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Aging-Resistant Organophosphate Bioscavenger Based on PEGylated Phe338Ala Human Acetylcholinesterase

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Running title: Protection against repeated OP exposure by modified HuAChE

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Abbreviations: AUC, area under curve; CaE, carboxylesterase; CBDP, cresylbenzodioxaphosphorin oxide; ChE, cholinesterase; DEFP, diethyl phosphorofluoridate; HuAChE, human acetylcholinesterase; BChE, butyrylcholinesterase; MEPQ, 7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide; MRT, mean residence time; PEG, polyethylene glycol; rHuAChE, recombinant human acetylcholinesterase; rHuBChE, recombinant human butyrylcholinesterase; OP, organophosphorous.

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Abstract

The high reactivity of ChEs toward OP compounds has led to propose recombinant ChEs as bioscavengers of nerve agents. The bioscavenging potential of recombinant ChEs can be enhanced by conjugation of PEG moieties, to extend their circulatory residence. However, the ability of exogenously administered ChEs to confer long-term protection against repeated exposures to nerve agents is still limited due to the aging process, whereby organophosphate-ChE-adducts undergo irreversible dealkylation, which precludes oxime-mediated reactivation of the enzyme. To generate an optimal AChE-based OP-bioscavenger, the F338A mutation, known to decelerate the rate of aging of AChE-OP conjugates, was incorporated into PEGylated human AChE. The PEGylated F338A-AChE displayed unaltered rates of hydrolysis, inhibition, phosphylation and reactivation and could effectively protect mice against exposure to soman, sarin or VX. Unlike PEGylated WT-AChE, the PEGylated F338A-AChE exhibits significantly reduced aging rates following soman inhibition and can be efficiently reactivated by the HI-6 oxime, both *in-vitro* and *in-vivo*. Accordingly, oxime administration to PEG-F338A-AChE pretreated mice enabled them to withstand repeated soman exposure (5.4 and 4 LD_{50}/dose), while same regime treatment of non-PEGylated F338A-AChE or PEGylated WT-AChE pretreated mice failed to protect against the second challenge, due to rapid clearance or irreversible aging of the latter enzymes. Thus, judicious incorporation of selected mutations into the AChE mold in conjunction with its chemical modification, provides means to engineer an optimal ChE based OP-bioscavenger in terms of circulatory longevity, resistance to aging, and reduced doses required for protection even against repeated exposures to nerve agents, such as soman.

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Introduction

Acetylcholinesterase plays a central role in the cholinergic system, where it hydrolyses the neurotransmitter acetylcholine, thereby terminating nerve impulse transmission. This enzyme is the major target for organophosphorous nerve agents such as soman, sarin and VX, which irreversibly phosphylate the catalytic serine residue within the enzyme active site. Inactivation of AChE leads to continuous stimulation of cholinergic receptors, resulting in symptoms of toxicity such as salivation, tremors, and miosis and in severe cases, respiratory paralysis and death. Current drug treatment against nerve agent exposure is based on administration of cholinolytic drugs (e.g. atropine) and oximes, which displace the phosphyl group to restore AChE activity and anticonvulsants, which reduce OP-intoxication associated convulsions. Yet, while the combined post-exposure treatment is effective in preventing lethality, it does not prevent severe incapacitation including behavioral difficulties and permanent brain damage (Dawson RM, 1994).

The high reactivity of ChEs toward OP-nerve agents has led to propose their use as exogenous biological scavengers for the sequestering of toxic OP agents before they reach their physiological target (Raveh et al., 1993, 1997; Lenz et al., 2007; Cohen et al. 2006; Kronman et al. 2007). Being a stoichiometric scavenger, it was estimated that about 200mg of exogenous enzyme are needed to protect adult humans against $2LD_{50}$ of soman (Ashani et al., 1998). This requirement for large quantities of enzyme encouraged the development of production systems for the generation of recombinant ChEs at large-scale (Kronman et al., 1992; Fischer et al., 1993; Masson et al., 1993). However, pharmacokinetic studies revealed that these recombinant enzymes display rapid clearance profiles, which limit their use as efficient bioscavengers that could confer long-term protection (Kronman et al., 1995). To overcome this limitation, chemical modification of recombinant human-AChE (rHuAChE) via controlled appendage of polyethylene glycol (PEG) to lysine side chains was performed (Cohen et al., 2001, 2004). The PEGylated rHuAChE exhibited very long retention times in

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mice and monkeys and allowed effective protection of mice against OP-nerve agents (Cohen et al., 2004, 2006; Kronman et al., 2007).

Upon exposure to OP-agents, phosphylation of the inhibited AChE-active site serine results in the formation of a stable conjugate, which can further undergo a unimolecular process (Fleisher and Harris, 1965), resulting in an "aged" enzyme form which is refractive to oxime reactivation (Scheme 1, see also Shafferman et al., 1996). This aging process is most pronounced for OPs containing a branched alkyl group, such as soman (Barak et al., 1997). Inhibition of exogenously administered scavenger ChEs, by “aging”, limits their ability to confer long-term protection against repeated exposures to nerve agents. Such a limitation may be alleviated by the development of AChE-based bioscavengers with reduced aging rates, which can thereby be effectively reactivated by oximes.

Over the past two decades, extensive research was carried out in order to understand the basic aspects of the mechanism of catalysis, phosphylation and aging of AChE. Such studies included X-ray crystallography (Sussman et al., 1991; Millard et al., 1999), site directed mutagenesis and molecular modeling together with kinetic studies of the AChE mutants with substrates and reversible inhibitors (Ordentlich et al., 1993, 1996; Radic et al., 1993; Barak et al., 1994; Shafferman et al., 1996, 2005). These studies revealed several functional subsites in the AChE active center gorge that are essential for accommodation of the various groups of AChE ligands, as well as for the pronounced activity of the enzyme (reviewed in Shafferman et al., 2005). Among other findings, these studies allowed to identify the amino acid residues that play a role in the enzyme aging process (Ordentlich et al., 1993, 1996). Most notably, replacement of the Phe^{338} by alanine (F338A-AChE) resulted in a pronounced decrease (160-fold) in the rate of aging following soman inhibition (Shafferman et al., 1996) as compared to the WT enzyme (Grosfeld et al., 1996). Consequently, the adduct of F338A HuAChE with soman could be reactivated much in the same way as adducts of HuAChE with sarin.

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In the present study, we generated a PEGylated human AChE enzyme based upon the F338A mutant (PEG-F338A AChE), which exhibits an improved pharmacokinetic profile, and could be effectively reactivated following inhibition by various OP compounds. In a series of *in-vivo* experiments, we demonstrate that PEG-F338A AChE, unlike PEG-WT AChE, effectively protects mice against repeated soman exposure in conjunction with oxime treatment. Thus, incorporation of the F388A mutation into the PEGylated-AChE template may provide the means to generate a superior prophylactic agent that can confer long-term protection against OP compound exposures.

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Materials and methods

Production of enzymes

Transfection of the human embryonal kidney cell line, HEK-293, with expression vectors coding for the C-terminus truncated WT or F338A HuAChE enzymes (Grosfeld et al., 1996; Cohen et al., 2001) and the generation of stable cell clones expressing high levels of recombinant product, were described previously (Kronman et al., 1992). AChEs were purified as described earlier (Kronman et al., 1995). Attachment of PEG chains to primary amines of AChEs was performed using succinimidyl propionate-activated methoxy-PEG (SPA-PEG; Nektar Inc.) as described previously (Cohen et al., 2001). Briefly, purified AChE (5 μM) was incubated with PEG-20000 at a ratio of 30:1 (mol/mol) $[PEG]_0/[AChE\ primary\ amines]_0$ in 50 mM borate buffer, pH 8.5, for 2 h at room temperature. The chemically modified products were dialyzed extensively against PBS and visualized on 6% SDS/PAGE gels.

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 as described in previously reported procedures (Levy and Ashani, 1986; Cohen et al., 2006). The purity of the OPs (>95%) was determined by ^{1}H and ^{31}P NMR spectroscopy and by a titration with a defined amount of HuAChE. Stock solutions were kept at $-20^{0}C$, and diluted in 0.9% saline to the desired concentration, prior to use.

Kinetic Studies

HuAChE activity was assayed according to Ellman et al. (1961) in the presence of 0.2mg/ml BSA, 0.3 mM DTNB, 50 mM sodium phosphate buffer (pH 8.0), and 0.5 mM ATC at 27°C

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and monitored with a Thermomax microplate reader (Molecular Devices). Measurements of phosphylation rates were carried out by monitoring residual activity (E) at various timepoints, following incubation of the enzyme in the presence of at least four different concentrations of OP-inhibitor (I). The apparent bimolecular phosphylation 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 $\ln\{E/[I_0 - (E_0 - E)]\}$ versus time. Stereoselectivity of the enzymes toward soman was determined by active site titrations, comparing residual activities of enzymes inhibited by the appropriate racemic phosphonate to that of MEPQ (Shafferman et al., 1996).

Measurements of aging rates were performed essentially as described previously (Shafferman et al., 1996), where the reactivatable fraction of the OP-inhibited AChEs was determined following reactivation with 0.5mM HI-6. The activity of the reactivated enzyme (E_r) was routinely corrected for the inhibitory effect of the reactivator. The first-order rate constants of aging (k_a) were determined from the slopes of $\ln(E_r/E_{0)}$ against time.

Measurements of the reactivation kinetics rates were performed as described previously (Grosfeld et al., 1996). Briefly, reactivation reactions of the OP-inhibited AChEs (in 50mM phosphate buffer, pH 8.0) were initiated by the addition of HI-6 at various concentrations (0.005-1mM) and incubated at 27^{o}C. Regeneration of AChE activity was then monitored, and compared to noninhibited enzyme preparations which served as control for the expected enzyme activity at maximal reactivation. For the *in vitro* reactivation of soman-inhibited AChE, heparinized blood of CBDP-treated mice was spiked with either PEGylated WT or F338A AChE (0.8μM) and then inhibited with soman, at a concentration (1.6μM) that leads to 90% inactivation. HI-6 (0.5mM) was added to the enzyme/soman mixture after incubation for 20 minutes at 37^{o}C. Reactivation of AChE in the blood samples was determined as described above.

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Animal studies

Male outbred ICR mice (Charles River Laboratories) were maintained at 20–22^{0}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 *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.

In vivo reactivation of the soman-inhibited AChEs was initiated 20 min after soman intoxication, by the intravascular administration of HI-6 (50mg/kg), a well-tolerated dose without any signs of distress. Residual AChE activity was determined in blood samples withdrawn at various timepoints throughout the experiments.

For challenge experiments, mice intravenously administered with AChEs were exposed intravenously to OPs. In the case of exposure to soman or sarin, mice were pretreated with CBDP (2mg/kg) one hour prior to challenge to inhibit endogenous carboxylesterases (CaEs), as described previously (Cohen et al. 2006). In experiments where repeated soman challenge was performed, second administration of CBDP (1mg/kg) was performed immediately prior to AChE treatment. Mice were monitored for toxic signs and mortality for four hours following OP administration.

Clearance experiments in mice (3 to 6 mice per enzyme sample) were carried out essentially as described previously (Cohen et al., 2006). Mice were injected intravenously with 200 units/mouse of native or PEGylated AChEs, in 0.2 ml PBS. At different time points, blood samples (5µl) were drawn from the tail vein, diluted 20-fold in PBS, and centrifuged for three minutes at 3000 rpm for the removal of red blood cells. Residual AChE activity in the serum was measured and values were corrected for background hydrolytic activity in the blood. Pharmacokinetic parameters were calculated using the PK solutions software (Summit research services).

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Results

Reactivity of PEGylated F338A-AChE towards active-center inhibitors and OP agents

F338A mutated HuAChE was conjugated to polyethylene glycol under conditions that should promote high level PEGylation without compromising the catalytic activity of the chemically-modified enzyme product (Cohen et al., 2001). Kinetic analyses of PEGylated F338A-AChE, allowed us to determine that this version of the enzyme hydrolyzes ATC with a K_m value of 0.2mM, similar to its nonPEGylated counterpart (0.14mM; Table 1). Likewise, the PEGylated and nonPEGylated F338A AChE exhibit comparable values of k_{cat} and k_{app}. These values were also similar to those exhibited by both the non-PEGylated and PEGylated versions of the WT enzyme (Table 1). Thus, the appendage of PEG chains to F338A AChE did not deleteriously affect the catalytic efficiency of the enzyme.

We next determined the inhibition kinetic constants of PEG F338A AChE towards several AChE active-center inhibitors. PEGylation of both F338A AChE and WT enzymes did not affect their interactions with edrophonium, both exhibiting K_i values (1.2μM) similar to those of the non-modified enzymes (0.8μM; Table 1). Likewise, binding of PEG chains to the enzyme surface did not affect the inhibition constant of PEG-F338A-AChE or PEG-WT-AChE towards the peripheral anionic site ligand, propidium, or the *bis*-quarternary inhibitor, BW284C51, and in both cases the measured K_i values were in the same range as determined earlier for the nonPEGylated enzyme forms (Table 1).

The kinetic properties of F338A AChEs were further examined by determining the reactivity of this enzyme towards various OP compounds. Determination of the apparent bimolecular rate constant (k_i) of PEG-F338A AChE for soman phosphylation, demonstrated that the chemically-modified enzyme retained its reactivity toward soman (Table 2). Likewise, the inhibition rate constant of the PEG-F338A AChE toward sarin was found to be similar to that of the non-PEGylated F338A AChE form and of WT AChE (Table 2). In the case of VX, the

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reactivity of both non-PEGylated and PEGylated F338A AChE were also similar, yet these values were found to be slightly lower (~4-fold) than those displayed by the nonPEGylated and PEGylated WT enzymes (Table 2). This difference in reactivity towards VX has been observed previously, and can be related to a minor disruption within the active center environment caused by the Phe^{338} to Ala replacement, which affects the accommodation of positively charged OP molecules such as VX, within the active center (Ordentlich et al., 2005). PEGylation of F338A AChE did not reduce its reactivity towards the organophosphate compound, paraoxon, as compared to its non-PEGylated counterpart (Table 2). The reactivity of PEG-F338A-AChE was about 10-fold lower than that of PEG-WT-AChE, analogous to the difference in reactivities towards paraoxon of the non-modified forms of F338A-AChE and WT-AChE (Table 2). This was previously shown to be related specifically to this organophosphate compound, where in the generation of the AChE-OP adduct, the leaving group is aromatic. Indeed, in the case of diethyl phosphorofluoridate (DEFP), which generates the same AChE-adduct as paraoxon but where the leaving group is smaller (fluoride in the case of DEFP *vs.* p-nitrophenol in the case of paraoxon), the rate of phosphylation is not affected by the F338A mutation (Ordentlich *et al*., 1996).

AChEs are characterized by a pronounced stereoselectivity toward various OP agents such as soman and sarin (de Jong et al., 1988). To determine that stereoselectivity is not affected in the modified enzyme, active site titration analysis of PEG-F338A AChE and PEG-WT-AChE was performed with soman. In parallel, active site titration analysis of both enzymes was performed with MEPQ, an OP inhibitor known to react with AChE at a 1:1 stoichiometric ratio (i.e., both the P_S and P_R enantiomers react equally with AChE). By comparing the intercept for zero enzymatic activity with soman to that obtained with MEPQ (Figure 1A), we could determine a 1:2 stoichiometry for the interactions with soman of the PEGylated forms of the WT and F338A AChEs. Thus, the PEGylated F338A mutated version of AChE retains its pronounced stereoselectivity towards the toxic soman diastereomers.

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As mentioned above, WT-AChE undergoes rapid aging in the presence of soman (Barak et al., 1997). In contrast, the F338A-AChE mutant exhibits a pronounced decrease in its rate of aging following soman inhibition (Shafferman et al., 1996). To verify whether the PEGylated version of F338A AChE retains this unique trait, the first-order rate constant (k_a) of aging was determined for the soman-inhibited PEGylated AChE enzymes (WT and F338A), by measuring the enzyme activity restored in the presence of the oxime HI-6, which is known to be a potent reactivator of soman adducts (De Jong and Kossen, 1985, Dawson RM, 1994). PEGylated WT AChE was rapidly aged (Fig. 1B; inset), displaying an apparent k_a value of $95x10^3$ min^{-1}. In contrast, the aging rate of the soman-inhibited PEG-F338A AChE was found to be ~170-fold slower than that of PEG-WT enzyme (Fig. 1B), exhibiting an apparent k_a value of 0.55 $x10^3$ min^{-1}. These results are in good correlation with our previous data in which a 160-fold difference was found between the aging rates of the WT and the F338A AChE native forms (app. k_a values of 130 $x10^3$ min^{-1} and 0.8 $x10^3$ min^{-1}, respectively; Shafferman et al., 1996; Grosfeld et al., 1996).

The ability of oximes to effectively restore the enzymatic activity of OP-inhibited AChE depends not only on the rate of aging of the enzyme, but also on its reactivation kinetics. To quantify the reactivability of OP-inhibited F338A AChEs, we monitored the increase in enzyme activity over time following the addition of HI-6, and determined the apparent reactivation rate constant, k_r, and the dephosphorylation rate constant, k_2 (Table 3). Chemical modification of the F338A AChE by PEG conjugation did not altered its ability to interact with the HI-6, resulting in similar reactivation rates for both for the native (k_r =300 $M^{-1}min^{-1}$; k_2= $2.3x10^2$ min^{-1}) and the PEGylated (k_r =254 $M^{-1}min^{-1}$; k_2= $1.6x10^2$ min^{-1}) forms of this enzyme. In the case of WT AChEs, reactivation kinetics values could not be determined due to the rapid aging of its adducts with soman (see above). In contrast, the aging process of sarin and VX inhibited AChE is much slower (Barak et al., 1997; Worek et al., 2004) and

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therefore with these inhibitors, it was possible to determine and compare the reactivation rate constants of the F338A and WT AChEs.

Reactivation of sarin-inhibited WT and F338A AChEs proceeded at similar rates, with less than 4-fold and 2-fold differences in the k_r and k_2 values, respectively (Table 3). Similar results were observed for the VX-inhibited enzymes (less than 3-fold and 2-fold differences between the k_r and k_2 values of the WT and F338A AChEs). In all cases, the reactivation kinetic values of OP-inhibited PEGylated WT or PEGylated F338A enzymes were similar to those measured for their nonPEGylated counterparts (Table 3), attesting to the fact that PEG conjugation does not influence the reactivation process. It should be noted that there is an overall 10-fold decrease in the reactivation rates (k_r and k_2) of soman-inhibited F338A mutant as compared to sarin/VX-inhibited enzymes (Table 3). This considerable difference in the reactivation kinetics values may be attributed to the branched alkyl group of soman, which can interfere with the ability of HI-6 to remove the phosphyl residue from the active site of the enzyme (Barak et al., 1997).

Taken together, measurement of rate of hydrolysis, inhibition kinetics, phosphylation rates, stereoselectivity and rates of reactivation of OP-adducts, allowed us to determine that neither the introduction of the F338A mutation nor the chemical modification of the enzyme by PEG conjugation, deleteriously affected the bioscavenging properties of the enzyme. It should be noted that in many instances, the conjugation of PEG moieties to proteins was accompanied by a concomitant loss of their biological activity (Harris and Chess, 2003). We note that elaborate studies were indeed required to determine the exact conditions for performing efficient PEG conjugation without compromising the biological activity of ChEs (Cohen et al., 2001, 2007).

The only discernable difference between F338A-AChE and WT-AChE was their differential rate of aging. Controlled PEG conjugation of F338A-AChE did not affect its rate of aging, so that both the non-PEGylated and PEGylated versions of F338A-AChE are characterized by

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significantly lower rates of aging than WT-AChE. This low rate of aging may provide the enzyme with an advantageous trait, in terms of its ability to serve for prophylactic treatment of OP intoxication as demonstrated in the following section.

Reactivated PEG-F338A-AChE effectively protects mice against repeated OP-exposure

We next examined whether the slower aging rate of the F338A mutant (native and PEGylated form) will allow it to be more efficient than the WT AChE in providing long-term protection against repeated OP exposure in general, and soman in particular. To this end, we set out to perform a series of experiments in which we determined the pharmacokinetic profile of PEG-WT and F338A AChEs and their ability to serve for prophylaxis treatment against various OP-agents exposure. Finally, we compared the protective potential of these AChEs against repeated soman exposures in mice.

Pharmacokinetics of PEG-F338A AChE- PEG-F338A AChE, as well as its non-PEGylated counterpart, were administered *i.v.* to mice, and their pharmacokinetic profiles were determined (Fig. 2). PEG-F338A AChE displayed a mean residence time (MRT) value of ~2800 minutes (Table 4), at least 20-fold greater than the MRT value exhibited by the non-modified form of the enzyme (120 mins). Thus, in terms of its pharmacokinetic performance, the PEGylated version of F338A-AChE displays an extended circulatory lifetime, similar to that determined previously for the PEGylated version of WT AChE (Cohen et al., 2001; shown also in Fig.2 for comparison).

Prophylactic protection of mice against OP challenge- We next assessed the ability of PEG-F338 AChE to confer protection to mice against OP compound intoxication. Mice were administered either PEG-F338A AChE or PEG-WT AChE, and then exposed to lethal doses of soman, sarin or VX. In the past, we demonstrated that 1.2 nmol of PEG-WT AChE are required for the effective protection of mice against 2.5LD_{50} of soman (= 3 nmol soman/30 gr mouse; Cohen et al., 2006). Administration of the same amount of PEG-F338 AChE protected

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all of the mice challenged immediately after enzyme administration with $2.5LD_{50}$ soman (Table 5). Similarly, PEG-F338 AChE was as effective as PEG-WT AChE in its ability to confer protection against sarin or VX intoxication. Thus, mice administered with 2.6 or 3.2 nmol of PEG-F338 AChE, respectively, were fully protected against challenge of $2.5LD_{50}$ sarin (= 6.5 nmol sarin/30 gr mouse) or VX (=4 nmol VX/30 gr. mouse). In comparison to the AChE doses required for effective protection against soman or sarin intoxication (molar ratio of AChE:soman/sarin = 0.4:1), full protection against $2.5LD_{50}$ VX was achieved only when twice as high molar amounts of AChE were administered (molar ratio of AChE:VX = 0.8:1). This is due to the fact that AChE displays a marked stereoselectivity towards half of the enantiomers comprising racemic soman and sarin compounds yet interacts with both enantiomers of VX (Cohen et al., 2006, see also Fig. 1A). Taken together, these findings demonstrate that the PEG-F338A version of AChE is indeed as effective as PEG-WT AChE in its ability to protect mice against OP compounds.

HI-6 mediated reactivation of phosphorylated AChE derivatives- As stated earlier, we hypothesized that the slow aging rate of the soman-inhibited PEG F338A-AChE will allow restoration of its activity in the bloodstream by oxime reactivation, while the soman-inhibited WT AChE will remain refractive to this treatment. To examine this, we performed first an *in-vitro* experiment in which we measured the reactivation of soman-inhibited PEG F338A-AChE in blood samples. To this end, heparinized blood samples were removed from mice and spiked with either PEG WT-AChE or PEG- F338A-AChE (0.8μM). Soman was then added to the samples (final concentration = 1.6μM), and the reaction mixtures were incubated at 37^0C for 20 min. Under these conditions, 90% of both enzymes were inactivated, as judged by their diminished ability to hydrolyze ATC (Fig. 3). HI-6 (1.5mM) was then added and the catalytic activity of the two enzymes was monitored in samples removed at various time points. As expected, the soman-inhibited WT AChE remained unaffected throughout the experiment. In contrast, the inhibited F338A-AChE enzyme displayed a gradual increase in enzymatic

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activity, reaching 60% of input activity following 120 min of incubation in the presence of the oxime (Fig. 3). Thus, unlike in the case of WT-AChE, soman adducts of F338A-AChE remain susceptible to oxime-mediated reactivation in the blood samples, for extended periods of time.

To examine the ability to reactivate soman-inhibited F338A-AChE *in-vivo,* mice were administered either with PEG-WT-AChE or PEG-F338A-AChE (4 nmol/mouse) and 1 minute later exposed to the highest dose of soman (6.5 nmole/mouse, 5.4 LD_{50}) that is compatible with complete survival of the enzyme-administered mice. Measurement of AChE activity in blood samples removed 10 minutes later allowed us to determine that 70% of both enzyme forms were inactivated. HI-6 at a dose which is well tolerated, 50mg/kg, was then administered *i.v.* to the mice and enzyme reactivation was monitored in blood samples removed at various timepoints. In the case of the WT-AChE administered mice, the enzyme remained inhibited throughout the experiment, while in the case of the F338A-AChE-administered mice, the level of circulating active enzyme increased by 67% within 10 minutes following HI-6 administration, after which enzymatic activity remained constant (50% of input enzyme) up to 1 hour (Fig. 4A), probably due to the relatively rapid clearance of HI-6 from the circulation (Maxwell et al., 1993). Due to the elimination process of the PEGylated enzymes from the circulation, the levels of circulating PEGylated enzyme at the time of HI-6 administration were approximately 70% of input enzyme (Fig. 2), and therefore actually 50% of the inhibited PEG-F338A-AChE present at this timepoint can be expected to undergo reactivation by HI-6, which is in good agreement with the results obtained for *in-vitro* reactivation (Fig. 3). We thereby examined whether the increment in circulating active enzyme brought about by HI-6 reactivation of PEG-F338A-AChE, may protect the mice against an additional soman exposure.

Protection of mice against repeated soman exposure- To assess the ability of PEG-F338A-AChE to confer protection against repeated soman exposure, mice were administered

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PEGylated and non-PEGylated versions of both WT-AChE and F338A-AChE as above, and then exposed to 5.4LD_{50} of soman. The mice, all of which survived this soman challenge (Figure 4B, Soman A) and displayed no more than mild symptoms of OP-intoxication, were administered HI-6 twenty minutes later and after an additional 40 minutes, were re-exposed to a second soman challenge of 4LD_{50}. As expected, mice pretreated with the circulatory short-lived non-PEGylated versions of AChE succumbed to the second soman challenge (Figure 4B, Soman B) regardless of whether the WT or F338A version of the enzyme was administered. The PEGylated WT-AChE protected only 16% of the mice, even though this enzyme form exhibits long-term circulatory residence, so that at the time of the second soman challenge, 80% of the input enzyme still resides in the circulation (Figure 2). In contrast, 100% of the PEG-F338A-AChE pretreated mice survived the second soman challenge (Fig. 4B). Since the pharmacokinetics of PEG-F338A-AChE and PEG-WT-AChE are very similar (Fig. 2), the marked difference between the survival rates of the mice administered with these two enzyme forms necessarily stems from the differential ability of these enzymes to undergo reactivation by *in-vivo* oxime treatment (Fig. 4A). Based on these findings, it indeed seems that the *in-vivo* reactivation of soman-inhibited PEG-F338A AChE, can be harnessed to provide effective protection against multiple exposures to OP-compounds.

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Discussion

The utilization of recombinant ChEs as a source for OP bioscavengers, can serve not only to generate the large quantities of enzyme required for stoichiometric neutralization, but also provide the means to incorporate favorable characteristics into the enzyme fold by genetic engineering. For instance, amino acids involved in the aging of OP-ChE adducts, may be favorably altered by means of genetic engineering, to decelerate the irreversible inactivation of the bioscavenger. Extensive studies carried out in the past indeed allowed us to identify various amino acid residues that are involved in the aging of human AChE adduct with OPs. These include the W86 residue, a key element in the anionic binding subsite of the enzyme, E202, E450 and Y133 which are members of the H-bond network in the active center of the enzyme (Ordentlich et al., 1993; Shafferman et al., 1996), as well as F338, which is located within the aromatic patch lining the active center gorge of the enzyme (Ariel et al. 1998). Although replacement of any of these amino acids reduces the rate of aging of the HuAChE-OP adducts, only substitution of F338 results in a significantly lower rate of aging without compromising reactivity of the enzyme towards a wide variety of OPs (Shafferman et al., 1996 and Table 2 in present study). Therefore, from a practical point of view, replacement of F338 but not of the other residues involved in aging, can be utilized for designing an AChE-based bioscavenger of OP compounds, displaying improved biochemical traits. Indeed, here we demonstrate that incorporation of the F338A mutation into PEGylated HuAChE decelerates aging, so that the soman-inhibited enzyme can be reactivated by oximes *in-vivo* to an extent which enables mice to withstand repeated exposure to high doses of soman (e.g. 5.4 LD_{50} and then 4 LD_{50}). It should be noted in this context, that attempts to generate a recombinant BChE-based slow-aging enzyme by replacement of residue F329, which is analogous to F338 in HuAChE, were not successful and had a very limited effect on the rate of aging of BChE (Masson et al., 1997).

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An additional limitation in the exploitation of recombinant BChE as a bioscavenger of OP compounds, relates to the ability to extend its circulatory longevity. Recombinant ChEs are retained in the circulation of experimental animals for much shorter periods of time than native serum-resident ChEs (Kronman et al., 1995). We have shown in the past (Chitlaru et al., 2001) that recombinant AChE can be converted into a circulatory long-lived enzyme by the combined optimization of post-translation-related factors (sialic acid occupancy, enzyme tetramerization and glycan loading). However, obtaining a homogenous long-lived circulatory scavenger through such a biotechnological/biochemical route may be difficult. In parallel, we demonstrated that conjugation of PEG to lysine residues of rHuAChE or rHuBChE, can effectively extend their circulatory longevity, however, 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. In the case of recombinant AChE, conditions for high level PEGylation without compromising enzymatic activity were determined (Cohen et al., 2001) and the introduction of a set of defined lysine replacements was shown recently to give rise to a homogenous uniformly PEGylated enzyme product (Cohen et al., 2007). In contrast, PEGylation of rHuBChE resulted in the generation of a heterogeneous set of products, which unlike PEGylated rHuAChE, were eliminated more rapidly than native serum-derived BChE (Chilukuri et al., 2005). This limitation may stem from suboptimal PEGylation, which in turn may be related to the fact that BChE contains a high number of lysine residues (37 lysines) which may serve as target sites for PEG-conjugation (Cohen et al., 2007). Reducing the number of BChE lysines by mutagenesis, to allow for the generation of a more homogenous PEG-BChE preparation without seriously affecting enzymatic properties, might be a much more challenging task than that achieved in AChE.

Finally, 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

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since the latter, as opposed to the highly stereoselective AChE, is partially consumed by reaction with the nontoxic P_R enantiomers of soman and sarin (Cohen et al., 2006). If indeed 200mg BChE are required to protect humans against $2LD_{50}$ of soman, it appears that only 78mg of AChE will be needed to confer the same extent of protection (Cohen et al., 2006). The ability to reduce the quantities of exogenously administered protein when utilizing an AChE-based bioscavenger, and the relative facility to generate homogenous preparations of PEGylated enzyme of AChE source, serve as additional motives for preferring AChE over BChE as a therapeutic bioscavenger.

Altogether, we have tailored a specified form of human AChE that combines optimized kinetic and pharmacokinetic traits required for effective bioscavenging of OP compounds. By means of chemical conjugation of PEG to the AChE molecule, the pharmacokinetic properties of the enzyme can be improved so that the enzyme will reside in the circulation for sufficiently long periods of time, and thereby could be utilized for prophylactic treatment. The kinetic traits of the enzyme can also be tailored so that the enzyme will be able to cope more efficiently with OP-mediated intoxication. Specifically, substitution of phenylalanine^{338} by alanine within the active center of the AChE molecule can significantly reduce the rate of irreversible dealkylation (aging) of the AChE-based bioscavenger, without affecting the reactivity of the enzyme towards OP- agents. It thus appears that, to date, the PEG-F338A HuAChE is the most optimal OP-bioscavenger engineered for prophylactic treatment against OP-poisoning.

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Footnotes

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Legends to figures

Figure 1: Plots of active site titration of PEGylated WT- and F338A-AChEs and aging of their adducts with soman. *A.* Active site titration of PEGylated WT- and F338A-AChEs with soman and MEPQ. In all titration experiments, the concentration of the catalytic subunit of PEGylated WT- and F338-AChEs was 120 to 170 nM. The residual activity was determined after incubation at 27^0C in the presence of various concentrations of the different compounds for 60 min. *B.* Time course of aging of PEGylated WT- and F338A-AChEs with soman. PEG-WT-AChE or PEG-F338A-AChE (100 nM) was incubated with soman (200 nM) at 27^0C. HI-6 (0.5 mM) was added to samples removed at various timepoints and enzymatic activity (E_r) was determined after 120 minutes. Plots present the *ln* values of the fraction of reactivatable enzyme (E_r/E_0) versus enzyme/soman incubation times. *Inset*: Expansion of the scale of time for the fast aging of PEG-WT-AChE.

Figure 2: Circulatory clearance profiles of non-PEGylated and PEGylated F338A-AChEs. Non-PEGylated, PEGylated F338A-AChEs and PEGylated WT-AChE (100 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 time points were assayed for ChE activity and values were corrected for background hydrolytic activity in the blood. Values are presented as percent of input ChE determined 1 minute after enzyme administration.

Figure 3: *In-vitro* reactivation of soman-inhibited PEGylated WT- and F338A-AChEs. PEGylated AChEs and soman (0.8 and 1.6 μM, respectively) were added to heparinized blood samples removed from mice and incubated for 20 min at 37^0C, after which HI-6 (1.5mM) was added. AChE activity determined at various time points following the addition of HI-6 is expressed as percent of AChE activity measured in blood samples spiked with AChE (0.8 μM) only.

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Figure 4: *In-vivo* reactivation and protective potential against repeated soman exposure of WT- and F338A-AChEs. Mice were pretreated with non-PEGylated or PEGylated WT- and F338A AChEs (2 nmol/mouse, i.v., T=0), exposed to soman (4 nmol/mouse, i.v., T=1 min.), treated with HI-6 (50 mg/kg, iv, T=21 min.) and re-exposed to soman (4 nmol/mouse, i.v., T=61 min.). *A.* AChE activity was monitored in blood samples removed at various timepoints from mice pretreated with the PEGylated versions of AChE. Activity is expressed as percent of input values, determined immediately after administration of the exogenous PEG-AChEs. Times of AChE administration, first soman exposure (1^{st} soman), HI-6 treatment and second soman exposure (2^{nd} soman) are indicated by arrows. *B.* Percent survival following the first and second soman exposures of mice (n=6) pretreated with the different AChE enzyme forms.

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Table 1. ATC hydrolysis rates and inhibition constants (k_i) of WT- and F338A-AChEs[a].

	ATC			Edrophonium	Propidium	BW284C51
AChE species	K_m (mM)	k_{cat} ($x10^{-5}min^{-1}$)	k_{app} ($x10^{8}M^{-1}\ min^{-1}$)	K_i (μM)	K_i (μM)	K_i (μM)
WT[b]	0.14	4	29	0.75	1.4	10
F338A[b]	0.14	1.7	12	0.8	1.5	10
PEG-WT	0.17	6.4	38	1.1	1.6	12.5
PEG-F338A	0.2	2.7	14	1.2	1.1	16.5

[a] Values represents mean of triplicate determination with STD not exceeding 20%

[b] Values compiled from our previous studies (Ordentlich A et al, 1993) are presented here for comparison.

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Table 2. Apparent bimolecular rate constant for phosphylation (k_i) of WT- and F338A AChEs[a].

	Phosphylation k_i ($x10^{-4}$ M^{-1} min^{-1})			
AChE species	Soman	Sarin	VX	Paraoxon
WT[b]	8600	2350	11400	100
F338A[b]	9000	1450	2700	7
PEG-WT	7900	3100	8200	180
PEG-F338A	13000	2700	3000	8

[a] Values represents mean of triplicate determination with STD not exceeding 20%

[b] Values compiled from our previous studies (Ordentlich A et al, 1993, 1998) are presented here for comparison.

Table 3. HI-6 reactivation constants of OP-inhibited WT- and F338A-AChEs[a].

	Soman			Sarin			VX			Paraoxon		
AChE species	k_r (M^{-1} min^{-1})	$k_2 \times 10^2$ (min^{-1})	$K_r \times 10^5$ (M)[b]	k_r (M^{-1} min^{-1})	$k_2 \times 10^2$ (min^{-1})	$K_r \times 10^5$ (M)[b]	k_r (M^{-1} min^{-1})	$k_2 \times 10^2$ (min^{-1})	$K_r \times 10^5$ (M)[b]	k_r (M^{-1} min^{-1})	$k_2 \times 10^2$ (min^{-1})	$K_r \times 10^5$ (M)[b]
WT	ND	---	---	11800	36	3.1	14700	20	1.4	170[c]	6[c]	35[c]
F338A	300	2.3	7.8	3400	15	4.4	5900	10	1.7	80[c]	0.6[c]	7[c]
PEG-WT	ND	---	---	13000	28	2.1	5100	13	3.1	100	2.3	24
PEG-F338A	260	1.6	6.2	4200	10	2.3	4300	13	2.6	200	0.7	3.5

[a] Values represents mean of triplicate determination with STD not exceeding 20%

[b] K_r values were calculated from the ratio k_2/k_r

[c] Values compiled from our previous studies (Grosfeld et al., 1996) are presented here for comparison.

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Table 4. Pharmacokinetics parameters of F338A-AChE enzymes in mice blood[a]

	F338A-AChE	PEG-F338A-AChE
Distribution phase		
$t_{1/2\alpha}$ (min)	8 ± 2	46 ± 16
AUC (%)[b]	15	1
Elimination phase		
$t_{1/2\beta}$ (min)	75 ± 8	2130 ± 110
AUC (%)[b]	85	99
MRT (min)	120	2870

[a] Mice were intravenously injected with 100U, presented data is average ± STD of at least 3 mice for each enzyme.

[b] The percentage each term contributes to the area under the curve.

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Table 5. Survival rates of PEGylated WT- and F338A-AChE pretreated mice following exposure to different OP agents

	Administered AChE[a]		
Administered OP agent	None	PEG-WT	PEG-F338A
2.5 LD_{50} Soman[b]	0% (0/5)	100% (5/5)	100% (5/5)
2.5 LD_{50} Sarin[b]	0% (0/5)	100% (5/5)	100% (5/5)
2.5 LD_{50} VX	0% (0/5)	100% (5/5)	100% (5/5)

[a] animals were administered with 3 nmole/mouse AChE for soman challenge, 2.6 nmole/mouse AChE for sarin challenge and 3.2 nmole/mouse AChE for VX challenge. Survival is presented as percent of survived animal out of total

[b] Animals were pretreated with CBDP, one hour before enzyme administration.

Scheme 1

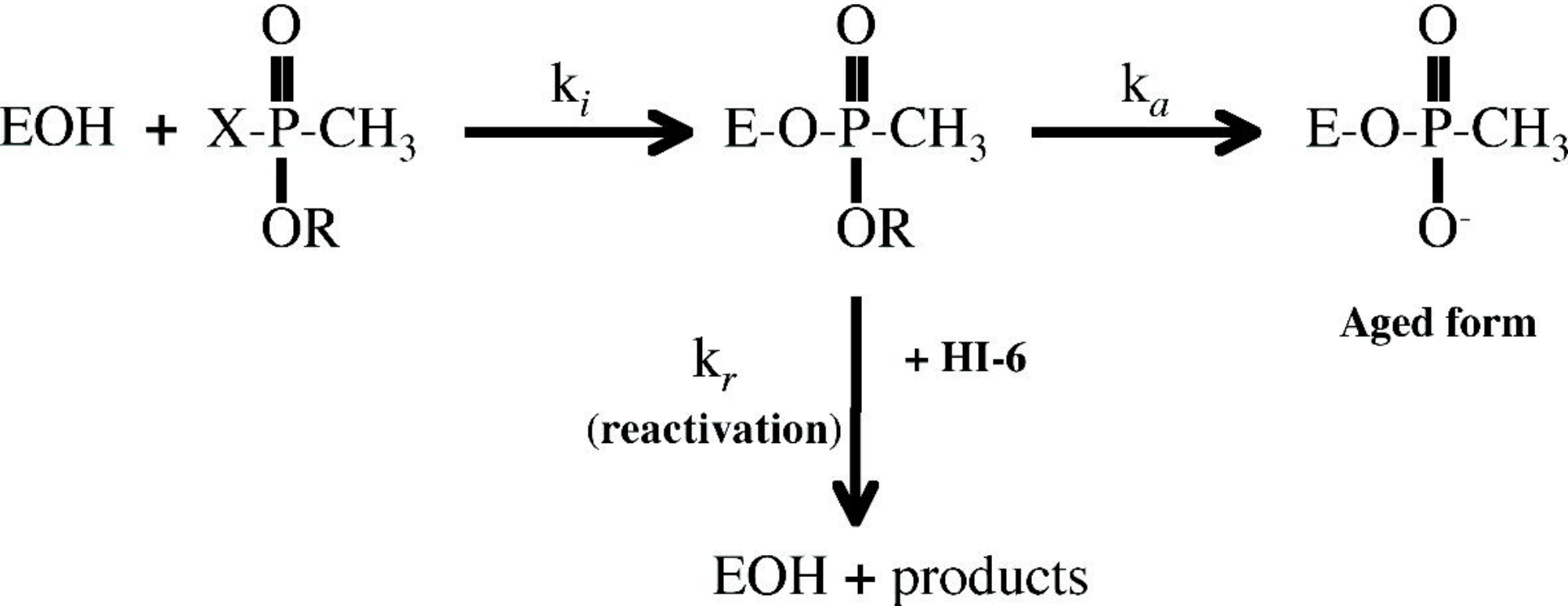

EOH + X-P-CH3
OR
ki
E-O-P-CH3
OR
ka
E-O-P-CH3
O-
Aged form
kr
(reactivation)
+ HI-6
EOH + products

Figure 1

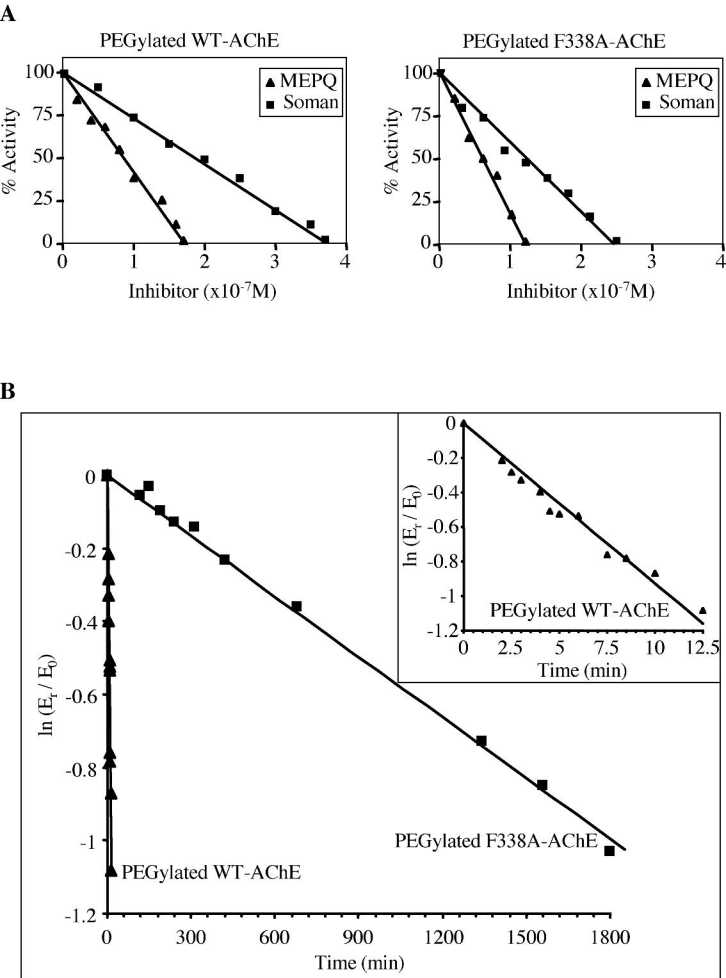

A
PEGylated WT-AChE
MEPQ
Soman
% Activity
Inhibitor (x10-7M)
PEGylated F338A-AChE
MEPQ
Soman
% Activity
Inhibitor (x10-7M)
B
ln (Et / E0)
PEGylated WT-AChE
PEGylated F338A-AChE
Time (min)
ln (Et / E0)
PEGylated WT-AChE
Time (min)

Figure 2

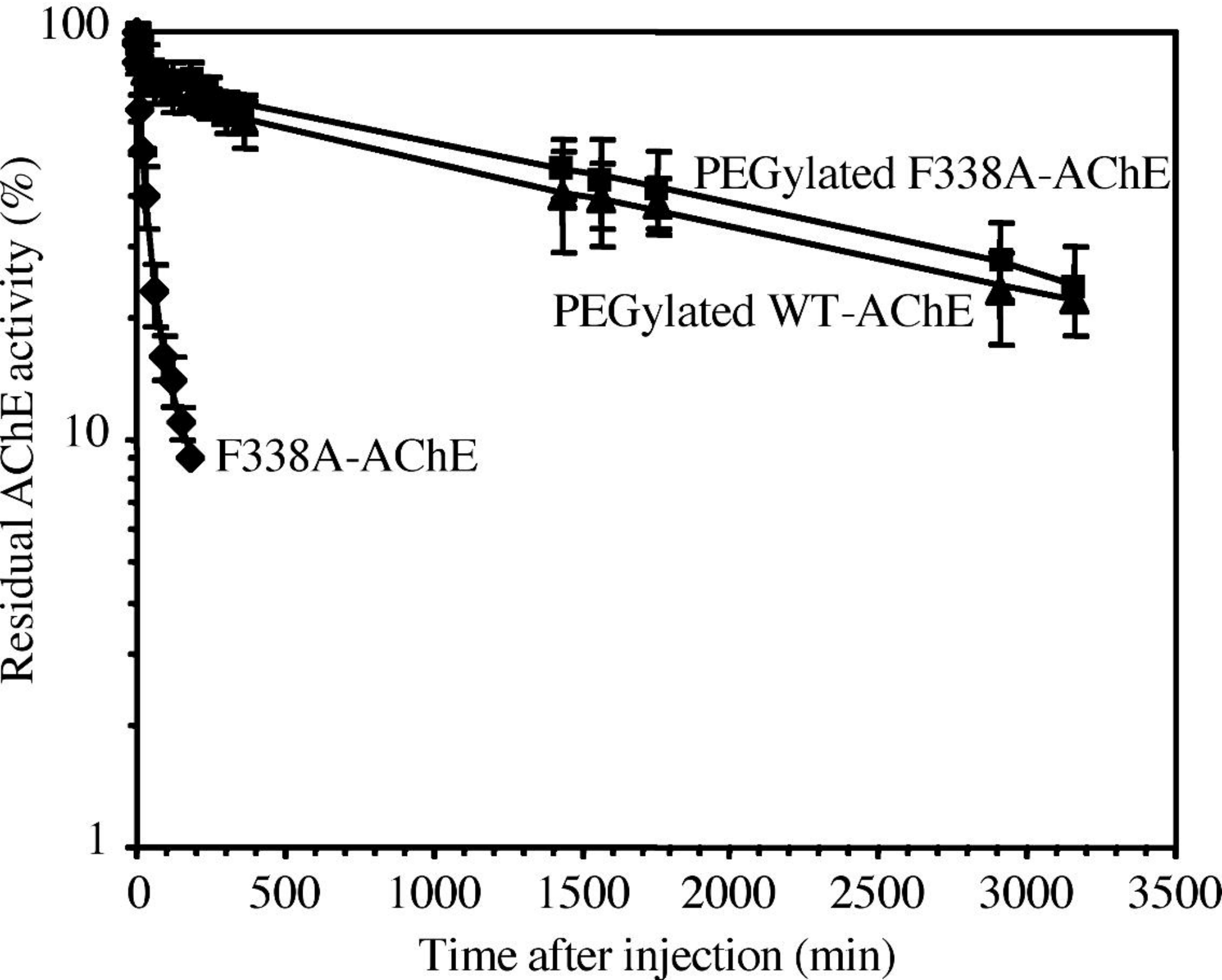

100
10
1
Residual AChE activity (%)
PEGylated F338A-AChE
PEGylated WT-AChE
F338A-AChE
0
500
1000
1500
2000
2500
3000
3500
Time after injection (min)

Figure 3

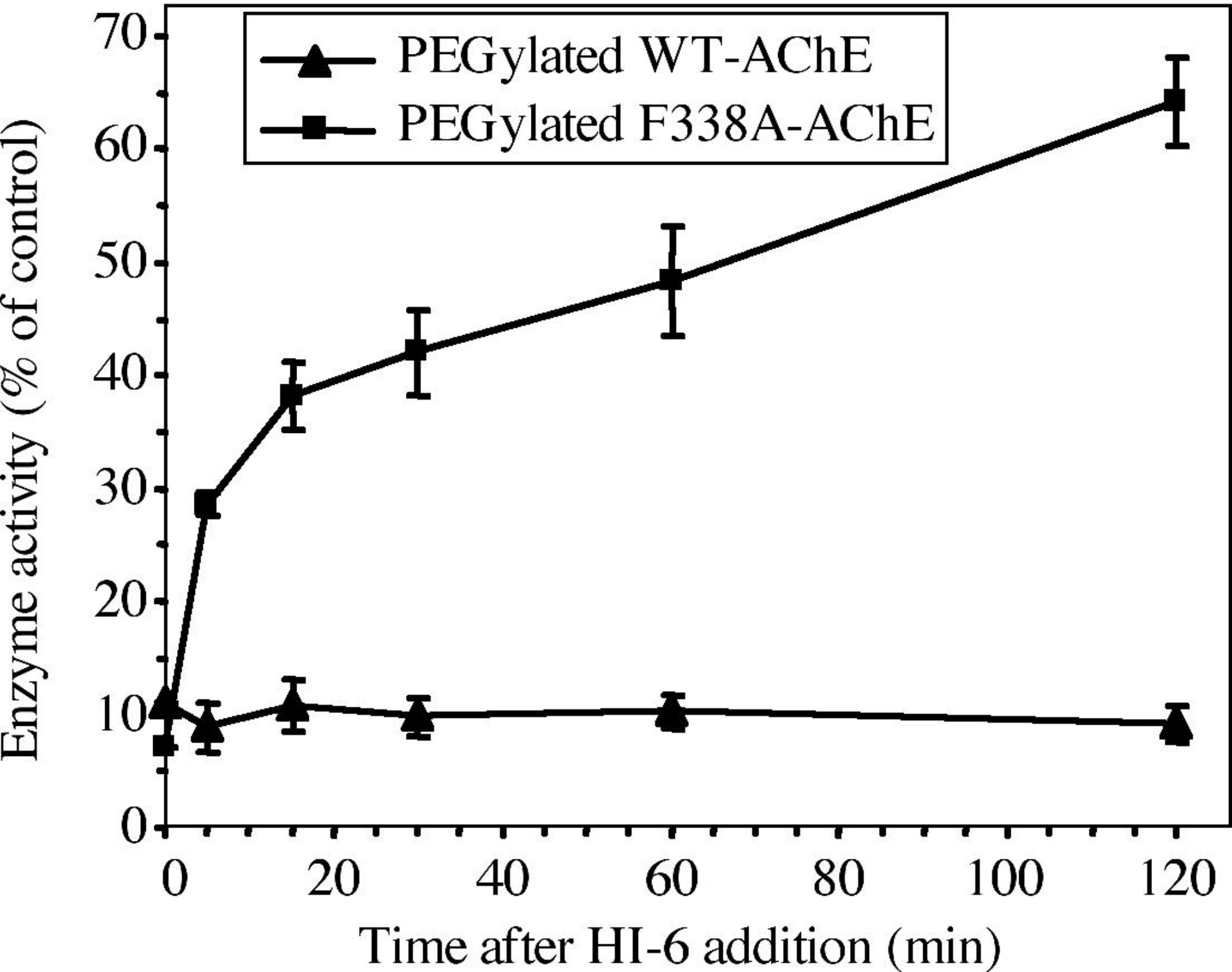
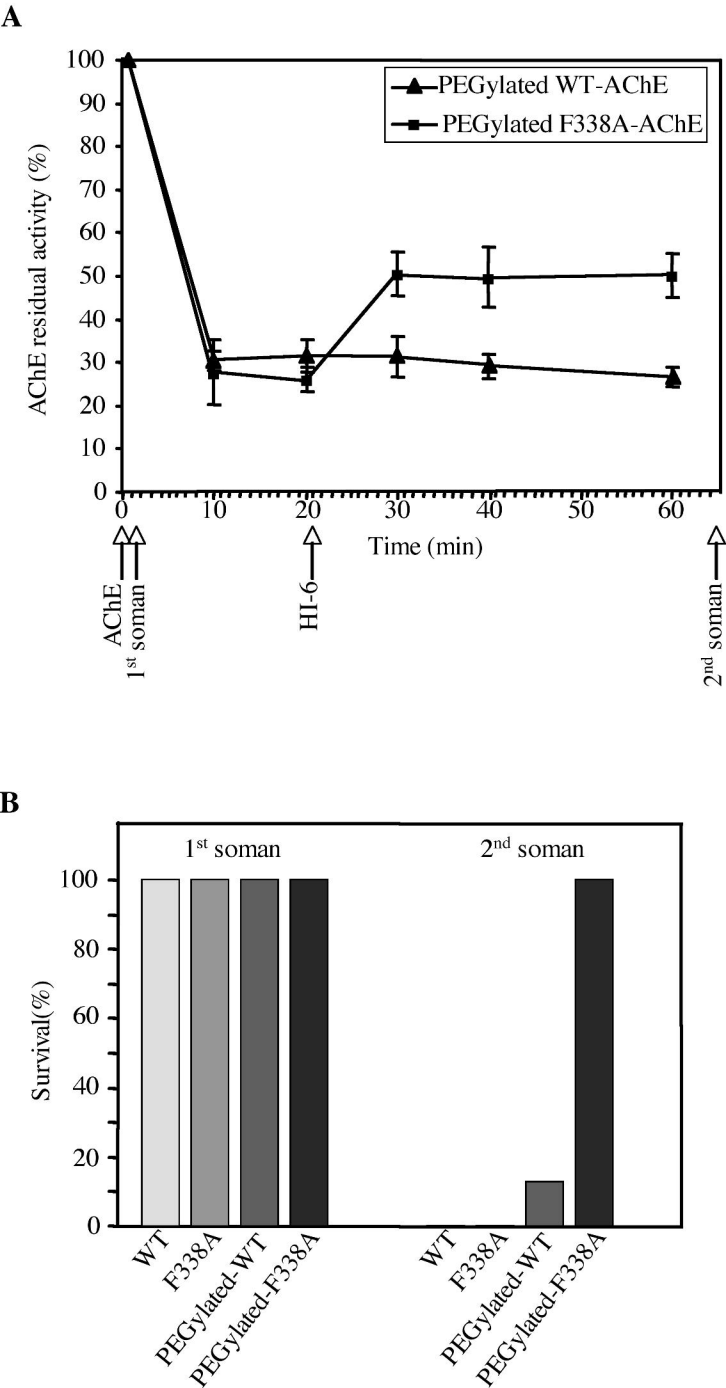

PEGylated WT-AChE
PEGylated F338A-AChE
Enzyme activity (% of control)
Time after HI-6 addition (min)
0
10
20
30
40
50
60
70
0
20
40
60
80
100
120

Figure 4


A
PEGylated WT-AChE
PEGylated F338A-AChE
AChE residual activity (%)
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
Time (min)
AChE
1st soman
HI-6
2nd soman
B
1st soman
2nd soman
Survival(%)
100
80
60
40
20
0
WT
F338A
PEGylated-WT
PEGylated-F338A
WT
F338A
PEGylated-WT
PEGylated-F338A