Comparison of PEGylated Recombinant Human Acetylcholinesterase and Serum Human Butyrylcholinesterase as Bioscavengers of Organophosphorous Compounds

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Running title: Cholinesterase-based organophosphate bioscavengers

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Number of text pages: 23

Number of Tables: 6

Number of Figures: 6

Number of references: 38

Number of words in Abstract: 250

Number of words in Introduction: 671

Number of words in Discussion: 1498

Abbreviations: AUC, area under curve; CaE, carboxylesterase; CBDP, cresylbenzodioxaphosphorin oxide; ChE, cholinesterase; CW, chemical warfare; EqBChE, equine serum butyrylcholinesterase; FBS, fetal bovine serum; HuAChE, human acetylcholinesterase HuBChE, human butyrylcholinesterase; MEPQ, 7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide; PEG, polyethylene glycol; rHuAChE, recombinant human acetylcholinesterase; rHuBChE, recombinant human butyrylcholinesterase; OP, organophosphorous.
Abstract

Comparative protection studies in mice demonstrate that on a mole basis, recombinant human acetylcholinesterase (rHuAChE) confers higher levels of protection than native human butyrylcholinesterase (HuBChE) against organophosphorous (OP) compound intoxication. For example, mice challenged with 2.5 LD$_{50}$ of sarin, soman and VX following treatment with equimolar amounts of the two cholinesterases, displayed 80, 100 and 100% survival respectively when pretreatment was carried out with rHuAChE and 0, 20 and 60% survival respectively, when pretreatment was carried out with HuBChE. Kinetic studies and active site titration analyses of the tested OP compounds with AChEs and BChEs from different mammalian species demonstrate that the superior in-vivo efficacy of acetylcholinesterases is in accordance with the higher stereoselectivity of AChE versus BChE towards the toxic enantiomers comprising the racemic mixtures of the various OP agents. In addition, we show that polyethylene glycol conjugated rHuAChE (PEGylated rHuAChE), which is characterized by a significantly extended circulatory residence both in mice and monkeys (Biochem. J. 357:795, 2001; Biochem. J. 378:117, 2004), retains full reactivity towards OP compounds both in-vitro and in-vivo and provides higher level of protection to mice against OP poisoning, as compared to native serum-derived HuBChE. In fact, PEGylated rHuAChE also confers superior prophylactic protection when administered intravenously or intramuscularly over 20 hours before exposure of mice to a lethal dose of VX (1.3-1.5 LD$_{50}$). These findings, together with the observations that the PEGylated rHuAChE exhibits unaltered biodistribution and high bioavailability, presents a case for utilizing PEGylated rHuAChE as a very efficacious bioscavenger of OP-agents.
Introduction

The primary role of acetylcholinesterase (acetylcholine acetylhydrolase, 3.1.1.7 AChE) is the termination of impulse transmission in cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine. A second acetylcholine hydrolysing enzyme, butyrylcholinesterase (alcholine acyl hydrolase 3.1.1.8, BChE), often appears together with AChE in various tissues. BChE differs from AChE in its substrate specificity and in susceptibility to inhibitors. Organophosphate (OP) compounds, such as the nerve agent sarin and soman, inhibit AChE and BChE irreversibly by rapid phosphonylation of the serine residue in the enzyme active site. The acute toxicity of these nerve agents is elicited in motor and respiratory failure following inhibition of AChE in the peripheral and central nervous system. Although the current drug treatment regimens against nerve agent exposure increased survival in intoxicated animals, they failed to prevent severe incapacitation, unless administered immediately after exposure.

The high reactivity of both acetylcholinesterase and butyrylcholinesterase towards CW nerve agents (e.g. soman, sarin, tabun, VX) or towards organophosphates compounds, led to propose these enzymes as potential bioscavengers for prophylactic treatment of OP-poisoning. Previous studies suggest that exogenous cholinesterase can serve as an effective therapeutic agent for sequestration of highly toxic OPs before they reach their physiological target (Raveh et al., 1993, 1997; Lenz et al., 2005 and references cited therein). Administration of native AChE (e.g. fetal bovine serum AChE, FBS-AChE) or BChE (e.g. equine serum butyrylcholinesterase, EqBChE; human serum BChE, HuBChE) protected the animals from a variety of highly toxic OPs without any toxic effects or performance decrements in both rodent and non-human primate models (Raveh et al., 1993, 1997; Lenz et al., 2005). Based on data from animal experiments, it was estimated that for human BChE, 200 mg would be required to provide protection for an adult human against 2LD50 of soman (Ashani et al., 1998).
The requirement for ChEs in large quantities encouraged the development of large-scale production systems for the generation of recombinant ChEs (Kronman et al., 1992; Fischer et al., 1993; Saxena et al., 1998), however pharmacokinetic studies have shown that recombinant enzymes generated in either bacterial or mammalian cells, are retained in the circulation of experimental animals for much shorter periods of time than native FBS-AChE or BChE (Kronman et al., 1995; Saxena et al., 1998). Extensive studies have shown that this problem can be effectively resolved by conversion of recombinant ChE into a circulatory long-lived enzyme by either optimization of post-translation modification (e.g. enzyme glycosylation, sialylation, subunit oligomerization, Kronman et al., 1995, 2000; Chitlaru et al., 1998, 2001, 2002; Duysen et al., 2002; Cohen et al., 2004) or by chemical modification of the enzyme through controlled appendage of polyethylene-glycol (PEG) side-chains (Cohen et al., 2001, 2004). Optimally PEGylated rHuAChE displayed very long circulatory residence in both mice and rhesus macaques, exceeding that of native serum derived cholinesterases (Cohen et al., 2004). However, the utilization of such a circulatory long-lived PEGylated version of recombinant human AChE as an in-vivo bioscavenger of OP-compounds, requires that additional conditions be met. First of all, the chemical modification of the enzyme should not alter its reactivity towards various OP-compounds. Furthermore, the PEGylated enzyme should display on one hand high bioavailability, and on the other hand, should not accumulate in vital organs where it may exert some negative biological effects.

In the present study we examined the ability of PEGylated rHuAChE to protect mice against OP-compound intoxication and monitored both circulatory buildup and subsequent biodistribution of the chemically modified recombinant enzyme. This line of studies allowed us to conclude that PEGylated recombinant human AChE is highly qualified for serving as a bioscavenger of OP-agents in-vivo. Moreover, by comparing the ability of serum BChE, rHuAChE and PEGylated rHuAChE to protect mice against OP-compound exposure, we could...
establish that on a molar basis, rHuAChE as well as PEGylated rHuAChE are superior to native BChE in their ability to protect mice, due to their higher stereoselectivity towards various OP agents. The high stereoselectivity of PEGylated rHuAChE, together with its extended circulatory stability, suggest that this enzyme form can serve as a superior prophylactic agent against OP intoxication.
Materials and Methods

Enzymes

Procedures of transfection of the human embryonal kidney derived cell line (HEK-293) with the expression vector of the C-terminus truncated HuAChE enzymes (Cohen et al., 2001) and the generation of stable cell clones expressing high levels of recombinant product, were described previously (Kronman et al., 1992). The method for purification of the secreted rHuAChE was described previously (Kronman et al., 1995). Human serum BChE was purified from outdated screened human plasma, obtained from local blood banks in Israel, as described previously (Kronman et al., 1995). FBS-AChE was purified similarly from fetal calf serum (Bet-Haemek, Israel).

Attachment of PEG chains to primary amines in rHuAChE was performed using succinimidyl propionate-activated methoxy-PEG (SPA-PEG; Nektar Inc.) as described previously (Cohen et al., 2001). Briefly, purified rHuAChE (5 µM) was incubated with PEG-20000 at a ratio of 30:1 (mol/mol) [PEG]/[AChE primary amines] in 50 mM borate buffer, pH 8.5, for 2 h at room temperature. The chemically modified products were dialyzed extensively against PBS and analyzed on 6% SDS/PAGE gels. The estimated molecular weight of PEGylated AChE is about 145kD (average of 4 PEG chains per AChE molecule).

Organophosphate Inhibitors

Soman (pinacolylmethyl phosphonofluoridate); Sarin (O-isopropyl methylphosphonofluoridate); VX (O-ethyl-S-(2-isopropylaminoethyl) methylphosphonothiolate), MEPQ (7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide) and CBDP (cresylbenzodioxaphosphorin oxide) were prepared according to previously reported procedures (Eto et al., 1962; Levy and Ashani, 1986; Ordentlich et al., 2004). Preparation of VX enantiomers was carried out as reported recently (Ordentlich et al., 2004).
The $P_S$-enantiomer of sarin was obtained in analogy to separation of soman by incubation of the racemic mixture with rabbit plasma (Benschop and De Jong, 1988) and the corresponding $P_R$-enantiomer by titration with HuAChE as suggested in Benschop et al., 2004. The purity of OPs (>95%) was determined by $^1$H and $^{31}$P NMR spectroscopy and by a titration of a known amount of HuAChE. Stock solutions were kept at -20°C, and diluted in 0.9% saline to the desired concentration. The toxicity of the diluted solutions was determined prior to each experiment by performing a dose response study in mice and calculating the LD$_{50}$ of these OPs, according to the Spearman and Karber method (Finney, D.J., 1964). The absolute configurations for the 4 diastereoisomers of soman $P_SC_S$, $P_S C_R$, $P_R C_S$, $P_R C_R$ were shown in the past (Ordentlich et al., 1999) to correspond to the older notations $C(+)P(-)$; $C(-)P(-)$; $C(-)P(+)$; $C(+)P(+)$. respectively.

**Kinetic Studies**

HuAChE activity was assayed according to the method of Ellman et al. (1961) in the presence of 0.1mg/mL BSA, 0.3 mM DTNB, 50 mM sodium phosphate buffer (pH 8.0), and various concentrations of ATC or BTC at 27°C and monitored with a Thermomax microplate reader (Molecular Devices). Enzyme concentration was determined by ELISA (Shafferman et al., 1992) and by active site titration using MEPQ (Shafferman et al., 1996).

Measurements of phosphorylation rates were carried out with at least four different concentrations of organophosphorus inhibitor (OP), and enzyme residual activity at various times was monitored. The apparent bimolecular phosphorylation rate constants ($k_i$) determined under pseudo first-order conditions were computed from the plot of slopes of $\ln(E)$ versus time at different inhibitor concentrations (Ordentlich et al., 1996, 1999). Rate constants under second order conditions were determined from plots of $\ln\{E/[OP_0 - (E_0 - E)]\}$ versus time. Interactions of HuAChE or PEG-HuAChE with the organophosphate compounds were analyzed as described previously (Ordentlich et al., 1996, 1999). Stereoselectivity of the enzymes toward various phosphonates was determined by active site titrations, comparing residual activities of enzymes.
inhibited by the appropriate racemic phosphonate to that of MEPQ (Shaffer{\textit{man et al.}, 1996}).

\textit{Animal procedures}

Male outbred ICR mice (Charles River Laboratories) were maintained at 20–22°C and a relative humidity of 50±10\% on a 12-h light/dark cycle, fed with commercial rodent chow (Koffolk Inc.) and provided with tap water \textit{ad libitum}. Treatment of animals was in accordance with regulations outlined in the USDA Animal Welfare Act and the conditions specified in Guide for Care and Use of Laboratory Animals (National Institute of Health, 1996). Animal studies were approved by the local ethical committee on animal experiments.

\textit{Challenge experiments}

Molar concentrations of ChEs were determined by active site titration with MEPQ (Levy and Ashani, 1986; Shaffer{\textit{man et al.}, 1996). ChEs were administered intravenously or intramuscularly to mice (5-10 per group) and 1 minute or 20-22 hours later, the mice were exposed intravenously to OPs. In the case of exposure to soman or sarin, mice were pretreated intramuscularly with CBDP (2mg/ml from freshly prepared 1mg/ml solution in DMSO) 1 hour before exposure to inhibit endogenous carboxylesterases (CaEs). The pharmacokinetic profile of HuBChE in mice pretreated with CBDP was virtually identical to that observed in mice which were not pretreated. Toxic signs and mortality were monitored for 24 hours following OP administration. In some experiments body weight was monitored daily for 7 days post exposure.

\textit{Pharmacokinetics of HuAChE in the circulation}

Clearance experiments in mice (3 to 6 mice per enzyme sample) were carried out essentially as described previously (Kronman et al., 2000, Chitlaru et al., 2001). Mice were injected either \textit{i.v.} or \textit{i.m.} with 200 units/mouse PEGylated rHuAChE, FBS-AChE or serum HuBChE in 0.2 ml PBS. Blood samples (5\textmu{l}) were drawn from the tail vein, diluted 20-fold in PBS, and
centrifuged for 3 minutes at 3000 rpm for the removal of red blood cells. Residual ChE activity in blood samples was measured at various time intervals and all values were corrected for background hydrolytic activity in the blood. Pharmacokinetic parameters were calculated using the WinNonLin software (Pharsight Corporation).

Tissue distribution

The C-terminus truncated HuAChE enzyme (ΔC-rHuAChE; Cohen et al., 2001) or PEGylated-rHuAChE (900U/mouse and 500U/mouse respectively) were administered i.v. to mice (15 mice per enzyme sample). At each time point, 3 mice were sacrificed and tissues were removed and homogenized in 9 volumes of ice-cold PBS/0.5% Tween-20. AChE activity was measured in the presence of 40µM iso-OMPA (tetraisopropylpyrophosphoramide), to inhibit endogenous BChE activity. The weight of the organs removed from mice were: liver: 540±70mg, kidney: 290±30mg, spleen: 120±20mg, brain: 430±30mg, heart: 170±20mg, lung: 220±20mg.
Results

PEGylated rHuAChE may serve as an efficient OP bioscavenger

Reactivity of PEGylated rHuAChE towards various OP compounds - The practical employment of ChEs as prophylactic agents against OP nerve agents requires that they can be administered hours or days prior to exposure, and therefore the relatively rapid elimination of recombinant ChEs from the circulation compromises the ability to utilize these enzyme forms as therapeutical bioscavengers (Kronman et al., 1995, 2000; Chitlaru et al., 1998; Saxena et al., 1998). However, this limitation in circulatory residence can be remedied by various post-translation modifications (Kronman et al., 2000; Chitlaru et al., 2001, 2002; Duysen et al., 2002) or by conjugation of PEG chains to lysine residues of recombinant AChE, resulting in its conversion into a circulatory long-lived molecule (Cohen et al., 2001, Cohen et al., 2004).

To examine the bioscavenging potential of PEG-modified AChE, recombinant HuAChE devoid of the C-terminal tail was subjected to controlled PEG-conjugation under conditions which allow loading an average of 4-to-5 PEG moieties per enzyme molecule, without affecting the catalytic activity of the enzyme (Cohen et al., 2001). Comparison of the inhibition constants ($k_i$ rate constant for phosphorylation, Table 1) of rHuAChE, rHuBChE and PEGylated rHuAChE towards various OP compounds demonstrated that the reactivity of the PEGylated rHuAChE towards sarin, soman, VX or tabun is very similar to that of rHuAChE, testifying to the fact that appendage of the PEG chains did not alter the ability of the enzyme to interact in an efficient manner with different OP agents.

Tissue distribution of exogenously administered PEGylated-AChE - In many animal species BChE serves as the major circulating ChE, while AChE is present at considerably lower levels or not at all (Li et al., 2000), raising the question whether administration of large amounts of recombinant AChE or its derivatives may result in altered disposition of the enzyme in various tissues. Furthermore, the presence of polyethylene glycol tails on ChEs may enhance their
accumulation in vital organs and thus may exert some negative biological effects. To address these issues, we conducted a comparative biodistribution analysis of the non-modified and PEGylated forms of C-terminal truncated rHuAChE. Both enzyme forms were administered intravenously to mice, and various organs (brain, liver, kidney, heart, spleen) as well as serum samples were removed at different time points and examined for exogenous AChE enzymatic activity. Taking into account the marked difference in circulatory residence times of the two enzyme forms (Fig. 1A), rHuAChE and PEGylated rHuAChEs were monitored until ninety percent of the corresponding enzyme forms were removed from the circulation (4 and 48 hours, for rHuAChE and PEGylated rHuAChE respectively). In the case of rHuAChE (Fig. 1B), exogenous enzyme levels in the liver were approximately 10% of that in the plasma only at 5 and 20 minutes following enzyme administration, and probably reflect rapid hepatic clearance of partially-sialylated rHuAChE within minutes of administration by the hepatic asialoglycoprotein receptor (Kronman et al., 1995, Chitlaru et al., 1998). Kidney levels of exogenous non-modified AChE were approximately 2% of plasma levels at all time points, most likely representing residual blood in this organ. AChE activity could not be detected at significant levels above endogenous background in the brain, heart or spleen of the mice at any of the time-points examined (always less than 1% of plasma levels). In the case of PEGylated rHuAChE (Fig. 1C), exogenous enzyme levels in all organs was very low at all time points and did not exceed 2% of plasma levels. Thus, exogenously administered recombinant human AChE enzyme did not accumulate in any of the organs examined, whether or not the enzyme was chemically modified by PEG-conjugation.
Comparison of the ability of AChE and BChE to neutralize OP-compounds in-vitro and in-vivo

Enhancement of OP toxicity in mice by CBDP- The therapeutic potential of cholinesterases as exogenous scavengers for sequestration of toxic OP-agents before they reach their physiological target has been examined in various animal models including mice, rats, guinea pigs and monkeys (Raveh et al., 1993, 1997; Lenz et al., 2005 and references cited therein). Small animals, such as mice, should be ideally suitable for carrying out wide-range comparative studies of the efficacies of different ChEs against a variety of OP agents. However, the susceptibility of mice to certain OP agents (e.g. soman, sarin) is considerably lower than that of primates due to the presence of high levels of endogenous carboxylesterases (CaEs) that bind these OP agents (Maxwell et al., 1987; Li et al., 2005).

Previous studies (Clement, 1984; Maxwell et al., 1987; Shapira et al., 1990) have shown that endogenous CaEs can be effectively inactivated in rats and mice by cresylbenzodioxaphosphorin oxide (CBDP) and thereby allow for lower quantities of sarin or soman for mice intoxication. However, when administered at high levels, CBDP inhibits cholinesterases as well, and the CBDP dose that would selectively inhibit CaEs in mice without affecting ChE activity has not been determined precisely (see Clement, 1984; Maxwell et al., 1987; Jimmerson et al., 1989). To determine exactly the amounts of CBDP that could be administered to mice without affecting exogenously administered cholinesterases, we first measured the bimolecular inhibition constants of CBDP towards AChE (1.4x10³ M⁻¹min⁻¹) and BChE (2x10⁴ M⁻¹min⁻¹, Fig. 2A). Since BChE is at least 20-fold more susceptible to inhibition than AChE, determination of the threshold level of CBDP that could effectively react with endogenous CaEs without affecting cholinesterase activity, can be monitored by examining the inhibition of endogenous BChE in mice treated with CBDP at various concentrations (Fig. 2B). At CBDP concentrations of 2mg/kg or less, endogenous BChE activity was only marginally affected (<2%, Fig. 2B). Furthermore, addition of...
purified BChE (1U/ml) to the serum samples removed from mice treated with CBDP within the range of 2-10 mg/kg demonstrated that the exogenous BChE was fully active and was not inhibited by the CBDP in a measurable manner (Fig. 2B, inset).

In line with these findings, bioscavenging studies of soman and sarin by AChE or BChE were conducted in the present study following pretreatment of mice with CBDP at a dose of 2 mg/kg one hour prior to challenge, since under these conditions the enzymatic activity of both endogenous cholinesterases and exogenously administered cholinesterases were not affected (see above). The LD$_{50}$ values of soman and sarin in mice pretreated with CBDP at 2 mg/kg were, as expected, considerably lower than without CBDP pretreatment (Table 2) and are similar to the corresponding LD$_{50}$ values determined in animal models devoid of serum CaEs, such as primates (Raveh et al., 1997). As expected, unlike in the case of soman and sarin, toxicity of VX in mice (LD$_{50}$ = 13.5 µg/kg) was not affected by CBDP treatment, since VX does not effectively bind to carboxylesterases (Maxwell, 1992). Based on these findings, experiments carried out with VX were performed without CBDP pretreatment.

**Comparison of the ability of AChE and BChE to neutralize soman in-vivo** - To compare the protective efficacy of exogenously administered AChE and BChE, mice were pretreated with equimolar amounts of either ChE and monitored for survival and toxic signs following OP-compound challenge. In an initial experiment, recombinant HuAChE and serum HuBChE were examined for their ability to protect mice against an exposure of 1 LD$_{50}$ of soman (1.2 nmole/mouse). Administration of both enzymes 1 minute prior to challenge at a dose of 1.2nmol/mouse (soman:ChE molar ratio =1; both OP and ChE administered i.v.), provided protection to all mice from lethality, while only 60% of the animals that were not pretreated with ChE, survived the 1 LD$_{50}$ soman challenge (Table 3). In contrast, equimolar amounts of recombinant HuAChE and serum HuBChE conferred different levels of protection to mice challenged with soman at a higher dose. Thus, when mice were challenged to 2.5LD$_{50}$ soman (3
nmole/mouse) after administration of ChEs at a dose of 1.2nmol ChE/mouse (soman:ChE molar ratio of 2.5), all rHuAChE-pretreated mice survived challenge and exhibited only mild signs of toxicity such as minor tremors and fasciculations (Table 3, lower panel), while 4 out of the 5 BChE-pretreated mice died within minutes. The single surviving BChE-pretreated mouse exhibited severe signs of toxicity, including tremors, Straub tail, salivation and respiratory distress. Control animals exposed to the 2.5LD50 soman challenge died within 2-3 minutes.

When mice pretreated with rHuAChE or serum HuBChE (1.2 nmole/mouse) were challenged with 2.1 LD50 soman, (2.5 nmole/mouse, soman:ChE ratio of 2.1) all 10 mice pretreated with rHuAChE survived, while only 5 out of 10 mice pretreated with serum HuBChE, survived challenge. rHuAChE-pretreated mice exhibited a reduction in body weight of no more than 5%, and regained their initial weight at day 4 post-challenge (Fig. 3). In contrast, serum HuBChE-pretreated mice that survived challenge exhibited a reduction in body weight of 10%, and reached their initial weights only on day 7 post-challenge.

The finding that, unlike AChE, BChE confers only partial protection to mice challenged with elevated levels of soman, prompted us to examine the ability of the two ChEs to protect mice from lethality at a wider range of soman:ChE ratios. To this end, mice were exposed to various soman doses 1 minute after administration of either rHuAChE or serum HuBChE at ChE doses of 0.9-1.3 nmole/mouse (Fig. 4). All BChE-pretreated mice survived challenge when the soman:BChE molar ratios were equal to or lower than 1.6. At higher soman:BChE ratios, the enzyme conferred partial protection only, and survival levels were linearly correlated to the molar ratio of OP compound to enzyme. Thus, only 80%, 60% and 20% of the mice survived challenge at soman:BChE ratios increasing from 1.8 to 2.0 and to 2.5, respectively (Fig. 4). In contrast, all AChE-pretreated mice survived challenge even when soman:AChE molar ratios were as high as 2.5. Lethality of AChE-pretreated mice could be observed only at higher soman:AChE ratios, and survival decreased in a linear manner, inversely correlating to the
soman:AChE values (Fig. 4). Thus, 20% survival was observed for AChE-pretreated mice, only at the high soman:AChE ratio of 4, equivalent to 4.0 LD₅₀. Taken together, though BChE and AChE equally provide full protection to mice at soman:ChE ratios of 1.6 or less, rHuAChE clearly confers a higher level of protection from lethality at higher soman:ChE ratios.

**Comparison of AChE and BChE reactivities toward soman, sarin and VX** – The most probable cause for the lower protective efficacy of BChE against soman intoxication observed in mice (see above) is its lower stereoselectivity towards OP-agents, as compared to AChE. The limited stereoselectivity of BChE towards the different stereoisomers of soman was manifested for both rHuBChE and equine serum BChE, and contrasts the marked stereoselectivity of the human or native bovine AChEs, (Table 4, Ordentlich et al., 1999, Ordentlich et al., 2004, Benschop et al., 1984, Benschop and DeJong, 1988, De Bisschop et al., 1991, Millard et al., 1998). Thus, unlike AChE, which is highly stereoselective towards the toxic P₅-diastereomer, BChE displays considerable reactivity toward both the toxic P₅ and the non-toxic P₆-diastereomers of soman. Judging by the phosphorylation bimolecular rate constants ($k_i$) of either recombinant HuBChE or native serum BChE (Table 4), it appears that as in the case of soman, BChEs have only a limited stereoselectivity (4-7-fold) toward the P₅-enantiomers of both sarin and VX. The low stereoselectivity of HuBChE towards sarin is, as in the case of soman, due to its higher reactivity toward the P₅-diastereomer as compared to that of HuAChE (150-fold). Consequently, HuBChE could be expected to react, at least partially, with this less toxic inhibitor.

In order to examine how the different stereoselectivities of AChE and BChE toward soman may have affected their relative efficacies as *in-vivo* bioscavengers, active site titration analyses with the racemic inhibitor were carried out at concentrations of enzymes and inhibitor simulating the estimated initial concentration ranges in plasma during the *in vivo* scavenging experiments in mice. Active site concentrations of the two ChEs were determined by titration with MEPQ, a
potent OP-inhibitor known to react with both AChE and BChE with 1:1 stoichiometry (Levy and Ashani, 1986; Shafferman et al., 1996). The intercepts for zero enzymatic activity, as observed in the actual titration profiles, were normalized to the corresponding intercepts determined in presence of MEPQ (Fig. 5). Under these experimental conditions, 2 moles of soman were neutralized by 1 mole of AChE while only 1 mole of the inhibitor was sequestered by 1 mole of BChE. These results are consistent with the stereoselectivities of the two enzymes toward the P₅-soman diastereomers, and appear to be directly correlated with the observed in vivo soman scavenging efficacies of AChE and BChE. As in the case of soman, active site titration analysis demonstrated that 2 moles of sarin were neutralized by 1 mole of AChE while only 1 mole of this inhibitor was sequestered by 1 mole of BChE. In contrast, the differential stereoselectivities of AChE and BChE towards VX, were less pronounced. Thus, 1.1 moles of VX were neutralized by 1 mole of AChE, while 1.0 mole of VX was neutralized by 1 mole BChE. Taken together, as in the case of soman, the active site titration profiles of sarin and VX with AChE and BChE are fully consistent with the measured phosphorylation rate constants (kᵢ) towards the corresponding enantiomers of these inhibitors. We therefore further examined whether the differential stereoselectivities of AChE and BChE towards sarin and VX, will also be reflected in the ability of the two enzymes to confer in-vivo protection against these two OP-inhibitors.

Comparison of the ability of AChE and BChE to neutralize sarin and VX in-vivo- In view of the similar profiles of the titration curves of soman and sarin (Fig. 5), the protective potential of AChE and BChE against sarin toxication was examined in mice at a sarin:ChE molar ratio of 2.5. To this end, rHuAChE or serum HuBChE (2.6 nmole/mouse) were administered to mice, and after 1 minute, the ChE-treated mice were challenged with 2.5LD₅₀ sarin (6.5 nmole/mouse). When serum HuBChE was administered, all mice died within minutes following exposure to sarin. In contrast, when rHuAChE was administered, 80% of the mice survived
challenge, yet they displayed severe toxic symptoms, including severe tremors and respiratory
distress (Table 5, upper panel). In an additional experiment, rHuAChE and serum HuBChE
were compared for their ability to protect mice against VX intoxication. In this case, greater
molar quantities of the ChEs (3.2 nmole/mouse, VX:ChE molar ratio =1.25:1) were
administered, since as suggested by the active site titrations, rHuAChE exhibited a lower
stereoselectivity towards VX, than to soman or sarin (see Table 4 and Fig. 5). Control animals
exposed to a 2.5 LD$_{50}$ VX challenge without ChE pre-treatment, died within 10-15 minutes
(Table 5, lower panel). All mice pretreated with rHuAChE survived and did not exhibit any
symptoms of toxicity. In contrast, serum HuBChE provided only partial (60%) protection
against this agent, while survivor animals displayed severe toxic symptoms. Taken together, the
set of bioscavenging experiments described above clearly demonstrates that rHuAChE is more
effective than serum HuBChE on a mole basis, in its ability to protect mice against soman, sarin
and even to some extent against VX poisoning.

The longevity of PEGylated rHuAChE makes it an effective prophylactic bioscavenger of OP-
compounds

Based on the similar reactivities of rHuAChE and its PEGylated form towards OP-agents (Table
1), one could expect that the circulatory stable PEGylated rHuAChE will be as effective as the
short-lived non-modified rHuAChE in its ability to neutralize OPs in-vivo. Indeed, when
PEGylated rHuAChE was administered to mice at a dose of 1.2 nmole/mouse and mice were
exposed after 1 minute to 2.5 LD$_{50}$ soman, the animals were fully protected and displayed no
more than minor symptoms of intoxication (Table 6A). Thus, the appendage of PEG moieties to
rHuAChE did not compromise the ability of the enzyme to effectively protect mice against
soman poisoning.
The therapeutic value of an OP-bioscavenger as a prophylactic agent in some realistic scenarios depends on its ability to provide protection even when it is administered a long time before the exposure to the CW agent. To examine the prophylactic potential of the PEG-modified AChE, equal amounts (3.2 nmole/mouse) of either rHuAChE, PEGylated rHuAChE, or serum HuBChE were administered to mice, and 22 hours later the mice were exposed to 1.5 LD₅₀ VX (Table 6B). All mice pretreated with the circulatory short-lived rHuAChE died within minutes of the challenge, like control mice, which were not pretreated with enzyme. This was as expected, since the non-modified version of rHuAChE, which is characterized by a circulatory half-life time value of 42 minutes, would be essentially eliminated from the bloodstream by the time of exposure to VX. In contrast, the PEGylated rHuAChE and serum HuBChE pretreated mice displayed 100% and 80% survival rates, respectively (Table 6B), demonstrating that recombinant AChE in its PEGylated version confers protection to mice against OP compounds even many hours after administration of the enzyme. To further assess the ability of these two enzyme forms to provide protection against multiple exposures to OPs, these mice were subjected 2 hours later to a second challenge of 0.9 LD₅₀ VX. Following re-exposure, 75% of the mice pretreated with serum BChE died, while all the PEGylated rHuAChE pretreated mice survived (Table 6B). Overall, during this experiment a total dose equal to 2.4 LD₅₀ of VX was administered to mice, and under these conditions, PEGylated rHuAChE conferred greater protection (100% survival) than HuBChE (25% survival).

All the series of experiments described above, were carried out with ChEs administered via the intravenous route. A more realistic approach to prophylactic treatment against OP-intoxication, however, would be to administer the therapeutic enzyme of choice, intramuscularly (i.m.). To evaluate the feasibility of intramuscular pretreatment with PEGylated rHuAChE, we first monitored the pharmacokinetic performance of i.m. administered PEGylated rHuAChE (Fig. 6). Following administration of 300U PEGylated rHuAChE by intramuscular injection to mice, an
initial phase of rapid increase in AChE activity in the serum was observed, followed by a moderate absorption phase, which reached peak levels ($t_{\text{max}}$) at 12 hrs. The rate of elimination from the serum of the PEGylated rHuAChE ($T_{1/2} = 29\pm3$ hrs) was very similar to that exhibited for this enzyme form following intravenous administration ($T_{1/2} = 26\pm2$ hrs). The bioavailability of the PEG-modified AChE was calculated to be 56%. In comparison, serum HuBChE and FBS-AChE displayed bioavailability values of 54% and 29%, respectively, following intramuscular administration to mice (Fig. 6, inset). Thus, PEG-conjugation does not seem to deleteriously affect the ability of the enzyme to be directed to the circulation. This relatively high bioavailability determined for PEGylated rHuAChE suggests that extravascular pretreatment with PEGylated rHuAChE may serve as an effective mode for protection against OP toxicity. To further examine this issue, mice were intramuscularly administered with 3 nmol of either serum HuBChE or PEGylated rHuAChE, and after 20 hours were challenged intravenously with 1.3 LD$_{50}$ VX (Table 6C). Nearly 90% of mice pretreated with PEGylated rHuAChE survived challenge and exhibited only mild symptoms of toxicity. In contrast, only 60% of the serum HuBChE-pretreated mice survived, while exhibiting severe signs of toxicity including tremors, Straub tail, salivation and respiratory distress.

In conclusion, it appears that appendage of PEG chains transforms rHuAChE into a very efficient bioscavenger, displaying a protective potential comparable and maybe even better than that of the native serum-derived HuBChE following i.m. administration.


Discussion

Various studies have demonstrated in the past that both AChE and BChE can potentially serve as bioscavengers of OP compounds (Lenz et al., 2005 and references cited therein). In the present study, comparative protection experiments, allowed us to determine that on a molar basis, AChE conferred a higher level of protection than BChE against soman and sarin intoxication (Fig. 4, Tables 3, 5A and 6A). This marked difference in the ability of AChE and BChE to provide effective protection, most likely stems from the higher stereoselectivity of AChE towards the P<sub>S</sub> stereoisomers of the racemic mixtures of soman and sarin (Table 4). Indeed, compilation of the apparent bimolecular rate constants of phosphonylation of different species of AChE (rHuAChE, native serum-derived bovine AChE) by purified stereoisomers demonstrates that this enzyme is characterized by a very high P<sub>S</sub>:P<sub>R</sub> phosphonylation rate constant ratios within the range of 5000 to about 60000. In contrast, the corresponding P<sub>S</sub>:P<sub>R</sub> phosphonylation rate constant ratios for different BChEs (rHuBChE, native serum-derived equine BChE) are considerably lower: 0.8 to 50-fold. Further support for the assumption that the differential stereoselectivity results in the higher consumption of BChE, was obtained from active site titration experiments carried out at enzyme and OP compound concentrations simulating the estimated initial values in the in-vivo protection experiments. In these experiments, neutralization of 1 mole of soman was effectively achieved by 0.5 mole of AChE, whereas neutralization of the same amount of soman by BChE required 1 mole of enzyme (Fig. 5), indicating that under these in-vitro conditions, AChE reacts effectively with only half of the stereoisomers, while BChE reacts at similar efficiencies with both the toxic and non-toxic stereoisomers comprising the racemic mixture of soman. A similar active site titration pattern was observed also when AChE and BChE were reacted with sarin (Fig. 5). We may therefore conclude that at a less than 50-fold difference between the phosphorylation rate constants of the P<sub>S</sub> and P<sub>R</sub> enantiomers (of soman and sarin), as observed for BChE (Table 4), effective
discrimination of the enantiomers under the experimental conditions is precluded. In contrast, the greater than 2000-fold difference between the phosphorylation rate constants of the $P_S$ and $P_R$ enantiomers of soman and sarin towards AChEs, may be sufficient to restrict in-vivo the reactivity of this enzyme to the $P_S$ enantiomer only. In line with these findings, we expected that the 115- to 200-fold difference between the phosphorylation rate constants of the $P_S$ and $P_R$ enantiomers of VX towards AChE (Table 4), may lead to a much less pronounced discrimination, in the reactivity of AChE towards the VX enantiomers in-vivo. Indeed, in the case of VX, the amount of AChE required for the in-vitro neutralization of the racemic mixture of VX was only slightly lower than that of BChE (Fig. 5). Furthermore, at a VX:ChE molar ratio of 1.25, AChE provided only slightly higher protection than BChE against VX intoxication in-vivo; 100% and 60% of AChE-pretreated and BChE-pretreated mice, respectively, survived challenge with 2.5 $LD_{50}$ VX.

Taken together, these experiments suggest that BChE is partially consumed by reaction with the nontoxic $P_R$ enantiomers of soman and sarin or the less toxic $P_R$ enantiomers of VX. This seems to be the major cause for the lower amount of AChE, versus BChE, required to confer protection against soman and sarin and even against VX. However, one should consider in scavenging studies, also the pharmacokinetic rates of the OP diastereoisomers themselves. It has been reported that the non-toxic enantiomers of soman are hydrolyzed by the plasma in-vitro at very high rates (De Jong et al, 1998). Yet, it was also shown by the same group, that in-vivo, about 50% of $^{14}C$ labeled $P_R$ diastereomers are eliminated by hydrolysis, while the rest is sequestered by covalent binding (Benschop and De Jong, 1991). Therefore, following administration of exogenous BChE, which displays very high affinity towards the $P_R$ enantiomer (Table 4), $P_R$ hydrolysis is not expected to significantly affect the differential in-vivo efficiencies manifested by AChE and BChE.
If indeed 200mg BChE are required to protect humans against 2LD$_{50}$ of soman (Ashani et al., 1998), it appears, based on the present study, that only 78mg of AChE will be needed to confer the same extent of protection. Taking into account the requirement to minimize quantities of exogenously administered protein in humans the advantage of AChE over BChE as a therapeutic bioscavenger is quite evident.

The use of cholinesterases in prophylactic treatments against OP agents, requires that these reside in the circulation for sufficiently long periods of time. Chemical modification of various recombinant proteins by covalent conjugation of PEG chains was shown to increase their circulatory residence (Harris and Chess, 2003), however, in many instances, the conjugation of PEG moieties to various proteins was accompanied by a concomitant loss of biological activity (Harris and Chess, 2003). We have demonstrated in the past, that under a certain set of conditions, rHuAChE can be efficiently PEGylated without compromising its catalytic activity, and that PEGylation of rHuAChE under these conditions resulted in a 50-fold increase in circulatory retention in mice as compared to the non-modified rHuAChE, displaying Mean Residence Time (MRT) values of 2100 minutes (Cohen et al., 2001). In an additional study, we demonstrated that the PEGylated rHuAChE displayed a 150-fold increase in circulatory retention relative to non-modified rHuAChE in rhesus macaques, exhibiting MRT values of approximately 10,000 minutes (Cohen et al., 2004).

In the present study it is shown that the reactivity of rHuAChE towards various OP agents (soman, sarin, tabun, VX) was not affected following its PEGylation (Table 1). The reactive PEGylated rHuAChE, by virtue of its improved pharmacokinetic performance, could efficiently protect mice from a VX challenge carried out 20 hours after administration of PEGylated rHuAChE. In fact, the prophylactic performance of PEGylated rHuAChE was even better than that of native serum-derived HuBChE, as manifested by the observation that upon re-exposure to VX, 75% of the BChE-pretreated mice died, while all of the PEGylated rHuAChE-pretreated
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mice survived (Table 6B). The superior protection of PEGylated rHuAChE was also manifested when this enzyme was administered intramuscularly to mice 20 hrs before VX challenge, resulting in nearly 90% percent survival of the animals. Similarly administration of native serum-derived HuBChE protected 60% of the mice (Table 6C). The marked difference in prophylactic efficacy of AChE versus BChE may be explained by the combined effect of the slightly higher stereoselectivity of the AChE towards VX (Table 4) and its better pharmacokinetic performance relative to native human BChE (MRT of PEGylated rHuAChE = 2100 min and of BChE = 1820 min; Fig. 6 and Raveh et al., 1993) in this animal model system.

In the present study, we demonstrate for the first time that recombinant AChE can be effectively employed as a soluble OP bioscavenger, conferring protection against soman, sarin and VX. Although soluble AChE is prevalent in the circulation of some animal species, its presence in the circulation of other mammals such as mice and primates is considerably lower than that of BChE (Li et al., 2000), and therefore one may speculate whether the administration of exogenous AChE would result in its altered disposition in various organs. We decided to test this possibility even though the recombinant HuAChE variant used in the present study is devoid of the C-terminal tail of the enzyme responsible both for is assembly into multiunit enzyme forms, as well as for its anchoring to biological membranes (Massoulie et al., 2005).

Extensive tissue distribution studies were therefore carried out to determine the fate of the exogenously administered rHuAChE and PEGylated rHuAChE. These studies clearly demonstrate that as in the case of the non-modified enzyme, PEGylated rHuAChE did not accumulate in any of the organs examined and did not affect tissue biodistribution (Fig. 1).

The generation of a bioactive PEGylated ChE is dependent on a delicate balance between efficient PEG-appendage and enzyme activity preservation, and therefore needs to be carried out in a highly controlled manner (Cohen et al., 2001). Recently, it was reported that PEG conjugation of rHuBChE resulted in the generation of a heterogeneous set of products
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(Chilukuri et al., 2005). Unlike PEGylated rHuAChE which exhibits a circulatory duration comparable or even exceeding that of native serum-derived BChE (Cohen et al., 2004), the reported PEGylated rHuBChE was eliminated much more rapidly than native serum-derived BChE. This limitation may stem from suboptimal PEGylation, which was shown to result in an insufficient extension of the circulatory life-time of cholinesterases (Cohen et al., 2001). Furthermore, one should note that PEGylated BChE products may be more prone to heterogeneity than PEGylated AChEs due to the fact that BChE contains many more lysine residues which may serve as candidate target sites for PEG-conjugation, than AChE.

In conclusion, the observation that the circulatory long lived PEGylated rHuAChE confers superior prophylactic protection to mice exposed to OP-compounds, together with the finding that the chemically modified enzyme displays unaltered biodistribution and high bioavailability, presents a case for utilizing PEGylated rHuAChE as a highly effective bioscavenger of CW OP-agents.

Acknowledgments

We thank our colleagues, Dr. Baruch Velan and Dr. Dov Barak for valuable suggestions and a careful reading of the manuscript. We thank Ms. Shirley Lazar and Ms. Dana Stein for excellent technical assistance.
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References:


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Footnotes

This work was supported in part by the U.S. Army Research and Development Command Contract DAMD17-03-C-0012 (to A.S.) and by a grant from Life Science Research Israel (Ltd.)
Legends to Figures

Fig. 1: Circulatory clearance profiles and tissue distribution of rHuAChE and PEGylated-rHuAChE. (A) Clearance profiles of non-modified and PEGylated rHuAChE. Exogenous rAChEs (200 U) were administered i.v. to 3 mice each, at levels that are at least 30-fold higher than background level. Blood samples withdrawn at various timepoints were assayed for AChE activity and values were corrected for background hydrolytic activity in the blood. Values are presented as percent of input AChE determined 1 minute after administration. (B, C) Non-modified (B) or PEGylated-rHuAChE (C) were administered i.v. to mice and at each time point, tissues from 3 mice were removed and AChE activity was measured in tissue homogenates.

Fig. 2: Reactivity of CBDP towards ChEs, and its effect on ChE activity in mice. (A) HuAChE and HuBChE were reacted with CBDP at various concentration. The apparent bimolecular phosphorylation rate constants (k_i) determined under pseudo first-order conditions were computed from the plot of slopes of ln(residual enzyme activity) versus time at different inhibitor concentrations. Values represent means of triplicate determination with standard deviations not exceeding 20%. (B) Residual endogenous BChE was measured in blood samples removed from mice at 1 hour after administration of CBDP at various doses (5 mice per dose). Values are presented as percent of activity measured in blood samples of naïve animals. Vertical dotted line represents the CBDP dose selected for performing protection experiments against soman and sarin (2mg/kg). Inset: Purified BChE (1U/ml) was added to serum samples removed from mice pretreated with CBDP within the range of 2-10 mg/kg and BChE activity was determined.

Fig. 3: Toxic effects in ChE-pretreated mice following exposure to soman. rHuAChE or serum HuBChE (1.2 nmole/mouse) were administered to mice which were then exposed to soman (2.1 LD_{50}). Body weight of survivor mice was monitored for 7 days following challenge.
Body weight of control mice treated with PBS without subsequent exposure to soman is shown for comparison.

**Fig. 4: Survival of AChE- and BChE- pretreated mice following exposure to soman at a wide range of soman to ChE molar ratios.** rHuAChE or serum HuBChE (0.9-1.3 nmole/mouse) were administered to CBDP-pretreated mice and mice were then exposed to soman at various doses. Each point is based on 5-10 mice per group. Values are presented as percent of surviving mice for each soman to ChE molar ratio.

**Fig. 5: Active site titration of rHuAChE and serum HuBChE with soman, sarin, VX and MEPQ.** In all titration experiments the concentration of the catalytic subunit of rHuAChE and HuBChE was 60-80nM. The residual activity was determined after incubation at 27°C in the presence of various concentrations of the different compounds for 30 and 60 minutes.

**Fig. 6: Circulatory clearance profiles of PEGylated-rHuAChE administered by different routes.** PEGylated AChE (200U) was administered *i.v.* and *i.m.* to 3 mice each. Blood samples withdrawn at various timepoints were assayed for AChE activity and values were corrected for background hydrolytic activity in the blood. Values are presented as AChE units per ml blood. Bioavailability of *i.m.* administered PEGylated rHuAChE was calculated as AUC_{i.m.}/AUC_{i.v.}

**Inset:** Clearance profiles of FBS-AChE and serum human BChE administered *i.m.* Bioavailability values of these two enzymes were calculated by comparing these clearance profiles to those obtained following *i.v.* administration of the corresponding enzyme forms (Raveh *et al.*, 1993; Kronman *et al.*, 2000).
Table 1: Rate constants of phosphorylation of rHuAChE, PEGylated- rHuAChE and serum HuBChE by various OP agents

<table>
<thead>
<tr>
<th>Agent</th>
<th>Non-modified HuAChE</th>
<th>PEGylated HuAChE</th>
<th>Serum HuBChE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarin</td>
<td>2280±90</td>
<td>3000±120</td>
<td>1200±90</td>
</tr>
<tr>
<td>Soman</td>
<td>8600±1900</td>
<td>7900±700</td>
<td>4000±250</td>
</tr>
<tr>
<td>VX</td>
<td>11300±760</td>
<td>8700±580</td>
<td>2300±130</td>
</tr>
<tr>
<td>Tabun</td>
<td>1500±60</td>
<td>1280±130</td>
<td>1600±80</td>
</tr>
</tbody>
</table>

* Values represent the mean of triplicate determinations with standard deviations.
Table 2: Determination of LD$_{50}$ values of various OP agents in mice following treatment with CBDP

<table>
<thead>
<tr>
<th>OP agent</th>
<th>LD$_{50}$ i.v. (µg/kg)</th>
<th>LD$_{50}$ i.v. following CBDP treatment (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VX</td>
<td>13.6 (12.9-14.3)</td>
<td>13.5 (12.6-14.4)</td>
</tr>
<tr>
<td>Soman</td>
<td>54.9 (52.0-57.9)</td>
<td>6.6 (5.9-7.4)</td>
</tr>
<tr>
<td>Sarin</td>
<td>82.9 (80.4-85.6)</td>
<td>13.8 (12.6-15.1)</td>
</tr>
</tbody>
</table>

Figures in parentheses are 95% confidence limits

OP agents were administered at various doses to 5-6 mice/per dose.
## Table 3: Survival of mice following ChE administration and exposure to 1 and 2.5 LD<sub>50</sub> of soman

<table>
<thead>
<tr>
<th>Administered soman</th>
<th>Administered ChE</th>
<th>rHuAChE</th>
<th>HuBChE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Administered enzyme (nmole/mouse)</td>
<td>-</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Administered soman (nmole/mouse)</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Survival (live/total)</td>
<td>60% (3/5)*</td>
<td>100% (5/5)</td>
<td>100% (5/5)</td>
</tr>
<tr>
<td>2.5 LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Administered enzyme (nmole/mouse)</td>
<td>-</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Administered soman (nmole/mouse)</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Survival (live/total)</td>
<td>0% (0/5)</td>
<td>100% (5/5)*</td>
<td>20% (1/5)**</td>
</tr>
</tbody>
</table>

* - Surviving animals displayed mild symptoms of toxicity: mild tremors, Straub tail

** - Surviving animals displayed severe symptoms of toxicity: severe tremors, Straub tail, salivation and respiratory distress
Table 4: Compilation of apparent bimolecular rate constants \((k_i)\) of phosphorylation of ChEs by enantiomers of VX, sarin and soman.

<table>
<thead>
<tr>
<th>ChE</th>
<th>VX</th>
<th>Sarin</th>
<th>Soman</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(k_i \times 10^{-4} \text{ M}^{-1} \text{ min}^{-1}) a</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(P_s)</td>
<td>(P_R)</td>
<td>(P_s/P_R) f</td>
</tr>
<tr>
<td>AChE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rHuAChE(^b)</td>
<td>13700</td>
<td>120</td>
<td>115</td>
</tr>
<tr>
<td>BoAChE(^c)</td>
<td>40000</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>BChE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rHuBChE(^d)</td>
<td>1600</td>
<td>280</td>
<td>6</td>
</tr>
<tr>
<td>EqBChE(^e)</td>
<td>670</td>
<td>170</td>
<td>4</td>
</tr>
</tbody>
</table>

a All values determined in the present study represent the mean of triplicate determinations with standard deviations not exceeding 20%

b Bimolecular rate constants for HuAChE with sarin were determined in the present study. Bimolecular rate constants for HuAChE with VX and soman are based on Ordentlich et al., 1999, 2004

c Bimolecular rate constants for BoAChE with VX, sarin and soman are based on Benschop et al., 1984 and Benschop and De Jong, 1988

d Bimolecular rate constants for HuBChE with sarin and VX were determined in the present study. Bimolecular rate constants for HuBChE with soman are based on Millard et al., 1998

e Bimolecular rate constants for EqBChE with sarin and VX were determined in the present study. Bimolecular rate constants for EqBChE with soman are based on Ordentlich et al., 1999

f \(P_s/P_R\) = ratio of bimolecular rate constants of phosphorylation of the stereoisomers. In the case of soman, values were determined for the pairs \(P_sC_S/P_RC_S\) and \(P_sC_R/P_RC_R\)

g The bimolecular rate constant for rHuBChE with the \(P_RC_S\) stereoisomer of soman was not determined experimentally. However, since the bimolecular rate constants of phosphorylation of the stereoisomers of the two BChEs presented were in most cases very similar, we used for computation, the corresponding \(k_i\) value determined for EqBChE as the value of the \(P_RC_S\) stereoisomer of soman for rHuBChE

h The bimolecular rate constant for other native serum-derived BChEs (human, canine and porcine) with stereoisomers of soman are similar (De Bisschop et al., 1991) with the \(P_s/P_R\) values in the range of 2.5 to 120
Table 5: Survival of mice following ChE administration and exposure to sarin and VX

<table>
<thead>
<tr>
<th>Administered OP agent</th>
<th>Administered ChE</th>
<th>None</th>
<th>rHuAChE</th>
<th>HuBChE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2.5 LD₅₀ Sarin</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Administered enzyme (nmole/mouse)</td>
<td>-</td>
<td>2.6</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>Administered OP (nmole/mouse)</td>
<td>6.5</td>
<td>6.5</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>Survival (live/total)</td>
<td>0% (0/5)</td>
<td>80% (4/5)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0% (0/5)</td>
</tr>
<tr>
<td><strong>2.5 LD₅₀ VX</strong></td>
<td>Administered enzyme (nmole/mouse)</td>
<td>-</td>
<td>3.2</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>Administered OP (nmole/mouse)</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Survival (live/total)</td>
<td>0% (0/5)</td>
<td>100% (5/5)</td>
<td>60% (3/5)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Animals challenged with sarin were pretreated with CBDP 1 hour prior to enzyme administration.

<sup>b</sup> Surviving animals displayed severe symptoms of toxicity: severe tremors, Straub tail, salivation and respiratory distress.
Table 6: Protection of mice against OP agents by PEGylated rHuAChE

A. Survival of mice following exposure to 2.5 LD<sub>50</sub> soman<sup>a</sup>

<table>
<thead>
<tr>
<th>Administered ChE</th>
<th>None</th>
<th>rHuAChE</th>
<th>PEG-rHuAChE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Administered enzyme (nmole/mouse)</td>
<td>-</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Administered soman (nmole/mouse)</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Survival (live/total)</td>
<td>0% (0/5)</td>
<td>100% (5/5)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>100% (5/5)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

B. Survival of mice exposed to VX 20-22 hrs after intravenous administration of ChEs

<table>
<thead>
<tr>
<th>Administered ChE</th>
<th>None</th>
<th>PEG-rHuAChE</th>
<th>HuBChE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; exposure (1.5 LD&lt;sub&gt;50&lt;/sub&gt; : 20h)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Administered enzyme (nmole/mouse)</td>
<td>-</td>
<td>3.2</td>
<td>3.2</td>
</tr>
<tr>
<td>Administered VX (nmole/mouse)</td>
<td>2.4</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>Survival (live/total)</td>
<td>0% (0/5)</td>
<td>100% (5/5)</td>
<td>80% (4/5)</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; exposure (0.9 LD&lt;sub&gt;50&lt;/sub&gt; : 22h)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Administered VX (nmole/mouse)</td>
<td>-</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Survival (live/total)</td>
<td>-</td>
<td>100% (5/5)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25% (1/4)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

C. Survival of mice exposed to 1.3 LD<sub>50</sub> VX 20 hrs after intramuscular administration of ChEs

<table>
<thead>
<tr>
<th>Administered ChE</th>
<th>None</th>
<th>PEG-rHuAChE</th>
<th>HuBChE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Administered enzyme (nmole/mouse)</td>
<td>-</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Administered VX (nmole/mouse)</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Survival (live/total)</td>
<td>0% (0/8)</td>
<td>88% (8/9)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>57% (4/7)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Animals challenged with soman were pretreated with CBDP 1 hour prior to enzyme administration

<sup>b</sup> Surviving animals displayed mild symptoms of toxicity: mild tremors, Straub tail

<sup>c</sup> Surviving animals displayed severe symptoms of toxicity: severe tremors, Straub tail, salivation and respiratory distress
Figure 2

A.  

**HuBChE**  

\[ k_i = 20000 \text{ M}^{-1}\text{min}^{-1} \]

**rHuAChE**  

\[ k_i = 1400 \text{ M}^{-1}\text{min}^{-1} \]

B.  

Residual Endogenous BChE Activity (%)

Exogenous BChE Activity (%)

CBDP (mg/kg)

CBDP (x10^-6 M)
Figure 3:

![Graph showing body weight (% of start) over days after treatment for Control, AChE, and BChE groups. The graph includes error bars indicating variability.]
Figure 4:

![Graph showing % Survival vs. Soman/ChE (mole/mole) for HuBChE and rHuAChE](image-url)
Figure 5

**rHuAChE**

- MEPQ
- VX
- Sarin
- Soman

**HuBChE**

- MEPQ
- VX
- Sarin
- Soman

% Activity vs. [Organophosphate] (M)
Figure 6