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# Acetylcholinesterase-Fc Fusion Protein (AChE-Fc): A Novel Potential Organophosphate Bioscavenger with Extended Plasma Half-Life

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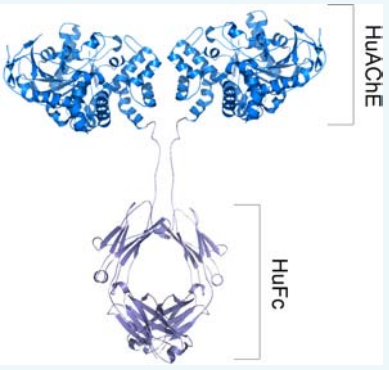
**ABSTRACT:** Acetylcholinesterase (AChE) is the physiological target of organophosphate nerve agent compounds. Currently, the development of a formulation for prophylactic administration of cholinesterases as bioscavengers in established risk situations of exposure to nerve agents is the incentive for many efforts. While cholinesterase bioscavengers were found to be highly effective in conferring protection against nerve agent exposure in animal models, their therapeutic use is complicated by short circulatory residence time. To create a bioscavenger with prolonged plasma half-life, compatible with biotechnological production and purification, a chimeric recombinant molecule of HuAChE coupled to the Fc region of human IgG1 was designed. The novel fusion protein, expressed in cultured cells under optimized conditions, maintains its full enzymatic activity, at levels similar to those of the recombinant AChE enzyme. Thus, this novel fusion product retained its binding affinity toward BW284c5 and propidium, and its bioscavenging reactivity toward the organophosphate-AChE inhibitors sarin and VX. Furthermore, when administered to mice, AChE-Fc exhibits exceptional circulatory residence longevity (MRT of 6000 min), superior to any other known cholinesterase-based recombinant bioscavengers. Owing to its optimized pharmacokinetic performance, high reactivity toward nerve agents, and ease of production, AChE-Fc emerges as a promising next-generation organophosphate bioscavenger.




## INTRODUCTION

Organophosphate (OP) compounds are a diverse group of chemicals that include, among others, insecticides, antihelminitics, and nerve agents. The acute toxicity of OP compounds is due to the irreversible inhibition of acetylcholinesterase (AChE) leading to the accumulation of acetylcholine in the synaptic clefts, thereby disrupting the cholinergic neurotransmission. Post-exposure treatment against OP poisoning includes the administration of atropine that blocks the acetylcholine receptor, thereby preventing nerve and muscle activation, the use of oximes to reactivate the OP-inhibited AChE and antianxiety drugs to prevent seizures.[1,2] Pyridostigmine bromide is an FDA approved nerve agent prophylactic antidote that reversibly inhibits AChE and competes with the nerve agents for the active site of the enzyme. Yet, the current pre- and post-exposure treatment against OP poisoning may, at best, increase survival, but fail to reduce post-exposure incapacitation and irreversible brain damage.[1,2] It was therefore proposed to use exogenously AChE or butyrylcholinesterase (BChE) as potential stochiometric bioscavenger that may sequester the toxic OPs in the bloodstream before they can reach their physiological target. Indeed, previous studies demonstrated that exogenous BChE can serve as an effective therapeutic agent for prophylactic treatment against nerve agent exposure.[3,4] An essential therapeutic requirement from prophylactic OP bioscavengers is a long plasma half-life, preferably of several days. Native human BChE (HuBChE), purified from human plasma, possesses long retention time, yet its limited availability and purification high costs render it economically prohibited. Over the years, several attempts were made to produce recombinant AChE and BChE (rAChE and rBChE, respectively) in different expression systems.[2] However, pharmacokinetic studies revealed that these recombinant enzymes exhibit fast clearance rates.[5] To overcome this drawback, chemical modifications or fusion of cholinesterases (ChEs) to carrier proteins and peptides were attempted, resulting in extension of the plasma half-life (from minutes to hours and days).[5] The most successful modification published so far is the conjugation of polyethylene glycol (PEG) side-chains to recombinant human AChE (rHuAChE) or BChE (rHuBChE)[6−8] increasing their mean residence time to 3000 min (as compared to 120 min for the nonmodified enzymes). It was also shown that rHuAChE is more stereoselective than HuBChE and therefore upon administration of equivalent doses, rHuAChE provides better protection against nerve agent exposure compared to HuBChE.[7] Moreover, being stochiometric scavengers, it was estimated before that approximately 80 mg and 200 mg of exogenously administered AChE or BChE, respectively, are needed to protect human adults against $2LD_{50}$ of soman.[7,9] Nevertheless, from a pharmaceutical point of view, the production of PEGylated bioscavenger in clinically relevant doses may pose many challenges as to its purification and production.[10] Therefore, there is an unmet need for a circulatory long-lasting recombinant bioscavenger.

Fc-based fusion proteins are composed of the immunoglobulin Fc domain linked to a protein of interest.[11] The presence

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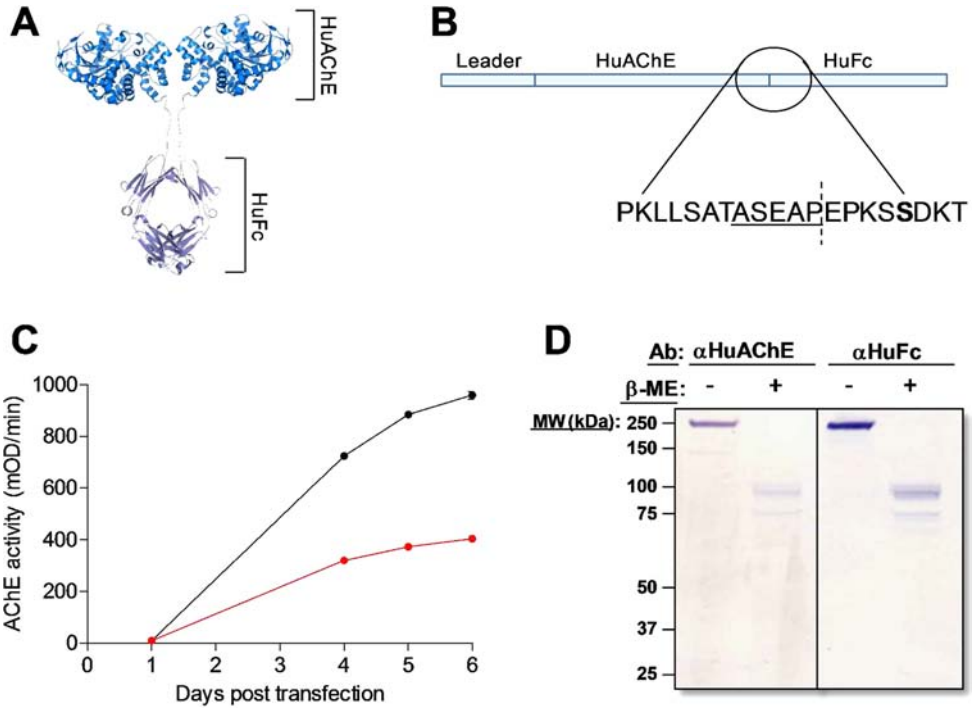


**Figure 1.** Construction and expression of AChE-Fc. (A) Schematic illustration of the AChE-Fc homodimer fusion protein. The model composed of the artificially linked crystal structures of HuAChE (Blue; PDB 1B41) and the HuFc fragment (Purple; PDB 2IG2 + 1FC2). (B) Amino acid sequences at the HuAChE and HuFc fusion site, emphasizing the short peptide linker (underlined) and the serine residue (bold) that replaced the cysteine. (C) Expression profile of AChE-Fc generated using transient expression of HEK293 cells that transfected with vector containing either kappa-light chain (Black) or HuAChE (Red) signal peptides. Points are average ± SEM of triplicate analyses. (D) Aliquots of cell culture supernatant were analyzed on SDS-PAGE in the absence or the presence of $\beta$-ME. Western blot analysis was performed using polyclonal antibodies directed against either HuAChE or HuFc.

of the Fc domain endows the fused protein with increased plasma half-life (days to weeks), owing to its interaction with the neonatal Fc-receptor (FcRn) that mediates the IgG salvation process.[11] Other benefits to the use of Fc-fusion proteins are the fact that they can be easily manufactured in high yields in mammalian cell culture facilities, and purified by standard Fc-based affinity chromatography (e.g., protein-A).[12] We hypothesized that by linking HuAChE to human Fc we can create a long-lived recombinant bioscavenger amenable with its production in cultured cells. To test this hypothesis, a vector encoding a chimeric molecule of HuAChE coupled to the Fc region of human IgG1 was created. The AChE-Fc fusion protein was expressed in cultured cells, its reactivity toward OP and nerve agents was evaluated, and a detailed study of its circulatory pharmacokinetic in mice was performed.

## RESULTS AND DISCUSSION

**Construction and Expression of the Fusion Protein.** As depicted in the schematic diagram of the AChE-Fc fusion protein (Figure 1A), the designed protein comprises a homodimer of two HuAChE enzymatic moieties fused to a dimer of human Fc (HuFc), resulting in a 250 kDa molecule. The following aspects were considered in designing and constructing the vector encoding for the AChE-Fc fusion protein: (i) The native form of HuAChE contains an elongated collagen-like tail unit that mediates its assembly into tetramers of catalytic subunits.[13] It was previously reported that by replacing the 40 amino acids that form the collagen-like structure at the C-terminal end of the HuAChE (positions 544−583) with the ASEAP pentapeptide (to generate a derivative of the H-subunit of HuAChE[6,14]), a fully active recombinant HuAChE monomer was generated. Accordingly, this modified version of the enzyme was incorporated in the fusion protein. (ii) We addressed the question of whether a peptide spacer should be incorporated at the junction site between the HuAChE and the HuFc fragment. Such a short linker may provide some structural flexibility while being beneficial in preserving the catalytic characteristics of the fusion protein. As indicated, the recombinant HuAChE monomer exhibits a short five-amino-acid (ASEAP) peptide that replaced the original tail.[14] Since the addition of this peptide had no effect on the HuAChE enzymatic activity, it was used as a linker between the HuAChE and the HuFc fragment (Figure 1B). In addition, the HuAChE was linked to the hinge region of IgG1, allowing extra flexibility at the fusion site. Furthermore, the cysteine that facilitates the covalent linkage at the hinge region between the heavy and the light chains of IgG1 was replaced by a serine (Figure 1B), in order to prevent nonspecific bonding.

Since the fusion protein was designated to be expressed in mammalian cells and secreted into the culture medium, an appropriate signal peptide was included at the N-terminus of the molecules. On the one hand, the Kappa light-chain signal peptide sequence is often used in vectors designed to express Fc-fusion proteins in mammalian cultured cells. On the other hand, it was shown previously that HuAChE signal peptide can induce a very efficient secretion of the soluble form of rHuAChE in tissue culture.[14] We therefore decided to construct two versions of AChE-Fc expression vectors, with signal peptides of either the Kappa light-chain or the HuAChE, and to compare the secretion levels of the two chimeric proteins. To this end, cells were transfected with either of the


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Table 1. Rate Constants of ATC Hydrolysis, Enzyme Inhibition, and Phosphylation

| | ATC | | | $K_i$ | | | |
|---|---|---|---|---|---|---|---|
| | $K_m$ (mM) | $K_{cat}$ ($\times 10^{-5}$ $min^{-1}$) | $K_{app}$ ($\times 10^8$ $M^{-1}$ $min^{-1}$) | propidium[a] ($\mu$M) | BW284c5[a] (nM) | VX[b] ($\times 10^5$ $M^{-1}$ $min^{-1}$) | Sarin[b] ($\times 10^5$ $M^{-1}$ $min^{-1}$) |
| rHuAChE | 0.19 | 5.2 | 27 | 12.5 | 17.8 | 450 | 10.3 |
| AChE-Fc | 0.18 | 4.5 | 25 | 11.1 | 19.2 | 400 | 11.5 |

[a]Enzyme inhibition constant. [b]Apparent bimolecular rate constant for phosphylation.

two vectors and the amount of secreted AChE-Fc in the culture medium was monitored. It was found that both vectors could induce the secretion of the active fusion protein (Figure 1C), yet the levels obtained by transfecting with the vector containing the Kappa light-chain signal peptide sequence were 2-fold higher. It was therefore decided to continue with the vector containing the Kappa light-chain signal peptide sequence. Next, we sought to confirm that the secreted protein indeed comprised both AChE and Fc and that it was secreted as a dimer. To this end, a sample of the culture medium was analyzed by SDS-PAGE under nonreducing conditions, and further subjected to Western blot analysis using antibodies against either HuAChE or HuFc (Figure 1D). Indeed, both antibodies identically visualized a distinct band with a molecular weight of about 250 kDa, indicating that the fusion protein is composed of HuAChE and HuFc and that it was secreted solely as a dimer. Under reducing conditions, it could be verified that the dimers were separated to two monomers of approximately 100 kDa. It should be noted that under reducing condition a slight degradation of the rHuAChE is observed, a phenomenon that was previously documented.[14,15]

**Enzymatic Characterization of AChE-Fc.** The practical use of the AChE-Fc fusion protein as a prophylactic agent against OP nerve agents requires retention of the catalytic activity of the HuAChE enzyme, and therefore detailed kinetic studies and binding properties were performed. The fusion protein was purified from the cell culture supernatant using procainamide affinity chromatography and the specific activity of the purified fusion protein was determined by MEPQ titration.[7] The rate constant of ATC hydrolysis ($K_m$) was found to be similar to that of the rHuAChE enzyme (Table 1). Likewise, both AChE-Fc and rHuAChE exhibit comparable values of $k_{cat}$ and $k_{app}$. To further evaluate whether the structure of the enzymatic moiety remained intact, interactions of AChE-Fc with propidium, a peripheral anionic site ligand, and with the bis-quaternary inhibitor BW284C5, whose binding site spans both the peripheral and the active-center gorge,[13,16] were measured. Indeed, the fusion protein displayed high affinity toward these two ligands, exhibiting values that were similar to these of the recombinant enzyme (Table 1). These results encouraged us to examine the bioscavenging potential of AChE-Fc toward various nerve agents. To this end, the reactivity of the fusion protein toward sarin, a representative of the "G-agent" organophosphates, was determined. The apparent bimolecular rate constant ($k_i$) of AChE-Fc toward sarin was found to be $11.5 \times 10^5$ $M^{-1}$ $min^{-1}$, indicating that it retained its full bioscavenging activity as the rHuAChE (Table 1). Similarly, both AChE-Fc and rHuAChE exhibit similar inhibition rate constants toward VX, a charged organophosphate of the "V-agents", with $k_i$ values of 400 and $450 \times 10^5$ $M^{-1}$ $min^{-1}$, respectively (Table 1). Taken together, these results indicate that the AChE-Fc fusion protein maintained its reactivity toward various ligands and organophosphates and strengthen the notion that it may serve as a valuable prophylactic agent against exposure to nerve agents.

**Pharmacokinetics of AChE-Fc in the Circulation of Mice.** To determine the pharmacokinetic characteristics of the fusion protein, it was first necessary to establish a specific and sensitive assay that will enable monitoring of the circulatory levels of the AChE-Fc with minimal interferences from endogenous AChE that naturally resides in the blood. To this end, a functional capture ELISA assay was designed in which plates are coated with antibodies against the human Fc for immobilization of the AChE-Fc present in the blood samples (Figure 2). Plates are then washed, and a specific AChE substrate[17] is added for colorimetric kinetic detection of the enzyme. The assay, which exhibits a clear dose response between the concentration of AChE-Fc and color formation, is highly sensitive, allowing detection of the fusion protein at the sub-picomolar concentration range (Figure 2). Owing to the specificity of the assay format, control naïve serum samples did not induce any color formation (virtually zero background). These results indicated that the novel assay can be used to monitor the AChE-Fc levels in mouse serum.

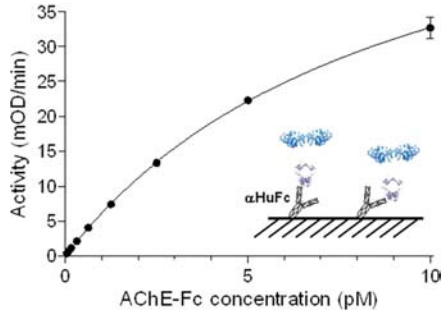


**Figure 2.** Functional AChE-Fc capture ELISA. Plats were coated with anti-HuFc specific antibody, followed by the addition of increasing amounts of AChE-Fc fusion protein (as illustrated in the inset). The plates were then washed, AChE specific substrate (ATC) was added, and the enzymatic activity was measured.

AChE-Fc as well as rHuAChE were administered intravenously to mice, and their pharmacokinetic profiles were determined (Figure 3). As expected, the recombinant enzyme cleared rapidly from the bloodstream in a biphasic behavior, with a rapid and short distribution phase (half-life of 1 min accounting for 6% of the area under curve; AUC), followed by a longer elimination phase ($\beta$) with half-life of about 30 min (Table 2). The rHuAChE mean residence time (MRT) value of 40 min is in good agreement with previously reported data.[6] The AChE-Fc, however, exhibited a totally different profile with an extremely extended circulatory lifetime. The half-life of AChE-Fc in the distribution phase is about 50 min followed by much longer elimination half-life (4000 min), about 100-fold slower than that of the recombinant enzyme and the overall calculated MRT value is about 6000 min. The fact that the


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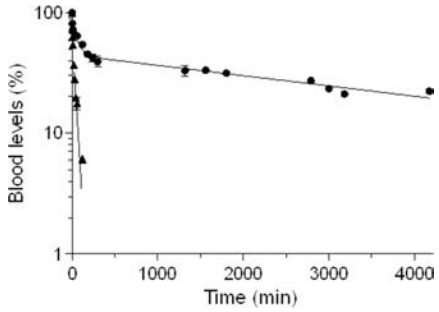


**Figure 3.** Enzyme pharmacokinetics in mice circulation. rHuAChE (triangles) and AChE-Fc (circles) were administered intravenously to mice ($n = 3$). Blood samples were drawn at various time points and the levels of each enzyme were determined. Values are presented as percentages of maximum blood levels ($C_{max}$), and points are average ± SEM.

**Table 2. Pharmacokinetic Parameters of Enzymes in Mouse Circulation Following Intravenous Administration**[a]

| | rHuAChE | AChE-Fc |
|---|---|---|
| distribution phase | | |
| $t_{1/2\alpha}$ (min) | 1 ± 0.4 | 47 ± 18 |
| AUC (%)[b] | 6 | 1 |
| elimination phase | | |
| $t_{1/2\beta}$ (min) | 29 ± 3 | 4020 ± 800 |
| AUC (%)[b] | 94 | 99 |
| MRT (min) | 40 ± 4 | 5800 ± 1100 |

[a]Presented data is average ± SEM of 3 mice for each enzyme. [b]Percentage each term contributes to the area under the curve.

distribution phase accounts for 99% of the total AChE-Fc AUC indicates high bioavailability of the enzyme as was also demonstrated in a previous study where PEGylation of rHuAChE markedly increased its bioavalibilty.[18]

Depending on the therapeutic protein that is fused to the Fc, steric hindrance between the effector molecule and the Fc may occur, thus adversely affecting the interaction with FcRn and consequently failing to extend the half-life of the hybrid molecule.[19] It was also shown recently that the interaction of the effector molecule with its natural serum carrier proteins may govern the FcRn-mediated salvation process, thus preventing significant extension of the Fc-fusion protein residence time in the circulation compared to the unmodified protein.[20] In the study documented in this report, a large effector molecule (AChE) is fused to the Fc without affecting the interaction with FcRn, as demonstrated by its extended plasma half-life. The half-life value of the AChE-Fc in mouse circulation (4000 min) is in good agreement with previous reports of several Fc fusion proteins, in which the half-life values in the circulation mice were shown to range 960−3200 min.[19,21−23] Moreover, pharmacokinetic studies performed in rabbits using the cAb29 antibody that shares the exact Fc as the AChE-Fc also demonstrated a similar half-life value of 3500 min.[24]

In the past, several attempts were made to extend the serum residence time of AChE and BChE, including optimization of post-translation modification and PEGylation.[5−7,18] Indeed, a marked prolongation of the enzyme's serum half-life was achieved, especially for the chemically modified PEG-versions of rHuAChE that demonstrated a half-life of 2000 min in mouse circulation.[7,18] Interestingly, efforts to prolong the serum life span of BChE by creating an albumin-fusion protein succeeded to extend its circulatory half-life to 2000 min in pigs,[25] yet in a murine model the half-life of the BChE-albumin was only 500 min.[26] Since albumin shares the same salvation process as the Fc (by binding to the FcRn) it was expected that the albumin-BChE fusion proteins will exhibit longer plasma residence time, as shown here for the AChE-Fc. A possible explanation for this phenomenon is either that BChE interferes with the albumin-FcRn interactions or that its blood levels are controlled by a specific clearance pathway that bypasses the FcRn mediated salvation process.

One of the major issues faced so far in the development of a bioscavenger is large-scale production. For example, the purification of native HuBChE from plasma (which is a limited resource) is both very expensive and fraught with inherent safety issues associated with blood-based pharmaceutical products. Chemical modifications (such as polysialylation or PEGylation) of recombinant AChE or BChE are also viable alternatives, yet they also pose a biotechnological challenge for their large-scale production. For example, variation in the numbers and positions of the attached PEG chains shows the need to develop specific separation and purification processes and unique analytical methods.[10] In contrast, from the biotechnological standpoint, the fact that AChE-Fc can be readily expressed in cultured cells renders it an appealing candidate. Moreover, AChE-Fc can be readily purified using procainamide based affinity chromatography, therefore simplifying the downstream manufacturing process.

In conclusion, we developed a novel nerve-agent bioscavenger that is based on chimeric recombinant molecule of HuAChE coupled to the Fc region of human IgG1. The novel fusion protein, expressed in cultured cells under optimized conditions, is highly compatible with biotechnological manufacturing that will enable its large-scale production and purification. AChE-Fc exhibits exceptional circulatory residence longevity when compared to other Fc-fusion proteins in general and superior to any other known cholinesterase-based recombinant bioscavengers in particular. By combining the required activity toward OP compounds with optimized pharmacokinetics traits, AChE-Fc seems to be a very promising next-generation bioscavenger that should be further evaluated in animal models.

## ■ MATERIALS AND METHODS

**Organophosphate Inhibitors.** Sarin (*O*-isopropyl methylphosphonofluoridate); VX (*O*-ethyl-*S*-(2-isopropylaminoethyl) methylphosphonothiolate), and MEPQ (7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide) were prepared as described in previously reported procedures.[7,27] The purity of the OPs (>95%) was determined by $^{1}H$ and $^{31}P$ NMR spectroscopy. Stock solutions were kept at −20 °C, and diluted in sodium phosphate buffer to the desired concentration, prior to use.

**Preparation of Plasmid Encoding AChE-Fc.** A mammalian expression vector containing the heavy chain of human IgG1 under the control of the CMV promoter[28] was digested with the SacI/AleI restriction enzymes (Thermo Scientific, USA). This restriction removed the variable region, CH1, and hinge regions and the heavy-chain leader. A synthetic sequence designed to include a truncated HuAChE (where the C-terminus 40 amino acids were removed), a spacer containing the amino acids ASEAP, and the hinge region of IgG1 (SP-AChE-spacer-hinge) was synthesized by IDT (Integrated DNA


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technologies Inc., USA). A second sequence was synthesized to include an N-terminal and C-terminal truncated HuAChE, where the N-terminal signal peptide was also removed, and replaced by the Kappa light-chain signal peptide (K-AChE-spacer-hinge). These two sequences were also digested with the same restriction enzymes and then ligated, each, to the digested vector, resulting in cloning of the synthetic sequences N-terminal to the Fc portion.

**Enzyme Expression and Purification.** A C-terminus truncated rHuAChE was collected from the supernatant of the stably expressed HEK293-AChE cells.[14] For the production of AChE-Fc, HEK293 cells (Life technologies, USA) cultured in serum-free media were transiently transfected with a plasmid encoding for the fusion protein and the supernatant was collected after a week. Both secreted enzymes were purified from the supernatant by adsorption to a procainamide-Sepharose 4B column (4000 units/mL resin) as described before.[5] Briefly, following adsorption the column was first washed with 50 mM sodium phosphate buffer containing 1 mM EDTA followed by a second with the same buffer containing 1 mM EDTA and 400 mM NaCl. Enzymes were then eluted with 20 mM decamethonium (Sigma) in 50 mM sodium phosphate buffer containing 1 mM EDTA, followed by extensive dialysis against PBS. The concentration of each enzyme was determined using MEPQ titration, as described before.[29]

**Western Blot.** Samples of HEK293 cells supernatant containing AChE-Fc were boiled in sample buffer (Bio-Rad) with or without $\beta$-mercaptoethanol ($\beta$-ME) and loaded onto a 4−12% precasted SDS-PAGE gel (Bio-Rad). The samples were blotted to a nitrocellulose membrane which was then blocked with PBST (0.05% Tween 20, 2% BSA in PBS) and incubated with murine anti-HuAChE antibody[14] or with goat-anti-Human Fc antibody (Sigma). The membrane was washed, incubated with detecting antibody, and developed using the Supersignal West Pico chemiluminescent substrate (Thermo scientific).

**Kinetic Studies.** Enzyme activity was assayed according to Ellman et al.[17] in the presence of AChE substrate buffer (0.1 mg/mL BSA, 0.3 mM DTNB, 50 mM sodium phosphate buffer (pH 8.0), and 0.5 mM ATC) at 27 °C and monitored with a Thermomax microplate reader (Molecular Devices). Measurements of phosphylation rates were carried out by monitoring residual activity ($E$) at various time points, 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.[30] Rate constants under second-order conditions were determined from plots of $\ln\{E/[I_0 - (E_0 - E)]\}$ versus time.

**ELISA.** Capture ELISA was performed to determine the presence of active AChE-Fc in the various samples. Maxisorp 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated overnight with 5 $\mu$g/mL of anti-human Fc $F(ab)_2$ fragment (Sigma; 50 $\mu$L/well) in $NaHCO_3$ buffer (50 mM, pH 9.6), washed, and blocked with PBST at room temperature for 1 h. Samples were serially diluted in PBST, added to the coated plates, and incubated for 1 h at 37 °C. Plates were then washed with PBST, incubated with the AChE substrate buffer, and color formation was monitored as described above.

**Pharmacokinetic Experiments.** Female 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 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. Pharmacokinetic experiments in mice (3 mice per enzyme sample) were carried out essentially as described previously.[7] Mice were injected intravenously with rHuAChE (to reach 30-fold increase over endogenous background levels, 70 units in 0.2 mL PBS were administered[7]) or with AChE-Fc (3 units in 0.2 mL PBS). At different time points, blood samples (5 $\mu$L) were drawn from the tail vein, diluted 20-fold in PBS, and centrifuged for three min at 3000 rpm for the removal of red blood cells. The levels of rHuAChE in each sample were determined as described above and expressed as the percent of the initial concentration at time zero (background levels of endogenous AChE activity were subtracted from all measurements). The levels of AChE-Fc in each sample were determined using the capture ELISA described above and values are expressed as the percent of the initial concentration at time zero. Pharmacokinetic parameters were calculated using *Prism* software (GraphPad Software Inc., USA).

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### Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS

AChE, acetylcholinesterase; BChE, butyrylcholinesterase; OP, organophosphate; FcRn, neonatal Fc-receptor; HuAChE, human acetylcholinesterase; HuBChE, human butyrylcholinesterase

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