Supporting Information

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SI Materials and Methods

Plant Production of Human Cholinesterases (ChEs). We have recently described the generation of transgenic *Nicotiana benthamiana* plants harboring WT plant-derived human butyrylcholinesterase (pBChE) (1) and plant-derived read-through isoform of human acetylcholinesterase (pAChE-R) (2, 3). Both proteins were targeted for retention in the endoplasmic reticulum by a C-terminal SEKDEL peptide. Plants were grown in a US Department of Agriculture approved facility, and were harvested 8–12 wk after germination. Soil and unprocessed plant material were autoclaved prior to their composting. Detailed protocols for the purification of pBChE (1) and pAChE-R (2, 3) were previously described. The G117H/E197Q variant of pBChE was transiently expressed in wild-type plants using the MagnICON vector system (4) and was purified following a similar protocol to used for WT pBChE.

Fractionation of BChE Molecular Species. Plant-derived BChE oligomers were fractionated based on their native molecular mass using size-exclusion HPLC. The Alliance HPLC System (Waters) equipped with a Shodex KW-803 column (8 × 300 mm; Kawasaki) was used. The mobile phase solution contained 20 mM Na_2HPO_4/NaH_2PO_4 buffer, pH 8.0, 200 mM NaCl, and 0.04% NaN_3, and 0.5 mL samples were collected at a flow rate of 0.5 mL/ min. Molecular mass standards (β-amylase, 200 kDa, and BSA, 66 kDa) were separately resolved. Relative amounts of BChE molecular species were calculated based on the area under the curve in the chromatogram.

For sucrose gradient fractionation, aliquots of pBChE or human serum BChE (hBChE) were applied to 5–15% sucrose gradients prepared in 50 mM NaPi, pH 8.0. The gradients were centrifuged (SW41Ti rotor, Beckman) at 75,000 × *g* for 18 h at 4 °C.

PEG Conjugation. Conjugation of WT pBChE or pAChE-R (2) to PEG was performed essentially as described (5). Briefly, azide-free enzyme preparations (5 μM) were incubated with succinimidyl propionate-activated methoxy-PEG (Nektar, Inc.) in 50 mM NaPi buffer, pH 8.0 at a 50:1 molar ratio (PEG:primary amine, latter is 37 based on available lysines and N terminus) at 25 ºC. PEG with average molecular mass of either 5 kDa (PEG-5K) or 20 kDa (PEG-20K) was used. Incubation was for 2 h [PEG-5K(p) and PEG-20K] or for 4 h to ensure reaction completion [PEG5K (c)]. The reaction mixture was then diluted with PBS (10 vol), dialyzed overnight against PBS at 4 ºC in a Slide-A-Lyzer 20,000 molecular weight cutoff (Pierce), then concentrated to >1 mg/mL in a 50,000 molecular weight cutoff centrifugal concentrator.

SDS-PAGE and Immunoblot Analyses. Protein preparations were resolved by SDS-PAGE on 6% (PEG-pBChE conjugates) or 8% gels (all other preparations), and were stained with GelCode SilverSNAP Stain Kit II (Pierce), or transferred to a PVDF membrane and immunodecorated with rabbit polyclonal anti-hBChE Abs (the generous gift of Oksana Lockridge, University of Nebraska Medical Center, Omaha, NE). HRP-conjugated goat-anti-rabbit IgGs (Santa Cruz Biotechnology) and the ECL-plus kit (Amersham) were used for detection. Total soluble protein was determined as described (6).

Enzymatic Assays. All assays were run at 25 ºC and at pH 7.4. BChE activity was assayed by a modified Ellman assay (2, 7) with butyrylthiocholine iodide (BTCh, Sigma) as substrate. To determine the K_m, activity was measured in the presence of varying concentrations of BTCh, the results were plotted, and nonlinear regression was applied (GraphPad Prism ver 4.0, GraphPad Software). Inhibition curves were obtained by incubating pBChE (29.7 mU/mL, one unit of enzyme will hydrolyze 1.0 μmol of butyrylthiocholine to thiocholine and butyrate per minute) and hBChE (30.0 mU/mL) for 30 min with the indicated concentrations of paraoxon (POX) followed by a modified Ellman assay with 1 mM BTCh. IC_{50} values were determined by fitting the results to the equation

$$[\text{residual BChE activity}] = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{[\text{POX}] - \log \text{IC}_{50}}}$$

using GraphPad Prism.

Organophosphorous compounds (OP) nerve agents tabun (ethyl N,N-dimethylphosphoramidocyanidate), sarin [GB, 2-(fluoro-methylphosphoryl)oxypropane], soman [GD, 2-(fluoro-methyl-phosphoryl)oxy-3,3-dimethyl-butane], cyclosarin [(fluoro-methyl-phosphoryl)oxycyclohexane], and VX (*O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate) were obtained from the US Army Edgewood Chemical Biological Center and diluted into appropriate buffers before storage or use. The second-order inhibition rate constant (k_i) of racemic VX and racemic soman was determined with both WT and G117H/E197Q pBChE using a modified Ellman assay (Reiter, 2008 #4461). Residual pBChE activity was determined at several time points following incubation with nerve agent in defined excess concentrations (8) that were sufficiently high to establish pseudo-first-order reaction conditions in all cases except for WT pBChE with soman, for which the biomolecular rate could only be approximated. Oximes used were HI-6 [1-2-hydroxyiminomethyl-1-pyridino-3-(4-carbamoyl-1-pyridino-2-oxapropane dichloride)]; 2-PAM (pyridine-2-aldoxime methochloride); MMB-4 [1,1′-methylenebis[4-[(hydroxyimino) methyl] pyridinium] dichloride)]; TMB-4 [(1,1′-trimethyl bis-[4-formyl pyridinium chloride] dioxime)]; HS-6 [(2-hydroxyiminomethylpyridinium)-3-(3-carbamoyl-pyridinium)-2-oxa-propane dichloride)].

In Vivo Experiments. To test protection from a POX challenge, FVB/N mice (6–8 wk-old male, 23–30 g, Charles River Laboratories) were i.v. injected (tail vein) with various amounts (0–2.5 mg/
animal) of pBChE in 100 μL of 0.9% saline, and actual serum BChE levels were determined at 5 min after injection (blood drawn by tail nick) and results and actual enzyme doses were determined. At 10 min after pBChE injection, mice were challenged with POX (14–19 μg/animal, i.v.) and then observed for 15 min. Molar concentrations were calculated taking body weight into account and assuming blood constitutes 7% of body weight. Molar ratios ([BChE]/[POX]) of the administered doses were calculated and ranged between 0 and 0.51. Symptom ranking was 0, asymptomatic; 1, decreased motor activity (lack of investigation of surroundings, retreating to a single area in cage); 2, tremors or fasciculation; 3, convulsions involving persistent flexion of the spine; 4, death (defined by lack of motor activity, pallor, and absence of cardiac auscultation for 1 min). No supportive measures were given, and mice that displayed severe signs of poisoning at 15 min after challenge were euthanized.

The protective efficacy of WT pBChE against GD was assessed in male Hartley guinea pigs (Hartley, 250–350 g; Charles River

Laboratories) injected with 26.15 mg/kg of enzyme via carotid catheter, followed 5 min later by exposure (s.c.) to $2 \times LD_{50}$ (56 μg/kg) of GD. Animals were observed for at least 2 h, and survival was scored at 2 and 24 h after exposure. For efficacy experiments using G117H/E197Q pBChE, the same experimental paradigm was used, and animals were exposed to $2 \times LD_{50}$ of GD, VX (18 μg/kg), or GB (87 μg/kg) or $4.5 \times LD_{50}$ of GD (112 μg/kg). GB, GD, and VX were diluted to between 0.3 and 1.0 mM in saline, and were injected subcutaneously into guinea pigs in volumes of 20–200 μL per animal.

To determine circulatory biological half-life of pBChE in guinea pigs ($N = 2$ per dose), we injected animals with pBChE at 17.6 or 8.8 mg/kg through a carotid catheter. Blood samples were collected through toenail clips at the indicated times, then analyzed for BChE activity. Endogenous BChE activity was subtracted from the data shown. Pharmacokinetics studies of pBChE in mice were conducted as previously described (3).

Experiments were approved by the Institutional Animal Care and Use Committees of Arizona State University and of the US Army Medical Research Institute for Chemical Defense.

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