

# Recombinant Human Butyrylcholinesterase as a Therapeutic Agent to Counteract the Effects of VX Toxicity in Domestic Swine

CC. Tenn\*, JR. Mikler, I. Hill, K. Weatherby, M. Garrett, N. Caddy  
C. Stewart, CN. Karatzas<sup>1</sup> Y. Huang<sup>2</sup>, J. Troyer<sup>3</sup>, PM. Lundy and  
TW. Sawyer

Casualty Management Section, DRDC Suffield  
P.O. Box 4000, Station Main,  
Medicine Hat, Alberta  
Canada T1A 8K6

<sup>1</sup>CNKonsulting and MDDI Ltd.  
251 Sherwood Road,  
Beaconsfield, Quebec,  
Canada H9W 2H4

<sup>2</sup>PharmAthene Canada Inc.  
7150 Alexander-Fleming  
Montreal, Quebec  
Canada H4S 2C8

<sup>3</sup>PharmAthene ,Inc.  
One Park Place  
Suite 450  
Annapolis, MD 21401

**\*Corresponding Author:**

Casualty Management Section  
DRDC Suffield  
P.O. Box 4000, Station Main  
Medicine Hat, Alberta Canada T1A 8K6  
Tel (403) 544-5096  
Fax: (403) 544-4714  
Email: catherine.tenn@drdc-rddc.gc.ca

## ABSTRACT

The efficacy of a recombinant human butyrylcholinesterase (rBChE) in protecting against topically administered VX was examined in anaesthetized domestic swine. Animals exposed to 2LD<sub>50</sub> of VX showed signs of organophosphate poisoning and died within 2—3 hours. When a single dose of rBChE was administered 15 min post VX exposure, survival times increased but the animals died before the end of the six-hour test period. In an attempt to increase the effectiveness of rBChE, the dose was administered in 5 equal portions at various times post-agent. The animals survived with little or no signs of poisoning. Using this multiple rBChE dosing regimen, animals were monitored for 7 days following VX exposure. The VX exposed and rBChE treated animals were compared to swine that received rBChE only or were exposed to VX and treated with the current standard of treatment for nerve agent poisoning: 2PAM and atropine. Animals were monitored for clinical signs, body weight and cognitive function. Blood samples were taken to determine biochemistry, electrolytes and cholinesterase levels. None of the surviving animals displayed any sign of cognitive impairment, moreover no hematological or chemical abnormalities were observed. These results demonstrated that rBChE was effective in protecting the animals against a lethal dose of VX.

## INTRODUCTION

Organophosphate (OP) compounds are potent neurotoxins that can be used as chemical nerve agents, making them potential threats in both military and civilian situations. The major mechanism of toxicity is the inhibition of acetylcholinesterase (AChE), which is responsible for degradation of the neurotransmitter, acetylcholine. This affects both muscarinic and nicotinic receptors in the central and peripheral nervous systems, causing hypersecretion, tremors, convulsions, respiratory distress and death [Weinbroum, 2005]. The current standard of treatment for nerve agent poisoning is the administration of a drug combination that includes an anti-muscarinic agent, such as atropine, an oxime reactivator (e.g., pralidoxime chloride [2PAM]) and an anticonvulsant, such as diazepam [Dawson, 1994; Kassa, 2002; Shih et al., 1991]. However, this treatment regimen has limitations, as it only addresses the symptoms of OP exposure once they become apparent, making the efficacy of the antidotes dependent on the timing of symptom presentation. As an alternative treatment, the use of enzyme bioscavengers is currently being explored. Bioscavengers react directly with the nerve agent before it inhibits AChE at physiological target sites, thus preventing the availability of the poison and the resulting toxicity [Lenz et al., 2005; Raveh et al., 1993; 1997]. Cholinesterases, particularly butyrylcholinesterase (BChE), are among the enzymes that are being examined as potential bioscavengers for the prophylactic treatment of OP toxicity [Doctor and Saxena 2005; Lenz et al., 2005; Allon et al., 1998; Raveh et al., 1997]. While BChE is capable of hydrolyzing acetylcholine and other choline esters, its exact physiological function remains unknown [Darvesh et al., 2003]. In recent studies, human serum BChE was found to provide full protection against multiple LD<sub>50</sub>s of nerve agents in guinea pigs and

non-human primates [Doctor and Saxena, 2005; Lenz et al., 2005]. Although the use of human serum BChE shows promise for human use, the practicality of this approach is limited by the difficulties in producing sufficient quantities for civilian and military needs. A recombinant dimer form of human BChE (rBChE) that is expressed in the milk of transgenic goats [Cerasoli et al., 2005; Huang et al., 2007] represents a novel solution to this limitation since it can be produced in large quantities.

Due to its low volatility, the nerve agent VX (O-ethyl-S-[2 (diisopropylamino) ethyl] methylphosphonothioate) is considered to be primarily a contact poison. The effect of topical VX exposure was reported to have a delayed onset [Hamilton et al., 2004] thus providing an opportunity for therapeutic intervention. In the past, others have used bioscavengers (including rBChE) as a pretreatment for nerve agent poisoning [Doctor et al., 1991; Lenz et al., 2005; 2007; Raveh et al., 1993; 1997; Saxena et al., 2006] while in this study we have examined the use of rBChE as a therapeutic agent against topically applied VX in anaesthetized domestic swine. The domestic swine was used in this study since it is commonly recognized as a suitable model for human skin absorption studies [see for example Duncan et al., 2002; Singh et al., 2002; Schmook et al., 2001]. Previous studies have used this model system to examine skin penetration, as well as the distribution and toxicity of VX when applied topically [Hamilton et al., 2004]. The efficacy of rBChE as a therapeutic treatment for VX exposure was compared to that of the current standard of treatment for nerve agent: 2PAM and atropine.

## MATERIALS AND METHODS

### Experimental animals

Castrated male Yorkshire-Landrace cross pigs weighing approximately 15 kg were purchased from a local supplier. The animals were housed in groups of six per pen in a temperature controlled area with a 12:00/12:00 hour light/dark cycle (lights on at 6:00 am). The animals had free access to water and were fed twice per day. Swine were allowed to acclimatize for at least one week prior to experimental use. Animals weighed  $20.0 \pm 1.5$  kg at the time of surgery. In conducting this research, the authors adhered to the "Guide to the Care and Use of Experimental Animals" and "The Ethics of Animal Experimentation" published by the Canadian Council on Animal Care.

### Anesthesia

The animals were anesthetized using inhalation induction with 5% isoflurane in a carrier gas of 100% oxygen ( $O_2$ ) at a flow rate of  $8 \text{ L}\cdot\text{min}^{-1}$ . Post-induction, the animals were placed in the dorsal recumbent position on a heated operating table. Once the animals were intubated, the isoflurane concentration was reduced to 3% in 100%  $O_2$  at a flow rate of  $1 \text{ L}\cdot\text{min}^{-1}$ . Core body temperature was maintained at  $38.5 \pm 1.2^\circ\text{C}$ . Once the instrumentation was finished, isoflurane was maintained at a rate of 2% in room air supplemented with

oxygen to a fractional inspired oxygen (FiO<sub>2</sub>) of 0.3. The animals received 0.9% normal saline via an IV line for fluid replacement. A cystostomy was performed in order to monitor urinary output. The animals were allowed to stabilize for at least 30 min, during which time steady-state anesthesia (SSA) was established. Continuous physiological parameters were monitored while the animals were under anesthesia. Blood samples for hematology, electrolytes and cholinesterase (ChE) levels were obtained at induction of general anesthesia (immediately after placement of the anesthetic mask an intravenous line was established in one of the swine's ears and a blood sample was drawn), at SSA and at various time points during the 6 or 6.5 hours under anesthesia and one week after treatment for the recovery experiments. ChE activities were determined by a slightly modified radiometric method of Johnson and Russell [Johnson et al., 1975]. Briefly, the radiolabeled substrate was tritiated acetylcholine iodide (crystals) (Perkin Elmer, Boston, MA), which was prepared in 50% ethanol. The cold substrate used was acetylcholine chloride (Sigma-Aldrich, St. Louis, MO). Each of the blood samples collected was analyzed in triplicates.

### **Pharmacokinetics of rBChE**

To determine the pharmacokinetics of rBChE (without VX exposure), 9.5 mg/kg of material was injected intravenously and blood ChE activity was monitored as a function of time. ChE activity data were analyzed assuming one compartmental distribution using PK Solutions, Non-Compartmental Pharmacokinetic Data Analysis Software Version 2 (Summit Research Services, Montrose, CO).

### **Organophosphate nerve agent and rBChE dosing regimen**

The VX used in this study was synthesized and purified (>98%) by the Canadian National Single Small Scale Facility at DRDC Suffield. Once the animals reached SSA, a drop of VX equivalent to a 2LD<sub>50</sub> (as previously determined — unpublished data) was applied topically to the ventral surface of one ear. Initial studies conducted found the 1LD<sub>50</sub> to be 77 µg/kg. Subsequent experiments carried out just before the recovery studies with rBChE began showed that the LD<sub>50</sub> had dropped slightly to 62.25 µg/kg. Thus, a dose of 124.5 µg/kg ( 2LD<sub>50</sub>) were used in the recovery studies. In order to determine the optimal rBChE dosing regimen, studies were conducted using a 1:1 stoichiometric ratio of rBChE:VX, applied intravenously either as a single dose or in multiple doses. In the first set of experiments, three animals were given a single bolus dose of 46.1 mg/kg of rBChE applied 15 min following VX application. In the second set of experiments, three animals received the full 1:1 stoichiometric dose of rBChE that was divided into five equal doses and administered 15, 75, 135, 195 and 255 min after VX was administered.

### **Behavioral task**

The training task is one that was developed by Alam [Alam et al., 2005], where the swine rely on visual cues such as colour, as well as their strong “rooting behavior”. The task was carried out as follows: the animal was placed in

a training arena with three boxes containing food. Each box had a different colour lid: white, yellow or blue. The blue box was the only one that could be opened by the animal. The animal was expected to go directly to the blue box and open it to get to the food, without attempting to open the other two boxes. The boxes were placed in three of the possible four corners of the training arena and at each session were rotated into different positions. The time taken to complete the task during the session was monitored for each animal (maximum time was 300s). A composite performance score was calculated as follows:

+2 = opens blue box

+1 = touches blue box but does not open it

+1 = does not approach the other two boxes

+0 = smells either of the other two boxes

-1 = tries to open either of the other two boxes

The maximum score possible was +3 (opening of blue box without moving towards the other two boxes). Each false attempt was scored as a negative mark, of which there was no maximum. The session ended as soon as the blue box was opened or at 300s. Training consist of one trial per day for five consecutive days and only animals that reached the criterion score of at least +2 within 60s were used in the recovery experiment.

### **rBChE and 2PAM-atropine treatment in swine exposed to topical VX**

After five days of training, the animals that made the behavioral criterion score were placed into a study that monitored their recovery for seven days following various treatments. On treatment day, the swine were anaesthetized, and once SSA was achieved, the animals were exposed to one of the four following conditions: one group consisting of three swine was given VX and left untreated (VX) and a second group consisting of four swine received intravenously five equal doses of the rBChE at 15, 75, 135, 195 and 255 min after VX was administered (VX/rBChE). From the dosing regimen studies described above, based on protective efficacy over the initial six-hour period that was observed in animals treated with five equal doses of rBChE, this dosing schedule was used for recovery experiments. In addition to the multiple dosing regimen, to increase the likelihood that the animals would survive the entire testing period (7 days), a slightly increased stoichiometric ratio (1.1:1 rBChE:VX) was utilized. The third group consisting of three animals received the same dosage regimen of rBChE but no nerve agent (rBChE). The fourth group consisting of four animals was administered 2PAM and atropine after VX exposure. These animals were injected intramuscularly with three equal doses of 2PAM (600 mg/injection) and atropine (2 mg/injection) at 30, 90 and 180 min after VX was administered (VX/2PAM-atropine). The times selected for the 2PAM-atropine treatment were based on previous studies we have conducted in this model system (unpublished data). The first injection was given when signs of nerve agent poisoning were first observed and ChE activity fell to approximately 20% of the baseline level. The remaining injections were given to maintain the animal under stable conditions throughout the six-hour test period. This dosing strategy 'mimics' the antidotal regimen used in a field situation, i.e.,

autoinjectors are administered when the first signs of nerve agent poisoning are observed and then thereafter to stabilize the casualty.

The nerve agent (2LD<sub>50</sub>) was applied topically to the ventral surface of one ear of the swine. The animals were then monitored for 6.5 hours after VX was administered. During the last 30 min under anesthesia, the VX-exposed ear was decontaminated with Reactive Skin Decontamination Lotion, RSDL. Following the decontamination period, the animals were returned to a recovery pen in the swine housing area. The animals were monitored closely until they appeared to be physiologically stable and were then left alone to fully recover. If an animal showed any signs of respiratory or acute cardiopulmonary crisis, it was immediately euthanized. On the second, third, fourth and seventh day after VX exposure, the animals were retested on the previously learned task. The animals were sacrificed after testing on the seventh day and a comprehensive post-mortem was carried out.

### **Enzyme Kinetics**

Enzyme kinetic experiments were conducted, using a modification of Wilson [Wilson et al., 1999, 2002] method, to determine the K<sub>i</sub> values of VX in whole blood and in a solution of purified rBChE. All solutions were prepared in 50 mM Na/K phosphate buffer (pH 7.4). Frozen whole blood samples obtained from three non-anaesthetized animals were thawed and incubated with VX at 0, 1, 2, 5, and 10 nM. The purified rBChE (0.94 nM) was incubated with 0.94, 1.94 and 4.7 nM VX. VX at a concentration of 130 nM in either blood or rBChE solution was used to provide complete ChE inhibition, which served as the control (blank) sample. The samples were incubated for 45 min at 37°C. After the incubation period, the samples were transferred to a 96 well plate and diluted 1:200 with a final concentration of 0.25 mM 6,6'-dithiodinicotinic acid and a series of acetylthiocholine concentrations of 0.125, 0.25, 0.5, 1.0 and 1.5 mM in a total volume of 0.2 mL. The plate was incubated at 37°C and the absorbance read at 340 nm every 5 min for 30 min using a Bio Tek FL600 Microplate Fluorescence Reader (MTX Lab Systems, Inc., Vienna, VA) plate reader. The K<sub>i</sub>, V<sub>max</sub> and K<sub>m</sub> were determined by non-linear regression of enzyme velocities at various substrate concentrations assuming a non-competitive inhibition using GraphPad Prism 5.00 for Windows (GraphPad Software, San Diego, CA). Data were analyzed by a two-tailed unpaired t-test. A value of  $p \leq 0.05$  was considered significant between groups.

## **RESULTS**

### **Pharmacokinetics of rBChE and Dosing Regimen Optimization**

The animals that were exposed to VX and received no treatment exhibited signs of poisoning, including fasciculations, mastication, salivation and miosis. These animals stopped breathing approximately 1-2 hours after VX exposure and

had to be placed on a ventilator for the remaining six-hour test period. Figure 1 shows that the blood ChE activity in animals exposed to a 2LD<sub>50</sub> dose of VX was dramatically inhibited. Blood ChE activity at the time of anesthetic induction was considered as control levels (100%). All other time points at which blood ChE activity was measured were presented as a percentage of the control values. At 2—3 hours post-VX exposure the ChE activity had declined to approximately 16 ± 2% of control values. The enzyme activities at the time of anesthetic induction were calculated and expressed as the amount of substrate (ACh) hydrolyzed over time per mL of whole blood. The baseline level of AChE was 5.01 ± 0.69 μmol/min/mL.

Figure 2 shows the blood ChE activity following a bolus IV injection of rBChE. The rBChE dose was 9.5 mg/kg, which represents 20% of the amount required to stoichiometrically bind 2LD<sub>50</sub> VX in a 1:1 ratio. After injection of rBChE, blood ChE activity increased immediately to approximately 12 times that of control values and then rapidly decline. The baseline AChE activity was 5.08 ± 1.07 μmol/min/mL. The half-life of rBChE was found to be only 54.1 ± 9.1 min (mean ± SEM, from 3 animals).

When a single bolus dose of rBChE was administered 15 min after VX treatment, the signs of toxicity were delayed. The animals went into respiratory distress and stopped breathing approximately four hours post agent exposure. These animals had no ventilatory support but would spontaneously breathe for a while. However, they eventually all died five hours into the test period. Figure 3 shows the dramatic increase in ChE activity that occurred immediately after a dose of rBChE (46.1 mg/kg) was administered to these animals. Enzyme activity increased over 60 times, but fell towards baseline values over the next several hours. The baseline AChE activity was 5.08 ± 1.07 μmol/min/mL. In an attempt to lengthen the residency time of rBChE in the bloodstream and prolong survival over the six-hour test period, the single bolus dose was divided into five equal portions and administered at various time points as described in the Methods section. This dosing regimen was found to completely protect the animals for the entire test period (data not shown). Predicated on these results, the multiple dosing regimen and a slightly higher rBChE:VX ratio (1.1:1) was selected to ensure the animals survived the seven-day recovery studies.

### **Efficacy of rBChE and 2PAM-atropine in swine exposed to topical VX**

Table 1 illustrates the therapeutic efficacy of rBChE and 2PAM-atropine in anaesthetized swine exposed to VX. Animals exposed to a 2LD<sub>50</sub> dose of VX and left untreated, exhibited signs of nerve agent poisoning with death occurring 2—3 hours later. All animals in the VX/rBChE group showed little or no signs of nerve agent toxicity and survived until the end of the experiment (one week post-treatment). Animals exposed to VX and given the current standard of treatment; 2PAM-atropine also survived and displayed minimal signs of toxicity. Two of the three animals in the rBChE control group displayed mild fasciculations. It is not clear whether this was due to the anesthetic, as mild fasciculations are routinely

observed in anaesthetized animals that have not been exposed to nerve agent. In all surviving animals, toxicity signs cleared within 24 hours.

### **Effect of rBChE and 2PAM-atropine treatment on blood ChE activity in swine exposed to topical VX**

Figure 4 demonstrates the effect of rBChE and 2PAM-atropine treatment on blood ChE activity in anaesthetized swine exposed to topically applied VX. Blood ChE values increased significantly with the administration of each dose of rBChE. The enzyme activity in the rBChE group of animals increased approximately fourfold and rapidly fell to approximately half of that within the hour. For the VX/rBChE animals, the blood ChE values decreased even more so, but still remained significantly above the baseline values. After 6.5 hours under anesthesia, ChE activity for VX/rBChE swine declined more rapidly than the rBChE group of animals. For the VX/2PAM-atropine group of animals, the enzyme activity fell below that of baseline and remained low throughout the period in which they were anaesthetized. One week post-treatment, there was no difference between any of the surviving animals in terms of ChE activity; the enzyme activity for all three groups returning to baseline levels. The baseline levels of AChE activities were  $5.75 \pm 1.2$ ,  $5.05 \pm 1.18$  and  $5.46 \pm 1.37$   $\mu\text{mol}/\text{min}/\text{mL}$  for the rBChE, VX/rBChE and VX/2PAM-atropine group of animals, respectively.

### **Effect of rBChE and 2PAM-atropine treatment on cognitive function, body weight and hematological parameters of swine exposed to topical VX**

Animals that were trained pre-exposure and survived the experimental treatment, performed well on the previously learnt task when tested for several days post VX exposure (figure 5). One week post-treatment, there was no difference between the groups in terms of the performance score or the time taken to complete the task.

There was very little effect of either rBChE or 2PAM-atropine treatment on the body weight of swine exposed to VX (data not shown). Forty-eight hours after treatment, the animals had lost only a small amount (approximately 5%) of their body weight. The loss of weight could be attributed to approximately 24 hours without food while the animals were either in the operating room or recovering. In subsequent days, the animals gained weight at a rate comparable to that of the rBChE animals.

There were no hematological or chemical abnormalities observed during the 6.5 hours under anesthesia or one week later in any of the surviving animals (table 2). In addition, no gross pathology was observed in any of the surviving animals (data not shown).

## Enzyme kinetics of VX in whole blood and rBChE solution

The first kinetic parameters of VX were examined in swine whole blood, as well as in a solution of purified rBChE, at the same  $V_{max}$  values (whole blood:  $44.71 \pm 2.65$  absorbance units/min versus rBChE:  $43.13 \pm 1.53$  absorbance units/min). The  $K_i$  for rBChE was significantly lower than that for whole blood ( $1.02 \pm 0.07$  nM versus  $2.45 \pm 0.36$  nM, respectively;  $p < 0.05$ ). In contrast, the substrate (acetylthiocholine) had a higher affinity for whole blood than for the rBChE solution (whole blood:  $2.11 \pm 0.12$  vs. rBChE:  $5.65 \pm 0.71$ ,  $p < 0.05$ ).

## DISCUSSION AND CONCLUSIONS

The effect of topically applied VX in swine is characterized by a latent onset of the signs of poisoning which include mastication, salivation, fasciculations and apnea (Hamilton et al., 2004). Animals that were exposed to VX and did not receive any medical countermeasures, developed signs of toxicity and died within 2—3 hours. In contrast, animals exposed to VX and treated with five equal doses of rBChE at one-hour intervals showed little or no signs of OP poisoning and survived for the entire one-week test period.

In determining the therapeutic efficacy of rBChE against  $2LD_{50}$  VX, the dosage regimen was based on either a 1:1 (a single bolus dose or 5 equal treatments; 6 hour survival) or a 1.1:1 (a bolus dose divided into 5 equal treatments; 7 day survival) stoichiometric ratio with respect to the binding of rBChE to VX. Examination of the blood ChE activity in the rBChE animals showed a sharp increase shortly after every administration, with a sharp decrease within the hour. This demonstrates the short circulatory half-life of rBChE in swine, as compared to that of human plasma-derived BChE, several days [Ostergaard et al., 1988]. Although the half-life of the rBChE tested in this study was relatively short, a single bolus dose significantly delayed signs of toxicity and respiratory distress as compared to the VX group of animals. In order to lengthen the residency time in the circulatory system, the rBChE dose was divided into 5 equal portions. This dosing strategy provided significant protection in the animals exposed to the nerve agent, dramatically decreasing signs of poisoning and completely protecting against lethality. These results indicate that although the circulating blood concentration of rBChE was lower than in a single bolus dose, it was still therapeutically effective when the administration was modified so as to achieve equilibrium with the rapid clearance from the circulatory system.

In an attempt to explain the onset of signs in VX/rBChE animals despite higher than control ChE activity, enzyme kinetic experiments were conducted. These studies were carried out to determine if the VX-induced inhibition of swine whole blood ChE was significantly different than that obtained in solutions of purified rBChE. Using a modified Wilson procedure, VX dilutions in whole blood and purified rBChE were selected to produce a similar final  $V_{max}$ . Acetylthiocholine was used as the substrate in this assay to determine the combined activity of all ChEs present in the whole blood and in the rBChE solution. The  $K_i$  for the rBChE solution was considerably smaller than for blood

suggesting that the purified enzyme was inhibited at lower concentrations of VX. However, the substrate affinity for whole blood was significantly higher than for the rBChE solution. These results are in agreement with previous kinetic data that found human ChE sequestered VX more rapidly than swine ChE [Aurbek et al., 2006]. Based on these results, rBChE should be more effective at scavenging VX than the endogenous ChE in swine blood. However, in the VX animals treated with a single bolus injection of rBChE, the onset of toxicity occurred when ChE activity was higher than the initial control values. Based solely on the enzyme kinetic data, toxicity was expected to occur when blood ChE activity is well below control levels. The observation of toxicity despite higher than control blood ChE activity suggests that unless rapidly scavenged by high levels of rBChE, continued entry of VX into the systemic circulation via the contaminated skin depot or spontaneous reactivation of inhibited enzyme can rapidly distribute into rBChE inaccessible compartments [Thierman et al., 2007]. Five hours after VX application the skin depot would be expected to be depleted [Chilcott et al., 2005] and the rate of spontaneous activation is insufficient to induce toxic levels of VX.

For the VX/2PAM-atropine group of animals, despite the fact that the ChE activity fell below that of the baseline values and remained below throughout the period in which they were under anesthesia, these swine survived the exposure to a lethal dose of VX and did not exhibit signs of OP poisoning to the extent that the VX group did. It is possible that although blood ChE levels were low in the VX/2PAM-atropine animals, the reactivation of tissue ChE by the oxime was enough to minimize the toxic effects of VX. One week post-treatment, there were no differences between any of the surviving groups of animals in terms of ChE activity; the enzyme activity for all three groups returned to baseline levels.

In conclusion, these results demonstrate the efficacy of rBChE as a therapeutic agent to counteract the effects of VX toxicity in anaesthetized swine. While the majority of studies carried out so far have examined various bioscavengers, including rBChE, as a prophylaxis against nerve agent exposure [Doctor et al., 1991; Raveh et al., 1993; 1997; Saxena et al., 2006; Lenz et al., 2005; 2007], to our knowledge, this is the first study that shows rBChE may also be used as a therapeutic (post-exposure) countermeasure against OP poisoning. Furthermore, rBChE was found to be comparable as a treatment regimen to that of the current standard of treatment for nerve agent poisoning. Both treatment regimes resulted in survival and in a reduction in the signs of OP poisoning as compared to the VX group of animals. In addition, none of the surviving animals showed any sign of cognitive impairment, and, no hematological or chemical abnormalities were observed. While the rBChE effectively protected the animals against VX exposure, due to its short half-life multiple doses were required. Recently, it was reported that the form of rBChE used in this study could be modified with polyethylene glycol (PEG), which extends the half-life in the circulatory system to one that is comparable to human plasma-derived BChE [Huang et al., 2007]. Thus, a product such as the PEGylated rBChE, which possesses a longer half-life, should greatly improve the therapeutic value of this approach since it will sequester any slow leaching VX from the exposure site,

whereas the current standard of treatment would have to be continuously administered in a hospital setting.

## TABLES

**Table 1. Therapeutic efficacy of rBChE and 2PAM-atropine in anaesthetized swine exposed to topical VX.**

Group	Challenge	Treatment	Survivors (7 days)	Signs of OP poison
1	2LD <sub>50</sub> VX			mastication, salivation, fasciculations, erythema, miosis, and apnea.
2	none	rBChE	3/3	mild fasciculations*
3	2LD <sub>50</sub> VX	rBChE	4/4	mild signs of mastication, fasciculations, erythema and miosis*
4	2LD <sub>50</sub> VX	2PAM-atrop	4/4	mild erythema*

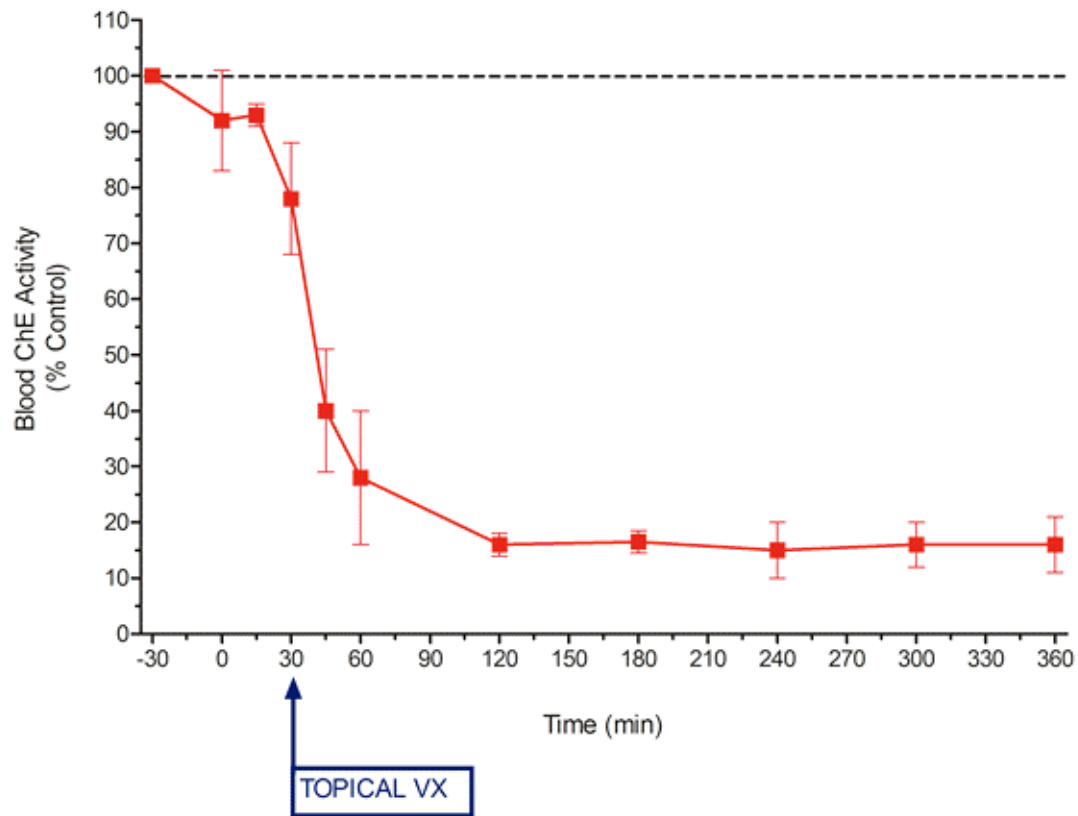
\*All signs cleared within 24 hours. Survivors refer to the number to animals in that particular group that survived the entire seven-day recovery experiment. Animals were challenged with 2LD<sub>50</sub> (124.5 µg/kg) dose of VX.

**Table 2. Selected hematological and chemical parameters of anaesthetized swine exposed to topical VX and treated with either rBChE or 2PAM-atropine**

Parameter	Group	SSA	6 hours	7 day
<b>WBC (109/L)</b>	rBChE	11.7 ± 3.9	10.9 ± 5.1	15.5 ± 1.9
	VX/rBChE	13.1 ± 2.2	12.0 ± 2.6	15.7 ± 4.9
	VX/2PAM-atropine	12.2 ± 2.2	11.9 ± 2.0	14.1 ± 2.4
<b>Hematocrit (%)</b>	rBChE	29.4 ± 3.5	26.9 ± 0.9	32.7 ± 4.2
	VX/rBChE	26.9 ± 4.1	24.6 ± 2.9	32.3 ± 3.5
	VX/2PAM-atropine	26.2 ± 1.6	24.6 ± 0.9	29.8 ± 3.1
<b>Na (mmol/L)</b>	rBChE	143.3 ± 1.5	141.6 ± 2.1	146.6 ± 3.5
	VX/rBChE	141.5 ± 1.3	141.3 ± 1.0	148.7 ± 3.8
	VX/2PAM-atropine	141.5 ± 0.6	140.5 ± 2.1	144.3 ± 0.6
<b>K (mmol/L)</b>	rBChE	4.3 ± 0.1	4.4 ± 0.3	4.3 ± 0.2
	VX/rBChE	4.4 ± 0.1	4.4 ± 0.3	4.3 ± 0.5
	VX/2PAM-atropine	4.6 ± 0.2	4.5 ± 0.1	4.1 ± 0.3
<b>Cl (mmol/L)</b>	rBChE	105.3 ± 1.5	104.3 ± 2.5	105.0 ± 2.6
	VX/rBChE	104.5 ± 1.3	103.7 ± 1.3	105.3 ± 2.5
	VX/2PAM-atropine	104.7 ± 2.4	104.0 ± 2.2	103.3 ± 0.6

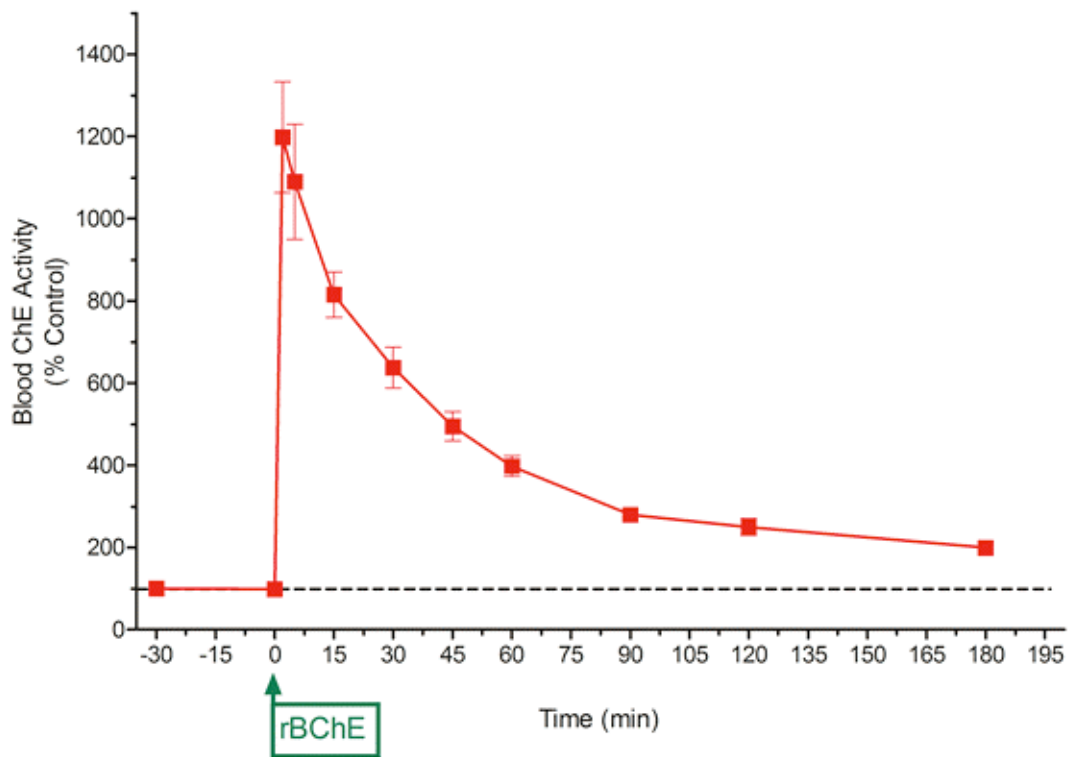
Data presented as group mean ± SD for 3-4 animals/group. SSA = steady state anesthesia, Na = sodium; K = potassium, Cl = chloride. Animals were exposed to a 2LD<sub>50</sub> (124.5 µg/kg) dose of VX.

## FIGURES



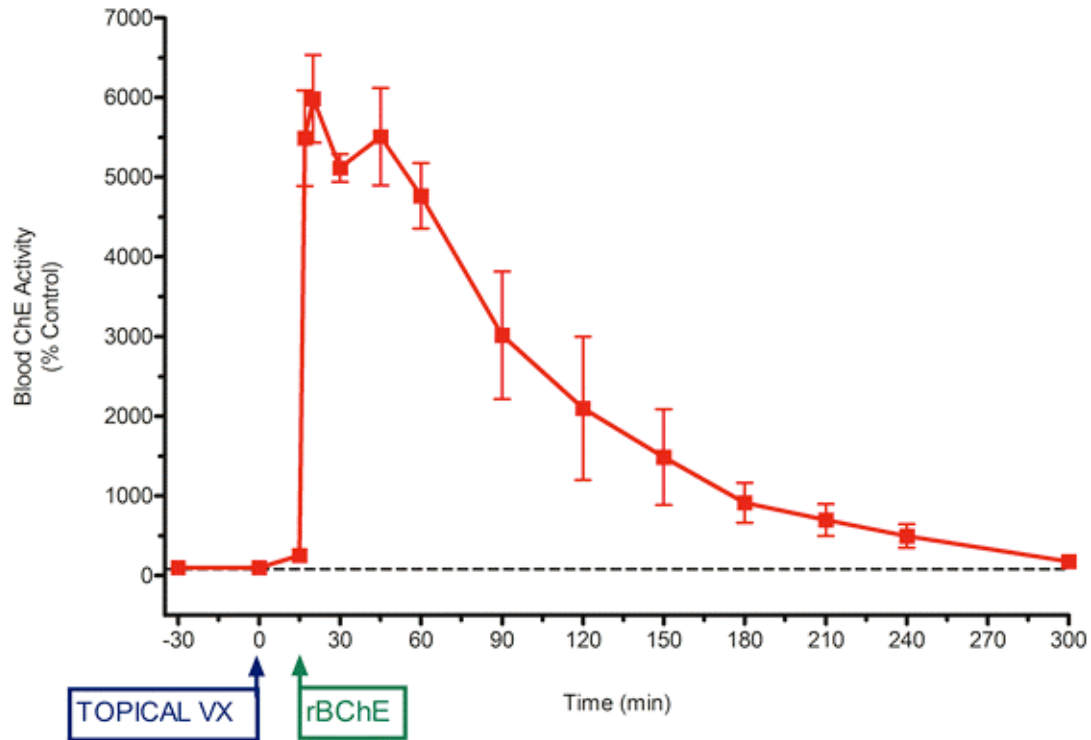
**Figure 1. Effect of VX applied topically to the ear of anesthetised swine on blood cholinesterase (ChE) activity**

ChE activity at the time of anesthetic induction was considered as control (100%). Subsequent ChE activity is presented as a percentage of control. A 2LD<sub>50</sub> dose of VX (154 ug/kg) was applied at time zero. Data points indicate time at which blood samples were taken and represent the mean  $\pm$  SEM for 3 animals.



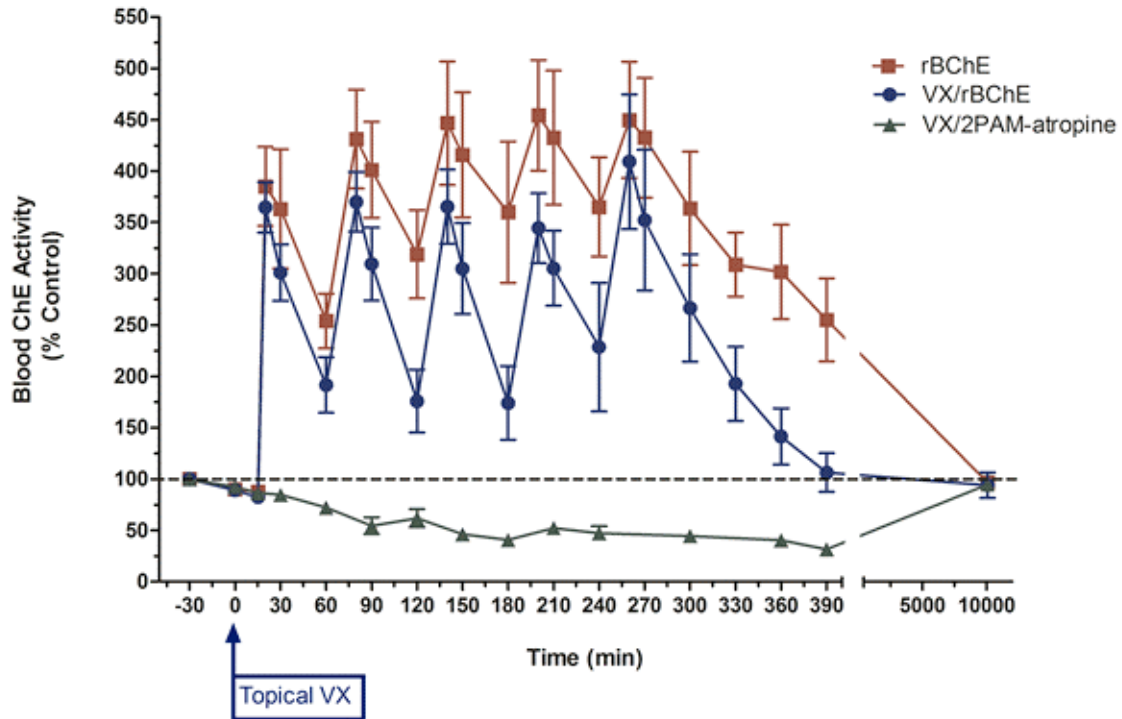
**Figure 2. Effect of a single bolus injection of rBChE on cholinesterase (ChE) activity**

ChE activity at the time of anesthetic induction was considered as control (100%). Subsequent ChE activity is presented as a percentage of control. The rBChE dose (9.5 mg/kg) represents 20% of the dose required to stoichiometrically bind 2LD<sub>50</sub> VX (154 µg/kg) in a 1:1 ratio assuming all VX enters the blood stream. Data points indicate time at which blood samples were taken and represent the mean ± SEM for three animals.



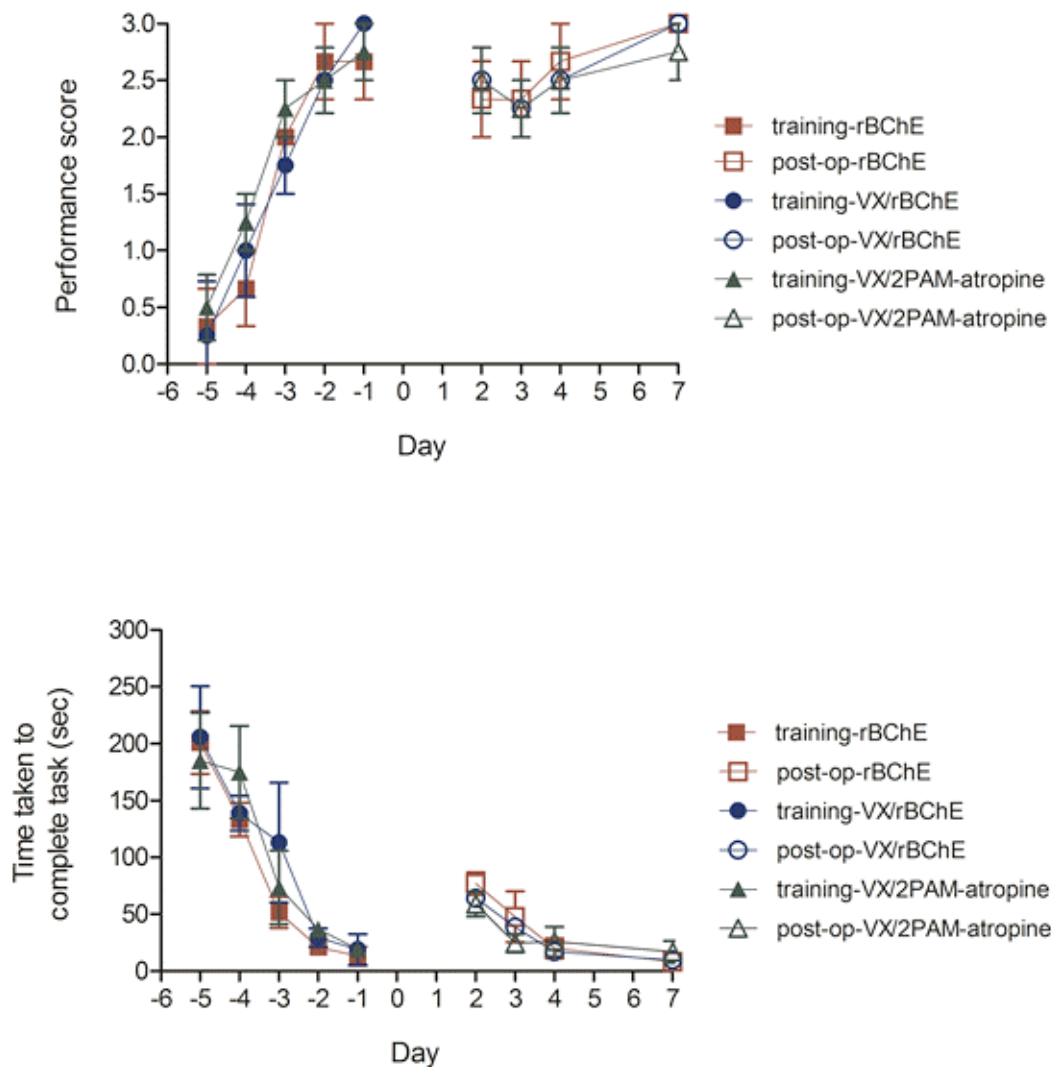
**Figure 3. Effect of delayed rBChE administration on blood cholinesterase (ChE) activity following topical exposure of 2LD<sub>50</sub> VX**

Cholinesterase activity at the time of anesthetic induction was considered as control (100%). Subsequent ChE activity is presented as a percentage of control. VX treatment 2LD<sub>50</sub> VX (154 µg/kg) was applied to the ear at time zero. A single bolus injection of rBChE (46.1 mg/kg) was administered 15 min post VX exposure. The rBChE dose represents 100% of the dose required to bind 2LD<sub>50</sub> VX in a 1:1 ratio assuming all VX enters the blood stream. Data points indicate time at which blood samples were taken and represent the mean ± SEM for three animals.



**Figure 4. Effect of rBChE and 2PAM-atropine treatment on blood cholinesterase (ChE) activity in anesthetised swine exposed to topically applied VX**

Cholinesterase activity at time of anesthetic induction was considered as control (100%). Subsequent ChE activity is presented as a percentage of control. A dose of  $2LD_{50}$  VX (124.5 ug/kg) was applied at time zero. The VX/rBChE animals received intravenous injections of rBChE were administered in five equal doses at 15, 75, 135, 195 and 255 min post VX (at a 1.1:1 full stoichiometric ratio with VX). The rBChE animals received five equal doses of rBChE but no nerve agent at the times stated above. The VX/2PAM animals were exposed to VX and then treated with 2PAM and atropine in three equal intramuscular injections at 30, 90 and 180 min post-VX. Data points indicate time of blood sampling for each animal. Mean  $\pm$  SEM for 3—4 animals/group.



**Figure 5. Cognitive behavioural testing Performance score (top) and time taken to complete the task (bottom).**

The animals were trained for five consecutive days (day -5 to -1) to achieve a criterion score of at least 2 within 60 sec. On day zero, the day of exposure, the animals were anaesthetized and exposed to 2LD<sub>50</sub> of VX (124.5 µg/kg) topically and treated either with rBChE or 2PAM and atropine (2PAM-atropine). The animals were evaluated for their capacity to remember the previously learned task on day 2, 3, 4 and 7 after treatment. See “Methods” section for description of the task and how the performance score was calculated. Data are presented as mean ± SEM for 3-4 animals/group.

## REFERENCES

- Alam, H.B., Chen, Z., Ahuja, N., Chen, H., Conran, R., Ayuste, E.C., Toruno, K., Ariaban, N., Rhee, P., Nadel, A., Koustova, E. (2005). Profound hypothermia protects neurons and astrocytes, and preserves cognitive functions in a swine model of lethal haemorrhage. *J. Surg. Res.* 126, 172-181.
- Allon, N., Raveh, L., Gilat, E., Cohen, E., Grunwald, J., Ashani, Y. (1998). Prophylaxis against soman inhalation toxicity in guinea pigs by pretreatment alone with human serum butyrylcholinesterase. *Toxicol. Sci.* 43, 121-128.
- Aurbek, N., Thiermann, H., Szincz, L., Eyer, P., Worek, F. (2006). Application of kinetic-based computer modelling to evaluate the efficacy of HI-6 in percutaneous VX poisoning. *Toxicology* 224, 74-80.
- Cerasoli, D.M., Griffitys, E.M., Doctor, B.P., Saxena, A., Fedoroko, J.M., Greigh, N.H., Yu, Q.S., Huang, Y., Wilgus, H., Karatzas, C.N., Koplovits, I., Lenz, D.E. (2005). In vitro and in vivo characterization of recombinant human butyrylcholinesterase (Protexia) as a potential nerve agent bioscavenger. *Chem.-Biol. Interact.* 157, 363-365.
- Chilcott, R.P., Dalton, C.H., Hill, I., Davidson, C.M., Blohm, K.L., Clarkson, E.D., Hamilton, M.G. (2005). In vivo skin absorption and distribution of the nerve agent VX (O-ethyl-S-[2(diisopropylamino)ethyl]methylphosphonothioate) in the domestic white pig. *Human Exp. Toxicol.* 24, 347-352.
- Darvesh, S., Hopkins, D.A., Geula, C. (2003). Neurobiology of butyrylcholinesterase. *Nature Review Neurosci.* 4, 131-138.
- Dawson, R.M. (1994). Review of oximes available for treatment of nerve agent poisoning. *J. Appl. Toxicol.* 14(5), 317-331.
- Doctor, B.P., Raveh, L., Wolfe, A.D., Maxwell, D., Ashani, Y. (1991). Enzymes as pre-treatment drugs for organophosphate toxicity. *Neurosci. Behav. Rev.* 15, 123-128.
- Doctor, B.P., Saxena, A. (2005). Bioscavengers for the protection of humans against organophosphate toxicity. *Chem.-Biol. Interaction* 157-158, 167-171.
- Duncan, E.J., Brown, A., Lundy, P., Sawyer, T.W., Hamilton, M., Hill, I., Conley, J.D. (2002). Site-specific percutaneous absorption of methyl salicylate and VX in domestic swine. *J. Appl. Toxicol.* 22, 141-148.
- Hamilton, M.G., Hill, I., Conley, J., Sawyer, T.W., Caneva, D.C., Lundy, P.M. (2004). Clinical aspects of percutaneous poisoning by the chemical warfare agent VX: effects of application site and decontamination. *Mil. Med.* 169, 856-862.

Huang, Y.J., Huang Y., Baldassarre, H., Wang, B., Lazaris, A., Leduc, M., Bilodeau, A.S., Bellemare, A., Cote, M., Herskovits, P., Touati, M., Turcotte, C., Valeanu, L., Lemee, N., Wilgus, H., Begin, I., Bhatia, B., Rao, K., Neveu, N., Brochu, E., Pierson, J., Hockley, D.K., Cerasoli, D.M., Lenz, D.E., Karatzas, C.N., Langermann, S. (2007). Recombinant human butyrylcholinesterase from milk of transgenic animals to protect against organophosphate poisoning. Proc. Natl. Acad. Sci. USA 104(34), 13603-13608.

Johnson, C.D., Russell, R.L. (1975). A rapid, simple radiometric assay for cholinesterase, suitable for multiple determinations. Analyt. Biochem. 64, 229-238.

Kassa, J. (2002). Review of oximes in the antidotal treatment of poisoning by organophosphorus nerve agents. J. Toxicol. Clin. Toxicol. 40(6), 803-816.

Lenz, D.E., Maxwell, D.M., Koplovitz, I., Clark C.R., Capacio, B.R., Cerasoli, D.M., Federko, J.M., Luo, C., Saxena, A., Doctor, B.P., Olson, C. (2005). Protection against soman or VX poisoning by human butyrylcholinesterase in guinea pigs and cynomolgus monkeys. Chem.-Biol. Interactions 157-158, 205-210.

Lenz, D.E., Yeung, D., Smith, J.R., Sweeney, R.E., Lumley, Cerasoli, D.M. (2007). Stoichiometric and catalytic scavengers as protection against nerve agent toxicity: A mini review. Toxicology 233, 31-39.

Ostergaard, D., Viby-Mogensen, J., Hanel, H., Skovgaard, L.T. (1988). Half-life of plasma cholinesterase. Acta Anaesth. Scand. 32, 266-269.

Raveh, L., Grunwald, J., Marcus D., Papier, Y., Cohen, E., Ashani, Y. (1993). Human butyrylcholinesterase as a general prophylactic antidote for nerve agent toxicity. In vitro and in vivo quantitative characterization. Biochem. Pharmacol. 45(12), 2465-2474.

Raveh, L., Grauer, E., Grundwald, J. Cohen, E., Ashani, Y. (1997). The stoichiometry of protection against soman and VX toxicity in monkeys pre-treated with human butyrylcholinesterase. Toxicol. Appl. Pharmacol. 145, 43-53.

Saxena, A., Sun, W., Luo, C., Meyers, T.M., Koplovitz, I., Lenz, D.E., Doctor, B.P. (2006). Bioscavengers for protection from toxicity of organophosphorus compounds. J. Mol. Neurosci. 30, 145-148.

Schmook, F.P., Meingassner, J.G., Billich, A. (2001). Comparison of human and animal skin in *in-vitro* percutaneous absorption. Int. J. Pharm. 215, 51-56.

Shih, T-M., Koviak, T.A., Capacio, B.R. (1991). Anticonvulsants for poisoning by the organophosphorus compound soman: pharmacological mechanisms. Neurosci. Bio-behav. Rev. 15, 349-362.

Singh, S., Zhao, K., Singh, J. (2002). *In vitro* permeability and binding of hydrocarbons in pig ear and human abdominal skin. *Drug Chem. Toxicol.* 25, 83-92.

Thiermann, H., Szincz, L., Eyer, P., Felgenhauer, N., Zilker, T., Worek, F. (2007). Lessons to be learnt from organophosphorus pesticide poisoning for the treatment of nerve agent poisoning. *Toxicology* 233, 145-154.

Weinbroum, A.A. (2005). Pathophysiological and clinical aspects of combat anticholinesterase poisoning. *Br. Med. Bull.* 72, 119-133.

Wilson, B.W., Padilla, S., Henderson, J.D., Brimijion, S., Dass, P.D., Elliot, G., Jaeger, D., Lanz, D., Pearson, R., Spies, R. (1996). Factors in standardizing automated cholinesterase assays. *J. Toxicol. Environ. Health.* 48:187-195.

Wilson, B., Henderson, J., Ramirez, A., O'Malley, M. (2002). Standardization of clinical cholinesterase measurements. *Int. J. Toxicol.* 21:385-388.