Development of a Prophylactic Butyrylcholinesterase Bioscavenger to Protect Against Insecticide Toxicity Using a Homologous Macaque Model

Yvonne Rosenberg, Xiaoming Jiang, Lingjun Mao, Segundo Hernandez Abanto, Keunmyoung Lee
PlantVax Inc.
USA

1. Introduction

Organophosphorus (OP) and carbamate pesticides are extensively used to control agricultural, household and structural pests. Each year approximately 5.6 billion pounds of pesticides are used worldwide potentially exposing ~1.8 billion people who use pesticides to protect the food and commercial products that they produce (Alavanja, 2009). Although unintentional occupational poisonings represent only a small number, estimated to be ~10% (Litchfield, 2005) or 25 million agricultural workers globally (Jeyaratnam, 1990), large scale exposure of both civilian and military personnel has become an ever increasing threat, as a result of deliberate insecticide contamination of the environment and critical water supplies by terrorists. In this context, pesticide use is one of only two exposures consistently identified by Gulf War epidemiologic studies to be significantly associated with the multisymptom illness profiles described as Gulf War illness (Cao et al., 2011). Pesticide use has also been associated with neurocognitive deficits and neuroendocrine alterations in Gulf War veterans in clinical studies conducted following the end of the war.

While OP nerve agents and WHO Class I and Class II OP pesticides constitute a diverse group of chemical structures, all potentially exhibit a common mechanism of toxicity, that is, active site phosphorylation of acetylcholine (AChE) resulting in AChE inhibition and accumulation of acetylcholine, overstimulation of cholinergic receptors, and consequent clinical signs of cholinergic toxicity such as seizures, brain damage and cognitive and behavioural defects (Millard et al., 1999; Rosenberry et al., 1999; Colosio et al., 2009). The relationship between AChE inhibition and symptoms showed that prevalence ratios were significantly >1 for respiratory, eye and central nervous system symptoms for workers with >30% inhibition (Ohayo-Mitoko et al., 2000). More recent studies indicate that insecticide exposure to DFP (diisopropyl fluorophosphate) causes a prolonged increased in hippocampal neuronal Ca++ plateau which may underlie morbidity and mortality (Deshpande et al., 2010). These findings are consistent with those indicating persistent changes in locus ceruleus noradrenergic neuronal activity and lasting changes in this brain area after removal of the insecticide chlorpyrifos oxon; reminiscent of the lasting cognitive...
symptoms of Gulf War illness in soldiers exposed to these compounds (US DOD, Pesticides-Final Report, 2003). Currently, the standard (approved) treatment for acute OP pesticide poisoning involves administration of intravenous (iv) atropine and an oxime e.g. obidoxime, pralidoxime to reactivate inhibited AChE (Worek et al., 2010). However, the effectiveness and safety of oxime administration in acute OP pesticide-poisoned patients has been challenged and a recent clinical trial showed no clinical benefits and a trend towards harm in all sub-groups, despite clear evidence that these doses reactivated AChE in the blood (Buckley et al., 2011).

An efficacious prophylactic therapeutic treatment for preventing insecticide poisoning that can bind and scavenge the OP before it reaches and targets AChE in neuromuscular junctions is therefore a high priority. The leading candidate of this type is native (plasma) butyrylcholinesterase (BChE) whose potent OP bioscavenging ability has been demonstrated in many animal models and against varied OP neurotoxins (Doctor et al., 2001; Lenz et al., 2001). While several new catalytic and other stoichiometric enzymes also exhibit this ability (Lenz et al., 2007), based on availability, broad spectrum efficacy, stability and safety (Sun et al., 2005), BChE is the most advanced in terms of development of a human treatment. In Turkey, frozen plasma (BChE levels of 3,000 - 5,700 units) given as an alternative or adjunctive treatment with atropine and oximes, has been shown to prevent mortality and intermediate syndrome in acutely insecticide-exposed and hospitalized individuals (Güven et al., 2004). Currently, BChE also finds use as a treatment of cocaine overdose and for the alleviation of succinylcholine-induced apnea.

Structurally, BChE (also known as pseudocholinesterase or non-specific cholinesterase) is a serine esterase (MW=345,000) comprised of four identical subunits each containing 574 amino acids, held together by non-covalent bonds, with 36 carbohydrate chains (23.9% by weight) (Lockridge, 1990; Nachon et al., 2002). BChE is found in all species at levels of 1-20 ug/ml in plasma (Rosenberg, unp. data) and is also abundant in liver, intestine and lung. Recombinant (r) human butyryl-cholinesterase (HuBChE), like the native form, is also a potent bioscavenger of OP neurotoxins (Doctor et al., 2001; Lenz et al., 2001; Raveh et al., 1997) but its development as a human treatment for pesticide exposure has been disadvantaged by: (i) poor in vivo stability (bioavailability) of the unmodified forms and the presence of potentially immunogenic glycans using certain expression systems (ii) a 1:1 stoichiometry between the enzyme and OP (Raveh et al., 1997) and (iii) the high LD50 of insecticides (ug-mg/kg levels). This necessitates the delivery of large, costly, rBChE doses to detoxify exposed individuals which is problematic when intramuscular (im) or subcutaneous (sc) injections are the chosen routes of delivery. In this chapter, we shall describe our experience of how the chemistry, glycosylation, chemical modification, animal model and route of administration may reduce or enhance the potential of BChE bioscavengers as prophylactic therapeutic human antidotes for OP insecticide exposure.

2. Production of tetrameric and monomeric forms of rMaBChE and rHuBChE

Macaque (Ma) and human (Hu) BChE molecules are very similar molecules differing by only 22 amino acids and sharing ~96% DNA sequence identity, critical glycosylation sites, cysteines and disulfide bridging (Boeck et al., 2002; Rosenberg et al., 2010). Thus, most anti-BChE antisera react with both molecules. Native HuBChE and MaBChE in plasma are composed predominantly of tetramers (98%) with the tetramerization domain being located within the last 40 C-terminal residues of each monomeric subunit (534-574) (Blong et al.,
In human serum, the association of lamellipodin proline rich peptides with the monomeric chains results in the formation of BChE tetramers (Li et al., 2008). Recombinant BChE produced in mammalian cells, in contrast, has only 10-20% tetrameric forms and therefore optimal tetramerization requires the addition of either poly(L-proline) to the culture medium or co-expression of the full length BChE monomers with the proline-rich attachment domain (PRAD) of CoQ gene (Altamirano & Lockridge, 1999).

To date, rHuBChE and rMaBChE molecules have been produced in transgenic mammalian cells (Chilukuri et al., 2008; Rosenberg et al., 2010), goat milk (Huang et al., 2007) and in plants (Geyer et al., 2010; Jiang, unpub. data). Our approach has been to utilize two expression systems for the production of rMaBChE and rHuBChE. Initially, Chinese hamster ovary cells (CHO) were used because of their human-like glycosylation. More recently, a transient plant expression platform was adopted to increase the yield and reduce the time and cost of producing rBChE. Although CHO cells and plants are able to produce significant levels of tetrameric BChE molecules (Li et al., 2008; Geyer et al., 2010), in the present studies, co-transfection of the BChE and PRAD genes has been shown to increase both levels of tetramerization and yields in each expression system. While the CHO cell expression of recombinant proteins is very well established, recent innovations in transient plant expression systems e.g. Bayer’s Magnifexfection system (Gleba et al., 2005) and the Cow Pea Mosaic Virus Hyper-translatable Protein Expression System (PBL Technology) (Sainsbury et al., 2008) have been shown to be some of the most rapid, cost effective and productive expression systems in existence; capable of producing grams of recombinant proteins in weeks (Goodin et al., 2008).

| CHO-derived (Stable Transfection)* | Plant-derived (Transient Transfection)* |
|-----------------------------------|-----------------------------------------|
| rMaBChE## | rHuBChE | rMaBChE# |
| N. tobacum | N. benthamiana |
| Monomeric | Tetrameric | Monomeric | Tetrameric | Monomeric | Tetrameric | Tetrameric |
| 8U/ml (9mg/L) | 25U/ml (28mg/L) | 16 U/ml (22.9mg/L) | 45 U/ml (64.3 mg/L) | 60 U/gm (66.6 mg/kg) | 140 U/gm (155.5 mg/kg) | 400 U/gm (444 mg/kg) |

*All tobacco plants and leaves from Nicotiana tobacum and N. benthamiana were transfected using Agrobacterium-mediated infiltration

* CHO supernatants and whole leaf extracts are prepared for purification.

* BChE activity is determined spectrophotometrically (Grunwald at al., 1997), using butyrylthiocholine (BTC) (0.5 mM each) as substrate. One unit of enzyme activity is the amount required to hydrolyze 1 μmol substrate/ min. One mg MaBChE has 900 units of activity and one mg HuBChE has 700 units.

Table 1. Expression levels of different forms of rBChE using CHO-and plant-based expression systems.

In addition to the tetrameric forms, a truncated monomeric form of rBChE (MW=81KDa) that is incapable of oligomerization has also been produced by the insertion of a stop codon at G534 resulting in a monomeric form lacking 41 C-terminal residues (Blong et al., 1997). The smaller monomeric molecules may more rapidly gain access to the blood from muscle or lungs (depending on the route of delivery) with transiently higher bioavailability in the plasma, which would be advantageous in emergency situations that require real time responses and rapid treatment or booster administrations.
3. In vitro biological properties of rMaBChE

To test the chemical properties of CHO- and tobacco-derived rMaBChE, inhibition and reactivation assays using diisopropyl fluorophosphate (DFP) and paraoxon (diethyl 4-nitrophenyl phosphate) have been performed with and without the oxime 2-PAM (pyridine-2-aldoxime methochloride)(Luo et al., 2008). DFP is an OP compound that has been used as an experimental insecticide agent in neuroscience because of its ability to inhibit cholinesterase and cause delayed peripheral neuropathy. Paraoxon is an insecticide and will be described in detail in a later section. Following purification of the CHO supernatant and the plant leaf extract using procainamide sepharose, rMaBChE molecules conjugated with polyethelene glycol (PEG) using succinimidyl-propionate-activated methoxy-PEG-20K (SPA-PEG-20K; Nektar Inc., Birmingham, AL) or Sunbright ME-200HS 20K PEG (NOF, Tokyo, Japan) (Chilukuri et al., 2008a; Cohen et al., 2001) to test the effects of PEGylation on enzyme plasma stability. Initially, the biochemical properties of both the unmodified and PEGylated forms of both monomeric and tetrameric rMaBChE were examined using DFP inhibition; bimolecular rate constants \( k_{1} \times 10^{7} \) M\(^{-1}\) min\(^{-1}\) for inhibition of all the recombinants forms ranging from 2.58 - 2.23 \( \times 10^{7} \) M\(^{-1}\) min\(^{-1}\) which were indistinguishable from the well characterized native HuBChE (2.29 +/- 0.1) and native MaBChE (2.22 +/- 0.1) (data not shown).

3.1 Inhibition and reactivation of plant derived CHO-derived and plant-derived rBChE by paraoxon

The kinetics of inhibition of both plant-derived and CHO-derived rMaBChE by paraoxon were further examined as shown in Fig. 1A. At low paraoxon concentrations (0.01 and 0.02uM), the reciprocal value of \( E_{t}/E_{t,0} \) was highly correlated with the reaction time; the reaction rate constant of plant-derived rMaBChE at 0.01uM paraoxon being slightly faster than that of CHO-derived MaBChE (0.035 M\(^{-1}\)min\(^{-1}\) vs 0.022 M\(^{-1}\)min\(^{-1}\) respectively). These values follow the simple 2nd-order (reciprocal) model.

Fig. 1. Inhibition kinetics of plant- and CHO-derived* rMaBChE by different concentrations of paraoxon (0.01uM - 0.10uM) A: Percent inhibition of BChE by paraoxon. (Percent BChE activity was obtained by dividing the BChE activity with paraoxon at each time point with control BChE activity at the same time point. B: Reciprocal plot of BChE inhibition by paraoxon.
3.2 Reactivation of paraoxon-inhibited plant-derived rMaBChE by 2-PAM
Since a 1 hour incubation of 0.016 uM plant-derived MaBChE (1.2U/ml) with 0.02uM paraoxon resulted in 80-90% inhibition of the enzyme (Fig. 1A), the same conditions (incubation of paraoxon with rMaBChE at a final enzyme concentration of 0.04-0.05uM), was used to prepare inhibited rMaBChE. Reactivation of inhibited rMaBChE was then initiated by adding different concentrations of 2-PAM (0.4mM-6.4mM) for various times (Fig.2). The kinetics of reactivation of paraoxon-inhibited CHO- and plant-derived rMaBChE were found to follow the simple first-order (mono-exponential) model.

Fig. 2. Reactivation kinetics of paraoxon inhibited plant- and CHO-derived MaBChE by 2-PAM. A, C: Plant-derived MaBChE; B, D: CHO-derived MaBChE; A and B: Direct plot of the time course vs % reactivation; C and D: Semi-logarithmic plot of time course of reactivation. For inhibition controls, inhibited BChE was incubated with reaction buffer without 2-PAM. Triplicate BChE assays were performed at the times indicated.
The results indicate that both paraoxon-inhibited plant- and CHO-derived rMaBChE showed very similar patterns of reactivation by different concentrations of 2-PAM (Fig. 2A, 2B) with nearly 100 % reactivation of each rMaBChE form being achieved by 24 hours at >1.60 mM 2-PAM; the $k_{app}$ values of CHO-rMaBChE ranging from 0.0014 to 0.004 min$^{-1}$ and plant-rMaBChE from 0.0013 to 0.0051 min$^{-1}$ (Fig. 2C, 2D). The reactivation $k_{app}$s at each 2-PAM concentration was linear when plotted against 2-PAM concentration (mM) expressed logarithmically.

4. In vivo testing of rBChE

In the area of insecticide exposure/contamination, there is a high likelihood that agricultural workers or military personnel will be exposed multiple times during their lives and thus multiple prophylactic treatments must be considered a possibly. This is often problematic since administration of heterologous HuBChE into macaques or other species eg mice has been shown to generate anti-BChE antibody responses and rapidly eliminate enzyme on repeated injections (Matzke et al., 1999; Chiluluri et al., 2008b; Sun et al., 2009). Thus, in vivo retention times of exogenously administered recombinant proteins can only be accurately assessed using homologous systems (rMaBChE $\rightarrow$ macaques and rHuBChE $\rightarrow$ humans) in which antibodies or other immune responses are not induced. In this context, homologous BChE enzyme has been shown to have a long half-life (8-12 days) with no adverse effects and no immunogenicity following either (i) transfusions of human plasma into humans (ii) daily administrations of partially purified native HuBChE into humans for several weeks (Jenkins et al., 1967; Cascio et al., 1988) or (iii) injection of purified native MaBChhE or PEG-rMaBChE into macaques (MRT= 200-300 h)(Rosenberg et al., 2002, 2010). These data are in contrast to exogenously administered heterologous HuBChE which displayed a rapid clearance in macaques (MRT = 33.7 h) (Raveh et al., 1989). While the choice of the animal model for PK, immunogenicity and efficacy testing is always important, the animal species utilized for the evaluation of an efficacious human cholinesterase bioscavenger is critical, since potential treatments against OP toxicity cannot be tested in humans and will require extensive testing in animal models and the Animal Rule (CFR 601.90 for biologics) for regulatory approval.

4.1 Pharmacokinetics of clearance in rodent and macaque models

Pharmacokinetic profiles following administration of biologics in many rodent and primate species are used to indicate the periods after administration that such biologics are likely to exhibit optimal benefit or protection. An efficacious therapeutic for preventing OP poisoning is a molecule that: (i) can bind and scavenge the OP before it reaches the targeted AChE in neuromuscular junctions and (ii) has the ability to remain at therapeutic levels in the blood for prolonged periods to counteract a known or impending OP exposure. The in vivo parameters generally used to assess PK performance after administration are mean retention time (MRT), maximal concentration (Cmax), time to reach maximal concentration (Tmax), elimination half life (T1/2) and area under the plasma concentration curve extrapolated to infinity (AUC).
Generally pharmacokinetics of recombinant molecules differs considerably depending on the structure, glycosylation, size, route of administration, immunogenicity, and animal model utilized. Interestingly, despite protein sequence identity, rBChE proteins, similar to many other recombinant biologics, have been shown to be rapidly cleared following injection (Saxena et al., 1998; Cohen et al., 2006) in contrast to the good plasma stability of native BChE. Thus, rBChE molecules require post-translational modification to provide protection as therapeutic scavengers. A common means of increasing the radius of the target molecule permitting slower renal clearance and prolonging plasma retention is by PEG conjugation. This has been successfully used with proteins, peptides, oligonucleotides and antibody fragments to improve pharmacokinetic and immunological profiles (Kang et al., 2009). Accordingly, both monomeric and tetrameric forms of rMaBChE have been conjugated with 20KD PEG (without interference of in vitro biological properties) and the pharmacokinetic profiles of the unmodified and PEG-conjugated rMaBChE forms compared in monkeys and mice (Rosenberg et al., 2010).

Figure 3 shows the PK profiles in 24 monkeys following iv injection of 1.2 -3 mg/kg of unmodified or PEG-rMaBChE and illustrates several aspects of BChE clearance: (i) PEG-rMaBChE exhibits good stability in the lower range of the native form; the hierarchy of clearance being native BChE ~ PEG-rMaBChE >>> unmodified monomeric rMaBChE > unmodified tetrameric rMaBChE. (ii) Surprisingly, five of the monkeys demonstrated unexpected dramatic decreases in BChE levels (shown in bold between days 150 and 230 days post injection). In each case, these decreases always occurred immediately after the weekend treatment of the grass surrounding the animal facility and presumably resulted from exposure of the housed monkeys to insecticide; highlighting the unintentional consequences of routine insecticide use on plasma BChE activity and (iii) despite very poor retention of the unmodified monomeric rBChE, administration of the PEGylated monomeric rMaBChE showed overlapping pharmacokinetic parameters with the larger PEG-rMaBChE tetrameric form despite lack of oligomerization.

Importantly, the extended circulatory retention afforded by PEG conjugation of rMaBChE in monkeys (injected iv) was not observed in mice (injected ip) where unmodified and modified monomeric and tetrameric rMaBChE all exhibited the same high MRT and T1/2 (Rosenberg et al., 2010). This indicates that, depending on the parameter measured, the mouse model does not accurately predict the outcome in monkeys with MRT and T1/2 values appearing to be less predictive indicators of circulatory stability in macaques than parameters such as AUC and Cmax. Similar differential pharmacokinetic behaviour was observed following the administration of recombinant rhesus (Rh) and HuAChE in mice and monkeys (Cohen et al., 2004).

These studies highlight the potential problems inherent in choosing an animal model to test human biologics. Notwithstanding the differences in pharmacokinetic behaviour of the same protein in different species and the high potential for immunogenicity in rodents due to the evolutionary distance between rodents and humans, other influences may also play a role in the circulatory stability of proteins following even the first injections into heterologous species. Table 2 shows the pharmacokinetic parameters (MRT, Cmax, Tmax, T1/2 and AUC) following injection of different forms of BChE into several different animal species determined from the time course curve of blood BChE concentrations and using a Windows-based program for non-compartmental analysis. Several conclusions can be made.
Fig. 3. Pharmacokinetics of clearance following iv injection of 1.2 - 3.0 mg/kg rMaBChE into 24 monkeys. Each line represents a single monkey. Different forms of rMaBChE were used except for 4 macaques receiving native BChE.

For example, while the Cmax following first injections appear to be similar in any animal model at comparable doses, the AUC, MRT and T1/2 are often significantly higher in homologous systems (e.g. PEG-rMaBChE into macaques and native mouse (Mo) BChE into mice) than heterologous injections (native HuBChE into monkeys or mice or PEG-rHuBChE into monkeys). This indicates that heterologous proteins, even when PEGylated and given at a time when anti-BChE titers are absent or low, appear to be eliminated faster than homologous proteins suggesting that pharmacokinetic parameters are less than optimal in all heterologous systems.

It should also be noted, that while PEG conjugation markedly improves the pharmacokinetic profile of therapeutic rMaBChE and other biologics, effects relating to immunogenicity have been mixed. Thus, reduced immunogenicity has been observed following PEGylation of enzymes, cytokines and hormones, while administration of PEGylated interferon-β1a to monkeys actually resulted in increased immunogenicity (Pepinsky et al., 2001). In the case of rHuBChE produced in HEK-293 cells, PEGylation failed to eliminate immunogenicity in mice as demonstrated by the rapid clearance of a repeat 100U injection of (heterologous) PEG-rHuBChE, coincident with induction of high levels of serum anti-BChE antibody (Sun et al., 2009). Likewise, when tested in a sandwich ELISA, the presence of 4–7 PEG molecules per rMaBChE monomer did not prevent the binding of BChE epitopes to either an anti-BChE MAb or a polyclonal rabbit anti-BChE antibody when antigen concentrations were increased to as little as 4–8 U/ml (Rosenberg et al., 2010) which, as mentioned above, is in the range of BChE in normal plasma. These studies raise the question whether chemical modification by PEG will be able to mask any “foreign” rBChE epitopes, such as non-human glycans, sufficient to prevent humoral immune responses and also highlights the importance of using homologous animal models to perform in vivo PK, immunogenicity and efficacy testing.
Development of a Prophylactic Butyrylcholinesterase Bioscavenger to Protect Against Insecticide Toxicity Using a Homologous Macaque Model

| Human and Mouse BChE | BChE | Dose [Units, mg, mg/kg] | Animal | Route | MRT (hr) | AUC (U/ml.h) | Cmax (U/ml) | Tmax (hr) | T1/2 (hr) |
|----------------------|------|------------------------|--------|-------|----------|-------------|------------|----------|----------|
| natHuBChE (Raveh,1997) | 11.5 mg (8,000 U) | Monkey | iv | 33 | 710 |
| natHuBChE | 11.5 mg (8,000 U) | Monkey | im | 582 | 16.2 | 9.5 |
| natHuBChE (Lenz,2005) | 5.25 mg/kg (12,000 U) | Monkey | im | 2576 | 21 | 9.27 | 79.3 |
| 8.75 mg/kg (20,000 U) | Monkey | im | 3822 | 33 | 10.3 | 73.5 |
| natHuBChE (Sun, 2005) | 34 mg/kg (30,000 U) | Monkey | iv | 73 | 16,538 | 222 | 0 | 37 |
| natHuBChE (Sun, 2009) | 100 U | Mouse | im | 48 | 1,500 | 19 | 21 |
| natMaBChE * | 100 U | Mouse | im | 73 | 2,500 | 25 | 24 |

| Monkey BChE | BChE | Dose [Units, mg, mg/kg] | Animal | Route | MRT (hr) | AUC (U/ml.h) | Cmax (U/ml) | Tmax (hr) | T1/2 (hr) |
|-------------|------|------------------------|--------|-------|----------|-------------|------------|----------|----------|
| natMaBChE * | 3 - 5 mg/kg (7,000 U) | Monkey | iv | 191 |
| (Rosenberg, 2002) | 1.3 - 1.65 mg/kg (3,000 U) | Monkey | iv | 50 |
| natMaBChE (unpub)* | 1.8 mg/kg | Monkey | iv | 142 | 2950 | 27 |
| natMaBChE* | 1.8 mg/kg | Monkey | iv | 142 | 4010 | 37 |
| (Rosenberg, 2010) | | | | | | | | | |
| natMaBChE* | 2.9 mg/kg | Monkey | iv | 224 | 4431 | 38 | 143 |
| 2.9 mg/kg | Monkey | iv | 307 | 4299 | 40 | 126 |
| 1.9 mg/kg | Monkey | iv | 200 | 2097 | 26 | 157 |
| PEG-rMaBChE* | 2.9 mg/kg | Monkey | iv | 168 | 2141 | 33 | 112 |
| (Rosenberg, 2010) | 2.9 mg/kg | Monkey | iv | 223 | 3312 | 39 | 85 |
| 1.9 mg/kg | Monkey | iv | 134 | 1724 | 24 | 97 |
| PEG-rMaBChE (unpub)* | 3.0 mg/kg | Monkey | iv | 4359 | 51 |
| PEG-rHuBChE (unpub)* | 3.0 mg/kg | Monkey | iv | 1101 | 40 |

MRT: mean retention time, Cmax: maximal concentration, Tmax: time to reach maximal concentration, T1/2: elimination half life, AUC: area under the plasma concentration curve extrapolated to infinity.

nat: native, Mon: monomeric, Tet: tetramer.

Table 2. Pharmacokinetic parameters of different forms of BChE in homologous* and heterologous systems.

4.2 The role of glycosylation and oligomerization on pharmacokinetics

The BChE molecule is a soluble protein, protected from proteolysis by a heavy sugar coating from nine N-linked glycans (Li et al., 2008). N-glycosylation is one of the major post-translational modifications of proteins and can be critical to their bioavailability. Importantly, while the first steps in the N-glycosylation pathway, leading to the formation of oligomannnosidic structures, are conserved in plants and animals, the final steps in the formation of complex N-glycans may differ with the expression system used. Thus, in contrast to native HuBChE molecules which have highly sialylated bi- and triantennary type glycans (Saxena et al., 1998; Kolarich et al. 2008) containing the N-acetyl neuraminic acid (NANA, NeuAc) form of sialic acid (Varki, 2001), rHuBChE molecules may exhibit undersialylated or immunogenic non-human glycan structures that accelerate in vivo clearance,
due to rapid uptake by asialoglycoprotein and mannose receptors in the liver or by antibody-mediated mechanisms (Park et al., 2005). For example, CHO cells produce recombinant proteins which contain human-like glycans that may be undersialylated, compared to those produced in livestock systems which append the non-human galactose-α1,3-galactose and the N-glycolyl neuraminic (NGNA, NeuGc) form of sialic acid (Chung et al., 2008; Diaz et al., 2009) and those produced in plants which are non-sialylated and append the non-human β1,2 xylose and α1,3 fucose containing glycans (Altmann, 2007).

The relationship between sialic acid levels and oligomerization of recombinant molecules with their circulatory longevity has been extensively studied. For example, administration to mice of recombinant bovine and rhesus acetylcholinesterase (rBoAChE, rRhAChE) as well as plant-derived rHuBChE have supported the idea that pharmacokinetic behaviour is governed by hierarchical rules (Kronman et al., 2000); efficient enzyme tetramerization and high sialic acid occupancy both being required for optimal plasma retention. However, other data from monkey and mice studies do not closely obey these classical rules for circulatory retention. For example: (i) the requirement for tetramerization of rAChE molecules was less important when performed in macaques rather than mice (Cohen et al., 2004) (ii) CHO-derived monomeric PEG-rMaBChE resulted in high MRT when injected into in monkeys (Fig.3, Rosenberg et al., 2010) and (iii) the MRT and T1/2 of unmodified and PEG-modified monomeric rMaBChE were both unexpectedly high following injection into mice; PEG-conjugation offering no significant advantages.

While the short lived circulatory retention of asialylated BChE attests to the importance of sialylation in retention/clearance, the degree to which sialic acid occupancy is required does not always seem straightforward. Thus, although the rapid clearance of monomeric (13% non-sialylated) and tetrameric (25% nonsialylated) rMaBChE in monkeys, compared to the native or PEGylated forms, has been thought to result from undersialylation, glycan analysis by MALDI-TOF of the highly stable native HuBChE and MaBChE proteins indicates that these also contain a significant percentage of nonsialylated or undersialylated proteins. For example, native HuBChE contains 23% monosialylated glycans (99.9% NANA) and a significant percentage of non-sialylated glycans (Kolarich et al., 2008) while native MaBChE is comprised of 21.3% non-sialylated glycans and 21.8% monosialylated glycans (99.9% NGNA) (Rosenberg, unp. data). This means that heterologous animal models invariably involve the administration of native or CHO-derived human proteins containing NANA into animals containing the NGNA form of sialic acid (monkeys, rodents). These findings showing either high percentages of undersialylated glycans in the stable native proteins and those showing lower pharmacokinetic parameters following heterologous injections, raise the interesting question as to whether the type of sialic acid type as well as the degree of sialic acid occupancy may determine the rate of clearance of recombinant glycoproteins.

It is also important to note that recent engineering of different expression systems is now permitting the production of glycoproteins with human-like glycans. For example, while the inability to perform appropriate N-glycosylation has been a major limitation of plants as expression systems, these are being overcome by new approaches involving the generation of knockout or knockdown plants that: (i) completely lack xylosyl transferase (XylT) and fucosyl transferase (FucT) activity (Strasser et al., 2004) and accumulate high amounts of human-like N-glycan structures that contain no 1,2-xylose or core a1,3-fucose (ii) lack complex N-glycans resulting from the inactivity of N-acetylglucosaminyltransferase 1 (GnT1) (Strasser et al, 2005; Wenderoth & von Schaewen, 2000) and (iii) contain glycans
terminating in sialic acid (Paccalet et al., 2007; Castilho et al., 2010). In addition, different glycoforms of plant derived proteins can be generated by protein targeting to different compartments (i) cytosol (aglycosylated) (ii) ER (high mannose) or (iii) secreted into the apoplast (complex) (Stoger et al., 2005).

4.3 Effects of the route of administration on pharmacokinetics

As mentioned, delivery of PEG-rBChE as a pre-exposure modality is disadvantaged by its large size and a 1:1 stoichiometry between the enzyme and OP requiring high doses due to the high LD$_{50}$ of many insecticides (ug-mg/kg levels). The route of systemic delivery of high doses of native BChE (MW~350KDa) and tetrameric PEG-rMaBChE (MW>800KDa) will determine the pharmacokinetics (PK) of clearance and is critical to efficacy and safety. Currently very little monkey data exists on the delivery of a stoichiometrically equivalent dose of PEG-rBChE calculated to protect against a known LD$_{50}$ of a toxic OP insecticide. Although immediate release requiring intravenous (iv) injection may be necessary in certain high threat situations, these are usually impractical in the field. Needleless cutaneous delivery via the dermis and epidermis (chemical mediators, electroporation) appear quite promising, but are unlikely to deliver high doses. Thus, self-administered transdermal injections through the skin either by subcutaneous (sc) or intramuscular (im) routes have been the approaches most commonly used; virtually all human vaccines currently on the market being administered via these routes. Traditionally, autoinjectors, devices for im delivery of a self administered single dose of a drug are used in the military to protect personnel from chemical warfare agents and are currently used to deliver morphine for pain and atropine, diazepam and 2-PAM-Cl for first-aid against nerve agents. For this reason, most animal protection studies with OP bioscavengers have routinely been delivered im to rodents (Lenz et al., 2005; Mumford et al, 2010; Saxena, et al., 2011). Despite all the pharmacokinetics data generated using im and sc routes of delivery of many drugs and biologics, little is known about the factors that govern the rate and extent of protein absorption from the injection site and the role of the lymphatic system in the transport of large molecules to the systemic circulation. With smaller molecules, the time to maximal concentration is usually shorter following im injections compared to sc injections where absorption is slow and prolonged and accounts for the lag in entering the blood. However with larger therapeutic molecules (MW>16KDa), the lymphatics are thought by some groups to be the primary route of absorption from sc (and im) injection sites. Large molecules are thought to exit the interstitium via cleft like openings into the lymph and enter the systemic circulation via the thoracic duct (Supersaxo et al., 1990; Porter et al., 2001; McLennan et L., 2006). To assess the effects of different routes of delivery, pharmacokinetic behaviour using different doses of PEG-rMaBChE tetrameric molecules was compared in monkeys following im and sc injections.

4.3.1 Intramuscular delivery of PEG-rMaBChE

Four monkeys each received an im injection of either 2.5 or 3 mg/kg of PEG-rMaBChE. As shown in Fig. 4, unlike the delivery of the smaller native HuBChE which appear to behave uniformly following im injection (Lenz et al., 2005), the much larger PEG-conjugated form exhibits very variable results when delivered into the muscle with Tmax values in the 4 macaques having values of 8, 24, 48 and 48 hr respectively; the 8-hour peak looking more like an iv injection than an im injection. It is not clear whether this more rapid exit from the
muscle injection site into the blood reflects a more vascularised muscle or whether im delivery has more potential to damage blood vessels and promote faster draining. It is clear however that delivery of large doses of a therapeutic such as PEG-rHuBChE will require many im injections to achieve required peak values and will increase the likelihood of targeting a blood vessel. The stoichiometric dose of BChE required to protect humans against 2 LD$_{50}$ of soman has been considered to be 3 mg/kgm (200 mg/70 kg); the antidotal efficacy of BChE being contingent upon both the rate of OP detoxification and its levels in blood (Raveh, 1997; Ashani & Pistinner, 2004). It would be unlikely that Cmax values (20 and 23 U/ml at 3 mg/kg and 17 and 10 U/kg at 2.5 mg/kg) following im administration would be sufficient for protection. In addition, the variable times of peak enzyme make it difficult to choose a time for prophylactic dosing.

4.3.2 Subcutaneous delivery of PEG-rMaBChE

Extensive pharmacokinetics have been performed on many well known biologics in monkeys and humans, either PEGylated or unmodified, using the sc routes of delivery (Boelaert et al., 1989; Ramakrishnan et al., 2003; Heatherington et al., 2001; Radwanski et al., 1987; Mager et al., 2005), although extrapolation from these studies may be problematic because all used considerably smaller molecules than native or PEG-rBChE. Generally, sc injections have been the delivery route of choice for compounds with limited oral bioavailability, as a means of modifying or extending the release profiles of these molecules, or as a means of delivering drugs that require large quantities (Yang, 2003) since larger volumes may be injected. In one case, a highly concentrated form of a therapeutic requiring large doses for its effects has be prepared as a crystalline and successfully delivered sc in a small volume (Yang et al. 2003).

Fig. 4. Pharmacokinetic profiles of PEG-rMaBChE delivered by im injection. Four monkeys were injected into the thigh muscles using a 1-ml syringe.

Figure 5 shows the pharmacokinetic profiles following sc delivery of the tetrameric PEG-rMaBChE at 2.5, 3 and 5 mg/kg. Tmax values were all consistently ~48 hrs, regardless of the
dose. However, while Cmax was generally associated with dose, there was a good deal of overlap between the 3 mg/kg and 5mg/kg doses; the larger doses being retained at higher levels in the blood for many days. This once again raises the question as to whether a high dose of very large molecules can leave the site of the sc injection and enter the blood at levels required for protection. By contrast 3 mg/kg delivered iv reaches a peak of >50 U/ml. It is important to note that despite the apparent low bioavailability of sc administered proteins compared to those given intravenously (17-65%), sc delivery often produces equivalent efficacy to iv administration and is assumed to be due to prolonged absorption leading to reduced receptor saturation.

![Pharmacokinetics of PEG-rMaBChE delivered by sc injection. Eight monkeys were injected with the doses indicated in 2-4 ml sc between the shoulder blades.](image)

A direct comparison of the pharmacokinetic parameters following im versus sc injections of 4 monkeys at does of 2.5 mg/kg and 3 mg/kg is shown in Table 3 and indicates that the im and sc values are quite similar. Overall, the results indicate that for a very high MW protein such as PEG-rMaBChE or PEG-rHuBChE, neither im or sc administrarion are optimal to achieve good plasma retention with high PK parameters. For this reason, a different non-parenteral route of delivery via the lung, where the high MW becomes an advantage, is now the choice route of delivery.

| Parameters         | Subcutaneous injection | Intramuscular injection |
|--------------------|------------------------|-------------------------|
|                    | Four individual monkeys| Four individual monkeys |
| MRT (h)            | 62.23 90.12 110.2 73.4 | 49.37 60.99 58.6 108.0 |
| T1/2 (h)           | 25.2 42.3 77.8 37.8   | 23.3 19.4 24.0 58.7    |
| Cmax (U/ml)        | 19.6 18.3 12.3 11.0   | 23.1 20.3 16.5 9.8    |
| AUC (U/ml·h)       | 1706 1856 1489 1128   | 1762 1675 1089 1367   |

Table 3. Comparison of the pharmacokinetics parameters four following sc and im injections performed in parallel.
4.4 Protection studies with PEG-rMaBChE

Many studies have demonstrated efficacy of native HuBChE, both pre-and post-exposure, in rodents and monkeys to protect against OP nerve agents delivered by sc injection, iv injection or vapour. (Lenz et al., 2005; Sun et al., 2008; Saxena et al., 2011; Mumford et al, 2010). Protection has also been shown in mice and guinea pigs using PEG-rBChE produced in goat and plants (Huang et al. 2007, Geyer et al., 2010). However, very few studies have utilized the non-human primate monkey model for assessing insecticide toxicity and none have used respiratory exposure.

Two types of protection studies using different routes of delivery are currently being performed to assess the ability of BChE to protect against toxicity resulting from exposure to the insecticide paraoxon.

1. Aerosolized PEG-rMaBChE 1 hr prior to aerosolized paraoxon exposure.
2. Intravenous delivery of PEG-rMaBChE 1 hr prior to sc delivery of paraoxon.

4.4.1 Paraoxon

The majority of OP insecticides are lipophilic, not ionised, and are absorbed rapidly following inhalation or ingestion (Vale, 1998). Dermal absorption is slower and can be prevented by removing clothes and bathing, but severe poisoning may still ensue if exposure is prolonged. Respiratory pesticide exposure by inhalation of powders, airborne droplets or vapours is particularly hazardous because pesticide particles can quickly enter the bloodstream via the lungs and cause serious damage. Under low pressure, droplet size is too large to remain airborne. However, when high pressure, ultra low volume application (ULV) or fogging equipment is used for agricultural purposes, respiratory exposure is increased due to the production of mist- or fog-size particles, which can be carried on air currents for a considerable distance (Armed Forces Pest Management Board Technical Guide No. 13). Small children are highly vulnerable because they breathe in greater volumes of air, relative to their body weight, than adults.

![Fig. 6. Chemical structure of parathion and paraoxon.](image)

Paraoxon is the active metabolite of the inactive parathion (Fig. 6) produced by a sulfur-for-oxygen substitution carried out predominantly in the liver by the mixed-function oxidases (Dauterman, 1971). It was chosen for these studies because it inhibits AChE, BChE and carboxylesterase (Levine, 2006), it has a relatively low LD$_{50}$, and low volatility and stability in aqueous solution. Parathion has probably been responsible for more cases of accidental
poisoning and death than any other OP insecticide (Lotti & Moretto, 2005) and was recently phased out of use in the US. In humans, parathion is absorbed via skin, mucous membranes, and orally and is rapidly metabolized to paraoxon which can result in headaches, convulsions, poor vision, vomiting, abdominal pain, severe diarrhea, unconsciousness, tremor, dyspnea and finally lung edema as well as respiratory arrest. Symptoms of severe poisoning are known to last for extended periods of time, sometimes months. Additionally, peripheral neuropathy including paralysis is noticed as late sequelae after recovery from acute intoxication (http://extoxnet.orst.edu/pips/parathio.htm). Parathion has been extensively used for committing suicide and potentially for the deliberate killing of people.

4.4.2 Aerosolized PEG-rMaBChE protection against aerosolized paraoxon exposure

As an alternative to delivering high doses of a large molecule into the systemic circulation by sc or im routes, studies are currently being performed using aerosol therapy for delivering rBChE directly to the lung in order to create an effective “pulmonary bioshield” that will detoxify incoming inhaled insecticide in situ and prevent or reduce respiratory toxicity. This takes advantage of the large size of the molecule which will be retained in the lung due to its inability to pass through the lung endothelium into the blood. In this context, inhalation serves as a major means of intoxication because of rapid accesses of the OP to the blood. An efficient pre-exposure pulmonary therapeutic in the form of aerosolized PEG-rBChE could be delivered before a known use/release of insecticides and prevent the lung damage and delayed neuropathy often associated with exposure, while reducing the need for post-exposure atropine and oximes.

Maxwell et al. (2006) have recently shown that for OP compounds (including the insecticides paraoxon, DFP and dichlorvos) the primary mechanism of in vivo toxicity is the inhibition of AChE and the residual unexplained variation in OP toxicity represents <10% of the total variation in toxicity. Almost all of the variation in the LD$_{50}$ of OP compounds in rats was explained by the variation in their in vitro rate constants for inhibition of AChE. Thus, to develop a paraoxon/monkey animal model for aerosolized insecticide exposure and to avoid unnecessary stressing and killing of monkeys in developing the model, the dose of aerosolized paraoxon required to achieve a ~50% inhibition of RBC AChE and serum BChE has been used initially as a readout for toxicity and a basis from which to analyze protection by CHO-derived rMaBChE. Thus, paraoxon which is not neutralized in the lung will enter the blood and can be measured by the inhibition of AChE and BChE activity in lysed whole blood using using a modified assay (Ellman et al, 1961) with 5,5'-dithiobis(2-nitrobenzoic acid), the substrate acetyl-thiocholine (ATC) and 20uM ethopropazine to inhibit BChE activity.

Initially, the dose of aerosolized paraoxon required to produce ~50% inhibition of red blood cell (RBC) AChE and BChE in the circulation was first determined in mice prior to the macaque studies. The LD$_{50}$ of paraoxon in rodents has been established using oral, percutaneous (pc) and subcutaneous (sc) routes (mice: 760 ug/kg orally; 270 - 800 ug/kg sc and for rats: 1800 ug/kg orally and 200 - 430 sc (reviewed in Levine, 2006; Villa et al., 2007). Milatovic et al. (1996) showed that a single acute injection of 0.09, 0.12, or 0.19 mg/kg paraoxon in rats, representing 40% LD$_{50}$, 52% LD$_{50}$ and 83% LD$_{50}$ respectively, did not produce signs of cholinergic hyperactivity. In the present study, the effective dose of aerosolized paraoxon resulting in 50% inhibition in mice was found to be 150-180 ug/kg which is less toxic than the parenteral route. In addition, aerosolized BChE given 24 hr prior
to the paraoxon significantly reduced the AChE inhibition (our unpub. data). Rodents contain a high endogenous levels of CaE, another stoichiometric OP scavenger (Dirnhuber et al. 1979) and are known to be ~10-fold less sensitive to soman than non-human primates (Maxwell et al., 2006). Accordingly, a dose of 15 ug/kg of aerosolized paraoxon has been shown to result in 50-60% RBC AChE inhibition and preliminary data indicate that PEG-rMaBChE, delivered as a pre-exposure aerosol one hour prior to exposure, can totally reduce this inhibition in a dose-dependent manner.

4.4.3 Intravenous PEG-rMaBChE protection against subcutaneous paraoxon exposure

These studies are being formed to compare routes of delivery with efficacy of protection and indicate that while paraoxon delivered sc is also more toxic than as an aerosol, complete protection can be achieved by PEG-rMaBChE pretreatment.

5. References

Alavanja, M. C. (2009). Pesticides Use and Exposure Extensive Worldwide. Rev Environ Health, 24(4):303-9.
Altamirano, C. V., Lockridge, O. (1999). Association of tetramers of human butyrylcholinesterase is mediated by conserved aromatic residues of the carboxy terminus. Chem Biol Interact. 119-120:53-60.
Altmann, F. (2007). The role of protein glycosylation in allergy. Int Arch Allergy Immunol. 142(2):99-115.
Ashani, Y., & Pistinner, S. (2004). Estimation of the upper limit of human butyrylcholinesterase dose required for protection against organophosphates toxicity: a mathematically based toxicokinetic model. Toxicol Sci. 77(2):358-67.
Blong, R. M., Bedows, E., & Lockridge, O. (1997). Tetramerization domain of human butyrylcholinesterase is at the C-terminus. Biochem J. 327 (Pt 3):747-57.
Boeck, A. T., Schopfer, L. M., & Lockridge, O. (2002). DNA sequence of butyrylcholinesterase from the rat: expression of the protein and characterization of the properties of rat butyrylcholinesterase. Biochem Pharmacol. 63(12):2101-10.
Boelaert, J. R., Schurgers, M. L., Matthys, E. G., Belpaire, F. M., Daneels, R. F., De Cre, M. J., & Bogaert, M. G. (1989). Comparative pharmacokinetics of recombinant erythropoietin administered by the intravenous, subcutaneous, and intraperitoneal routes in continuous ambulatory peritoneal dialysis (CAPD) patients. Perit Dial Int. 9(2):95-8.
Buckley, N. A., Eddleston, M., Li, Y., Bevan, M., & Robertson, J. (2011). Oximes for acute organophosphate pesticide poisoning. Cochrane Database Syst Rev. 16;(2):CD005085. Review.
Cao, J. L., Varnell, A. L., & Cooper, D. C. (2011). Gulf War Syndrome: A role for organophosphate induced plasticity of locus coeruleus neurons. Nature Precedings: hdl:10101/npre.2011.6057.1: Posted 23 Jun 2011.
Cascio, C., Comite Ghiara, M., Lanza, G., & Ponchione, A. (1988). Use of serum cholinesterases in severe organophosphorus poisioning. Our experience. Minerva Anestesiol. 54(7-8):337-8.
Castilho, A., Strasser, R., Stadlimann, J., Grass, J., Jez, J., Gattinger, P., Kunert, R., Quendler, H., Pabst, M., Leonard, R., Altmann, F., & Steinkellner, H. (2010). In planta protein sialylation through overexpression of the respective mammalian pathway. *J Biol Chem*. 285(21):15923-30.

Chilukuri, N., Sun, W., Naik, R. S., Parikh, K., Tang, L., Doctor, B. P., & Saxena, A. (2008a). Effect of polyethylene glycol modification on the circulatory stability and immunogenicity of recombinant human butyrylcholinesterase. *Chem Biol Interact*. 175(1-3):255-60.

Chilukuri, N., Sun, W., Parikh, K., Naik, R. S., Tang, L., Doctor, B. P., & Saxena, A. (2008b). A repeated injection of polyethyleneglycol-conjugated recombinant human butyrylcholinesterase elicits immune response in mice. *Toxicol Appl Pharmacol*. 231(3):423-9.

Chung, C. H., Mirakhur, B., Chan, E., Le, Q. T., Berlin, J., Morse, M., Murphy, B. A., Satinover, S.M., Hosen, J., Mauro, D., Slebos, R. J., Zhou, Q., Gold, D., Hatley, T., Hicklin, D. J., & Platts-Mills, T. A. (2008). Cetuximab-induced anaphylaxis and IgE specific for galactose-alpha-1,3-galactose. *N Engl J Med*. 358(11):1109-17.

Cohen, O., Kronman, C., Chitlaru, T., Ordentlich, A., Velan, B., & Shafferman, A. (2001). Effect of chemical modification of recombinant human acetylcholinesterase by polyethylene glycol on its circulatory longevity. *Biochem J*. 357(Pt 3):795-802.

Colosio, C., Tiramani, M., Brambilla, G., Colombi, A., & Moretto, A. (2009). Neurobehavioural effects of pesticides with special focus on organophosphorus compounds: which is the real size of the problem? *Neurotoxicology*. 30(6):1155-61.

Dauterman, W. C. (1971). Biological and nonbiological modifications of organophosphorus compounds. *Bull World Health Organ*. 44(1-3):133-50. Review.

Deshpande, L. S., Carter, D. S., Blair, R. E., & DeLorenzo, R.J. (2010). Development of a prolonged calcium plateau in hippocampal neurons in rats surviving status epilepticus induced by the organophosphate diisopropylfluorophosphate. *Toxicol Sci*. 116(2):623-31.

Diaz, S. L., Padler-Karavani, V., Ghaderi, D., Hurtado-Ziola, N., Yu, H., Chen, X., Brinkman-Van der Linden, E. C., Varki, A., & Varki, N. M. (2009). Sensitive and specific detection of the non-human sialic Acid N-glycoly neuraminic acid in human tissues and biotherapeutic products. *PLoS One*. 4(1):e4241.

Dirnhuber, P., French, M. C., Green, D.M., Leadbeater, L., & Stratton, J. A. (1979). The protection of primates against soman poisoning by pretreatment with pyridostigmine. *J Pharm Pharmacol*. 31(5):295-9.

Doctor, B. P., Maxwell, D. M., Ashani, Y., Saxena, A., & Gordon, R. K. (2001). New approaches to Medical Protection against Chemical Warfare Nerve Agents. Somani, SM and Romano, JA, Eds. CRC Press, New York, p191-214.
Ellman, G. L., Courtney, K. D., Andres, V. Jr., & Featherstone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol.* 7:88-95.

Geyer, B. C., Kannan, L., Garnaud, P. E., Broomfield, C. A., Cadieux, C. L., Cherni, I., Hodgins, S. M., Kasten, S. A., Kelley, K., Kilbourne, J., Oliver, Z. P., Otto, T. C., Pufferberger, I., Reeves, T. E., Robbins, N. 2nd., Woods, R. R., Soreq, H., Lenz, D. E., Cerasoli, D. M., & Mor, T. S. (2010). Plant-derived human butyrylcholinesterase, but not an organophosphorous-compound hydrolyzing variant thereof, protects rodents against nerve agents. *Proc Natl Acad Sci U S A.* 107(47):20251-6.

Gleba, Y., Klimyuk, V., & Marillonnet, S. (2005). Magnificient—a new platform for expressing recombinant vaccines in plants. *Vaccine.* 23(17-18):2042-8. Review.

Goodin, M. M., Zaitlin, D., Naidu, R. A., & Lommel, S. A. (2008). Nicotiana benthamiana: its history and future as a model for plant-pathogen interactions. *Mol Plant Microbe Interact.* 21(8):1015-26. Review.

Grunwald, J., Marcus, D., Papier, Y., Raveh, L., Pittel, Z., & Ashani, Y. (1997). Large-scale purification and long-term stability of human butyrylcholinesterase: a potential bioscavenger drug. *J Biochem Biophys Methods.* 34(2):123-35.

Güven, M., Sungur, M., Eser, B., Sari, I., & Altuntaş, F. (2004). The effects of fresh frozen plasma on cholinesterase levels and outcomes in patients with organophosphate poisoning. *J Toxicol Clin Toxicol.* 42(5):617-23.

Heatherton, A. C., Schuller, J., & Mercer, A. J. (2001). Pharmacokinetics of novel erythropoiesis stimulating protein (NESP) in cancer patients: preliminary report. *Br J Cancer.* 84 Suppl 1:11-6.

Huang, Y. J., Huang, Y., Baldassarre, H., Wang, B., Lazaris, A., Leduc, M., Bilodeau, A. S., Bellemare, A., Côté, M., Herskovits, P., Touati, M., Turcotte, C., Véleanu, L., Lemée, N., Wilgus, H., Bégin, 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 U S A.* 104(34):13603-8.

Jenkins, T., Balinsky, D., & Patient, D. W. (1967). Cholinesterase in plasma: first reported absence in the Bantu; half-life determination. *Science.* 156(783):1748-50.

Jeyaratnam, J. (1990). Acute pesticide poisoning: a major global health problem. *World Health Stat Q.* 43(3):139-44.

Kang, J. S., Deluca, P. P., & Lee, K. C. (2009). Emerging PEGylated drugs. *Expert Opin Emerg Drugs.* 14(2):363-80. Review.

Kolarich, D., Weber, A., Pabst, M., Stadlmann, J., Teschner, W., Ehrlich, H., Schwarz, H. P., & Altmann, F. (2008). Glycoproteomic characterization of butyrylcholinesterase from human plasma. *Proteomics.* 8(2):254-63.

Kronman, C., Chitlaru, T., Elhanany, E., Velan, B., & Shaffer, A. (2000). Hierarchy of post-translational modifications involved in the circulatory longevity of glycoproteins. Demonstration of concerted contributions of glycan sialylation and subunit assembly to the pharmacokinetic behavior of bovine acetylcholinesterase. *J Biol Chem.* 275(38):29488-502.
Kronman, C., Cohen, O., Raveh, L., Mazor, O., Ordentlich, A., & Shafferman, A. (2007). Polyethylene-glycol conjugated recombinant human acetylcholinesterase serves as an efficacious bioscavenger against soman intoxication. Toxicology. 233(1-3): 40-6.

Lenz, D. E., Broomfield, C. A., Maxwell, D. M., & Cerasoli, D. M. (2001). Nerve Agent Bioscavengers: Protection against High- and Low- Dose Organophosphorus Exposure. Somani, SM and Romano, JA, Eds. CRC Press, New York, p215-243.

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 Interact. 157-158:205-10.

Lenz, D. E., Yeung, D., Smith, J. R., Sweeney, R. E., Lumley, L. A., & Cerasoli, D. M. (2007). Stoichiometric and catalytic scavengers as protection against nerve agent toxicity: a mini review. Toxicology. 233(1-3):31-9. Review.

Levine, E. S. (2006). Nerve Agent Simulants: Can They Be Used as Substitutes for Nerve Agents in biomedical Research? Prepared for the U.S. Army Medical Research Institute of Chemical Defense under Contract No. GS-23F-8006H.

Li, H., Schopfer, L. M., Masson, P., & Lockridge, O. (2008). Lamellipodin proline rich peptides associated with native plasma butyrylcholinesterase tetramers. Biochem J. 411(2):425-32.

Litchfield, M. H. (2005). Estimates of acute pesticide poisoning in agricultural workers in less developed countries. Toxicol Rev., 24(4):271-8. Review.

Lockridge, O. (1990). Genetic variants of human serum cholinesterase influence metabolism of the muscle relaxant succinylcholine. Pharmacol Ther 47: 35-60.

Lotti, M., & Moretto, A. (2005). Organophosphate-induced delayed polyneuropathy. Toxicol Rev. 24(1):37-49. Review.

Luo, C., Tong, M., Maxwell, D. M., & Saxena, A. (2008). Comparison of oxime reactivation and aging of nerve agent-inhibited monkey and human acetylcholinesterases. Chem Biol Interact. 175(1-3):261-6.

Mager, D. E., Neuteboom, B., & Justo, W. J. (2005). Pharmacokinetics and pharmacodynamics of PEGylated IFN-beta 1a following subcutaneous administration in monkeys. Pharm Res. 22(1):58-61.

Mager, D. E., Woo, S., & Justo, W. J. (2009). Scaling pharmacodynamics from in vitro and preclinical animal studies to humans. Drug Metab Pharmacokinet. 24(1):16-24. Review.

Matzke, S. M., Oubre, J. L. Caranto, G. R., Gentry, M. K., & Galbicka, G. (1999). Behavioral and immunological effects of exogenous butyrylcholinesterase in rhesus monkeys. Pharmacol Biochem Behav. 62(3):523-30.

Maxwell, D. M., Brecht, K. M., Koplovitz, I., & Sweeney, R. E. (2006). Acetylcholinesterase inhibition: does it explain the toxicity of organophosphorus compounds? Arch Toxicol. 80(11):756-60.

McLennan, D. N., Porter, C. J., Edwards, G.A., Heatherington, A. C., Martin, S. W., & Charman, S. A. (2006). The absorption of darbepoetin alfa occurs predominantly
via the lymphatics following subcutaneous administration to sheep. *Pharm Res.* 23(9):2060-6.

Milatovic, D., & Dettbarn, W. D. (1996). Modification of acetylcholinesterase during adaptation to chronic, subacute paraoxon application in rat. *Toxicol Appl Pharmacol.* 136(1):20-8.

Millard, C. B., Kryger, G., Ordentlich, A., Greenblatt, H. M., Harel, M., Raves, M. L., Segall, Y., Barak, D., Shaferman, A., Silman, I., & Sussman, J. L. (1999). Crystal structures of aged phosphorylated acetylcholinesterase: nerve agent reaction products at the atomic level. *Biochemistry*, 38:7032-9.

Mumford, H., Price, M. E., Cerasoli, D.M., Teschner, W., Ehrlich, H., Schwarz, H.P., & Lenz, D. E. (2010). Efficacy and physiological effects of human butyrylcholinesterase as a post-exposure therapy against percutaneous poisoning by VX in the guinea-pig. *Chem Biol Interact.* 187(1-3):304-8.

Nachon, F., Nicolet, Y., Vigué, N., Masson, P., Fontecilla-Camps, J. C., & Lockridge, O. (2002). Engineering of a monomeric and low-glycosylated form of human butyrylcholinesterase: expression, purification, characterization and crystallization. *Eur J Biochem.* 269(2):630-7.

Ohayo-Mitoko, G. J., Kromhout, H., Simwa, J. M., Boleij, J. S., & Heederik, D. (2000). Self reported symptoms and inhibition of acetylcholinesterase activity among Kenyan agricultural workers. *Occup Environ Med.* 57(3):195-200.

Paccalet, T., Bardor, M., Rihouey, C., Delmas, F., Chevalier, C., D’Aoust, M. A., Faye, L., Vézina, L., Gomord, V., & Lerouge, P. (2007). Engineering of a sialic acid synthesis pathway in transgenic plants by expression of bacterial Neu5Ac-synthesizing enzymes. *Plant Biotechnol J.* 5(1):16-25.

Park, E. I., Mi, Y., Unverzagt, C., Gabius, H. J., & Baenziger, J. U. (2005). The asialoglycoprotein receptor clears glycoconjugates terminating with sialic acid alpha 2,6GalNAc. *Proc Natl Acad Sci U S A.* 102(47):17125-9.

Pepinsky, R. B., LePage, D. J., Gill, A., Chakraborty, A., Vaidyanathan, S., Green, M., Baker, D. P., Whalley, E., Hochman, P. S., & Martin, P. (2001). Improved pharmacokinetic properties of a polyethylene glycol-modified form of interferon-beta-1a with preserved in vitro bioactivity. *J Pharmacol Exp Ther.* 297(3):1059-66.

Porter, C. J., Edwards, G. A., & Charman, S.A. (2001). Lymphatic transport of proteins after s.c. injection: implications of animal model selection. *Adv Drug Deliv Rev.* 50(1-2):157-71. Review.

Radwanski, E., Perentesis, G., Jacobs, S., Oden, E., Affrime, M., Symchowicz, S., & Zampaglione, N. (1987). Pharmacokinetics of interferon alpha-2b in healthy volunteers. *J Clin Pharmacol.* 27(5):432-5.

Ramakrishnan, R., Cheung, W. K., Farrell, F., Joffe, L., & Jusko, W. J. (2003). Pharmacokinetic and pharmacodynamic modeling of recombinant human erythropoietin after intravenous and subcutaneous dose administration in cynomolgus monkeys. *J Pharmacol Exp Ther.* 306(1):324-31.

Raveh, L., Ashani, Y., Levy, D., De La Hoz, D., Wolfe, A. D., & Doctor, B.P. (1989). Acetylcholinesterase prophylaxis against organophosphate poisoning. Quantitative
correlation between protection and blood-enzyme level in mice. *Biochem Pharmacol.* 38(3):529-34.

Raveh, L., Grauer, E., Grunwald, J., Cohen, E., & Ashani, Y. (1997). The stoichiometry of protection against soman and VX toxicity in monkeys pretreated with human butyrylcholinesterase. *Toxicol Appl Pharmacol.* 145(1):43-53.

Rosenberg, Y. J., Luo, C., Ashani, Y., Doctor, B. P., Fischer, R., Wo1fee, G., & Saxena, A. (2002). Pharmacokinetics and immunologic consequences of exposing macaques to purified homologous butyrylcholinesterase. *Life Sci.* 72(2):125-34.

Rosenberg, Y. J., Saxena, A., Sun, W., Jiang, X., Chilukuri, N., Luo, C., Doctor, B. P., & Lee, K. D. (2010). Demonstration of in vivo stability and lack of immunogenicity of a polyethylene glycol-conjugated recombinant CHO-derived butyrylcholinesterase bioscavenger using a homologous macaque model. *Chem Biol Interact.* 187(1-3):279-86.

Rosenberry, T. L., Mallender, W. D., Thomas, P. J., & Szegletes, T. (1999). A steric blockade model for inhibition of acetylcholinesterase by peripheral site ligands and substrate. *Chem Biol Interact.* 119-120:85-97.

Sainsbury, F., Lavoie, P. O., D’Aoust, M. A., Vézina, L.P., & Lomonossoff, G. P. (2008). Expression of multiple proteins using full-length and deleted versions of cowpea mosaic virus RNA-2. *Plant Biotechnol J.* 6(1):82-92.

Saxena, A., Ashani, Y., Raveh, L., Stevenson, D., Patel, T., & Doctor, B. P. (1998). Role of oligosaccharides in the pharmacokinetics of tissue-derived and genetically engineered cholinesterases. *Mol Pharmacol.* 53(1):112-22.

Saxena, A., Sun, W., Fedorko, J. M., Koplovitz, I., & Doctor, B. P. (2011). Prophylaxis with human serum butyrylcholinesterase protects guinea pigs exposed to multiple lethal doses of soman or VX. *Biochem Pharmacol.* 81(1):164-9.

Stoger, E., Sack, M., Nicholson, L., Fischer, R., & Christou, P. (2005). Recent progress in plantibody technology. *Curr Pharm Des.* 11:2439-57.

Strasser, R., Altmann, F., Mach, L., Glössl, J., & Steinkellner, H. (2004). Generation of Arabidopsis thaliana plants with complex N-glycans lacking beta1,2-linked xylose and core alpha1,3-linked fucose. *FEBS Lett.* 561(1-3):132-6.

Strasser, R., Stadlmann, J., Svoboda, B., Altmann, F., Glössl, J., & Mach, L. (2005). Molecular basis of N-acetylglucosaminyltransferase I deficiency in Arabidopsis thaliana plants lacking complex N-glycans. *Biochem J.* 387(Pt 2):385-91.

Sun, W., Doctor, B. P., & Saxena, A. (2005). Safety and pharmacokinetics of human serum butyrylcholinesterase in guinea pigs. *Chem Biol Interact.* 157-158:428-9.

Sun, W., Doctor, B. P., Lenz, D. E., & Saxena, A. (2008). Long-term effects of human butyrylcholinesterase pretreatment followed by acute soman challenge in cynomolgus monkeys. *Chem Biol Interact.* 175(1-3):428-30.

Sun, W., Luo, C., Naik, R. S., Doctor, B. P., & Saxena, A. (2009). Pharmacokinetics and immunologic consequences of repeated administrations of purified heterologous and homologous butyrylcholinesterase in mice. *Life Sci.* 85(17-18):657-61.
Supersaxo, A., Hein, W. R., & Steffen, H. (1990). Effect of molecular weight on the lymphatic absorption of water-soluble compounds following subcutaneous administration. *Pharm Res.* 7(2):167-9.

Vale, J. (1998). Toxicokinetic and toxicodynamic aspects of organophosphorus (OP) insecticide poisoning. *Toxicol Lett.* 102-103:649-52.

Varki, A. (2001). N-glycolylneuraminic acid deficiency in humans. *Biochimie.* 83(7):615-22. Review.

Villa, A. F., Houze, P., Monier, C., Risède, P., Sarhan, H., Borron, S. W., Mégarbane, B., Garnier, R., & Baud, F. J. (2007). Toxic doses of paraoxon alter the respiratory pattern without causing respiratory failure in rats. *Toxicology.* 232(1-2):37-49.

Wenderoth, I., & von Schaewen, A. (2000). Isolation and characterization of plant N-acetyl glucosaminyltransferase I (GntI) cDNA sequences. Functional analyses in the Arabidopsis cgl mutant and in antisense plants. *Plant Physiol.* 123(3):1097-108.

Worek, F., Aurbek, N., Herkert, N. M., John, H., Eddleston, M., Eyer, P., & Thiermann, H. (2010). Evaluation of medical countermeasures against organophosphorus compounds: the value of experimental data and computer simulations. *Chem Biol Interact.* 187(1-3):259-64.

Yang, M.X., Shenoy, B., Disttler, M., Patel, R., McGrath, M., Pechenov, S., & Margolin, A. L. (2003). Crystalline monoclonal antibodies for subcutaneous delivery. *Proc Natl Acad Sci U S A.* 100(12):6934-9.
It is our hope that this book will be of interest and use not only to scientists, but also to the food-producing industry, governments, politicians and consumers as well. If we are able to stimulate this interest, albeit in a small way, we have achieved our goal.

How to reference
In order to correctly reference this scholarly work, feel free to copy and paste the following:

Yvonne Rosenberg, Xiaoming Jiang, Lingjun Mao, Segundo Hernandez Abanto, Keunmyoung Lee (2012). Development of a Prophylactic Butyrylcholinesterase Bioscavenger to Protect Against Insecticide Toxicity Using a Homologous Macaque Model, Insecticides - Basic and Other Applications, Dr. Sonia Soloneski (Ed.), ISBN: 978-953-51-0007-2, InTech, Available from: http://www.intechopen.com/books/insecticides-basic-and-other-applications/development-of-a-prophylactic-butyrylcholinesterase-bioscavenger-to-protect-against-insecticide-toxi

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821