

**Identification of Human Butyrylcholinesterase Organophosphate-Resistant Variants  
through a Novel Mammalian Enzyme Functional Screen**

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## **Running Title**

### **Molecular evolution of human BChE OP-resistant variants**

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#### **This manuscript contains:**

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238 words in Abstract;

745 words in Introduction;

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#### **Abbreviations:**

OP, organophosphate; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; ETP, echothiophate; GB, sarin; GD, soman; GF, cyclosarin; GA, tabun; VX, (S-[2-(diisopropylamino)ethyl]-O-ethyl methylphosphonothioate); ATC, acetylthiocholine iodide; BTC, butyrylthiocholine iodide; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); pAD, adenovirus plasmid vector; i.u., infection unit; MOI, multiplicity of infection.

## Abstract

Human butyrylcholinesterase (hBChE) is currently being developed as a detoxication enzyme for the catalytic hydrolysis or stoichiometric binding of organophosphates (OPs). Previously, rationally-designed hBChE mutants (i.e., G117H and E197Q) were reported in the literature and showed the feasibility of engineering OP hydrolytic functional activity into hBChE. However, the OP hydrolysis rate for G117H is too low for clinical utility. Additional OP-resistant hBChE variants with greater hydrolysis rates are needed as OP nerve agent countermeasures for therapeutic utility. As described herein, a directed molecular evolution process was used to identify amino acid residues that contribute to OP-resistant functional activity of hBChE variants. In this report, we describe the development and validation of a novel method to identify hBChE variants with OP-resistant functional activity (i.e., decreased rate of OP-inhibition). The method reported herein utilized an adenoviral protein expression system combined with a functional screening protocol of OP nerve agent model compounds that have been shown to have functional properties similar to authentic OP nerve agent compounds. The hBChE screening method was robust for transfection efficiency, library diversity, and reproducibility of positive signals. The screening approach not only identified the previously reported hBChE G117H variant, but also identified a series of additional hBChE variants, including hBChE G117N, G117R, E197C and L125V, that exhibited OP-resistant functional activities not reported before. The mammalian functional screening approach can serve as a cornerstone for further optimization and screening for OP-resistant hBChEs for potential therapeutic applications.

## Introduction

The primary physiologically relevant target of organophosphate (OP) nerve agents and pesticides in the central nervous system (CNS) is acetylcholinesterase (AChE), and a prominent target in the periphery is butyrylcholinesterase (BChE) (Gupta 2006). Currently available treatment for OP nerve agent poisoning includes combined administration of a cholinesterase (ChE) reactivator (i.e., an oxime), a muscarinic receptor antagonist (i.e., atropine), and an anticonvulsant (i.e., diazepam) (Fleisher and Harris 1965; Harris et al. 1966; Munro et al. 1990). These treatments only act in a competitive fashion and are not adequate because they do not prevent brain damage and incapacitation. One attractive approach is to administer a detoxication enzyme that not only binds the OP but also hydrolyzes OP nerve agents before they reach the CNS and cause irreversible damage. Human BChE (hBChE) represents an ideal enzyme for enzyme supplementation therapy because it doesn't require cofactors, is soluble and highly functional at the pH of the plasma, and the products of its action are generally non-toxic. Further, wild type hBChE has been developed as a catalytic or stoichiometric OP detoxication scavenger (Lenz et al. 2010). hBChE scavenges low doses of OP nerve agents by forming covalent bonds with these agents using a similar mechanism as AChE. Rationally-designed OP-resistant hBChE variants (i.e., G117H and E197Q) were reported and showed the feasibility of engineering OP hydrolytic functional activity into hBChE<sup>8</sup>. However, the OP hydrolysis rate of hBChE G117H is too low for clinical utility. OP-resistant hBChE variants with greater hydrolysis rates are needed as nerve agent countermeasures.

Previous biochemical efforts employed to search for a ChE designed to efficiently facilitate OP nerve agent hydrolysis have mainly focused on mutagenesis of selected amino acids based on X-ray structure analysis (Millard et al. 1995; Shafferman et al. 1996; Lockridge et al. 1997; Masson

et al. 1997; Millard et al. 1998; Schopfer et al. 2004). Those studies have provided insight regarding the interactions between OP compounds and cholinesterase enzymes. For example, introduction of a histidine side chain (i.e., G117H) near the active site S198 increased spontaneous enzyme dephosphorylation (Millard et al. 1995; Lockridge et al. 1997). Introduction of E197Q into an hBChE variant, was shown to slow down enzyme aging, a process where OP-adducts lose one or more of their alkyl moieties by spontaneous hydrolysis (Millard et al. 1998; Li et al. 2007). The hydrolysis rate for soman, sarin and VX is increased 200-, 1400-, and 4700-fold, respectively, for the G117H/E197Q double mutant variant (Millard et al. 1998). In addition, both D70 and W82 of hBChE were found to be important for irreversible aging (Masson et al. 1997). It was proposed that W86 of AChE contributes to the aging process by stabilizing the putative carbonium ion on the 1,2,2-trimethylpropyl moiety of soman, and E202 and F338 apparently contribute to the aging process by stabilizing the imidazolium ion of the catalytic triad H447 (Shafferman et al. 1996). Mutations of F338, W86, N74, and mutants of the hydrogen-bond network E202, E450, Y133 in AChE all showed resistance to aging (Shafferman et al. 1996). Apparently, the molecular basis for efficient ChE OP hydrolytic functional activity is distributed throughout a number of residues in the primary sequence of the protein.

As described herein, we employed a directed molecular evolution approach to identify residues of hBChE that can markedly improve its OP hydrolytic functional activity. In this report, we describe the development and validation of a novel screening method that identified hBChE variants with a decreased rate of inhibition by OPs. Because the use of chemical warfare agents is strictly regulated, we utilized nerve agent model compounds that have been shown to have similar functional properties to authentic OP compounds (Barakat et al. 2009; Gilley et al. 2009). OP model compounds not only served as a useful means to screen hBChE variants in molecular

evolution screening, but also was used in kinetic studies of variants identified (Ralph et al. 2011). The hBChE screening method was assessed for its robustness, including transfection efficiency, library diversity, and reproducibility of positive signals. The method not only could identify the known hBChE G117H variant from among the library members, but also identified G117N, G117R, E197C and L125V hBChE variants that have not been reported before. With the help of a newly developed His-tag protein purification procedure (Ralph et al. 2011), we investigated the kinetics of many identified hBChE variants. The molecular evolution screening method described herein thus can serve as a cornerstone for further optimization and screening for OP-resistant hBChEs in a mammalian protein expression system.

## **Methods**

### **Biological and chemical reagents**

Butyrylthiocholine iodide (BTC), acetylthiocholine (ATC) and 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO). BES-Thio (2, 4-dinitrobenzenesulfonyl fluorescein) was synthesized following procedures described previously (Maeda et al. 2005). Goat anti-rabbit antibodies conjugated with horseradish peroxidase enzyme (HRP) and Supersignal West Pico Chemiluminescent substrate was purchased from Pierce (Rockford, IL). HRP-conjugated goat anti-adenovirus serum was purchased from ViroStat (Portland, ME). Buffers and solvents were purchased from VWR Scientific, Inc. (San Diego, CA) in the highest purity commercially available. Molecular biology reagent was purchased from Life Technologies (Carlsbad, CA) unless otherwise specified. Highly purified hBChE, anti-BChE polyclonal antibodies, echothiophate iodide (2-[(Diethoxyphosphinyl)-thio-N,N,N-trimethylethanaminium iodide, ETP), and original wild type

hBChE cDNA constructs in pRC/CMV encoding the full length wild type hBChE, G117H single mutant, and G117H/E197Q double mutants were generously provided by Dr. Oksana Lockridge (University of Nebraska Medical Center, Omaha, NE). The nerve agent model compounds *S<sub>p</sub>*-*O*-cyclohexyl *S*-methyl methylphosphonothioate hydrochloride) (*S<sub>p</sub>*GF-Met), *R<sub>p</sub>*-*O*-cyclohexyl *S*-methyl methylphosphonothioate hydrochloride) (*R<sub>p</sub>*GF-Met), *S<sub>p</sub>*-*O*-isopropyl *S*-(2-trimethylammoniummethyl) methylphosphonothioate iodide) (*S<sub>p</sub>*GB-C) and *S<sub>p</sub>*GA-C were synthesized as previously described (Berman and Leonard 1989; Barakat et al. 2009). The nerve agent model compounds are toxic and should be handled with extreme care. Chemical waste containing these nerve agent model compounds was degraded by hydrolysis by overnight incubation with 2.5 M NaOH and 10% ethanol before disposal.

## Cell culture

HEK 293A cells were purchased from ATCC (Manassas, VA). HEK 293A cells were cultured in DMEM medium (Life Technologies) containing 8% FBS (Life Technologies) during passage and seeding. The media was exchanged to serum-free media when culture media was used for enzyme assays to avoid interference from bovine AChE from the serum. The adenovirus vector system is based on the adenovirus serotype 5 genome that contains no E1 and E2A regions (Gorziglia et al. 1996). For recombinant virus packaging, two cell lines were used, 293A and EC7, both of which carry the E1 and E2A gene. These features allowed the production of functional infective, yet replication-defective adenoviral vectors. The CHO-cTA-CAR suspension cell line was kindly provided by Dr. Ronald Gilbert (Biotechnology Research Institute, National Research Council, Montreal, Canada) (Gaillet et al. 2007). CHO-CAR suspension cells were cultured in CD-CHO medium (Life Technology).

## Construction of hBChE mutation libraries

One site-saturation mutation library (library 0) and six random mutation libraries (libraries 1-6), were constructed for the current study using PCR, cloning, and transfection procedures. First, mutations were introduced through PCR amplification using mutation primers (**Supplemental Table 1**). Site-saturation mutagenesis at DNA corresponding to amino acids G117 and E197 of *hBChE* was achieved through PCR using oligonucleotide primers listed in **Supplemental Table 1**. Three separate PCR reactions were done using the primer pairs KpnI\_F/117\_R, 117\_F/197\_R and 197\_F/XhoI\_R. The PCR products obtained were extracted from an agarose gel and the purified fragments were combined and used as templates in PCR reactions using primer pair KpnI\_F/XhoI\_R to produce full length hBChE genes. For random mutation library 1, two separate PCR reactions using primer pairs Kpn\_F/I\_R, and I\_F/Xho\_R were used. The PCR products obtained were extracted from the agarose gel and combined as a template in PCR reactions using primer pair KpnI\_F/XhoI\_R to produce a full length hBChE gene. Random mutation libraries 2-6 were generated through PCR as described above using corresponding primers listed in **Supplemental Table 1**.

The full length PCR fragments from each library were cloned into pENTR1A vector (Life Technologies) through KpnI and XhoI sites and transformed into the electro-competent 10B cells (Bio-Rad, Hercules, CA). Twenty random clones were sequenced to verify the introduction of the mutation. Approximated 5000 colonies from the pENTR1A cloning transformation were pooled for plasmid preparation to obtain mutation libraries in pENTR1A. A LR Clonase II (Life Technologies) recombination reaction was done between the pENTR1A-BChE library and the pAd/CMV/V5-DEST vector following the manufacturer's instructions (Life Technologies).



Recombination reaction products were transformed into electro-competent 10B cells (Bio-Rad). Twenty clones were randomly selected and sequenced to verify the presence of the mutation. Approximated  $10^5$  colonies from the recombination transformation were pooled for plasmid preparation to obtain the mutation library in pAD.

Generation of recombinant adenovirus using ViraPower Adenoviral Gateway Expression system was achieved following the manufacturer's instructions (Life Technologies). Briefly, adenovirus plasmids encoding hBChE genes were linearized with Pac I digestion and transfected into EC7 cells pre-seeded in 10 cm culture plates using Lipofectamine 2000. Recombinant virus generation was monitored based on the appearance of a cytopathic effect (CPE). Cells were lysed with three freeze-thaw cycles and recombinant viruses released into the medium were collected by filtration through a 0.22  $\mu$ m filter. The primary recombinant AD library stock was stored in -80 °C in small aliquots.

Recombinant virus encoding wild type (WT) hBChE and G117H variant enzymes were generated through subcloning hBChE gene fragments into *KpnI* and *XhoI* sites of pENTR1A, recombination into pAd/CMV/V5-DEST, and transfection after Pac I linearization was done as described for the library construction except that single colonies were isolated at each stage of the cloning process.

### **Virus titer determination and amplification**

For determination of virus titer, HEK 293A cells were seeded into 48 well plates and incubated overnight at  $2 \times 10^4$ /well in 0.2 mL 7% FBS/DMEM/well. Viral stocks were serial-diluted in 7% FBS/DMEM, and used to infect HEK 293A cells. At 48 hours post infection, the cells were rinsed with PBS and fixed by incubation with cold MeOH at -20°C for 20 min. Fixed cells were

probed with HRP-conjugated goat anti-Adenovirus serum (ViroStat, Portland, OR) diluted 1:2000 in PBS containing 1% BSA. After 1 hr incubation, the plate was thoroughly washed with PBS and stained using Metal Enhanced DAB Substrate Kit (Thermo Scientific, Rockford, IL) following the manufacturer's instructions. The number of stained cells was counted with a microscope to calculate the virus titer. Virus collected post transfection was generally in the range of  $10^7$ - $10^8$  infection unit/mL (i.u./mL).

### **Solid-phase screen for OP-resistant variants**

To screen for OP-resistant hBChE variants, HEK 293A cells were infected with a 0.02-0.002 multiplicity of infection (moi) recombinant AD library. At 24 h post infection, cells were washed with PBS to remove unbound virus and serum. The cells were then coated with 1% agarose in serum-free MEM pre-melted and cooled to 50°C. The agarose coating was allowed to solidify at room temperature, and the culture plates were incubated for 3 days (37 °C) to allow local virus amplification as well as virus-mediated hBChE library expression. Culture plates were incubated with 20-200  $\mu$ M of a specific OP for 2-6 h. Post OP inhibition, substrate BTC (200  $\mu$ M) and detection reagent (1  $\mu$ M DTNB or 1  $\mu$ M Bes-Thio) were applied in a top layer of the 1% agarose plates. Enzymes not inhibited by OP incubation that hydrolyzed BTC produced a yellow color signal. Single spots were readily identified by visualization by eye for the yellow color developed from the Ellman reagent-based detection. Agarose plugs containing adenovirus were cored out using a wide-orifice pipette tip and stored in PBS. Gel plugs served as a starting point for material in the following liquid-phase screening.

### **Liquid-phase screen for OP-resistant variants**

For confirmation of OP-resistant hBChE variants identified from the solid-phase screens, isolated virus was amplified by infecting HEK 293A cells. The hBChE gene products from the virus identified in the screen as “hits” were amplified through PCR using primer pairs KpnI\_F/Xho\_R and then prepared for sequence analysis. The enzyme variant that was identified as a “hit” was expressed through infection in 500  $\mu$ l CHO-CAR suspension cells at a density of  $5 \times 10^5$  cells/mL in a 96-well deep well format using amplified virus. Culture medium that contained expressed enzyme variants was assayed for BTC functional activity and OP inhibition resistant functional activity in clear bottom 96-well plates. Briefly, 5  $\mu$ L medium was incubated with a specified OP at different concentrations for different lengths of time. Residual enzyme activity was determined by addition of Ellman reaction mix to provide 1 mM BTC and 200  $\mu$ M DTNB in 50 mM sodium phosphate buffer pH 7.2. The incubation was followed photometrically. The slope of the absorbance change over 10 min for each incubation was monitored using a Victor<sup>2</sup> plate reader (Perkin Elmer, Waltham, MA) at 405 nm through continuous consecutive readings. For each OP-resistant variant candidate, the enzyme activities ( $OD_{405}/\text{min}$ ) with and without OP treatment were compared. Variants showing no changes or slight changes (usually, wild type hBChE was completely inhibited within 20 min in the presence of OP) were picked up as OP-resistant variants and were elected to proceed to the following ‘purification’ step.

### **Purification and functional activity of hBChE variants**

Each enzyme variant with OP inhibition resistant functional activity was verified by an aqueous-phase assay. Further validation and confirmation of the functional activity was done by cloning and expression of the desired gene in pAD. Specific enzyme variants were expressed through AD infection of 50 mL CHO-CAR cells as described above. The level of enzyme expression

was determined by BTC activity measurement. To harvest the secreted protein, cell pellet was removed by centrifugation at 30,000 x g for 30 minutes. The supernatant was concentrated by using a 10 KD MWCO filter (Millipore, Bedford, MA) and dialyzed with Buffer A (20 mM sodium phosphate pH 8, 1 mM EDTA) two to three times. The proteins were concentrated through filtration and then used for kinetic analysis using BTC as a substrate.

### **Adenovirus-mediated hBChE protein expression**

Human WT BChE and G117H variant enzymes were expressed in CHO adherent or CHO-cTA-CAR suspension cells cultured in the CD CHO medium including 50 µg/mL dextran sulfate and 5 mM glutamine (Ralph et al. 2011). Cells were cultured at a density of  $5 \times 10^5$  cells/mL and infected with recombinant adenovirus at 100 moi. Infected CHO-cTA-CAR cells were incubated at 37°C for 2 days, and then transferred to 30°C for 7 days. The functional activity of the expressed enzyme was monitored photometrically with an Ellman assay by examining aliquots from the medium. The Ellman assay consisted of incubation in the presence of 1 mM BTC and 200 µM DTNB in 50 mM sodium phosphate buffer (pH 7.2) (Ellman et al. 1961). Supernatant-containing secreted enzyme was separated from cells and cell debris through centrifugation at 30,000 x g for 30 min. The supernatant was concentrated using a 50 KD Amicon ultra centrifugal filter (Millipore) according to the manufacture's protocol using a J2-21M Induction Drive Centrifuge (Beckman Coulter, Fullerton, CA). The concentrated enzyme was washed three times with Tris-HCl buffer (40 mM, pH 7.4 at 25°C). Enzyme functional activity of the concentrated enzyme was determined by an Ellman assay as described above. Details about protein purification were previously reported (Ralph et al. 2011). In brief, His-tag truncated hBChE variants were purified using a Ni-NTA Superflow Resin (50% slurry) following protocols from the manufacturer (Qiagen, Valencia, CA).

## Enzyme assays

As previously described (Barakat et al. 2009), AChE and BChE functional activity was measured spectrophotometrically (Lambda 25, Perkin-Elmer, Palo Alto, CA) with an Ellman assay (Ellman et al. 1961). Briefly, ATC or BTC was used as a substrate for AChE and BChE, respectively, at 1 mM final concentration. The incubations were carried out in 50 mM potassium phosphate buffer (pH 7.2) at 25°C in the presence of 0.2 mM DTNB. Hydrolysis was followed continuously by monitoring absorbance at 412 nm. Functional activity was calculated using the molar extinction coefficient of 13,600 M<sup>-1</sup>cm<sup>-1</sup> (Ellman et al. 1961)

## Determination of inhibition rate constants ( $k_{inh}$ ) for AChE and BChE

To prevent loss of ChE enzyme activity during incubation, 30 µg/ml of bovine milk  $\beta$ -lactoglobulin was included in the enzyme-inhibitor incubation mixture. Nerve agent model compounds were placed in acetonitrile (ETP, Paraoxon) or DMSO (*SpGD*, *SpGB*, *SpGF*, or *SpGAc*) and then diluted in H<sub>2</sub>O before use. Final solvent concentration in the inhibition mixtures was < 5%. Control experiments showed no impact on enzyme activity with 5% solvent or less in the incubation mixture (i.e., BChE or AChE incubated with 30 µg/ml bovine milk  $\beta$ -lactoglobulin in 10 mM Tris buffer pH 7.6 at 25°C plus 5% acetonitrile or DMSO). The inhibition mixtures were incubated at 25°C, and aliquots were withdrawn at defined times (every 2 min) to determine functional activity. The rates of BTC hydrolysis were measured using an Ellman assay described above in Enzyme assays. The observed apparent rates of BTC hydrolysis were fit to a single-phase decay (using Prism software, GraphPad Prism, San Diego, CA) as a function of the incubation time with OP to yield apparent rates of inhibition ( $k_{inh}$ ).

## Data analysis

In the enzyme kinetic studies, the data was analyzed with GraphPad Prism version 5.01 (GraphPad Inc).

## Results

### Design and construction of highly diversified mutation libraries of human BChE

As shown in the cartoon model (**Figure 1A**), the active site of hBChE contains a traditional esterase catalytic triad S198-E325-H438 that lies near the bottom of a deep and narrow gorge (**Figure 1A**). Because a successful molecular evolution approach is dependent on the diversity and size of the random mutagenesis library, six critical structurally relevant regions were selected for targeted random mutagenesis (**Figure 1B**). The design of the libraries incorporated structural information known about hBChE (Cokugras 2003) and allowed generation of a set of focused mutation libraries. Together with previously identified mutation sites (i.e., G117 and E197), that were used as a positive control (library 0, an independent library distinct from libraries 1-6, **Figure 1B** to validate the overall functional screening process), we designed 7 libraries in total (**Figure 1B**) that covered different regions of the active site pocket of hBChE as shown in **Figure 1B**.

### PCR mutagenesis and library diversity validation

As shown in **Figure 2**, the hBChE library construction involved four stages: 1) introduction of mutations through PCR using doped oligonucleotides; 2) subcloning of PCR library fragments into the shuttle vector pENTR1A; 3) transfer of the mutation library from pENTR1A to pAD through recombination; and 4) packaging of the mutation libraries in pAD in recombinant AD particles through transfection. In stage 1, mutagenesis of the *hBChE* gene was done through PCR with primers that had proportionally doped nucleotides (i.e., 96% wild type, 4% NNK) to

introduce random amino acid substitutions into the target regions. Mutagenesis primers used were listed in **Supplemental Data Table 1**. As indicated in **Supplemental Data Table 1**, oligonucleotides synthesized were 96% wild type and 4% doped with NNK for each amino acid codon (i.e., MNN for antisense primers). In practice, the doped primers contained nucleotide changes that could be translated into approximately 1-2 random amino acid substitutions among the 120 targeted residues within each of the six primers. The diversity of library 0 was defined to be 1024 (i.e., saturation mutagenesis at 2 positions by incorporating NNK was  $32 \times 32 = 1024$ ). The target diversity of the mutation libraries 1-6 was also ~1000 (i.e., randomization of 20 residues through 1-1.5 mutations). The number of colonies recovered at each stage of the subcloning steps (i.e., stages 2 and 3) were >5000 colonies for library 0 and  $>10^4$  colonies for libraries 1-6 and ensured a good statistical coverage of the diversity of the libraries.

At stage 4, for adenovirus packaging, a titer of  $>10^7$  infection units (i.u.) was routinely achieved at 4 days post-transfection for a typical 10 µg linearized pAD library DNA. The results of parallel control transfection studies with a spike of 0.1% (g/g) of a pAD-LacZ construct allowed consistent recovery of pAD-LacZ. This result confirmed that >1000 virus packaging events/transfection was achieved with HEK293A cells to ensure that the complexity of the library was maintained at the recombinant virus stage. The diversity of the mutation library was also verified based on DNA sequence analysis of Stage 1 PCR products by sequencing random colonies from stage 2 and 3 subclones, and from sequencing DNA from random virus after limited dilution. Representative sequencing results (**Table 1**) showed that the mutation rate was 1.8% in nucleotides and 3.8% in amino acids.

### Functional screening for OP resistant hBChE variants

In the functional screening assay for hBChE variants possessing OP resistance (i.e., decreased rate of inhibition by OP), (steps 1-5, **Figure 3**), both ‘solid phase screening’ and ‘liquid phase screening’ techniques were used with full-length hBChE. In solid phase screening (step 1, **Figure 3**), gel plugs that gave bright yellow positive signals from the Ellman assay (i.e., plates were incubated with OP for 2 to 6 hours, followed by incubation with 1 mM butyrylthiocholine (BTC) and 1 mM of DTNB, that gave rise to the yellow color of 2-nitro-5-thiobenzoate when the disulfide bond of DTNB was cleaved by the thiol group of BTC) were identified and isolated by punching a hole on the agarose gel with large pore pipettes. To confirm the positive Ellman assay results from solid phase screening, a liquid-based method was developed. Virus particles isolated from gel plugs were used to infect cells cultured in 96 well plates to get amplified virus and then used to confirm the observed OP resistance in an Ellman assay. This method was referred to as ‘liquid screening’ (step 2, **Figure 3**). In liquid phase screening, amplified virus was serially diluted and used for functional BTC hydrolysis activity assays. The assays were done in the presence of 1 mM BTC and 1 mM DTNB for 10 min in 50 mM PBS buffer (pH 7.4). Virus from the incubation where enzyme was expressed that showed OP inhibition on the basis of the BTC assay was diluted, re-infected and amplified cells were used to isolate DNA. The viral DNA was sequenced to identify the sites where mutagenesis was observed (steps 3-5, **Figure 3**).

## Mutagenesis Studies

The initial hBChE molecular evolution library constructed, (i.e., library 0), was a site-saturated mutagenesis control library targeting two residues simultaneously (i.e., both G117 and E197 were mutagenized at the same time). Amino acid substitutions at these positions of hBChE, including G117H and G117H/E197Q have previously been shown to be associated with acquired OP-resistant activity as well as a low level of OP hydrolysis functional activity (Lockridge et al.



1997; Millard et al. 1998). Thus, positive identification of known variants from library 0 served as a validation of the entire design process. Two compounds used for screening of library 0 were nerve agent GF model compound enantiomers (i.e., *S<sub>p</sub>*GF-Met and *R<sub>p</sub>*GF-Met) (**Figure 4A and B**) that were described previously (Barakat et al. 2009; Gilley et al. 2009). These nerve agent model compounds recapitulate the behavior of authentic GF quite well with the exception that they are less toxic. In good agreement with the actual nerve agent enantiomers, the *Sp*GF-Met isomer preferentially inhibited hBChE compared to the *Rp*GF-Met enantiomer (Barakat et al. 2009). Briefly, the solid phase screen was done as follows: 20  $\mu$ M *S<sub>p</sub>*GF-Met was mixed with agarose and placed on a 10-cm plate (distributed uniformly over the plate) and incubated with library for 0 for 30 minutes, followed by incubation in the presence of 1 mM BTC and 1 mM DTNB for 2-6 hours. Gel spots showing a bright yellow color were judged to be positive and yellow plugs were isolated with large pore pipettes. The virus DNA was extracted from the plugs with a ViroStat kit and prepared for sequence analysis. After confirmation by sequence analysis of the viral DNA from the yellow plugs a few “hits” were identified (**Table 2**). Based on sequence analysis, some of the “hits” contained G117H mutations. To identify additional variants that were resistant to nerve agent model compound inhibition, libraries 1-6 were screened with *S<sub>p</sub>*GAc and the *S<sub>p</sub>* enantiomer of a nerve agent model compound for tabun (**Figure 4C**). In addition to some previously identified variants such as G117H, new variants such as G117N, G117R, E197C and L125V were identified during the screening of libraries 1-6 (**Table 2**).

In order to investigate the kinetics of the variants identified, significant amounts of hBChE variant protein was isolated and purified. However, purification of hBChE has been confounded due to low yields using currently available methodology that employ procainamide as the

binding ligand in affinity chromatography (Masson and Lockridge 2010). In addition, because hBChE variants can possess even lower affinity to procainamide than wild type enzyme a new expression and purification procedure was developed (Ralph et al. 2011). Thus, positive variants identified in the screens were cloned into pENTR1A and pAD vectors using a truncated version with a His-tag at the C-terminus. As reported previously, utilization of the His-tag facilitated protein production, purification, and robust kinetic studies (Ralph et al. 2011). By using this approach, relatively large amounts of highly purified hBChE variant protein were obtained that was used in kinetic studies.

### Kinetic studies of hBChE variants identified from screening libraries 0-6

As shown on the basis of the data in **Table 3**, hBChE variants including G117H, G117R, G117N, E197C, and L125V that were identified using the OP-resistance functional screening method described above were evaluated using *in vitro* kinetic studies. In contrast to the screening process described above, for kinetic analysis of the variants identified it was more expedient to use His-tag truncated hBChE variants. Highly purified His-tag truncated hBChE variants including G117H, G117R, G11N and E197C from the site-saturation mutagenesis library 0 were analyzed for the rate of substrate hydrolysis using a modified Ellman assay. Representative progress curves for hydrolysis of BTC for the identified variants G117H, G117R, G11N and E197C are shown in **Figure 5A-C** and representative progress curves for hydrolysis of acetylthiocholine (ATC) are shown in **Figure 5D-F**. Kinetic parameters for the variants identified were determined by fitting the observed rates of hydrolysis for both substrates to the Webb equation as described previously (Ralph et al. 2011). The hBChE variants G117R, G117N, and E197C (i.e.,  $K_m$  values = 490  $\mu$ M, 900  $\mu$ M and 210  $\mu$ M, respectively) showed a greater  $K_m$  for BTC relative to wild type hBChE (i.e.,  $K_m$  = 23  $\mu$ M) and the previously

characterized hBChE variant, G117H (i.e.,  $K_m = 220 \mu\text{M}$ , **Table 3**, (Lockridge et al. 1997)). Likewise, the  $K_m$  for hydrolysis of ATC was greater for the hBChE variants G117R, G117N, and G117H (i.e.,  $K_m = 600 \mu\text{M}$ ,  $190 \mu\text{M}$ , and  $1200 \mu\text{M}$ , respectively) but the  $K_m$  for the E197C variant was somewhat similar ( $K_m = 91 \mu\text{M}$ ) to wild type enzyme ( $K_m = 25 \mu\text{M}$ ). The greater  $K_m$  observed for hBChE variants with a mutation at 117 (i.e., G117R, G117H, and G117N) was likely due to steric hindrance of the amino acid residue of the variants (i.e., R117, H117 and N117) that afforded a decreased binding affinity for both ATC and BTC. The kinetic parameters for the L125V hBChE variant identified from random-mutagenesis library 2 (**Figure 1, Figure 6**) were determined as described above for the site-saturation variants. Compared to wild type hBChE ( $K_m = 23 \mu\text{M}$ ) the  $K_m$  for BTC hydrolysis was 65-fold greater for hBChE L125V ( $K_m = 1500 \mu\text{M}$ ). In contrast, the  $K_m$  for ATC hydrolysis by hBChE L125V was similar to that for wild type hBChE (**Table 3**).

hBChE variants identified in the screen (i.e., G117H, G117R, G117N, and L125V) were also evaluated for their resistance to inhibition by different OPs. Inhibition kinetics of hBChE variants (i.e., G117H, G117R, G117N, and L125V) were determined for several nerve agent model compounds and two OP pesticide compounds including *SpGD*, *SpGB*, *SpGAc*, *SpGF*, echothiophate and Paraoxon (Gilley et al. 2009). The hBChE 117 variants examined (i.e., G117H, G117N, G117R) possessed inhibition rate constants below the limit of detection for the assay (i.e.,  $0.02 \text{ min}^{-1} \mu\text{M}^{-1}$ ). Both the hBChE variants L125V and E197C had measureable  $k_i$  values that were lower than that for wild type BChE (**Table 3**).

## Discussion

In a previously published study, it was reported that rationally designed variants of hBChE (i.e., G117H and E197Q) could hydrolyze  $P_{R/S}C_R$ ,  $P_S C_S$ , and  $P_R C_S$  soman stereoisomers with apparent rate constants of 0.006, 0.077, and 0.128  $\text{min}^{-1}$ , respectively (Millard et al. 1998). Thus, while hBChE variants have the potential to show OP hydrolase functional activity (Millard et al. 1995; Millard et al. 1998), the OP hydrolysis rate for soman is too low for practical use or clinical utility. Previous efforts were focused on using a rational design based on the hBChE protein structure to design and develop an OP hydrolase (Shafferman et al. 1996; Lockridge et al. 1997; Saxena et al. 1997; Millard et al. 1998; Cokugras 2003).

Using a molecular evolution approach to efficiently identify OP-resistant variants, a screening method was designed and developed to begin to identify amino acid residues involved in hBChE OP hydrolase functional activity. As an unbiased approach, a directed molecular evolution screen is an ideal approach to identify individual and multiple amino acid residues that are required to markedly improve OP hydrolytic functional activity of hBChE. In contrast to evolution of bacterial OP hydrolase (Cho et al. 2002), molecular evolution of hBChE is more challenging because hBChE is a glycoprotein that requires mammalian cell expression for functional activity (Millard and Broomfield 1992). Accordingly, development of an efficient expression system to identify OP-resistant variants using a functional screening approach was developed in mammalian cells.

Thus, we developed a procedure that included: 1) high level protein expression in the intended screening system; 2) an efficient cloning method to construct the mutation library; and 3) an efficient gene recovery method so that after the functional screen, the identification of the DNA sequence and any variants could be readily accomplished. Our screening method used an adenoviral protein expression system combined with nerve agent model compound functional

screening to identify OP-resistant hBChE variants. Following the identification of positive variants, we used His-tag truncated versions of the variant hBChEs to facilitate the purification of mutant variants that are difficult to purify using traditional procainamide column chromatography (Ralph et al. 2011). This method therefore met all of the conditions required for an efficient screening system. Successful expression of active hBChE with an adenoviral vector has been reported (Gao et al. 2005) and we optimized the use of an adenovirus system to develop the functional screening approach reported herein.

An analysis of the literature did not reveal any reports of any similar attempts to identify OP-resistant variants using a screening procedure involving a mammalian protein expression system. Compared to a rational design approach, the molecular evolution approach is suitable for the screening of single or multiple amino acid variants that have novel functional activities. In mammalian protein expression systems, protein folding, post-translational modification, and glycosylation are efficient and correct, compared with a prokaryotic approach (Blagodatski and Katanaev 2011). It is therefore of a significant advantage to use a mammalian protein expression system and adenoviral approach to identify OP resistant variants in a molecular evolution screen. However, according to our experience, library packaging efficiency is still not ideal, with capacity reaching to just 1,000-2,000 variants/construction. This limitation limited the mutagenesis library diversity and therefore the final outcome of the mutation strategy.

One of the challenges in directed molecular evolution is that variants that have ideal functional activity are rarely present even with carefully designed libraries, because it is believed that novel function of a protein often occurs by incremental single amino acid changes (Tracewell and Arnold 2009). Based on the work reported here, this appears to be particularly true in the case of the hBChE variants identified herein. For example, the well-known variant G117H was

identified not only from the site saturation mutagenesis library (library 0) as expected, but also from library 1, which was irrelevant to amino acid position 117 (**Figure 1**).

Combined with available structural information for hBChE, we designed 6 libraries to cover most regions that were known to be important in the active site of hBChE or that impinged on the active site of hBChE (**Figure 1**). These regions not only included the gorge/substrate binding regions, but also included the active site itself that has the catalytic triad S198-D325-H438 and regions that can facilitate substrate binding (i.e., PAS/Omega loop and Acyl pocket). However, after functional screening, we identified only a handful of OP resistant variants (**Table 2, Figure 7**). Furthermore, most of them (11 out of 14) were recognized as previously reported mutation sites (i.e., G116, G117 or E197) (Millard et al. 1995; Lockridge et al. 1997), suggesting the importance of those single amino acids in mediating protein functional change during directed evolution. Amino acid 117 in hBChE is part of the oxyanion hole (i.e., G116, G117 and A199) that can stabilize the substrate or the transition state during ester hydrolysis (Lockridge et al. 1997).

When Gly 117 is replaced by Arg (G117R) or Asp (G117N), OP resistance is not as strong as when a His at position 117 is present. It is possible that G117H has stronger OP resistance due to its two hydrogen bonds with substrate instead of only one in the case of G117R or G117N (Lockridge et al. 1997; Schopfer et al. 2004), **Figure 7B-D**). Although most variants identified in this study are well known hBChE variants, we did identify some new variants (i.e., L125V, N68D) that have not been reported before. Although L125V showed an increased  $K_m$  when BTC was used as substrate compared to G117H (1500  $\mu$ M vs. 220  $\mu$ M respectively, **Table 3**), it showed a very low  $K_m$  (80  $\mu$ M vs. 1200  $\mu$ M respectively, **Table 3**) when ATC was used as substrate (**Table 3**). This suggests that L125V has a different mechanism in mediating substrate

hydrolysis compared to the G117H variant (Lockridge et al. 1997). The localization of L125 in hBChE close to the entry site to the gorge (**Figure 7E**) suggests that it might function in facilitating the initial binding of substrate. The data suggests that the screening platform is valid for identifying new variants in addition to previously identified variants (i.e., G117H) and the new variants can also serve as intermediates for future screening and optimization to ultimately afford additional novel variants.

Taken together, the method reported herein not only identified the previously reported hBChE G117H variant, but also identified G117N, G11R, E197C and L125V, hBChE variants that have not been reported before. The molecular evolution screening method can serve as a cornerstone for further optimization and screening for OP-resistant hBChEs in a mammalian protein expression system.

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### **Authorship contributions**

*Participated in research design:* Zhang and Cashman.

*Conducted experiments:* Zhang and Ralph.

*Contributed new reagents or analytic tools:*

*Performed data analysis:* Zhang, Chen, Ralph, and Dwyer.

*Wrote or contributed to the writing of the manuscript:* Dwyer, Chen, and Cashman.

## References

- Barakat, N.H., Zheng, X., Gilley, C.B., MacDonald, M., Okolotowicz, K., Cashman, J.R., Vyas, S., Beck, J.M., Hadad, C.M., and Zhang, J. 2009. Chemical synthesis of two series of nerve agent model compounds and their stereoselective interaction with human acetylcholinesterase and human butyrylcholinesterase. *Chem Res Toxicol* **22**(10): 1669-1679.
- Berman, H.A. and Leonard, K. 1989. Chiral reactions of acetylcholinesterase probed with enantiomeric methylphosphonothioates. Noncovalent determinants of enzyme chirality. *J Biol Chem* **264**(7): 3942-3950.
- Blagodatski, A. and Katanaev, V.L. 2011. Technologies of directed protein evolution in vivo. *Cell Mol Life Sci* **68**(7): 1207-1214.
- Cho, C.M., Mulchandani, A., and Chen, W. 2002. Bacterial cell surface display of organophosphorus hydrolase for selective screening of improved hydrolysis of organophosphate nerve agents. *Applied and environmental microbiology* **68**(4): 2026-2030.
- Cokugras, A.N. 2003. Butyrylcholinesterase: Structure and physiological importance. *Turk Biyokim Derg* **28**(Copyright (C) 2011 American Chemical Society (ACS). All Rights Reserved.): 54-61.
- Ellman, G.L., Courtney, K.D., Andres, V., Jr., and Feather-Stone, R.M. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* **7**: 88-95.
- Fleisher, J.H. and Harris, L.W. 1965. Dealkylation as a mechanism for aging of cholinesterase after poisoning with pinacolyl methylphosphonofluoridate. *Biochem Pharmacol* **14**(5): 641-650.



- Gaillet, B., Gilbert, R., Amziani, R., Guilbault, C., Gadoury, C., Caron, A.W., Mullick, A., Garnier, A., and Massie, B. 2007. High-level recombinant protein production in CHO cells using an adenoviral vector and the cumate gene-switch. *Biotechnol Prog* **23**(1): 200-209.
- Gao, Y., Atanasova, E., Sui, N., Pancook, J.D., Watkins, J.D., and Brimijoin, S. 2005. Gene transfer of cocaine hydrolase suppresses cardiovascular responses to cocaine in rats. *Mol Pharmacol* **67**(1): 204-211.
- Gilley, C., MacDonald, M., Nachon, F., Schopfer, L.M., Zhang, J., Cashman, J.R., and Lockridge, O. 2009. Nerve agent analogues that produce authentic soman, sarin, tabun, and cyclohexyl methylphosphonate-modified human butyrylcholinesterase. *Chem Res Toxicol* **22**(10): 1680-1688.
- Gorziglia, M.I., Kadan, M.J., Yei, S., Lim, J., Lee, G.M., Luthra, R., and Trapnell, B.C. 1996. Elimination of both E1 and E2 from adenovirus vectors further improves prospects for in vivo human gene therapy. *Journal of virology* **70**(6): 4173-4178.
- Gupta, R.C. 2006. *Toxicology of organophosphate and carbamate compounds*. Elsevier Academic Press, Amsterdam ; Boston.
- Harris, L.W., Fleisher, J.H., Clark, J., and Cliff, W.J. 1966. Dealkylation and loss of capacity for reactivation of cholinesterase inhibited by sarin. *Science* **154**(747): 404-407.
- Lenz, D.E., Clarkson, E.D., Schulz, S.M., and Cerasoli, D.M. 2010. Butyrylcholinesterase as a therapeutic drug for protection against percutaneous VX. *Chemico-biological interactions* **187**(1-3): 249-252.
- Li, H., Schopfer, L.M., Nachon, F., Froment, M.T., Masson, P., and Lockridge, O. 2007. Aging pathways for organophosphate-inhibited human butyrylcholinesterase, including novel

- pathways for isomalathion, resolved by mass spectrometry. *Toxicological sciences : an official journal of the Society of Toxicology* **100**(1): 136-145.
- Lockridge, O., Blong, R.M., Masson, P., Froment, M.T., Millard, C.B., and Broomfield, C.A. 1997. A single amino acid substitution, Gly117His, confers phosphotriesterase (organophosphorus acid anhydride hydrolase) activity on human butyrylcholinesterase. *Biochemistry* **36**(4): 786-795.
- Maeda, H., Matsuno, H., Ushida, M., Katayama, K., Saeki, K., and Itoh, N. 2005. 2,4-Dinitrobenzenesulfonyl fluoresceins as fluorescent alternatives to Ellman's reagent in thiol-quantification enzyme assays. *Angew Chem Int Ed Engl* **44**(19): 2922-2925.
- Masson, P., Froment, M.T., Bartels, C.F., and Lockridge, O. 1997. Importance of aspartate-70 in organophosphate inhibition, oxime re-activation and aging of human butyrylcholinesterase. *Biochem J* **325** ( Pt 1): 53-61.
- Masson, P. and Lockridge, O. 2010. Butyrylcholinesterase for protection from organophosphorus poisons: catalytic complexities and hysteretic behavior. *Arch Biochem Biophys* **494**(2): 107-120.
- Millard, C.B. and Broomfield, C.A. 1992. A computer model of glycosylated human butyrylcholinesterase. *Biochem Biophys Res Commun* **189**(3): 1280-1286.
- Millard, C.B., Lockridge, O., and Broomfield, C.A. 1995. Design and expression of organophosphorus acid anhydride hydrolase activity in human butyrylcholinesterase. *Biochemistry* **34**(49): 15925-15933.
- . 1998. Organophosphorus acid anhydride hydrolase activity in human butyrylcholinesterase: synergy results in a somanase. *Biochemistry* **37**(1): 237-247.

- Munro, N.B., Watson, A.P., Ambrose, K.R., and Griffin, G.D. 1990. Treating exposure to chemical warfare agents: implications for health care providers and community emergency planning. *Environ Health Perspect* **89**: 205-215.
- Ralph, E.C., Xiang, L., Cashman, J.R., and Zhang, J. 2011. His-tag truncated butyrylcholinesterase as a useful construct for in vitro characterization of wild-type and variant butyrylcholinesterases. *Protein Expr Purif* **80**(1): 22-27.
- Saxena, A., Maxwell, D.M., Quinn, D.M., Radic, Z., Taylor, P., and Doctor, B.P. 1997. Mutant acetylcholinesterases as potential detoxification agents for organophosphate poisoning. *Biochem Pharmacol* **54**(2): 269-274.
- Schopfer, L.M.B., A. T.; Broomfield, C. A.; Lockridge, O. 2004. Mutants of human butyrylcholinesterase with organophosphate hydrolase activity; evidence that His117 is a general base catalyst for hydrolysis of echothiophate. *Journal of Medical, Chemical, Biological and Radiological Defense* **2**.
- Shafferman, A., Ordentlich, A., Barak, D., Stein, D., Ariel, N., and Velan, B. 1996. Aging of phosphorylated human acetylcholinesterase: catalytic processes mediated by aromatic and polar residues of the active centre. *Biochem J* **318** ( Pt 3): 833-840.
- Tracewell, C.A. and Arnold, F.H. 2009. Directed enzyme evolution: climbing fitness peaks one amino acid at a time. *Curr Opin Chem Biol* **13**(1): 3-9.

## **Footnotes**

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## Legends for Figures

**Figure 1. A depiction of mutation libraries of hBChE.** **A.** Depiction of each designed mutation library around the active site of hBChE. Left panel, three-dimensional view of the active site of hBChE. Right panel, a cartoon model showing the active site of hBChE. **B.** List of libraries and their corresponding function. Library 0 is a site-saturated mutation library. Libraries 1-6 are randomized mutation libraries designed to cover 6 structurally relevant regions around the active pocket of hBChE. Each color represents a corresponding peptide sequence that was covered by each individual library. Green, library 1; orange, library 2; pink, library 3; blue, library 4; brown, library 5; light blue, library, 6.

**Figure 2. Overview of the hBChE library construction.** The mutation library construction was divided into four stages. **Stage 1:** Both ‘site-saturation mutagenesis’ (library 0) and ‘randomized mutagenesis’ (libraries 1-6) were done through PCR using designed primers. **Stage 2:** PCR products were then cloned into a pENTR1A vector to establish a mutation library. Twenty clones from the library were randomly picked and sequenced to verify the diversity of the library (Table 1). **Stage 3:** The mutation library in pENTR1A plasmid was then exchanged to destination vector ‘pAD/CMV-DEST’ to establish an adenoviral library by conducting a clonase reaction. **Stage 4:** After transfection of HEK 293A cells with a pAD library, the adenovirus library stock was prepared after packaging in HEK 293A cells.

**Figure 3. Functional screening of hBChE variants.** In functional screening of hBChE variants, both ‘solid phase’ screening and ‘liquid phase’ screening were used. In solid phase screening, a gel plug that had a positive signal (i.e., a yellow color, indicating a positive Ellman reaction) was isolated. To confirm the results from solid phase screening, virus particles isolated from gel

plugs were used to infect cells cultured in 96 well plates to confirm OP resistance. Viruses that were confirmed to be OP-resistant variants were further purified and sequenced to identify the site of mutation. Variants were subcloned, expressed, purified and studied further in *in vitro* incubations to characterize their kinetic parameters.

**Figure 4. Chemical structures of model compounds used in screening hBChE variants. A.** Chemical structure of *Sp*GF-Met. **B.** Chemical structure of *Rp*GF-Met. **C.** Chemical structure of *Sp*GAc.

**Figure 5. Substrate-dependence for hBChE variants G117H, G117N, G117R and E197C.** **A-C.** Substrate concentration-dependent rates of BTC hydrolysis for variants G117H, G117R, G117N and E197C monitored by UV-vis spectroscopy at 405 nm. **D-F.** Substrate concentration-dependent rates of ATC hydrolysis for variants G117H, G117R, G117N and E197C monitored by UV-vis spectroscopy at 405 nm.

**Figure 6. Kinetic studies of variant hBChE L125V. A.** Substrate concentration-dependent rates of BTC hydrolysis for hBChE L125V variant. **B.** Substrate concentration-dependent rates of ATC hydrolysis for the hBChE L125V variant.

**Figure 7. Structural models of hBChE variants.** Three-dimensional models of six hBChE variants identified in the molecular evolution screen and characterized by kinetics. **A.** Wild type hBChE, **B.** G117H, **C.** G117R, **D.** G117N, **E.** L125V, **F.** E197C. The catalytic triad of the hBChE substrate binding domain is shown as a yellow backbone on the left in every image.

Substrate is shown as gray in the center. The amino acid that underwent mutation is shown with a white backbone near the bottom right corner (**B**, **C** and **D**); right middle (**E**), and bottom left (**F**). Hydrogen bonds are shown in dashed lines.

Table 1. Results of Sequencing pAD library 1

Amino acid position	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	Mutant #	
Codon	AAA	AAT	GCC	ACT	GTA	TGG	ATA	TGG	ATT	TAT	GGT	GGT	GGT	TTT	CAA	ACT	GGA	ACA	TCA	TCT	TTA	CAT	DNA	A.A
Clone 1																							0	0
Clone 2			CC Ser															GCA Ala		GCT Ala			3	3
Clone 3		AA Lys			GTT Val											GCT Ala							3	2
Clone 4			GCT Ala																				1	0
Clone 5								AAG Lys										ACC Thr					3	1
Clone 6								GGG Arg					AGT Ser										2	2
Clone 7																							0	0
Clone 8																			TAT Tyr				2	1
Clone 9	AAG Lys																						1	1
Clone 10					ATT Ile																		1	1
Clone 11													GAT Asp										1	1
Clone 12																							0	0
Clone 13																							0	0
Clone 14											GGG Gly												1	0
Clone 15																					ATA Ile		-	1
Clone 16																				TGT Cys			2	2
Clone 17													TTA Leu					TTCA Phe					2	1
Clone 18													CTT Leu										1	1
Clone 19																							0	0
Clone 20	Poor sequence quality																					-	-	
																					Total	24	17	



**Table 2. Human BChE Variants identified in their respective libraries**

<b>Variant</b>	<b>Library</b>
G117H	lib0, lib1
G117R	lib0
G117N	lib0
E197C	lib0
L125V	lib1

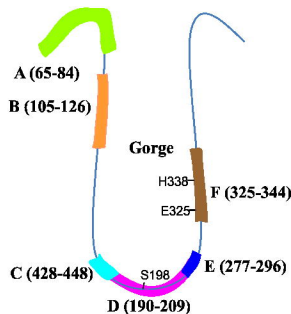
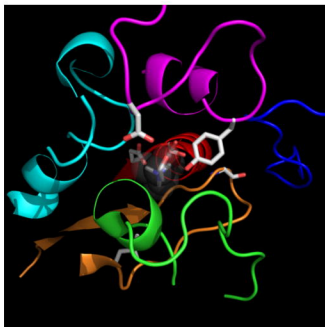
**Table 3. The effect of hBChE variants on kinetic parameters**

		<b>WT</b>	<b>G117R</b>	<b>G117H</b>	<b>G117N</b>	<b>E197C</b>	<b>L125V</b>
<b>BTC</b>	$K_m$ , $\mu\text{M}$	23 $\pm$ 12	490 $\pm$ 130	220 $\pm$ 10	900 $\pm$ 100	210 $\pm$ 40	1500 $\pm$ 100
	B	3.7 $\pm$ 0.7	1.3 $\pm$ 0.15	2.2	1*	1*	1*
	$K_{ss}$ , mM	0.74 $\pm$ 0.09	1	0.55 $\pm$ 0.06	NA	4.4 $\pm$ 0.7	NA
<b>ATC</b>	$K_m$ , $\mu\text{M}$	25 $\pm$ 17	600 $\pm$ 30	1200 $\pm$ 40	190 $\pm$ 90	91 $\pm$ 5	80 $\pm$ 20
	B	5 $\pm$ 2	1	2.7	5.7 $\pm$ 0.1	3.1 $\pm$ 0.1	3.1 $\pm$ 0.1
	$K_{ss}$ , mM	2.2 $\pm$ 0.6	NA	1.2 $\pm$ 0.2	12 $\pm$ 1	9 $\pm$ 1	9 $\pm$ 1
<b>ETP</b>	$k_i$ , $\text{min}^{-1}$ , $\mu\text{M}^{-1}$	2.0 $\pm$ 0.1	<0.02	<0.02	<0.02	1.5 $\pm$ 0.1	0.083 $\pm$ 0.004
<b>Paraoxon</b>	$k_i$ , $\text{min}^{-1}$ , $\mu\text{M}^{-1}$	1.2 $\pm$ 0.1	0.19 $\pm$ 0.01	<0.01	<0.01	0.7 $\pm$ 0.1	0.07 $\pm$ 0.01
<b>SpGD</b>	$k_i$ , $\text{min}^{-1}$ , $\mu\text{M}^{-1}$	0.20 $\pm$ 0.01	~0.001	<0.001	<0.001	0.076 $\pm$ 0.005	0.012 $\pm$ 0.004
<b>SpGB</b>	$k_i$ , $\text{min}^{-1}$ , $\mu\text{M}^{-1}$	0.12 $\pm$ 0.01	<0.004	<0.004	<0.004	0.22 $\pm$ 0.01	(0.007 $\pm$ 0.003)
<b>SpGAc</b>	$k_i$ , $\text{min}^{-1}$ , $\mu\text{M}^{-1}$	26 $\pm$ 1	<1	<1	<1	16 $\pm$ 1	1.8 $\pm$ 0.3
<b>SpGF</b>	$k_i$ , $\text{min}^{-1}$ , $\mu\text{M}^{-1}$	2.4 $\pm$ 0.1	<0.05	<0.05	<0.05	0.94 $\pm$ 0.02	0.15 $\pm$ 0.01

Data fit to the Webb equation (AKA modified Haldane equation) where B=1 yields the Michaelis-Menten equation. \* Indicated value is assigned as a constant before analysis.

# Figure 1

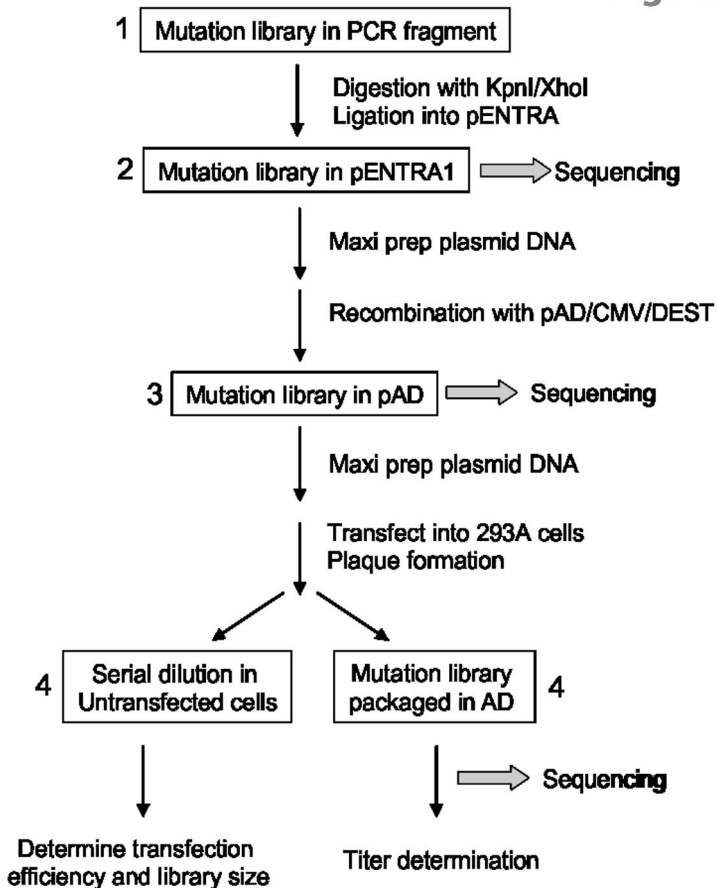
A.



B.

BChE region	Lib #	Residues	Functional Domain
B,D	0	117X,197X	SSM
A	1	65-84	PAS/ $\Omega$ loop
B	2	105-126	Gorge/Substrate binding
D	3	190-209	Active Site
E	4	277-296	Acyl pocket/Substrate binding
F	5	325-344	Gorge/Substrate binding
C	6	428-448	Gorge/Substrate binding

**Figure 2**



## Mutation library Construction

**Figure 3**

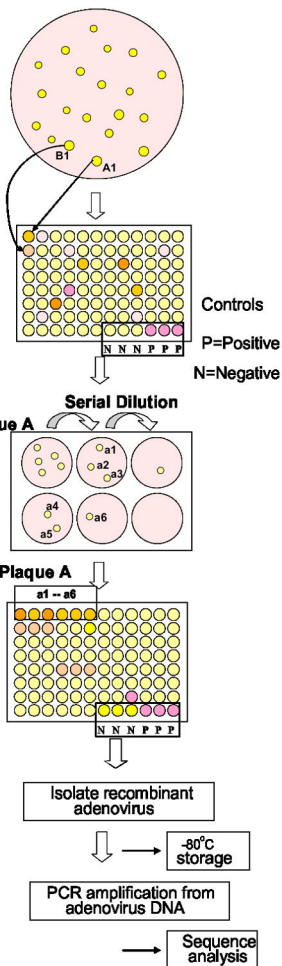
1. Solid-phase screening

2. Liquid-phase screening

3. Plaque purification

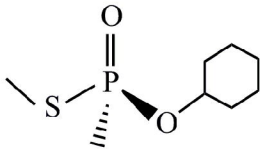
4. Confirm OP hydrolysis activity of single plaque

5. PCR evolved hBChE and sequence analysis



**Functional Screening**

A.



C.

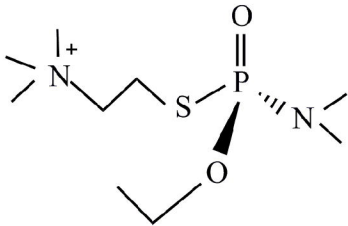
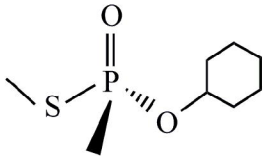


Figure 4

B.



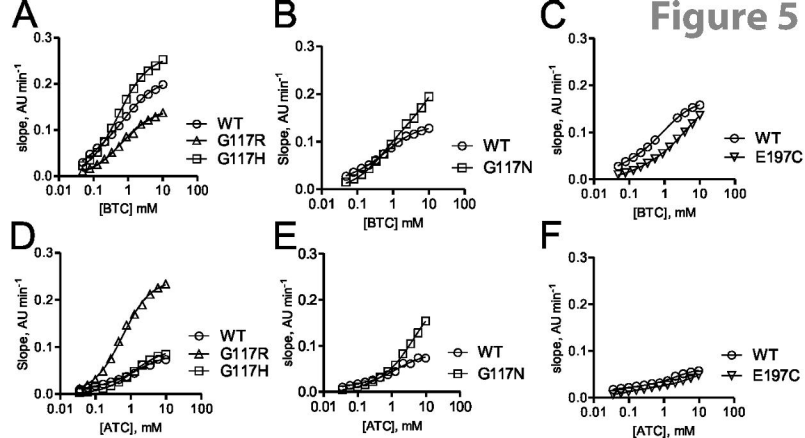
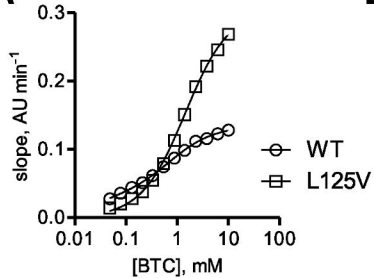
**Figure 5**

Figure 6

A



B

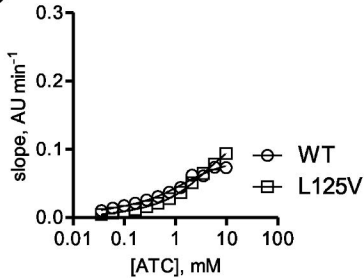




Figure 7

