Design, Preparation and Characterization of High-Activity Mutants of Human Butyrylcholinesterase Specific for Detoxification of Cocaine


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ABBREVIATIONS: BChE, butyrylcholinesterase; CocH, cocaine hydrolase; ACh, acetylcholine; AChE, acetylcholinesterase; FDA, U.S. Food and Drug Administration; MD, molecular dynamics; QM, quantum mechanics; QM/MM, hybrid quantum mechanics/molecular mechanics; TS1, transition state for the first chemical reaction step; DMEM, Dulbecco’s modified Eagle’s medium; TMB, 3, 3′, 5, 5′-Tetramethylbenzidine; ELISA, enzyme-linked immunosorbent assay; QFF, Q fast flow; NIH, National Institutes of Health; Aβ, β-amyloid; AD, Alzheimer’s disease; UNODC, United Nations Office on Drugs and Crime.
ABSTRACT

Cocaine is a widely abused drug without an FDA-approved medication. It is recognized a promising anti-cocaine medication to accelerate cocaine metabolism producing biologically inactive metabolites via a route similar to the primary cocaine-metabolizing pathway, i.e. cocaine hydrolysis catalyzed by butyrylcholinesterase (BChE) in plasma. An ideal, therapeutically valuable mutant of human BChE should have not only a significantly improved catalytic activity against (-)-cocaine, but also certain selectivity for (-)-cocaine over neurotransmitter acetylcholine (ACh) such that one would not expect systemic administration of the BChE mutant to interrupt cholinergic transmission. The present study accounting for the mutation-caused changes of the catalytic activities of BChE against both (-)-cocaine and ACh by means of molecular modeling and site-directed mutagenesis has led to identification of three BChE mutants that have not only a considerably improved catalytic efficiency against (-)-cocaine, but also the desirable selectivity for (-)-cocaine over ACh. Two representative BChE mutants have been confirmed to be potent in actual protection of mice from acute toxicity (convulsion and lethality) of a lethal dose of cocaine (180 mg/kg). Pretreatment with the BChE mutant (i.e. 1 min prior to cocaine administration) dose-dependently protected mice against cocaine-induced convulsions and lethality. In particular, all mice pretreated with the mutant (e.g. 0.02 mg or more A199S/F227A/S287G/A328W/E441D BChE) survived. The in vivo data reveal the primary factor, i.e. the relative catalytic efficiency, determining the efficacy in practical protection of mice from the acute cocaine toxicity and future direction for further improving the efficacy of the enzyme in the cocaine overdose treatment.
Introduction

Cocaine is a well-known, widely abused drug (UNODC, 2010). There is no FDA-approved medication specific for cocaine abuse treatment. The disastrous medical and social consequences of cocaine abuse have made a high priority the development of an anti-cocaine medication (Karila et al., 2008; Xi and Gardner, 2008). It would be an ideal anti-cocaine medication to accelerate cocaine metabolism producing biologically inactive metabolites via a route similar to the primary cocaine-metabolizing pathway, i.e. cocaine hydrolysis catalyzed by butyrylcholinesterase (BChE) in plasma (Carrera et al., 2004; Gorelick, 2008; Kamendulis et al., 1996; Landry et al., 1993; Meijler et al., 2005; Zhan et al., 2005). Unfortunately, wild-type BChE has a low catalytic activity against naturally occurring (-)-cocaine ($k_{cat} = 4.1$ min$^{-1}$ and $K_M = 4.5$ μM) (Darvesh et al., 2003; Gatley, 1991; Giacobini, 2003; Hamza et al., 2005; Sun et al., 2002a). It is interesting to design human BChE mutants that may be regarded as a cocaine hydrolase (CocH), with a significantly improved catalytic activity against (-)-cocaine.

It has been well-known that computational design of high-activity mutants of an enzyme is extremely challenging, particularly when the chemical reaction process is rate-determining for the enzymatic reaction (Gao et al., 2006; Gao and Zhan, 2005; Gao and Zhan, 2006). Generally speaking, for computational design of a mutant enzyme with an improved catalytic activity for a given substrate, one needs to design possible amino-acid mutations that can accelerate the rate-determining step of the catalytic reaction process (Hamza et al., 2005; Zhan and Gao, 2005; Zhan et al., 2003) while other steps of the reaction are not slowed down by the mutations. The detailed reaction pathway for BChE-catalyzed hydrolysis of (-)-cocaine was uncovered by extensive molecular dynamics (MD) simulations (Hamza et al., 2005; Zhan et al., 2003) and reaction coordinate calculations (Zhan and Gao, 2005; Zhan et al., 2003) using quantum mechanics (QM) and hybrid quantum mechanics/molecular mechanics (QM/MM). It has been known (Gao and Zhan, 2006; Hamza et al., 2005; Pan et al., 2005; Zhan et al., 2003) that the rate-determining step of (-)-cocaine hydrolysis catalyzed by the A328W/Y332A and A328W/Y332G mutants of BChE is the first step of the chemical reaction process. Therefore, starting from the A328W/Y332A or A328W/Y332G mutant, rational design of BChE mutants against (-)-cocaine has been focused on decreasing the energy barrier for the first reaction step without significantly affecting the other reaction steps. We have developed unique computational strategies and protocols based on the virtual screening of rate-determining transition states of the enzymatic reaction to design
enzyme mutants with improved catalytic activity (Pan et al., 2005; Pan et al., 2007; Pan et al., 2008; Yang et al., 2009; Zheng et al., 2008; Zheng et al., 2010). The computational design was followed by in vitro experiments, including site-directed mutagenesis, protein expression, and fast enzyme activity screening using the culture medium. The integrated computational-experimental studies have led to discovery of some BChE mutants with a significantly improved catalytic efficiency against (-)-cocaine (Pan et al., 2005; Pan et al., 2007; Pan et al., 2008; Yang et al., 2009; Yang et al., 2010; Zheng et al., 2008). One of our designed and discovered high-activity mutants of human BChE, i.e. the A199S/S287G/A328W/Y332G mutant (Pan et al., 2005), has been validated by an independent group of scientists (Brimijoin et al., 2008) who concluded that this mutant is “a true CocH with a catalytic efficiency that is 1,000-fold greater than wild-type BChE”.

Despite of the success of our previous integrated computational-experimental efforts, we did not account for substrate-selectivity of the enzyme in the design of BChE mutants. In particular, it is well-known that, as a cousin of acetylcholinesterase (AChE), BChE also catalyzes hydrolysis of neurotransmitter acetylcholine (ACh) (Gorelick, 1997). So far, ACh is the only known natural substrate of BChE in the body. An ideal, therapeutically valuable mutant of human BChE should have not only a significantly improved catalytic activity against (-)-cocaine, but also certain selectivity for (-)-cocaine over ACh. It is interesting to design mutants of human BChE with a significantly improved catalytic activity against (-)-cocaine without significantly increasing the catalytic activity against ACh compared to the wild-type enzyme. Our further integrated computational-experimental effort reported here has accounted for the mutation-caused changes of the catalytic activities of BChE against both (-)-cocaine and ACh, leading to identification of three BChE mutants that are satisfactory for both the catalytic activity and selectivity. Further, we have developed stable cell lines to express two representative mutants, one with a higher catalytic efficiency ($k_{cat}/K_M$) and the other with a larger catalytic rate constant ($k_{cat}$), in a relatively larger scale and employed the purified BChE mutants to test their in vivo potency in actual protection of mice from the acute cocaine toxicity. The in vivo data clearly demonstrate the relative importance of the $k_{cat}$ and $k_{cat}/K_M$ values of the enzyme in the cocaine detoxification.

Materials and Methods
In Vitro Activity Tests

Materials. Cloned pfu DNA polymerase and Dpn I endonuclease were obtained from Stratagene (La Jolla, CA). [3H](-)-cocaine (50 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA). All oligonucleotides were synthesized by the Integrated DNA Technologies, Inc (Coralville, IA). The QIAprep Spin Plasmid Miniprep Kit and Qiagen plasmid purification kit and QIAquick PCR purification kit were obtained from Qiagen (Santa Clarita, CA). Human embryonic kidney 293T/17 cells were from ATCC (Manassas, VA). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Fisher Scientific (Fairlawn, NJ). 3, 3′, 5, 5′-Tetramethylbenzidine (TMB) was obtained from Sigma (Saint Louis, Missouri). Anti-BChE (mouse monoclonal antibody, Product # HAH002-01) was purchased from AntibodyShop (Gentofte, Denmark) and goat anti-mouse IgG HRP conjugate was from Zymed (San Francisco, CA).

Site-directed Mutagenesis. Site-directed mutagenesis of human BChE cDNA was performed by using the QuikChange method (Braman et al., 2000). Further mutation(s) required to produce a new BChE mutant cDNA was/were generated from the cDNA corresponding to the A199S/S287G/A328W/Y332G mutant of human BChE in a pRc/CMV expression plasmid (Masson et al., 1999). Using plasmid DNA as template and primers with specific base-pair alterations, mutations were made by polymerase chain reaction with Pfu DNA polymerase, for replication fidelity. The PCR product was treated with Dpn I endonuclease to digest the parental DNA template. Modified plasmid DNA was transformed into Escherichia coli, amplified, and purified. The DNA sequences of the mutants were confirmed by DNA sequencing.

Protein Expression. Both the wild-type and mutants of human BChE were expressed and their enzyme activity against (-)-cocaine were assayed at the same time under the same experimental conditions; the wild-type was used a standard reference. The proteins (wild-type and mutants of BChE) were expressed in human embryonic kidney cell line 293T/17. Cells were grown to 80-90% confluence in 6-well dishes and then transfected by Lipofectamine 2,000 complexes of 4 μg plasmid DNA per each well. Cells were incubated at 37°C in a CO2 incubator for 24 hours and cells were moved to 60-mm culture vessel and cultured for four more days. The culture medium [10% fetal bovine serum in Dulbecco’s modified Eagle’s medium (DMEM)] was harvested for the BChE activity assays.
**Enzyme Activity Assays.** To measure (-)-coca and benzoic acid, the product of (-)-cocaine hydrolysis catalyzed by BChE, we used sensitive radiometric assays based on toluene extraction of [3H](-)coca labeled on its benzene ring (Sun et al., 2002b). In brief, to initiate the enzymatic reaction, 100 nCi of [3H](-)coca was mixed with 100 μl of culture medium. The enzymatic reactions proceeded at room temperature (25°C) with varying concentrations of (-)-coca. The reactions were stopped by adding 200 μl of 0.05 M HCl, which neutralized the liberated benzoic acid while ensuring a positive charge on the residual (-)-coca. [3H]benzoic acid was extracted by 1 ml of toluene and measured by scintillation counting. Finally, the measured (-)-coca concentration-dependent radiometric data were analyzed by using the standard Michaelis-Menten kinetics so that the catalytic parameters were determined along with the use of a well-established standard enzyme-linked immunosorbent assay (ELISA) protocol (Pan et al., 2008). The BChE activity assays with [3H]ACh are similar to the assays with [3H](-)coca. The primary difference is that the enzymatic reaction was stopped by addition of 200 μl of 0.2 M HCl containing 2 M NaCl and that the product was [3H]acetic acid for the ACh hydrolysis.

**Preparation of BChE Mutants in Large-scale**

**Materials.** Lentivirus related plasmids were kindly provided by Dr. Louis Hersh at College of Medicine, University of Kentucky. Plasmid purification kit was purchased from Qiagen Inc. (Valencia, CA). Q fast flow (QFF) anion exchanger was supplied by Amersham-pharmacia Biotech (Piscataway, NJ). Hypatite C (HTP) was supplied by Clarkson Chem. Co. (Williamsport, PA). Fibrous cellulose powder CF11 was from Whatman Co. (Sanford, ME). Nondenatured 4-20% gradient polyacrylamide gel, free style 293F cells, free style 293 expression medium, 5% penicillin and streptomycin, and 0.05% trypsin-EDTA were purchased from Invitrogen (Carlsbad, CA). Poly-L-proline was obtained from Sigma (Saint Louis, Missouri).

**Generation of recombinant lentivirus expressing mutants of human BChE.** The BChE mutant cDNAs in lentivirus plasmids were constructed in pCSC-SP-PW vector at ApaI and XhoI sites by PCR with BChE mutants in pRC/CMV plasmids as templates. Forward primer is gagggcccaaggtgcacggcccacgt (ApaI), and backward primer is ccgctcgagttagagacccacacaactttct (XhoI). The sequences of constructs were confirmed by DNA sequencing. To prepare
lentiviruses encoding BChE mutants, 293 FT cells were cultured in DMEM-10% FCS medium with 250 ng/ml G418 (Invitrogen). Cells were transfected at about 70% confluency by lentivirus plasmid encoding BChE (BChE/pCSC-SP-PW) or its mutants together with three other packaging plasmids, pMDL-gp.RRE, pRSV.Rev and pVSVG, at a mass ratio of 10.0:6.5:2.5:3.5. Transfection was achieved by calcium phosphate-mediated procedure. Briefly, for a 10 CM dish cells, total DNAs (about 22.5 μg) were mixed first, then 500 μl of 0.25 M freshly diluted CaCl₂ was added and mixed before another 500 μl of 2 × BBS solution was gently added and gently mixed. The mixture was incubated at room temperature for 15 minutes and then dropwisely added onto 10 CM dish. The cells were cultured in 3% CO₂ at 37°C. Culture medium was changed 12-16 hours post transfection and medium was collected for three times at a 24-hour interval beginning 24 hours after changing the medium post transfection. The medium was filtered through 0.45 μm cellulose acetate filter and spun in Beckman SW28 rotor at 19.4 K rpm for 2 hours at the room temperature to collect the virus particles. Lentivirus was suspended in Hank’s balanced salt solution and aliquoted in a small volume to store at -80°C.

**Expression of the BChE mutants.** Large-scale preparation of enzyme (BChE mutant) was first achieved by infecting 293F cells with lentivirus followed by resuspending attached 293F cells in suspension culture in free style 293 expression medium. 293F cells were routinely suspension cultured in serum-free medium according to manufacturer’s instruction at 8% CO₂, 37°C on orbit shaker at 125 rpm. The day before infection, cells were loaded at 1 × 10⁵/ml and cultured steadily in free style 293 expression medium with 1% FBS. Cells began to attach to plate soon after the change of culture condition. Lentivirus was then added to infect cells for 1 day with two intermittent additions of the virus. Infected cells were suspended by 0.05% trypsin-EDTA and seeded at 2-10 cells/well in 96-well plate in 1% FBS free-style medium again to culture for another 14-21 days without changing medium and shaking until single clones clearly appeared. Single-clone cell lines from 96-well plates were chosen to culture in 48-well plates, then 12-well plates and 6-well plates in 1% FBS free-style 293 expression medium. High expression single-clone cell lines were screened and selected by determining BChE activity in medium. Selected cell-line cells were then changed back to suspension culture and the culture volume increased from 6-well plate to 125-ml flask and a series of larger flasks and finally 2-L flask. Poly-L-proline was added at 1 μM to the free-style 293 expression medium. Culture medium was changed every 2-3 days and collected to store at 4°C in sterilized bottles for protein
purification. The enzyme, mostly in tetramers, was secreted in medium. The cultured medium contained 4 to 10 mg/L enzyme.

**Enzyme Purification.** Large-scale purification of each enzyme in medium was achieved by using an ion exchanger and adsorption chromatographies. Briefly, 8 liters of medium with a BChE mutant was diluted with the same volume of 20 mM Tris-HCl, pH 7.4. Equilibrated QFF anion exchanger was added to diluted medium in 1% of its volume and incubated at 4°C with occasional stirring for one hour. More than 95% enzyme activity was found to bind to the resin after this incubation. The suspension was then packed in a column (5 × 50 cm) and the medium was allowed to flow through rapidly with the aid of suction (50-100 ml/min). The QFF resin was repacked again in a washing buffer after all of the medium was excluded. Following washing the column with 20 mM Tris-HCl, pH 7.0 overnight at 4°C, the enzyme was eluted by 20 mM phosphate buffer, pH 7.0 plus 0.3 M NaCl. The eluate was desalted to 20 mM Tris-HCl, pH 7.0 by Millipore centrifugal filter device. The desalted eluate was applied to a Hypatic C (HTP) column (2.5 × 22 cm) which was packed with fibrous cellulose powder CF11 at a ratio of 1:1. The column was washed by 20 mM Tris-HCl, pH 7.0 and the enzyme was eluted by 10 mM sodium phosphate buffer, pH 7.0 containing 0.3 M NaCl. The purified enzyme was dialysed against PBS and stored at 4°C or -80°C. The entire purification process was carried out in cold room at 4°C. The purified enzymes were provided for *in vivo* activity tests described below.

**In Vivo Activity Tests**

**Subjects.** Male NIH-Swiss mice (27-30 g) were obtained from Harlan Sprague-Dawley Inc. (Indianapolis, IN) and were housed in groups of 4-6 mice per cage. All mice were allowed *ad libitum* access to food and water, and were maintained on a 12-h light-dark cycle with lights on at 6:30 AM in a room kept at a temperature of 21–22°C. Each mouse was used only once and each dosing condition contained 8 mice. Experiments were performed in the same colony room in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. The experimental protocols were approved by the University Committee on the Use and Care of Animals at the University of Michigan.

**Drugs.** Naturally occurring cocaine, *i.e.* (-)-Cocaine HCl (Tyco-Mallinckrodt, Hazelwood, MO), was dissolved in sterile water and was administered intraperitoneally at a volume of 0.01
ml/g. The BChE mutant was diluted to different concentrations in phosphate buffered saline and administered intravenously at a volume of 0.2 ml/mouse.

**Behavioral assay.** Cocaine-induced toxicity was characterized by the occurrence of convulsions and lethality. Cocaine-induced convulsions were defined as loss of righting posture for at least 5 sec with the simultaneous presence of clonic limb movements (Ko et al., 2007). Lethality was defined as cessation of observed movement and respiration. Following intraperitoneal administration of cocaine, mice were immediately placed individually in Plexiglas containers (16 x 28 x 20 cm high) for observation. The presence or absence of convulsions and lethality were recorded for 60 min following cocaine administration.

**Drug administration.** The mouse was placed in a small restraint chamber (Outer tube diameter: 30 mm, Inner tube diameter: 24 mm, Model #BS4-34-0012, Harvard Apparatus, Inc., Holliston, MA) that left the tail exposed. The tail was cleansed with an alcohol wipe, then a 30G1/2 precision glide needle (Fisher Scientific, Pittsburgh, PA) was inserted into one of the side veins for infusion. The intravenous injection volume of BChE mutant for different doses was 0.2 mL per mouse and it was given 1 min before intraperitoneal administration of cocaine 180 mg/kg (LD100). Sterile gauze and pressure were applied to the injection site to staunch the bleeding.

**Data analysis.** Data from the behavioral toxicity studies (i.e. % of mice showing affected responses) were analyzed with Fisher’s exact probability test. Protective effects of the BChE mutant were compared with those of phosphate buffered saline. The criterion for significance was set at p<0.05. The value of ED100conv was determined when 100% of mice showed convulsions. Such value was used to compare the degree of shifts of cocaine’s dose-response curve in subjects pretreated with different BChE mutants.

**Results**

**BChE Mutant Design: Insights from Molecular Modeling.** Our goal of the present study was to identify BChE mutants that have significantly improved catalytic activity against (-)-cocaine, without a significant change in the catalytic activity against ACh, compared to wild-type BChE in order to make sure that the cocaine detoxification with BChE mutants will not affect the cholinergic transmission. Based on the catalytic mechanisms for BChE-catalyzed hydrolyses of (-)-cocaine and ACh shown in Figure 1(A) and (B) (Gao et al., 2006; Gao and...
Zhan, 2005; Gao and Zhan, 2006; Zhan and Gao, 2005; Zhan et al., 2003), our rational design of BChE mutants in this study was focused on the hydrogen bonding interactions between the carbonyl oxygen of the substrate and the oxyanion hole. Our previous computational studies (Gao et al., 2006; Gao and Zhan, 2005; Gao and Zhan, 2006; Zhan and Gao, 2005; Zhan et al., 2003) have revealed that the fundamental reaction pathway for BChE-catalyzed hydrolysis of (-)-cocaine is similar to that for BChE-catalyzed hydrolysis of ACh in terms of the formation and breaking of covalent bonds during the reaction processes. As shown in Figure 1(A) and (B), for both (-)-cocaine and ACh, the BChE-catalyzed hydrolysis consists of acylation and deacylation. The acylation is initiated by the attack of the hydroxyl oxygen of Ser198 side chain at the carbonyl carbon of the substrate. While the hydroxyl oxygen of Ser198 side chain gradually approaches the carbonyl carbon of the substrate, the carbonyl oxygen of the substrate gradually becomes negatively charged and the hydroxyl hydrogen of Ser198 side chain gradually transfers to a nitrogen atom of His438 side chain. Thus, the carbonyl oxygen of the substrate forms stronger and stronger hydrogen bonds with the oxyanion hole (consisting of Gly116, Gly117, and Ala199) from the Michaelis-Menten complex to the transition state and to the intermediate during the acylation process. In this way, the hydrogen bonds between the carbonyl oxygen of the substrate and the oxyanion hole of BChE help to stabilize the transition state and, thus, to decrease the activation free energy of the BChE-catalyzed hydrolysis. The primary difference between the (-)-cocaine and ACh hydrolyses catalyzed by wild-type BChE exists in the number of potential hydrogen bonds between the carbonyl oxygen of the substrate and the oxyanion hole of the enzyme. The carbonyl oxygen of ACh can potentially form three hydrogen bonds with the oxyanion hole (two in the Michaelis-Menten complex and three in the transition state), whereas the carbonyl oxygen of (-)-cocaine can only potentially form two hydrogen bonds with the oxyanion hole of wild-type BChE (Gao and Zhan, 2005; Gao and Zhan, 2006). The mechanistic insights suggest that certain amino acid mutations that can increase a potential hydrogen bond between the carbonyl oxygen of the substrate and the oxyanion hole could significantly increase the catalytic activity of BChE.

In light of the above analysis, the desirable amino acid mutations on BChE should be those that can increase a potential hydrogen bond of the oxyanion hole with the carbonyl oxygen of (-)-cocaine, but not with the carbonyl oxygen of ACh. The BChE mutants corresponding to such type of desirable mutations may be expected to have a significantly improved catalytic activity.
against (-)-cocaine, without a significantly increased catalytic activity against ACh. Hence, the same computational modeling methods (including molecular dynamics simulations and energy minimizations using Amber program (Case et al., 2004) used in our previous studies (Gao and Zhan, 2005; Gao and Zhan, 2006; Pan et al., 2005) were employed, in the present study, to identify the desirable amino acid mutations on BChE. The computational modeling suggested that A199S/S287G/A328W/Y332G, A199S/F227A/S287G/A328W/E441D, and A199S/F227A/S287G/A328W/Y332G/E441D might be the desirable sets of mutations. Depicted in Figure 1(C) to (F) are the modeled structures of representative Michaelis-Menten complexes for the (-)-cocaine and ACh hydrolyses.

As seen in Figure 1(C), the carbonyl oxygen of (-)-cocaine can only have two potential hydrogen bonds with the NH groups of Gly117 and Ala199 backbones in the oxyanion hole of wild-type BChE; the hydrogen bond with Ala199 backbone is insignificant in the Michaelis-Menten complex, but it is expected to be significantly stronger in the transition state (TS1) (Gao and Zhan, 2005). For the (-)-cocaine hydrolysis catalyzed by each of these BChE mutants, the carbonyl oxygen of (-)-cocaine can have one more potential hydrogen bond with the hydroxyl group (OH) of Ser199 side chain, in addition to the two potential hydrogen bonds with the Gly117 and Ser199 backbones. Depicted in Figure 1(D) is the modeled structure of (-)-cocaine binding with the A199S/F227A/S287G/A328W/E441D mutant as an example; the modeled structures of (-)-cocaine binding with the other two mutants are similar to this one.

Compared to (-)-cocaine interacting with wild-type BChE, the carbonyl oxygen of ACh can have one more hydrogen bond with the NH group of Gly116 backbone, in addition to the two potential hydrogen bonds with the Gly117 and Ala199 backbones, as seen in Figure 1(E). One might expect that the same mutations which increase a potential hydrogen bond of the oxyanion hole with the carbonyl oxygen of (-)-cocaine also increase a potential hydrogen bond with the carbonyl oxygen of ACh. However, the modeled structures reveal that the expected additional hydrogen bond between the carbonyl oxygen of ACh and the hydroxyl group of Ser199 side chain does not exist in any of the modeled structures for ACh binding with the BChE mutants. Depicted in Figure 1(F) is the modeled structure of ACh binding with the A199S/F227A/S287G/A328W/E441D mutant as an example; the modeled structures of ACh binding with the other two mutants are similar to this one. These modeling results suggest that the A199S/S287G/A328W/Y332G, A199S/F227A/S287G/A328W/E441D, and
A199S/F227A/S287G/A328W/Y332G/E441D mutations should not significantly increase the catalytic activity of BChE against ACh, while significantly improving the catalytic activity against (-)-cocaine.

**In Vitro Activity of the BChE Mutants against (-)-Cocaine.** Based on the computational insights, we carried out *in vitro* experimental tests, including site-directed mutagenesis, protein expression, and *in vitro* enzyme activity assays, on the A199S/S287G/A328W/Y332G, A199S/F227A/S287G/A328W/E441D, and A199S/F227A/S287G/A328W/Y332G/E441D mutants of human BChE. To minimize the possible systematic experimental errors of *in vitro* kinetic data, we expressed the enzymes and performed kinetic studies with wild-type BChE and the mutants under the same conditions and compared the catalytic efficiencies of the mutants to the corresponding catalytic efficiencies of wild-type BChE against (-)-cocaine and ACh. Michaelis-Menten kinetics of the enzymatic hydrolysis of (-)-cocaine or ACh was determined by performing the sensitive radiometric assays using [3H](-)-cocaine (labeled on its benzene ring) or [3H]ACh (labeled on its acetyl group) with varying concentrations of substrate. Depicted in Figure 2 are the measured kinetic data. Summarized in Table 1 are the determined kinetic parameters.

The three mutants of human BChE collected in Table 1 all have a considerably improved catalytic efficiency against (-)-cocaine. The A199S/S287G/A328W/Y332G mutant has a 1,080-fold improved catalytic efficiency against (-)-cocaine ($k_{cat}/K_M = 9.9 \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$) compared to the wild-type enzyme ($k_{cat}/K_M = 9.1 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$). Both $k_{cat}$ and $K_M$ values of the A199S/F227A/S287G/A328W/E441D mutant against (-)-cocaine are significantly lower than the corresponding values of the A199S/S287G/A328W/Y332G mutant. Overall, the A199S/F227A/S287G/A328W/E441D mutant has a 1,730-fold improved catalytic efficiency against (-)-cocaine ($k_{cat}/K_M = 1.6 \times 10^9 \text{ M}^{-1} \text{ min}^{-1}$) compared to the wild-type enzyme. Starting from the A199S/F227A/S287G/A328W/E441D mutant, the additional Y332G mutation produces the A199S/F227A/S287G/A328W/Y332G/E441D mutant. The additional Y332G mutation significantly increases both the $k_{cat}$ and $K_M$ values of the A199S/F227A/S287G/A328W/E441D mutant against (-)-cocaine. Overall, the catalytic efficiency ($k_{cat}/K_M = 1.3 \times 10^9 \text{ M}^{-1} \text{ min}^{-1}$) of the A199S/F227A/S287G/A328W/Y332G/E441D mutant is slightly lower than that of the A199S/F227A/S287G/A328W/E441D mutant.
**In Vitro Activity of the BChE Mutants against Acetylcholine.** Regarding the catalytic activity of wild-type BChE against ACh, we obtained $k_{\text{cat}} = 86,000 \text{ min}^{-1}$ and $K_M = 148 \mu\text{M}$. The $K_M$ value of 148 µM is nearly identical to the $K_M$ value of 150 µM reported by Gao et al. (Gao et al., 2008). Our determined $k_{\text{cat}}$ value of 86,000 min$^{-1}$ is slightly larger than, but reasonably close to, the $k_{\text{cat}}$ value of 61,200 min$^{-1}$ reported by Gao et al. (Gao et al., 2008). It is remarkably notable in Table 1 that the same mutations that considerably improve the catalytic efficiency of BChE against (-)-cocaine do not increase the catalytic efficiency of BChE against ACh at all. Compared to the wild-type enzyme ($k_{\text{cat}}/K_M = 5.8 \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$), all of the mutants listed in Table 1 have significantly lower $k_{\text{cat}}$ and $K_M$ values against ACh. Overall, the catalytic efficiency ($k_{\text{cat}}/K_M = 5.4 \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$) of the A199S/F227A/S287G/A328W/E441D mutant against ACh is slightly lower than that of the wild-type enzyme (~0.93-fold), whereas the other two mutants even have a significantly lower catalytic efficiency against ACh (about one third).

Further, as seen in Table 1, the catalytic efficiency of wild-type BChE against ACh ($k_{\text{cat}}/K_M = 5.8 \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$) is ~640-fold higher than that of the same enzyme against (-)-cocaine ($k_{\text{cat}}/K_M = 9.1 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$). However, it is interesting to note that, for each of the three BChE mutants, the catalytic efficiency against (-)-cocaine ($k_{\text{cat}}/K_M = 9.9 \times 10^8 - 1.6 \times 10^9 \text{ M}^{-1} \text{ min}^{-1}$) becomes significantly higher than that of any enzyme (wild-type BChE or mutant) in Table 1 against ACh ($k_{\text{cat}}/K_M = 1.8 - 5.8 \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$). These BChE mutants not only have a considerably improved catalytic efficiency against (-)-cocaine, but also have the desirable selectivity for (-)-cocaine over ACh.

**In Vivo Activity of the BChE Mutants in Protection of Mice from Cocaine Toxicity.** The *in vitro* activity data discussed above suggest that all of the three BChE mutants have the desirable high catalytic efficiency and substrate selectivity for (-)-cocaine. Within the three promising BChE mutants, we expressed and purified the A199S/S287G/A328W/Y332G and A199S/F227A/S287G/A328W/E441D mutants in large-scale for the purpose of *in vivo* studies. We are interested in testing these two representative mutants *in vivo* because these two mutants both have their own unique mutation(s): Y332G mutation in the A199S/S287G/A328W/Y332G mutant; F227A and E441D mutations in the A199S/F227A/S287G/A328W/E441D mutant. It is interesting to know which mutant is actually more potent in the practical detoxification of cocaine.
For the large-scale expression and purification of these two BChE mutants, we developed the stable cell lines in 293F by using lentivirus plasmids encoding the cDNAs of the BChE mutants. Each mutant enzyme was purified by using an ion exchanger and adsorption chromatographies. The catalytic activities of the purified enzymes were confirmed by using the same in vitro assays mentioned above.

The purified mutant enzymes were used to study their in vivo activity in protection of male NIH-Swiss mice (27 – 30 g) from acute toxicity of a lethal dose of cocaine (180 mg/kg). Figure 3 compares the protective effects of the BChE mutants. Intraperitoneal administration of cocaine 180 mg/kg produced convulsions and lethality in all tested mice (n=8). Pretreatment with the BChE mutant (i.e. 1 min prior to cocaine administration) dose-dependently protected mice against cocaine-induced convulsions and lethality. In particular, the A199S/S287G/A328W/Y332G mutant of human BChE at the dose of 0.03 mg produced full protection in mice after receiving a lethal dose of cocaine 180 mg/kg (p<0.05). The A199S/F227A/S287G/A328W/E441D mutant of human BChE was more potent than the A199S/S287G/A328W/Y332G mutant. As seen in Figure 3, the A199S/F227A/S287G/A328W/E441D mutant at 0.02 mg also produced full protection in mice after receiving a lethal dose of cocaine 180 mg/kg (p<0.05). So, the minimum dose of the enzyme required to produce full protection of the mice from the acute toxicity of 180 mg/kg cocaine was determined to be 0.03 mg for the A199S/S287G/A328W/Y332G mutant and 0.02 mg for the A199S/F227A/S287G/A328W/E441D mutant.

**Discussion**

The present study accounting for the mutation-caused changes of the catalytic activities of BChE against both (-)-cocaine and ACh has led to identification of three BChE mutants that not only have a considerably improved catalytic efficiency against (-)-cocaine, but also have the desirable selectivity for (-)-cocaine over ACh. Development of the stable cell lines for large-scale expression of the BChE mutants has allowed us to confirm the in vivo potency of two representative BChE mutants in the actual protection of mice from the acute cocaine toxicity. The minimum doses of enzyme required to protect all of the mice from the acute toxicity of 180 mg/kg cocaine have been determined to be 0.02 and 0.03 mg for the A199S/F227A/S287G/A328W/E441D and A199S/S287G/A328W/Y332G mutants, respectively.
These mutants of human BChE provided significantly better in vivo protection of mice from the acute cocaine toxicity than the well-known cocaine esterase (CocE) and its mutants reported to date (Brim et al., 2010; Collins et al., 2009; Cooper et al., 2006; Gao et al., 2009; Ko et al., 2007; Narasimhan et al., 2010). The in vivo potency of these two mutants is roughly equivalent to that of the previously reported most efficient cocaine hydrolase (Zheng et al., 2008) which also provided the full protection of the mice at a similar dose (0.01 and 0.03 mg). But the present study accounted for the substrate selectivity for the first time.

We are not aware of any study determining whether administration of BChE produces significant decreases in ACh levels. Published case reports on clinical use of partially purified human BChE did not mention any adverse effects related to altered ACh activity (Gorelick, 2008). On the other hand, it has been known that BChE is over-expressed in Alzheimer’s disease (AD) patients (Guillozet et al., 1997) and the presence of BChE with β-amyloid (Aβ) plaques dramatically amplifies the toxicity of Aβ (Barber et al., 1996). In addition, increased BChE gene expression correlates with neurodegenerative disorders (Mack and Robitzki, 2000). In light of these published reports, without further studies, we do not know for sure that the administration of a BChE mutant with significantly increased activity against ACh will not cause any unexpected adverse effects for the patients, particularly those who also suffer from AD or other neurodegenerative disorders. In any case, it is reasonable to assume that the administration of a BChE mutant with substrate selectivity for (-)-cocaine over ACh will less-likely cause unexpected adverse effects.

Based on the in vitro activity data, the A199S/S287G/A328W/Y332G mutant has a significantly higher $k_{cat}$ value (3,060 min$^{-1}$) against (-)-cocaine than the A199S/F227A/S287G/A328W/E441D mutant ($k_{cat} = 1,730$ min$^{-1}$). On the other hand, the A199S/F227A/S287G/A328W/E441D mutant has a significantly lower $K_M$ value (1.1 µM) than the A199S/S287G/A328W/Y332G mutant ($K_M = 3.1$ µM). Overall, the catalytic efficiency ($k_{cat}/K_M$) of the A199S/F227A/S287G/A328W/E441D mutant is higher than that of the A199S/S287G/A328W/Y332G mutant. According to the relative magnitudes of the $k_{cat}$ values, when the (-)-cocaine concentration is much higher than the $K_M$ value such that the enzyme is nearly saturated with (-)-cocaine, the A199S/S287G/A328W/Y332G mutant is expected to be more effective than the A199S/F227A/S287G/A328W/E441D mutant. However, According to the relative magnitudes of the $k_{cat}/K_M$ values, when the (-)-cocaine concentration is much lower
than the $K_M$ value, the A199S/F227A/S287G/A328W/E441D mutant is expected to be more effective than the A199S/S287G/A328W/Y332G mutant.

The determined relative magnitudes of the minimum doses of the BChE mutants producing the full protection of mice from the acute cocaine toxicity clearly demonstrate that the catalytic efficiency (i.e., the $k_{cat}/K_M$ value) of the BChE mutant is the primary factor determining the in vivo potency in the practical protection of the mice from the acute cocaine toxicity, which contrasts with the usual perception that the catalytic rate constant ($k_{cat}$) is the primary factor. Further, due to the fact that the A199S/F227A/S287G/A328W/E441D mutant with a larger $k_{cat}/K_M$ value and a smaller $k_{cat}$ value (compared to the A199S/S287G/A328W/Y332G mutant) is more potent in the practical protection of the mice from the acute cocaine toxicity, the cocaine concentration in the blood of mice overdosed with 180 mg/kg cocaine was not higher than $K_M$ value (3.1 µM) of the A199S/S287G/A328W/Y332G mutant in the presence of the BChE mutant. In light of this finding, for further improvement of the efficacy in cocaine detoxification, future rational design of new BChE mutants should focus on the $k_{cat}/K_M$ value, rather than the $k_{cat}$ value alone.

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**Authorship Contribution**

*Participated in research design:* Zhan, Tai, Woods, and Ko.
*Conducted experiments:* Xue, Ko, Tong, Yang, Hou, Fang, Liu, and Zheng.
*Contributed new reagents or analytic tools:* Tai.
*Performed data analysis:* Zhan, Xue, Ko, and Zheng.
*Wrote or contributed to the writing of the manuscript:* Zhan, Xue, Tai, Ko, and Woods.
*Other:* Zhan, Woods, and Ko acquired funding for the research.
References


Footnotes

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Legends for figures

Figure 1. Schematic representation of hydrolyses of (-)-cocaine (A) and ACh (B) catalyzed by BChE mutant including the A199S mutation; (-)-cocaine binding with wild-type BChE (C) and the A199S/F227A/S287G/A328W/E441D mutant (D); and ACh binding with wild-type BChE (E) and the A199S/F227A/S287G/A328W/E441D mutant (F).

Figure 2. Plots of measured reaction rates (with error bars) versus the substrate concentration for the (-)-cocaine and ACh hydrolyses catalyzed by wild-type BChE and its mutants. The reaction rates were determined by using a sensitive radiometric assays based on toluene extraction of $[^3]$H(-)-cocaine labeled on its benzene ring. See Materials and Methods section for the details.

Figure 3. The potency of protective effects of the BChE mutants against cocaine-induced toxicity. “BChE mutant 12-4” represents the A199S/F227A/S287G/A328W/E441D mutant of human BChE, whereas “BChE mutant 14-3” refers to the A199S/S287G/A328W/Y332G mutant. The BChE mutant (mg/mouse) was administered intravenously 1 min before intraperitoneal administration of cocaine 180 mg/kg. Each data point represents the percentage of mice (n=8 for each dosing condition) exhibiting cocaine-induced convulsions or lethality. The asterisks represent a significant difference from the condition of mice pretreated with phosphate buffered saline (p<0.05). See Materials and Methods section for the details.
Table 1. Kinetic parameters determined for (-)-cocaine and acetylcholine (ACh) hydrolysces catalyzed by wild-type BChE and its mutants.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme&lt;sup&gt;a&lt;/sup&gt;</th>
<th>$K_M$ (µM)</th>
<th>$k_{cat}$ (min&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>$k_{cat}/K_M$ (M&lt;sup&gt;-1&lt;/sup&gt;min&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>RCE&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>(-)-cocaine</td>
<td>wild-type BChE&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.5</td>
<td>4.1</td>
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<td></td>
<td>A199S/S287G/A328W/Y332G&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.1</td>
<td>3,060</td>
<td>$9.9 \times 10^8$</td>
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<tr>
<td></td>
<td>A199S/F227A/S287G/A328W/E441D</td>
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<td>1,730</td>
<td>$1.6 \times 10^9$</td>
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<tr>
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<td>A199S/F227A/S287G/A328W/Y332G/E441D</td>
<td>3.5</td>
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<td>ACh</td>
<td>wild-type BChE</td>
<td>148</td>
<td>86,000</td>
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<td>1</td>
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<td>A199S/S287G/A328W/Y332G</td>
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<td>A199S/F227A/S287G/A328W/Y332G/E441D</td>
<td>75</td>
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<td>$1.8 \times 10^8$</td>
<td>0.32</td>
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<sup>a</sup> Unless indicated otherwise, all kinetic parameters listed in this table were determined in the present study.

<sup>b</sup> RCE refers to the relative catalytic efficiency ($k_{cat}/K_M$), i.e. the ratio of the $k_{cat}/K_M$ value of the mutant to that of wild-type BChE against the same substrate.

<sup>c</sup> Data for wild-type BChE from reference (Sun et al., 2002a).

<sup>d</sup> The kinetic parameters reported in reference (Yang et al., 2010). The experimental measurement in the present study was able to reproduce the kinetic parameters.
Figure 1
Figure 2
Figure 3

The graph shows the effects of different treatments on BChE mutant 14-3 in the presence of cocaine.

**Lethality (% Occurrence)**
- Vehicle
- BChE mutant 14-3
- BChE mutant 12-4

**Convulsions (% Occurrence)**
- Vehicle
- BChE mutant 14-3
- BChE mutant 12-4

The treatments include different concentrations of BChE mutant (mg, i.v.) and the addition of cocaine (+ Cocaine).

* indicates statistical significance compared to the control group.