	48h	7days
06SEP007																					
06SEP008																					
06SEP009																					
06SEP010																					
06SEP011																					
06SEP012			2	2	2	2	2	2	2	3	3	3	3	2							

(d) 100mg/kg CRM and 0.05mg/kg HUP

			1hr																	
	CRM		HUP	5	10	15	20	25	30	35	40	45	50	55	60	2h	3h	4h	5h	6h	24h	48h	7days
06SEP007																							
06SEP008																							
06SEP009																							
06SEP010																							
06SEP011																							
06SEP012																							

(e) 100mg/kg CRM and 0.25mg/kg HUP

				1hr																		
	CRM		HUP	5	10	15	20	25	30	35	40	45	50	55	60	2h	3h	4h	5h	6h	24h	48h	7days
06SEP025																							
06SEP026																							
06SEP027											2	3	3	2	2	2							
06SEP028																							
06SEP029										2													
06SEP030																							

(f) 8mg/kg GAL

		1hr																			
	GAL	5	10	15	20	25	30	35	40	45	50	55	60	2h	3h	4h	5h	6h	24h	48h	7days
06NOV005				2	2	2	3	2	2												
06NOV006				2	2	2	2	2	2												
06NOV007			3	3	3	3	2	2	2												
06NOV008			3	3	3	3	3	2	2												

(g) 100mg/kg CRM and 8mg/kg GAL

				1hr																	
	CRM		GAL	5	10	15	20	25	30	35	40	45	50	55	60	2h	3h	4h	5h	6h	24h	48h
06NOV001											2		2		2							
06NOV002									2													
06NOV003																						
06NOV004														2								

(h) 0.5mg/kg PHY and 5mg/kg SCO

					1hr																		
	PHY	5	10	SCO	5	10	15	20	25	30	35	40	45	50	55	60	2h	3h	4h	5h	6h	24h	48h
06NOV004			2	2	2	2	2	2	2	2	2	2	2										
06NOV007									2	2		2	2	2									
06NOV008				2	2		2		2	2													

Figure 1: Tolerability of pre-treatment regimens as assessed by observable signs. Each row represents an individual animal with the observed rating score indicated by the colour box under the observation period. Pre-treatment regimen is listed at the top of each group; (a) 100 mg/kg CRM (caramiphen), (b) 0.05 mg/kg HUP (huperzine), (c) 0.25 mg/kg HUP (huperzine), (d) 8 mg/kg GAL – (galantamine), (e) 100 mg/kg CRM and 0.05 mg/kg HUP, (f) 100 mg/kg CRM and 0.25 mg/kg HUP, (g) 100 mg/kg CRM and 8 mg/kg GAL, and (h) 0.5 mg/kg PHY and 5 mg/kg SCO (physostigmine and scopolamine) . Observed signs rating scores are represented by the colour in each box, white (1-normal), green (2-mild), yellow (3-moderate), red (4-severe) and black (5-deceased) with the time of the observation indicated at the top of each column.

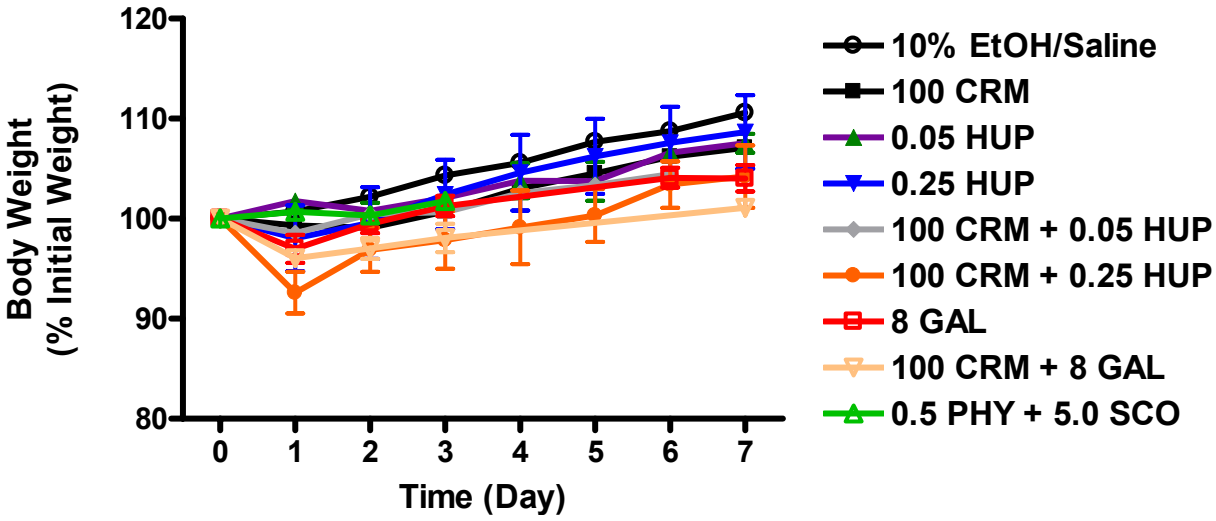

Figure 2: Effect of pre-treatment on guinea pig body weight gain. Animals were weighed every morning for 8 days. The initial body weight (100%) was the weight of the animal on day 0 prior to receiving pre-treatment. All subsequent weights were expressed as a percentage of the initial weight of the animal. The legend indicates the pre-treatment received. Values are the mean ± SEM for at least 3 animals.

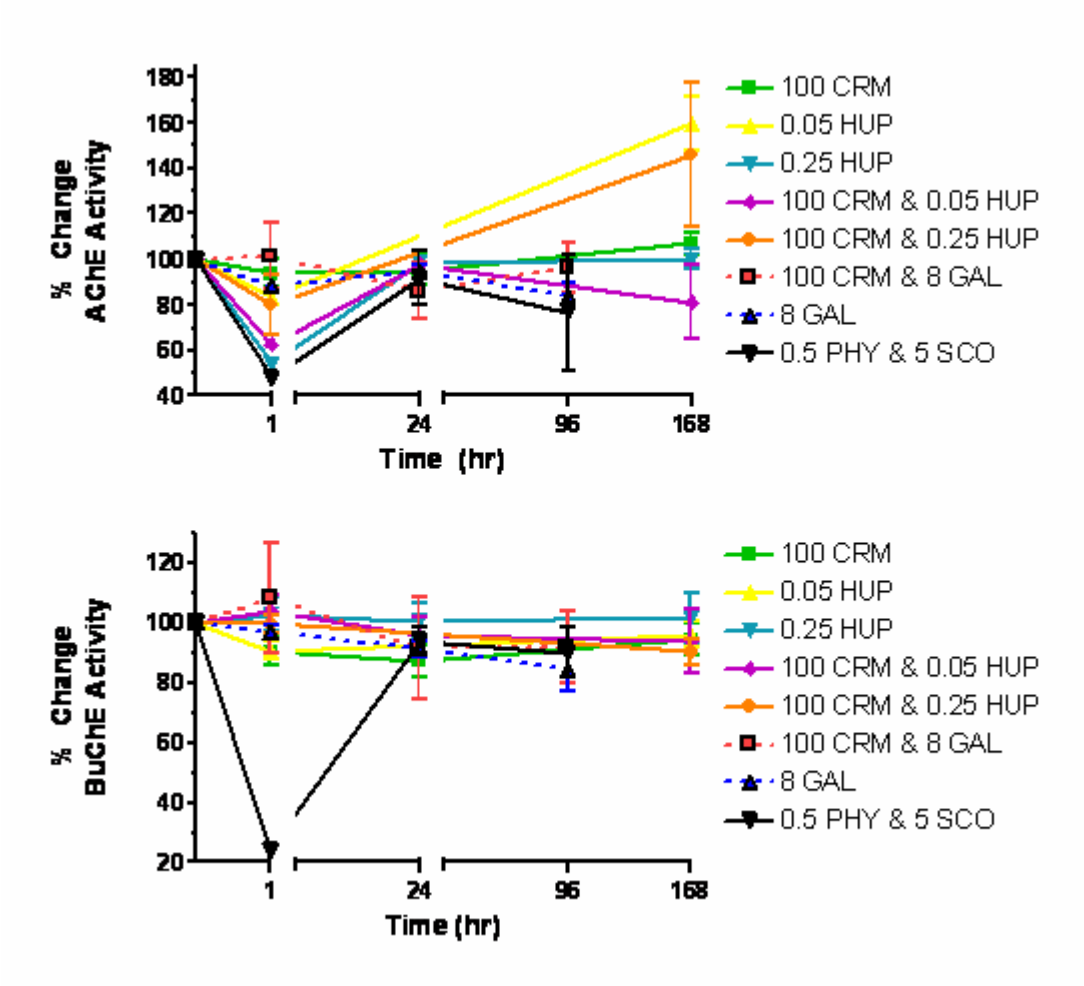

Figure 3: Effect of pre-treatment on whole blood acetylcholinesterase and butyrylcholinesterase activity. Cholinesterase activity was measured using a modified Ellman method. Control activity (100%) was determined in blood samples obtained from an ear vein on the morning before pre-treatment. Subsequent blood samples were obtained at 1, 24, 96 and 168 hours post initial pre-treatment. Pre-treatment regimens are indicated in the legend. Values are the mean ± SEM for at least 3 animals.

(a) Saline Controls & 1.5x LD_{50} GD

	1 Hr												2h	3h	4h	5h	6h	24h	48h
GD	5	10	15	20	25	30	35	40	45	50	55	60							
06JUL069		2	3	3	3	3	4	4											
06JUL073		3	4	4															
06JUL091					2	4	4												
06JUL095	2	3	4	4															
06JUL141				3	4	4													
06JUL142				3	3	4	4												
06JUL153			3	3	3	3	4	4											
06JUL154		2		3	3	4	4												
06JUL157				3	3	4	4												
06JUL158				3	4	4													

(b) 100 mg/kg CRM & 1.5x LD_{50} GD

	1 Hr												2h	3h	4h	5h	6h	24h	48h
GD	5	10	15	20	25	30	35	40	45	50	55	60							
06SEP013							2	2	2	2	3	3	2	2	3	3	3	4	
06SEP014											2	3	3	3	3	2	2	2	
06SEP015											2	3	3	3					
06SEP016								2	2	2	2	3	3	3	3	3	3	4	
06SEP017								2	2	2		2	2	2	2	2	2	3	
06SEP018											2	2	2	2	2	2	2		

(c) 0.05 mg/kg HUP & 1.5x LD_{50} GD

	1 Hr												2h	3h	4h	5h	6h	24h	48h
GD	5	10	15	20	25	30	35	40	45	50	55	60							
06SEP043									2	2	3	3	3	3	4				
06SEP044			2	3	4	4													
06SEP045											3	3	3	3	4				

(d) 0.25 mg/kg HUP & 1.5x LD_{50} GD

	2 Hr												3h	4h	5h	6h	7h	24h	48h
GD	5	10	15	20	25	30	35	40	45	50	55	60							
06SEP019	3	3	3	3	3	3	3	3	2	2	2	2	2	2	2	2	2		
06SEP020				2	2	2	3	3	3	3	3	3							
06SEP021	2	2	3	3	3	3	4	3	3	3	3	3	3	2	2	2	2	1	1
06SEP022				2	2	3	3	3	2	3	2	3							
06SEP023					2	2	2	2			2	2	2	2	2	2	2		
06SEP024	2	2	2	2	3	2	2	2	2	2	2		4						

(e) 8 mg/kg GAL & 1.5x LD_{50} GD

	1 Hr												2h	3h	4h	5h	6h	24h	48h
GD	5	10	15	20	25	30	35	40	45	50	55	60							
06NOV013	2	2									2	2							
06NOV014			2	2	2	3	4	4											
06NOV015	2	2	2	2	2	2	2	2	2	2	2	2							
06NOV016					2	2	3	3	3	4	4								
06NOV018	2	2	2	2	2	2	2	2	3	3	3	3	3						

(f) 0.05 mg/kg HUP, 100 mg/kg CRM & 1.5x LD_{50} GD

	1 Hr												2h	3h	4h	5h	6h	24h	48h
GD	5	10	15	20	25	30	35	40	45	50	55	60							
06SEP031													2	2					
06SEP032																			
06SEP033													2						
06SEP034																			
06SEP035													2						
06SEP036					2														

(g) 0.25mg/kg HUP, 100mg/kg CRM & 1.5x LD_{50} GD

	1 Hr												2h	3h	4h	5h	6h	24h	48h
GD	5	10	15	20	25	30	35	40	45	50	55	60							
06SEP037			2	2	2	2	2	2	2	2	2	2	2	2	2	2	2		
06SEP038			2	2	2	2	2	2	2	2	2	2	2	2	2	2	2		
06SEP039													3	3	3	3	3		
06SEP040		2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2		
06SEP041								2	2	2	2	2	2	2	2	2	2		
06SEP042														2	2	2	2		

(h) 8 mg/kg GAL, 100mg/kg & 1.5x LD_{50} GD

	1 Hr												2h	3h	4h	5h	6h	24h	48h
GD	5	10	15	20	25	30	35	40	45	50	55	60							
06NOV017		2	2																
06NOV019						2													
06NOV020					2														
06NOV021																			
06NOV022	2																		

(i) 0.5 mg/kg PHY, 5.0 mg/kg SCO & 1.5x LD_{50} GD

	GD	1 Hr 5	10	15	20	25	30	35	40	45	50	55	60	2h	3h	4h	5h	6h	24h	48h
06NOV009		2	2	2	2	2	2	2			2									
06NOV010			2	2	2		2													
06NOV011		2	2	2	2	2	2	2	2	2	2	2	2							
06NOV012		2	2	2		2				2	2	2	2	2						
06NOV005																				
06NOV006					2	2	2	2	2	2	2	2	2	2						

Figure 4: Efficacy of pre-treatment regimens against a subcutaneous 1.5x LD_{50} GD challenge as assessed by observable signs. Each row represents an individual animal with the observed rating score indicated by the colour box under the observation period. Pre-treatment regimen is listed at the top of each group; (a) no pretreatmen (control), (b) 100 mg/kg CRM (caramiphen), (c) 0.05 mg/kg HUP (huperzine), (d) 0.25 mg/kg HUP (huperzine), (e) 8 mg/kg GAL – (galantamine), (f) 100 mg/kg CRM and 0.05 mg/kg HUP, (g) 100 mg/kg CRM and 0.25 mg/kg HUP, (h) 100 mg/kg CRM and 8 mg/kg GAL, and (i) 0.5 mg/kg PHY and 5 mg/kg SCO (physostigmine and scopolamine) . Observed signs rating scores are represented by the colour in each box, white (1-normal), green (2-mild), yellow (3-moderate), red (4-severe) and black (5-deceased) with the time of the observation indicated at the top of each column.

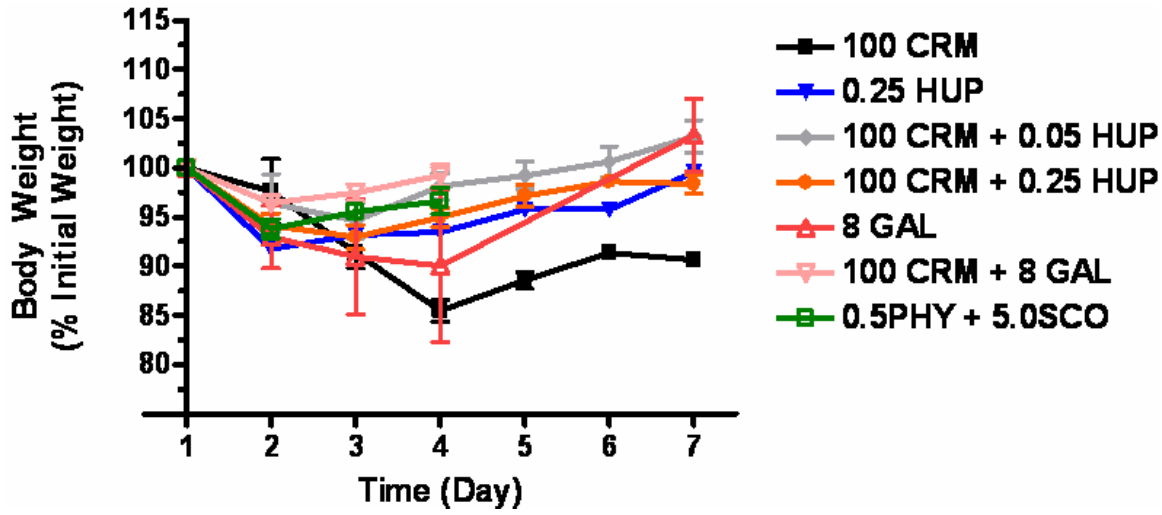

Figure 5: Efficacy of pre-treatment against soman challenge as assessed by body weight gain. Surviving animals were weighed every morning for 8 days. The initial body weight (100%) was the weight of the animal on day 0 prior to receiving pre-treatment. All subsequent weights were expressed as a percentage of the initial weight of the animal. The legend indicates the pre-treatment received. Values are the mean ± SEM.

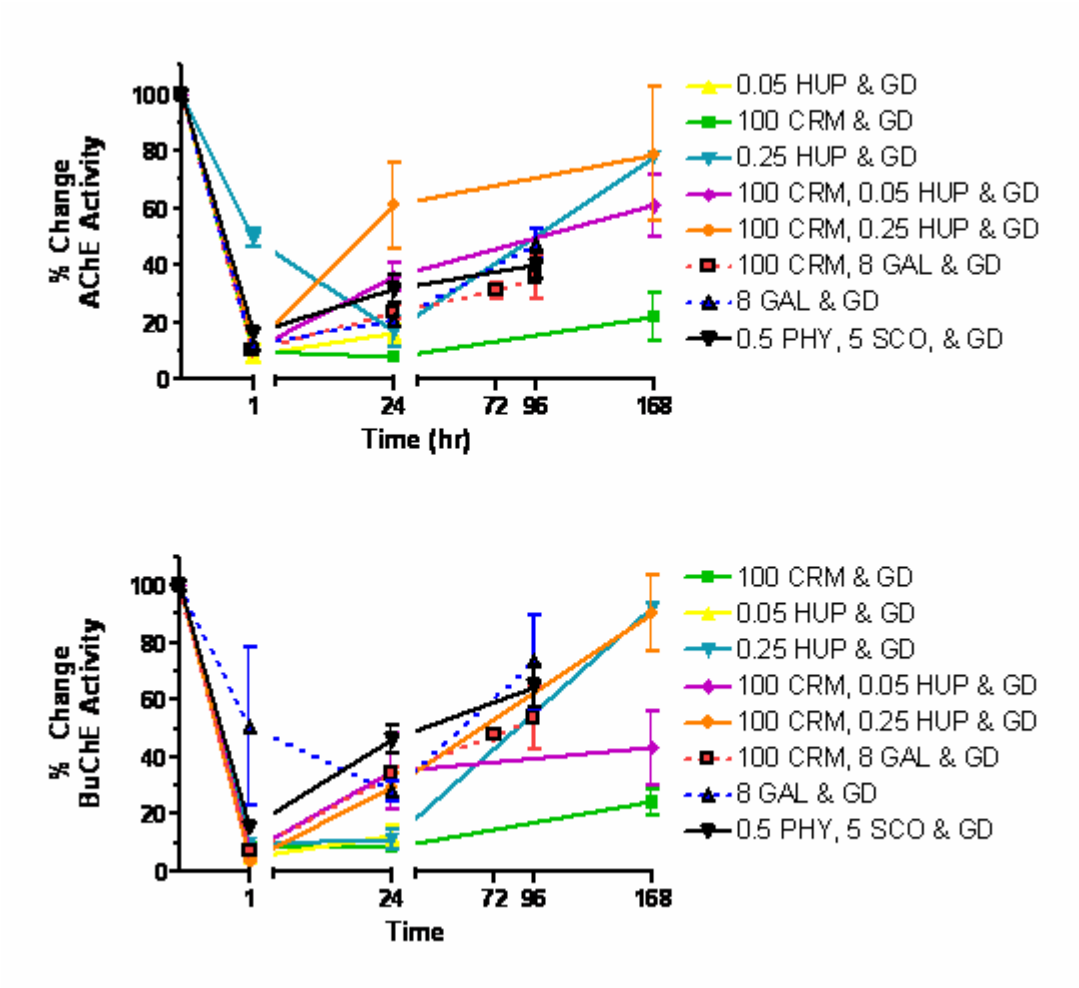

Figure 6: Whole blood acetylcholinesterase and butyrylcholinesterase activity following pre-treatment and soman exposure. Cholinesterase activity was measured using a modified Ellman method with acetylcholinesterase and butyrylcholinesterase activity being selectively assessed by the use of the substrates acetylthiocholine and butyrylthiocholine respectively. Control activity (100%) was determined in blood samples obtained from an ear vein on the morning before pre-treatment. Subsequent blood samples were obtained at 1, 24, and 96 or 168 hours post initial pre-treatment. Pre-treatment regimens are indicated in the legend. Values are the mean ± SEM.

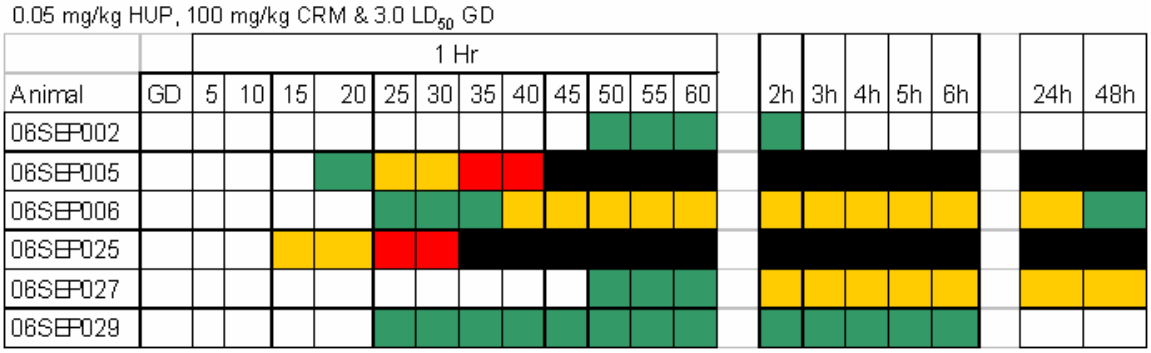

Figure 7: Efficacy of 0.05 mg/kg HUP and 100 mg/kg CRM pre-treatment against a subcutaneous 3.0x LD_{50} GD challenge as assessed by observable signs. Each row represents an individual animal with the observed rating score indicated by the colour box under the observation period. Observed signs rating scores are represented by the colour in each box, white (1-normal), green (2-mild), yellow (3-moderate), red (4-severe) and black (5-deceased) with the time of the observation indicated at the top of each column.

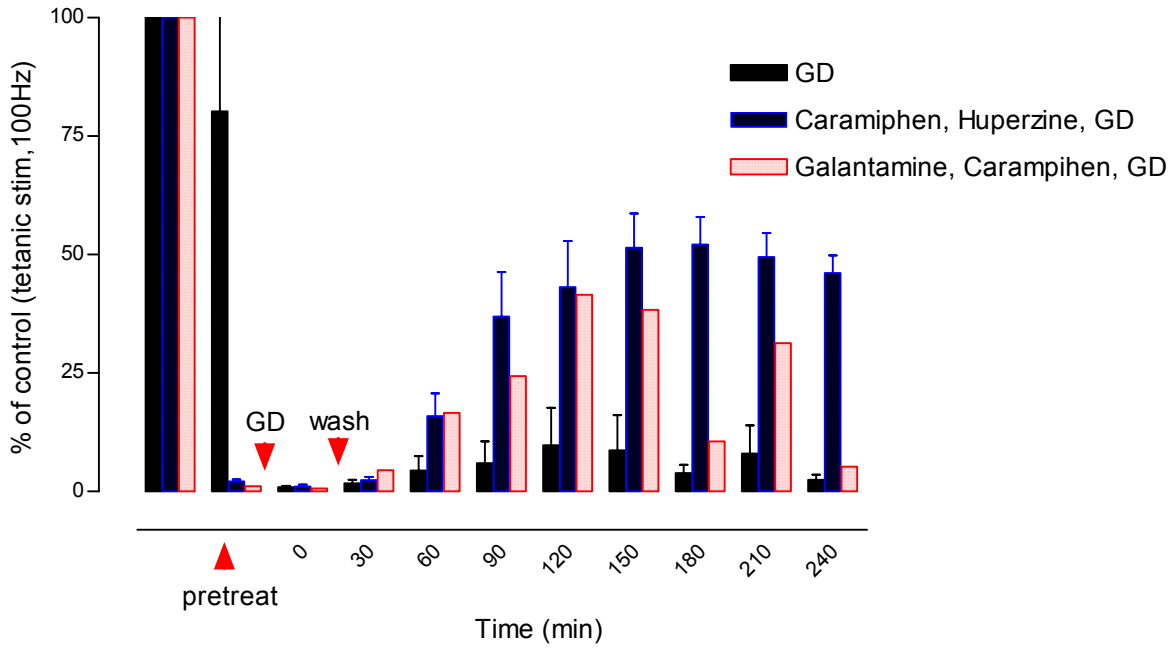

Figure 8: Hemi-diaphragm phrenic nerve functional *in vitro* study. Rat phrenic nerve hemi-diaphragms were stimulated with 100 Hz for 3 seconds at each time point. Diaphragms were pretreated with, Saline (control), 10 mM CRM + 3 mM HUP or 10 mM CRM + 10 mM GAL. Diaphragms were challenged with 1 nM soman (30 min after pretreatment).

6.0 REFERENCES

Albuquerque, E.X., E.F. Pereira, Y. Aracava, W.P. Fawcett, M. Oliveira, W.R. Randall, T.A. Hamilton, R.K. Kan, J.A. Romano, Jr. and M. Adler, 2006, Effective countermeasure against poisoning by organophosphorus insecticides and nerve agents, Proc Natl Acad Sci U S A 103, 13220.

Eckert, S., P. Eyer, H. Muckter and F. Worek, 2006, Kinetic analysis of the protection afforded by reversible inhibitors against irreversible inhibition of acetylcholinesterase by highly toxic organophosphorus compounds, Biochem Pharmacol 72, 344.

Ellin, R.I. and A. Kaminskis, 1989, Carbamoylated enzyme reversal as a means of predicting pyridostigmine protection against soman, J Pharm Pharmacol 41, 633.

Gordon, J.J., L. Leadbeater and M.P. Maidment, 1978, The protection of animals against organophosphate poisoning by pretreatment with a carbamate, Toxicol Appl Pharmacol 43, 207.

Harris, L.W., B.G. Talbot, W.J. Lennox, D.R. Anderson and R.P. Solana, 1991, Physostigmine (alone and together with adjunct) pretreatment against soman, sarin, tabun and VX intoxication, Drug Chem Toxicol 14, 265.

Koplovitz, I., L.W. Harris, D.R. Anderson, W.J. Lennox and J.R. Stewart, 1992, Reduction by pyridostigmine pretreatment of the efficacy of atropine and 2-PAM treatment of sarin and VX poisoning in rodents, Fundam Appl Toxicol 18, 102.

Laganiere, S., J. Corey, X.C. Tang, E. Wulfert and I. Hanin, 1991, Acute and chronic studies with the anticholinesterase Huperzine A: effect on central nervous system cholinergic parameters, Neuropharmacology 30, 763.

Lallement, G., V. Baille, D. Baubichon, P. Carpentier, J.M. Collombet, P. Filliat, A. Foquin, E. Four, C. Masqueliez, G. Testylier, L. Tonduli and F. Dorandeu, 2002, Review of the value of huperzine as pretreatment of organophosphate poisoning, Neurotoxicology 23, 1.

Lallement, G., J.P. Demoncheaux, A. Foquin, D. Baubichon, M. Galonnier, D. Clarencon and F. Dorandeu, 2002, Subchronic administration of pyridostigmine or huperzine to primates: compared efficacy against soman toxicity, Drug Chem Toxicol 25, 309.

Lallement, G., A. Foquin, F. Dorandeu, D. Baubichon and P. Carpentier, 2001, Subchronic administration of various pretreatments of nerve agent poisoning. II. Compared efficacy against soman toxicity, Drug Chem Toxicol 24, 165.

Lallement, G., J. Veyret, C. Masqueliez, S. Aubriot, M.F. Burckhart and D. Baubichon, 1997, Efficacy of huperzine in preventing soman-induced seizures, neuropathological changes and lethality, Fundam Clin Pharmacol 11, 387.

Layish, I., A. Krivoy, E. Rotman, A. Finkelstein, Z. Tashma and Y. Yehezkelli, 2005, Pharmacologic prophylaxis against nerve agent poisoning, Isr Med Assoc J 7, 182.

Leadbeater, L., R.H. Inns and J.M. Rylands, 1985, Treatment of poisoning by soman, Fundam Appl Toxicol 5, S225.

Lennox, W.J., L.W. Harris, D.R. Anderson, R.P. Solana, M.L. Murrow and J.V. Wade, 1992, Successful pretreatment/therapy of soman, sarin and VX intoxication, Drug Chem Toxicol 15, 271.

Philippens, I.H., M.J. Joosen and R.A. Vanwersch, 2005, Stress adversely affects efficacy of physostigmine-scopolamine pretreatment against soman in guinea pigs, Pharmacol Biochem Behav 82, 125.

Raveh, L., B.A. Weissman, G. Cohen, D. Alkalay, I. Rabinovitz, H. Sonego and R. Brandeis, 2002, Caramiphen and scopolamine prevent soman-induced brain damage and cognitive dysfunction, Neurotoxicology 23, 7.

Tang, X.C. and Y.F. Han, 1999, Pharmcological profile of Huperzine A, a novel acetycholinesterase inhibitor from chinese herb, CNS Drug Review 5, 281.

Tonduli, L.S., G. Testylier, C. Masqueliez, G. Lallement and P. Monmaur, 2001, Effects of Huperzine used as pre-treatment against soman-induced seizures, Neurotoxicology 22, 29.

Wetherell, J., T. Hall and S. Passingham, 2002, Physostigmine and hyoscine improves protection against the lethal and incapacitating effects of nerve agent poisoning in the guinea-pig, Neurotoxicology 23, 341.

Wilson, B.W., J.D. Henderson, A. Ramirez and M.A. O'Malley, 2002, Standardization of clinical cholinesterase measurements, Int J Toxicol 21, 385.