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HIGH-ACTIVITY MUTANTS OF HUMAN BUTYRYLCHOLINESTERASE FOR COCAINE ABUSE TREATMENT

Liu Xue
University of Kentucky, xueliu424@gmail.com

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Liu Xue, Student

Dr. Chang-Guo Zhan, Major Professor

Dr. Jim Pauly, Director of Graduate Studies

HIGH-ACTIVITY MUTANTS OF HUMAN BUTYRYLCHOLINESTERASE FOR COCAINE ABUSE TREATMENT

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Pharmacy
at the University of Kentucky

By Liu Xue

Lexington, Kentucky

Director: Dr. Chang-Guo Zhan, Professor of Pharmaceutical Sciences

Lexington, Kentucky

ABSTRACT OF DISSERTATION

HIGH-ACTIVITY MUTANTS OF HUMAN BUTYRYLCHOLINESTERASE FOR COCAINE ABUSE TREATMENT

Cocaine is a widely abused drug without an FDA-approved medication. It has been recognized as 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*. butyrylcholinesterase (BChE)-catalyzed hydrolysis. However, the native BChE has a low catalytic activity against cocaine. We recently designed and discovered a set of BChE mutants with a high catalytic activity specifically for cocaine. 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. Through integrated computational-experimental studies, several BChE mutants were identified to have not only a considerably improved catalytic efficiency against cocaine, but also the desirable selectivity for cocaine over ACh. 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, LD_{100}). Pretreatment with the BChE mutant (*i.e*. 1 min prior to cocaine administration) dose-dependently protected mice against cocaine-induced convulsions and lethality. 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.

For further characterization in animal models, we successfully developed high-efficiency stable cell lines efficiently expressing the BChE mutants by using a lentivirus-based repeated-transduction method. The large-scale protein production enabled us to further characterize the *in vivo* profiles of the BChE mutant concerning the biological half-life and potency in accelerating cocaine clearance. In particular, it has been demonstrated that the BChE mutant can rapidly metabolize cocaine and completely eliminate cocaine-induced hyperactivity in rodents, implying that the BChE mutant may be developed as a promising therapeutic agent for cocaine abuse treatment.

KEY WORDS: Enzyme, butyrylcholinesterase, protein drug, protein production, drug abuse

Liu Xue
Student's Signature

7/31/2013
Date

HIGH-ACTIVITY MUTANTS OF HUMAN BUTYRYLCHOLINESTERASE FOR COCAINE ABUSE TREATMENT

By

Liu Xue

Chang-Guo Zhan
Director of Dissertation

Jim Pauly
Director of Graduate Study

7/31/2013

This dissertation is dedicated to my dear family and friends.
Your support, encouragement and love, have sustained me throughout my life.

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CHAPTER 1 Cocaine Abuse And Therapeutic Treatment

Cocaine abuse is a worldwide social and public health problem. There were about 13~19 millions cocaine users aged 16-64 all around the world in 2010.[1] As one of the most reinforcing psychoactive substances, cocaine causes negative health consequences, loss in productivity, drug-related crime and deaths.[2-6] The use of cocaine resulted in 505,224 Emergency Department (ED) visits in the United States in 2011.[7] Unfortunately there is no FDA-approved medication specific for cocaine abuse treatment. In this chapter, I will briefly discuss the molecular mechanism of cocaine abuse, currently applied protocol for ED visits and ongoing researches for cocaine overdose and addiction treatment, and enzyme therapy as a novel anti-cocaine medication strategy.

1.1 The mechanism of cocaine action

Cocaine interacts with multiple crucial proteins in central and periphery systems and illicits a serial of physiological and psychological effects on the subjects that lead to addiction.[8] The chronic use of cocaine changes the structure and function of brain.[9] In the case of cocaine overdose, patients are in danger of severe traumas and even death.

1.1.1 Central nervous system and psychiatric effects

The rewarding effect of cocaine is associated with the interaction of cocaine with monoamine transporters, especially dopamine transporter (DAT).[6, 10] Cocaine acts primarily as a dopamine transporter blocker in the rewarding center of central never

system (CNS).[11] In normal dopaminergic neuron transmission, dopamine was released into synaptic cleft to stimulate the postsynaptic neuron as a neurotransmitter. During that, the excessive dopamine was reuptaked back to the presynaptic neuron by DAT for recycling or degradation. As a blocker/inhibitor of DAT, cocaine blocks the process of reuptaking dopamine. Therefore, excessive dopamine accumulated in the synaptic cleft prolongs the stimuli of the postsynaptic neuron.[10] Dopaminergic neurons are richly populated at the ventral tegamental area (VTA) in the midbrain.[12, 13] They project mainly to mesolimbic pathway (nucleus accumbens and other regions) and mesocortical pathway (prefrontal and insular parts of cerebral cortex).[10, 12-15] [16]Mesolimbic pathway is mostly referred as the rewarding pathway.[12, 13] Mesocortical pathway is involved in motivation and emotion response.[15]

Cocaine also interacts with other transporters and affected several pathways in neuron circuits. The use of cocaine changes the responsiveness of the brain to many neurotransmitters, such as norepinephrine, dopamine, serotonin, acetylcholine and gamma-aminobutyric acid (GABA).[8] Collectively, cocaine produces the feelings of euphoria, increased energy, motivation and excitement.

1.1.2 Acute toxicity/physiological effects

Besides monoamine transporters in CNS, cocaine also binds with muscarinic acetylcholine sigma receptors and several voltage-gated ion channels in periphery nervous system (Table 1-1.).[8] The collective short-term effect of cocaine includes increased heart rate, increased body temperature, elevated blood pressure, shortness of breath, and etc.

In the case of overdose, cocaine causes seizure and lethality. Most cocaine-overdosed patients are sent to the emergency department, because they are experiencing severe chest pain and/or heart attack. The actual half-life of cocaine depends on the dose used.[4] At low doses, the half-life of cocaine is 45-90 min in human body.[3] However, when the patients are overdosed, all the cocaine-metabolizing enzymes are saturated with cocaine and then the actual half-life of cocaine is proportional to the dose of cocaine.[6] The cocaine in the body would keep damaging patients cerebral vascular and cardiovascular systems and even put the lives of patients in danger.

Table 1-1. Inhibition constants of cocaine against functional proteins.[8]

Transporters/receptors/ion channels	K_i (μM)
Dopamine transporter	0.64
Serotonin transporter	0.14
Norepinephrine transporter	1.6
Muscarinic acetylcholine receptor (rat M2 heart)	19
Sigma receptor	6.7
Cardiac myocyte sodium channel	8
Cardiac potassium rectifier channel	5.6

1.1.3 Long-term effect

Blockage of monoamine transporters by cocaine leads to changes of gene expression in the brain. One-time use of cocaine will up-regulate DAT on the cell surface of dopaminergic neuron for months.[17] Meanwhile, dopamine receptor D2 on the postsynaptic neurons is decreased.[9] The overall neuron activity in the reward center is weakened. Cocaine use also changes the regional density of several other neurotransmitter receptors and monoamine transporters and influences a number of pathways.[8] Patients first feel impulsive to take cocaine (positive reinforcement), and soon become compulsive because of negative emotional state (negative reinforcement).[14] They reported anxiety, dysphoria and irritability when cocaine is not available. Chronic use of cocaine will constantly change the neuron circuits and functions of brain in the addicted patients. Thus, addiction to cocaine is developed and, once developed, very hard to get rid of.

1.2 On-going researches for cocaine overdose and addiction treatment

1.2.1 Cocaine addiction

There is no FDA-approved medication specific for cocaine abuse so far. Traditionally, to treat substance abuse, a pharmacodynamic approach is usually chosen, which aims to develop a small-molecule drug antagonizing the drug of abuse. A number of small-molecule drugs have been studied in clinical trials for the purpose of treating

cocaine addiction, including some drugs that have been marketed for other diseases. Despite decades of efforts, pharmacodynamic methods have not been successful yet for treatment of cocaine addiction or overdose.

1.2.1.1 Dopaminergic agents

Dopaminergic transmission is a major system that is involved in generating rewarding effect and maintaining drug use behavior. A number of anti-cocaine drugs that target DAT or dopamine receptor D1, D2 or D3 have been investigated.[18]

The binding site of cocaine in DAT is overlapped with the binding site of dopamine.[19-21] Therefore, it is very challenging to find a small molecule that can antagonize cocaine action on DAT without interferring the normal function of DAT and the downstream signaling. Methylphenidate and mazindol are dopamine reuptake inhibitors that have been used in studies of cocaine addiction medication.[18] Monoamine oxidase (MAO)-B inhibitors, dopamine receptor agonists and antagonists have also been studied since they are involved in dopaminergic transmission.[18] However, the studies have not yet proven their therapeutic values for anti-cocaine treatment.

1.2.1.2 GABAergic agents

GABA is the major inhibitory neurotransmitter in the central nervous system. GABA system modulates dopamine system and the effects of cocaine.[10, 13] GABA synapses coexist with dopaminergic neuron in VTA. Increase of GABA activity levels by

γ-vinyl-GABA injection attenuated cocaine-induced locomoter activity and dopamine release in nucleus accumbens.[18] Several drugs that activate the GABA system have been used in studies for anti-cocaine medication, including tiagabine, gabapentin and baclofen.[18] These studies suggest that the GABA system may be a target for cocaine addiction therapy. The therapeutic values of those drugs need to be evaluated further in clinical studies.

1.2.1.3 Adrenergic agents and vasodilators

Central noradrenergic system and its peripheral sympathoadrenal system mediate the physiological effects of cocaine, including increased heart rate, blood pressure and arousal. Studies suggested that β-adrenoceptor antagonists might attenuate the reinforcing effects of cocaine.[18] Vasodilators are also proposed because of their ability to lessen the vasoconstriction effect of cocaine on cerebral blood flow. [18] The therapeutic significances of adrenergic agents and vasodilators as anti-cocaine medication need to be investigated further.

1.2.2 Cocaine overdose

Currently used ED protocol for cocaine overdose is mainly focused on relief of cocaine-associated chest pain and heart attack.[22] Cocaine-induced chest pain is related to increased blood pressure and heart rate. Usually, intravenous benzodiazepine is given as early treatment. As a GABA enhancer, benzodiazepine relieves cocaine-induced chest

pain and dysfunction of cardiac system. Vasodilator nitroglycerin is also used to reverse the chest pain and control hypertension induced by cocaine. When patients do not respond to neither benzodiazepine nor nitroglycerin, calcium channel blocker would be considered.[22] Since the interaction of cocaine with several transporters/receptors/voltage-gated channels is complex and involves several organ systems[8], so single pharmarcodynamic treatment has limited effects to reverse all the cocaine actions in the case of ED treatment. It is also warned that beta-blockers and some calcium channel blockers should never be used in acute situation because of contraindication.[22]

1.3 Pharmacokinetic approach for cocaine abuse treatment

Since traditional pharmacodynamic approach is not successful to antagonize cocaine toxicity yet, novel pharmacokinetic approach has received increasing attention.[6] Pharmacokinetic approach for drug abuse treatment is to accelerate drug clearance or alter the distribution of the abused drug. Pharmacokinetic agents are usually macromolecules such as antibody or enzyme. They do not travel through the blood-brain barrier (BBB), so they are not expected to interrupt normal neuron transmissions. Antibody and vaccine therapies are proposed that antibodies are able to bind with cocaine molecules and prevent them from entering the brain. The approach can be achieved through active prophylaxis (vaccine) or passive prophylaxis (monoclonal antibody

produced in another host).[23] The shortcoming of antibody/vaccine therapy is that each antigen-binding site of the antibody can only bind with one cocaine molecule. So in the case of cocaine overdose, a large amount of antibody would be needed to overcome cocaine toxicity. Compared with that, enzyme therapy is much more efficient. An enzyme molecule not only can bind with cocaine, but also break it down. After the reaction products are released from the active site of the enzyme, the enzyme molecule is free to bind with and metabolize another cocaine molecule. Thus, one enzyme molecule can keep metabolizing cocaine molecules as long as it retains its activity.

One question often being asked is: for overdose treatment, how can systematic administration of a cocaine-metabolizing enzyme clear the cocaine molecules that have already reached the CNS. The fact is that, because cocaine can cross the BBB back and forth rapidly, cocaine concentrations in the brain and plasma can quickly reach the equilibrium as demonstrated in the positron emission tomography (PET) imaging studies.[24] If cocaine is being metabolized in the circulation system, the equilibrium is shifted and cocaine in the brain moves back to the plasma. Eventually cocaine in CNS can be cleared by exogenous enzyme administrated into the plasma.

For addiction patients, the brains have the ability to recover back to normal when kept away from further cocaine effect. The recovery of the brain function of addicted patients back to normal is a slow process.[17] Addiction treatment requires the cocaine-metabolizing enzyme to have a sufficiently high activity to completely suppress the cocaine effect in the CNS.[5] The threshold concentration of cocaine in brain required to

produce physiological effects has been estimated to be about 0.22 μM.[25] If sufficient enzymatic activity stays in the plasma, the enzyme may keep cocaine under the threshold to illicit physiological effects by rapid metabolism of cocaine and prevent cocaine from reinforcing the functional changes of addiction.[3-6]

1.4 Cocaine metabolism in physiological condition

The major cocaine metabolism pathway is enzymatic hydrolysis at either benzoyl ester or methyl ester.[4] Hydrolysis of cocaine at benzoyl ester generates ecgonine methyl ester (EME), whereas cocaine hydrolysis at methyl ester yields benzoylecognine (BE). The primary cocaine-metabolizing enzyme is butyrylcholinesterase (BChE) that catalyzes the reaction of cocaine hydrolysis at the benzoyl ester to produce benzoic acid and EME. Liver carboxylesteras 1 and 2 hydrolyze cocaine at methyl ester group and benzoyl ester group, respectively. In addition, P450 oxidation accounts for 5% of cocaine metabolism and produces norcocaine. EME appears to be the least pharmacologically active metabolite, and has the effect of vasodilation that counteracts with cocaine. BE and norcocaine have similar effects as cocaine, including vasoconstriction and lowering the threshold of seizer. Therefore, BChE-catalyzed cocaine hydrolysis is a safe and natural pathway of cocaine metabolism.

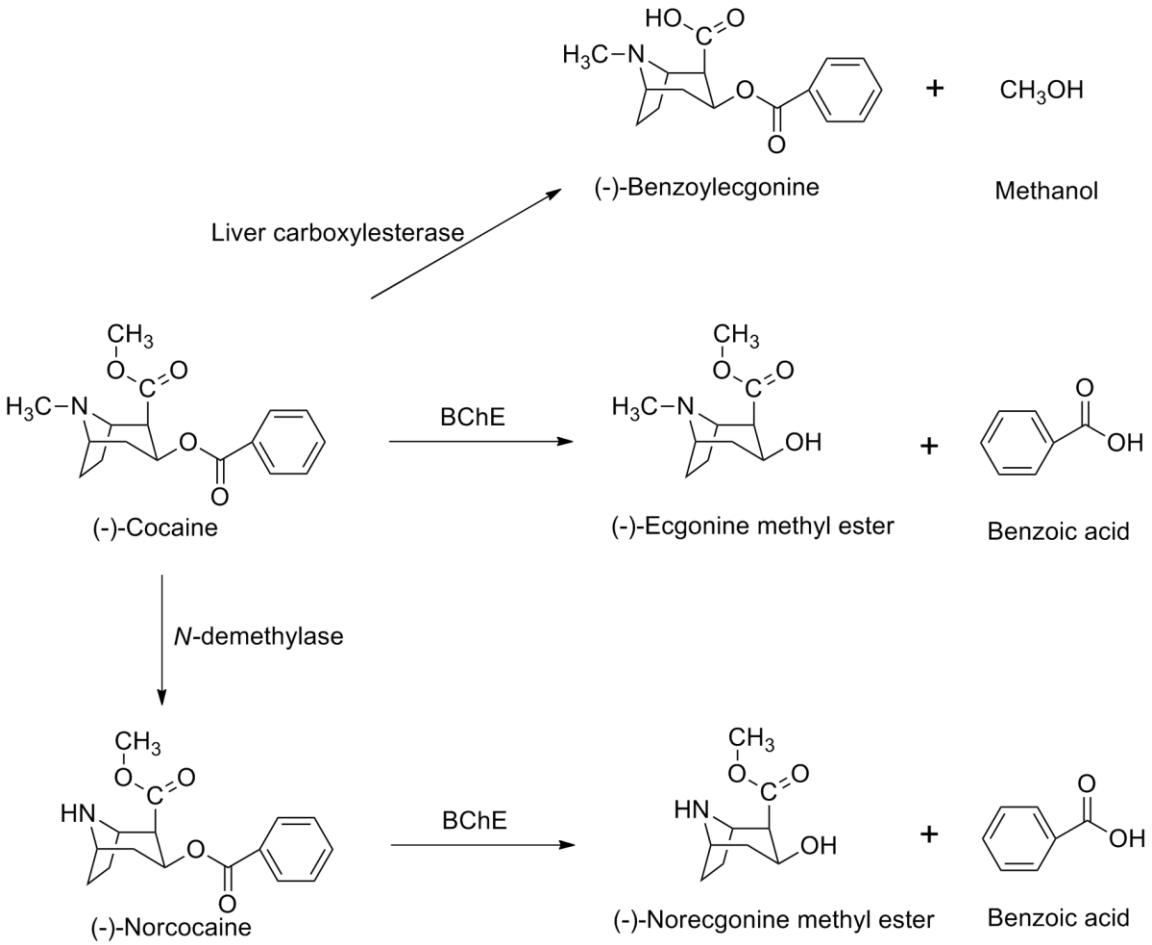

Figure 1-1. Cocaine metabolic pathways in physiological condition

1.5 Human butyrylcholinesterase

Human butyrylcholinesterase (BChE) gene has been found expressed in many tissues and organs including plasma, liver, kidney, brain, muscle, skin and etc.[26] BChE is a cousin of acetylcholinesterase (AChE). BChE was first called psedocholinesterase because of its ability to hydrolyze acetylcholine (ACh). However, complete inhibition of

BChE activity has no effect on muscle contraction.[26] So it is not the main function of BChE to hydrolyze ACh. The function of BChE is still not clear yet. People lacking BChE activity do not display any abnormality.[27] It has been proposed to be a housekeeping enzyme since it is responsible for detoxification of a broad spectrum of toxins including succinylcholine, organophosphates, and cocaine.[27-29]

Use of BChE as an exogenous enzyme has several advantages. First, BChE is the principal enzyme to hydrolyze cocaine in human body and produce biological inactive metabolites. Second, BChE is from a human source, so the problem of immune response can be avoided. Third, BChE is a thermostable enzyme and has a long half-life (~12 days) in the body.[30] Fourth, over 65 different naturally occurring variants of human BChE have been identified, and none of them is antigenic.[31, 32] Last, BChE has been in clinical use for a long time (for other therapeutic purposes) with no side effect reported (Table 1-2).

Table 1-2. Clinical applications of human BChE.[32, 33]

Substrate / Target	**Clinical Use For**
Succinylcholine	Metabolism of anesthesia
Organophosphates	Prevention of pesticide or nerve gas poisoning
Carbamate toxin	Detoxification of insecticide
Mivacurium chloride	Metabolism of muscle relaxant

Unfortunately, native human BChE has a low catalytic activity against naturally-occurring (-)-cocaine. The activity of BChE against (-)-cocaine is 2000-fold lower than that against the synthetic (+)-cocaine.[34] (+)-Cocaine is biological inactive, and it can be metabolized quickly by BChE. The fact is that at a very low dose of 0.2 mg/kg (+)-cocaine can be cleared from the plasma by BChE within seconds, whereas (-)-cocaine at the same low dose has a half-life of 47 min.[35] PET mapping (+)- and (-)-cocaine binding in baboon CNS suggest that (+)-cocaine has no uptake in CNS, whereas the effect of (-)-cocaine peaks in minutes primarily at striatum.[36] If BChE can be re-engineered to obtain activity against (-)-cocaine as high as it does against (+)-cocaine, it would rescue/protect cocaine-abused patients against (-)-cocaine toxicity.

1.6 Mechanisms of cocaine hydrolysis catalyzed by BChE

The detailed reaction pathway for BChE-catalyzed hydrolysis of (-)-cocaine was uncovered by extensive molecular dynamics (MD) simulations [37, 38] and reaction coordinate calculations [38, 39] using quantum mechanics (QM) and hybrid quantum mechanics/molecular mechanics (QM/MM). First, the binding of cocaine to BChE leads to the formation of a prereactive complex. For (+)-cocaine and (-)-cocaine, the substrates need to rotate about 90^{o} during the change from the non-prereactive enzyme-substrate (ES) complex to the prereactive ES complex. (-)-Cocaine needs to rotate more than (+)-

cocaine. The molecular docking and MD simulation demonstrated that (-)-cocaine or (+)-cocaine first slides down the substrate-binding gorge to bind to W82 and stands vertically in the gorge between D70 and W82 in the non-prereactive complex and rotates to a position in the catalytic site within a favorite distance for nucleophilic attack and hydrolysis by S198 (prereactive complex). Following the formation of prereactive ES complex, the entire chemical reaction that consists of four steps (ES→TS1→INT1→TS2→INT2→TS3→INT3→TS4→EB depicted in Figure 1-1) takes place.

Combined computational and experimental data have been shown that the rate-determining step for BChE-catalyzed (-)-cocaine hydrolysis is the formation of prereactive ES complex of BChE-(-)-cocaine, whereas the rate-determining step for (+)-cocaine hydrolysis by BChE is the chemical reaction. The structural and mechanistic similarities between (+)-cocaine and (-)-cocaine in the BChE-catalyzed hydrolysis indicate the chances of improving the catalytic activity of BChE against (-)-cocaine.

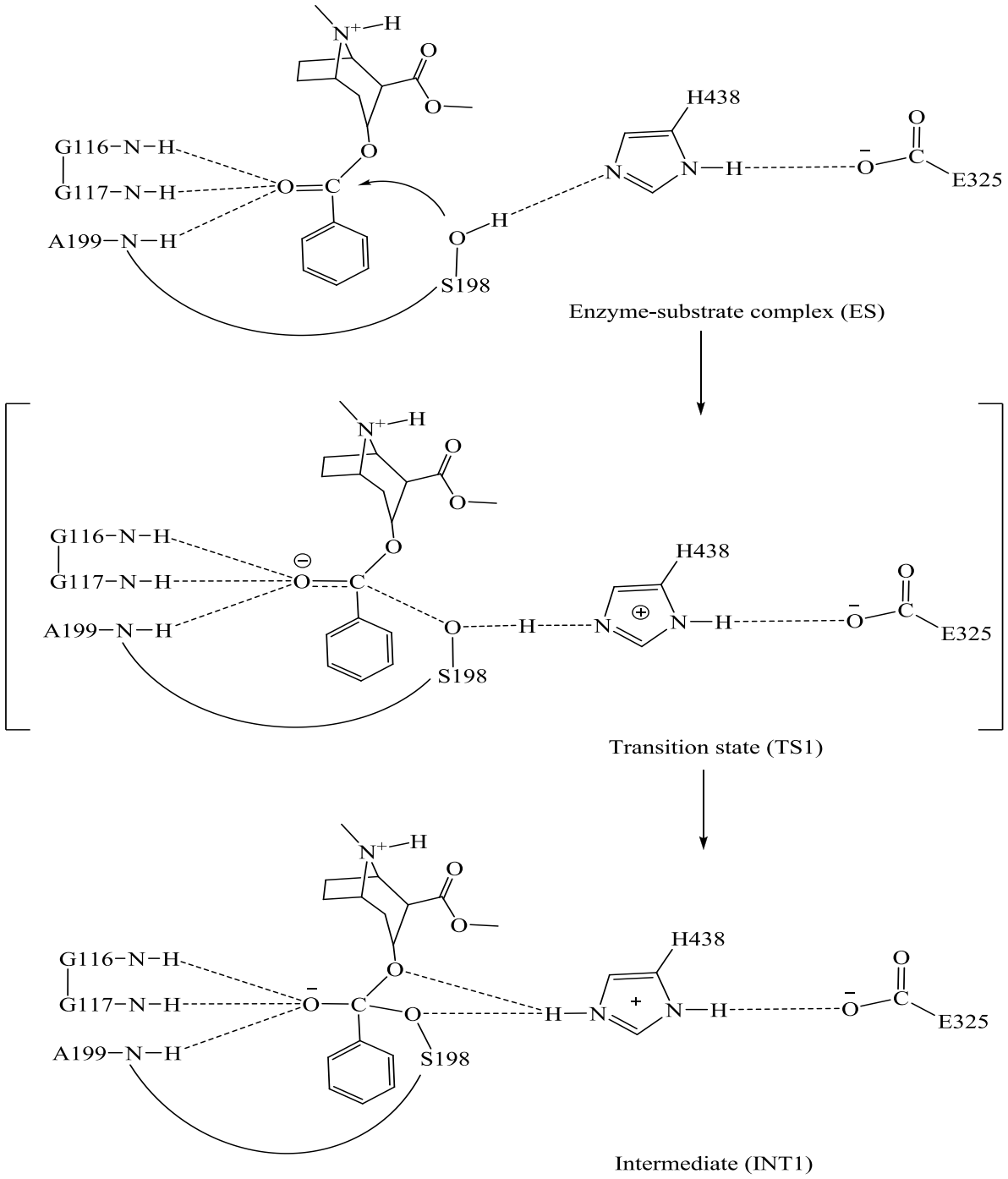

Figure 1-2(A). Reaction pathway for BChE-catalyzed (-)-cocaine hydrolysis of (-)-cocaine

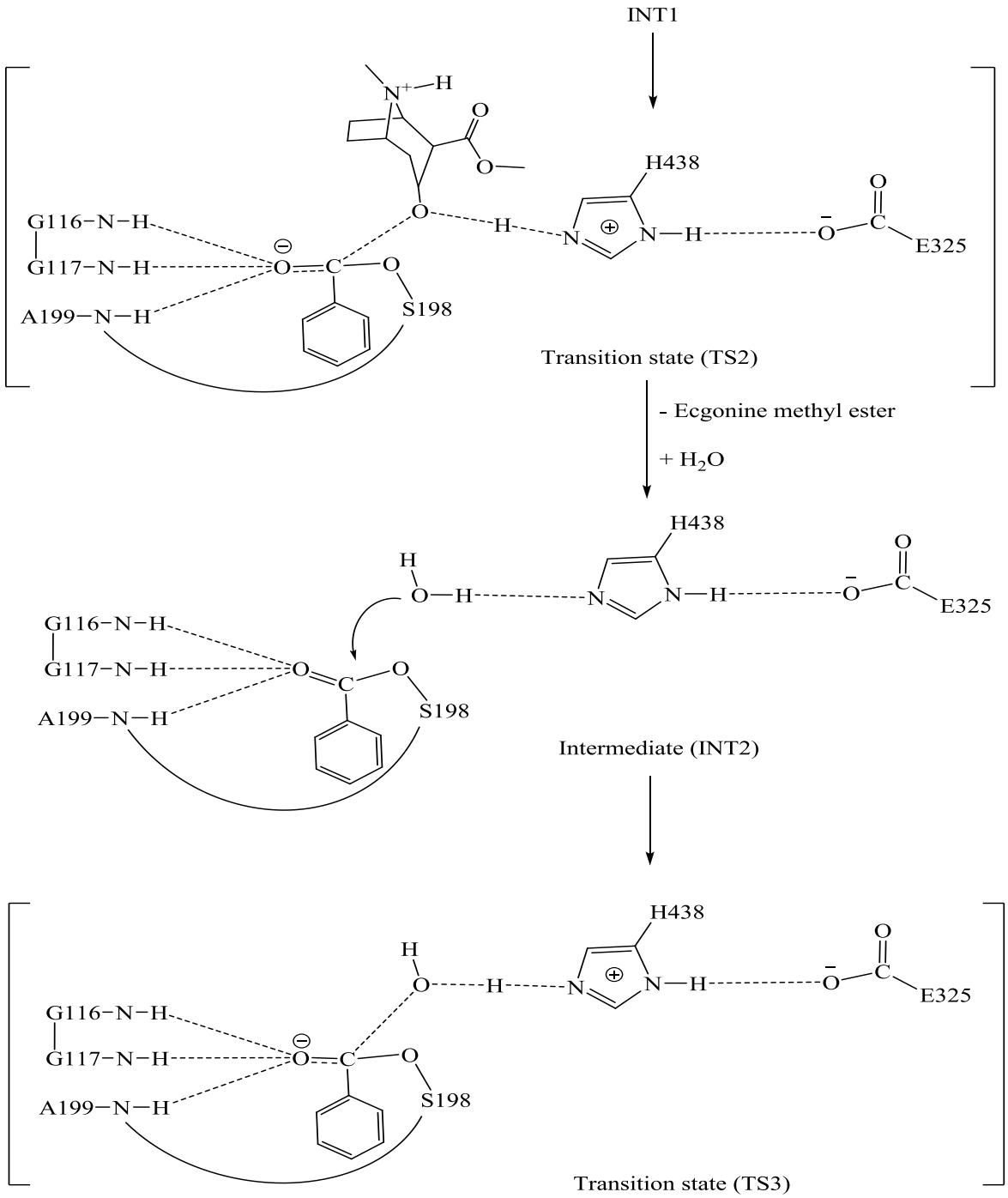

Figure 1-1(B). Reaction pathway for BChE-catalyzed (-)-cocaine hydrolysis of (-)-cocaine

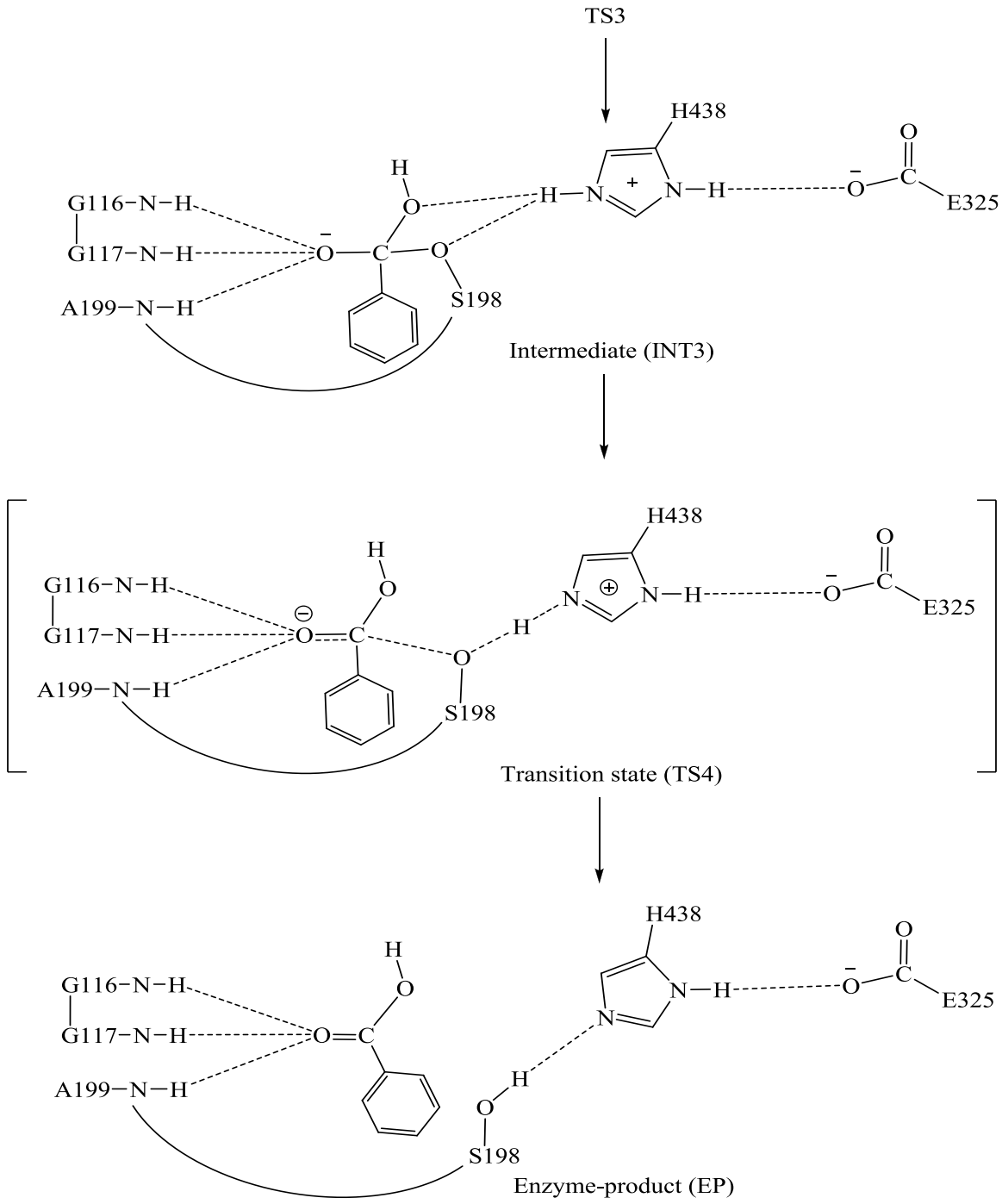

Figure 1-1(C). Reaction pathway for BChE-catalyzed (-)-cocaine hydrolysis of (-)-cocaine

1.7 Summary

Cocaine is one of the most reinforcing drugs of abuse. Unfortunately, there is no FDA-approved effective treatment for cocaine addiction or overdose. Enzyme therapy is a promising approach to treat cocaine abuse. As a good candidate for this purpose, human BChE has several therapeutic potentials, but is limited by its low activity against (-)-cocaine. The knowledge of the detailed mechanism of cocaine hydrolysis by BChE provides a foundation for reengineering human BChE as a potential anti-cocaine therapeutic enzyme. Next chapter is a brief discussion of our rational design and discovery of high-activity and high-selectivity mutants of human BChE.

CHAPTER 2 Design, Preparation And Characterization Of High-Activity Mutants Of Human Butyrylcholinesterase Specific For Detoxification Of Cocaine

2.1 General consideration of the BChE mutant design

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.[40-42] 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 [37-39] 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 [37, 38] and reaction-coordinate calculations [38, 39] using quantum mechanics (QM) and hybrid quantum mechanics/molecular mechanics (QM/MM). It has been known [37, 38, 40, 43] 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. [3, 43-46] 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. [3, 43-47] One of our designed and discovered high-activity mutants of human BChE, *i.e*. the A199S/S287G/A328W/Y332G mutant (denoted as CocH1 for convience) [43], has been validated by an independent group of scientists [48] 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, the early stage of the BChE mutant design did not account for the substrate-selectivity of the enzyme. In particular, it is well-known that, as a cousin of acetylcholinesterase (AChE), BChE also catalyzes hydrolysis of neurotransmitter acetylcholine (ACh) [49]. 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[2, 50] described in this chapter has accounted for the mutation-caused changes of the catalytic activities of BChE against both (-)-cocaine and ACh, leading to identification of a series of BChE mutants that are satisfactory for both the catalytic activity and selectivity.

The main results described in this chapter have been published.[2, 50] Below we briefly discuss the methods and the main discoveries.

2.2 Materials and methods

2.2.1 Materials

Cloned *pfu* DNA polymerase and *Dpn I* endonuclease were obtained from Stratagene (La Jolla, CA). [^{3}H](-)-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).

2.2.2 Site-directed mutagenesis

Site-directed mutagenesis of human BChE cDNA was performed by using the QuikChange method [51]. Further mutation(s) required to produce a new BChE mutant cDNA was/were generated (one mutation at a time) from the cDNA corresponding to the mutant CocH1 (A199S/S287G/A328W/Y332G) of human BChE in a pRc/CMV expression plasmid [52]. 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.

2.2.3 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 (HEK293T/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 CO_2 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.

2.2.4 Enzyme activity assays

To measure (-)-cocaine and benzoic acid, the product of (-)-cocaine hydrolysis catalyzed by BChE, we used sensitive radiometric assays based on toluene extraction of [^{3}H](-)-cocaine labeled on its benzene ring *(53)*. In brief, to initiate the enzymatic reaction, 100 nCi of [^{3}H](-)-cocaine was mixed with 100 μl of culture medium. The enzymatic reactions proceeded at room temperature (25°C) with varying concentrations of (-)-cocaine. 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 (-)-cocaine. [^{3}H]benzoic acid was extracted by 1 ml of toluene and measured by scintillation counting. Finally, the measured (-)-cocaine 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 *(45)*. The BChE activity assays with [^{3}H]ACh are similar to the assays with [^{3}H](-)-cocaine. 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 [^{3}H]acetic acid for the ACh hydrolysis.

2.3 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 *(38-42)*, 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 *(38-42)* 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 Figures 2-1 and 2-2, 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 *(40, 41)*. 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.

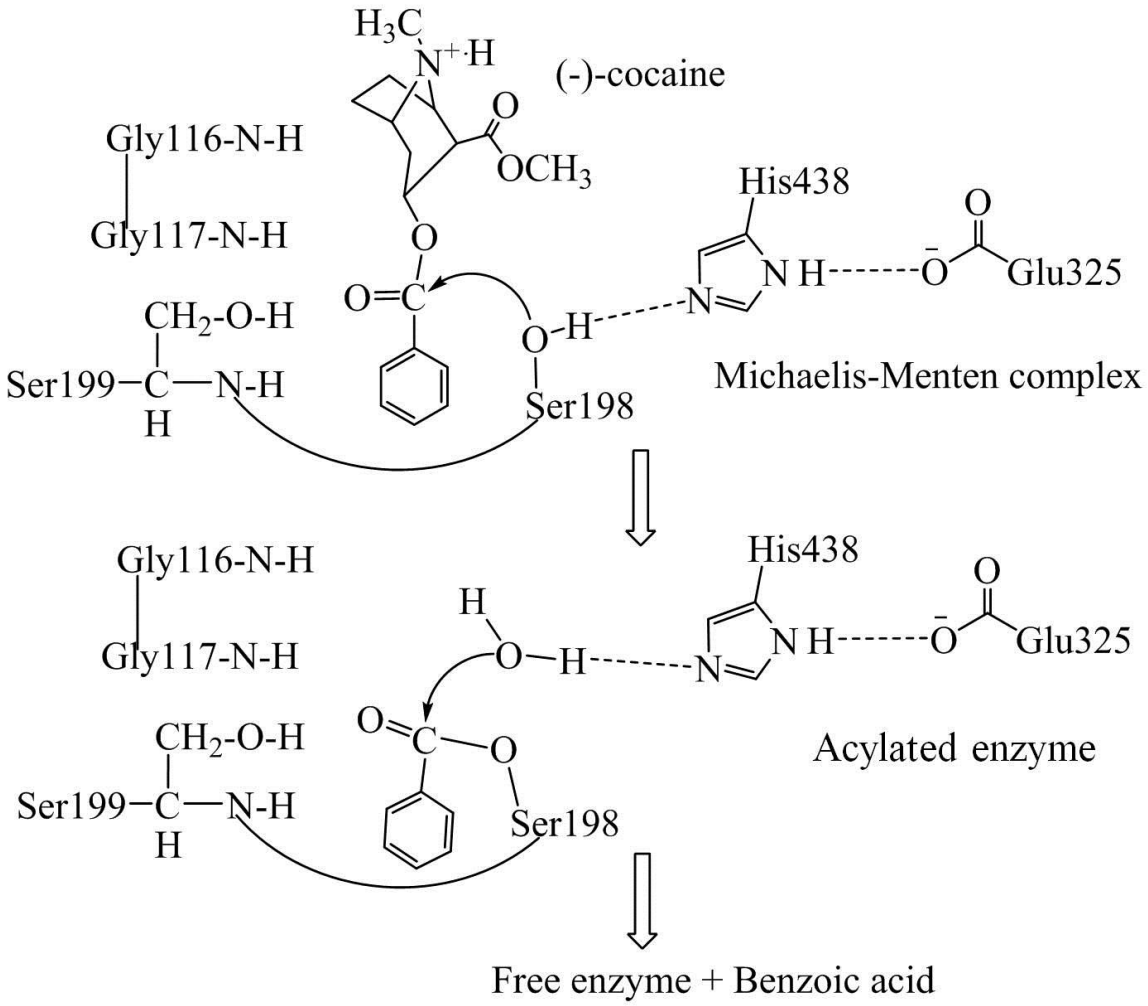

Figure 2-1. Schematic representation of hydrolysis of (-)-cocaine catalyzed by a BChE mutant including the A199S mutation

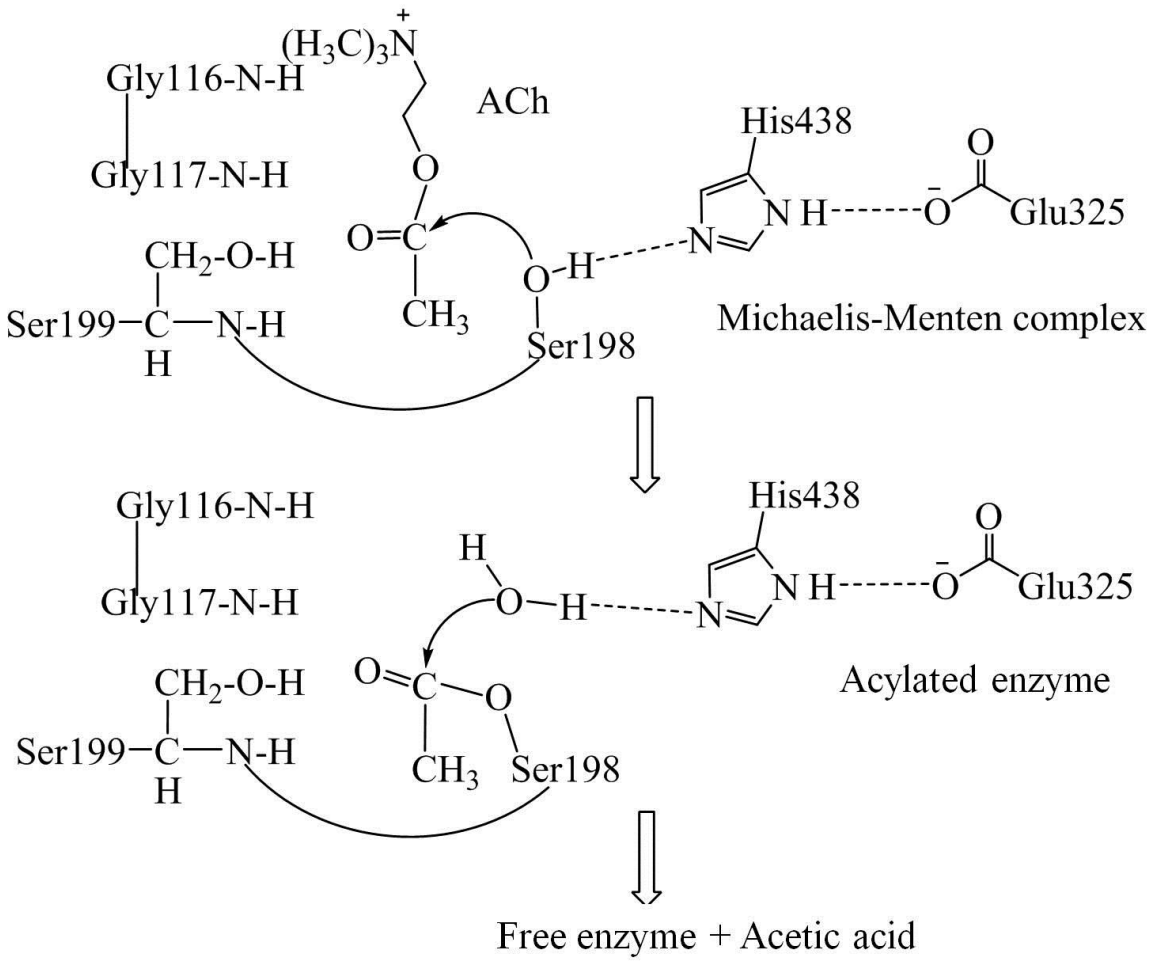

Figure 2-2. Schematic representation of hydrolysis of acetylcholine catalyzed by a BChE mutant including the A199S mutation

In light of the above analysis, the desirable amino-acid mutations on BChE should be those that can potentially increase a 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 [54] used in our previous studies [40, 41, 43] were employed, in the study described in this chapter, to identify the desirable amino-acid mutations on BChE. The computational modeling suggested that the A199S/S287G/A328W/Y332G mutant (CocH1), the A199S/F227A/S287G/A328W/E441D mutant (denoted as CocH2 for convenience), the A199S/F227A/S287G/A328W/Y332G/E441D mutant (denoted as E13-1), and the A199S/F227A/S287G/A328W/Y332G mutant (denoted as CocH3), might be the desirable sets of mutations. Depicted in Figures 2-3 to 2-6 are the modeled structures of representative Michaelis-Menten complexes for the (-)-cocaine and ACh hydrolyses.

As seen in Figure 2-3, 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) [41]. 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 2-4 is the modeled structure of (-)-cocaine binding with CocH2 as an example; the modeled structures of (-)-cocaine binding with the other three mutants are similar to this one.

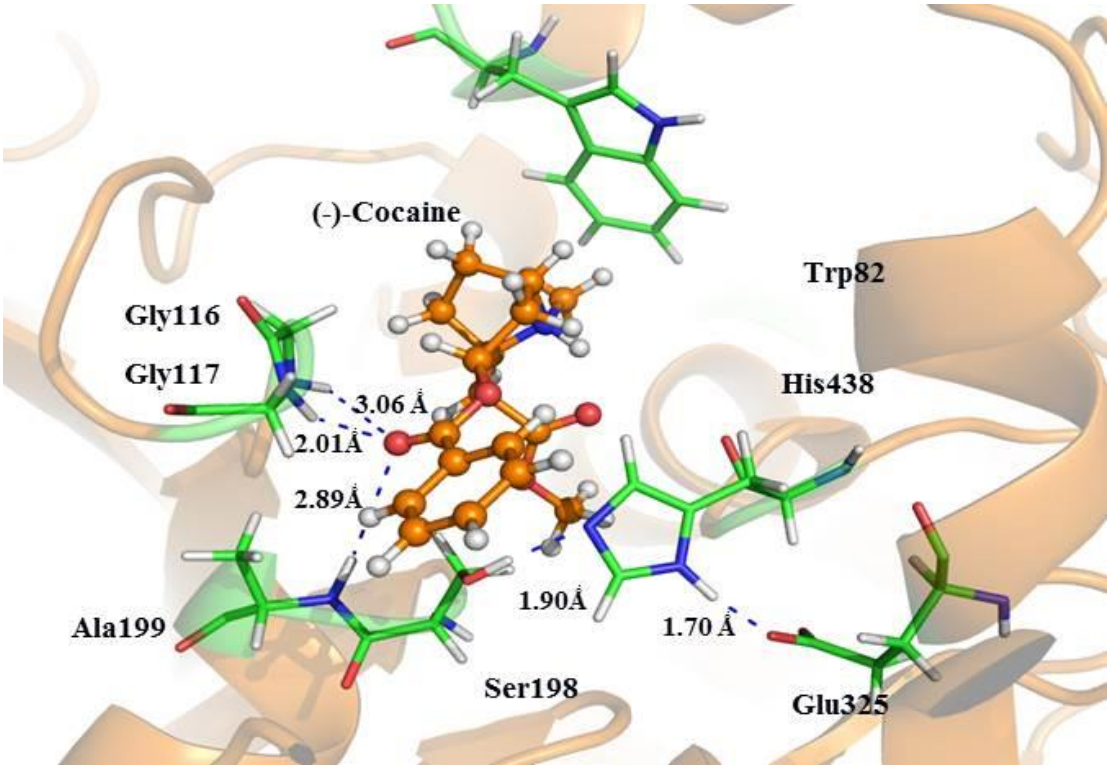

Figure 2-3. Demonstration of (-)-cocaine binding with wild-type BChE in the prereactive ES complex.

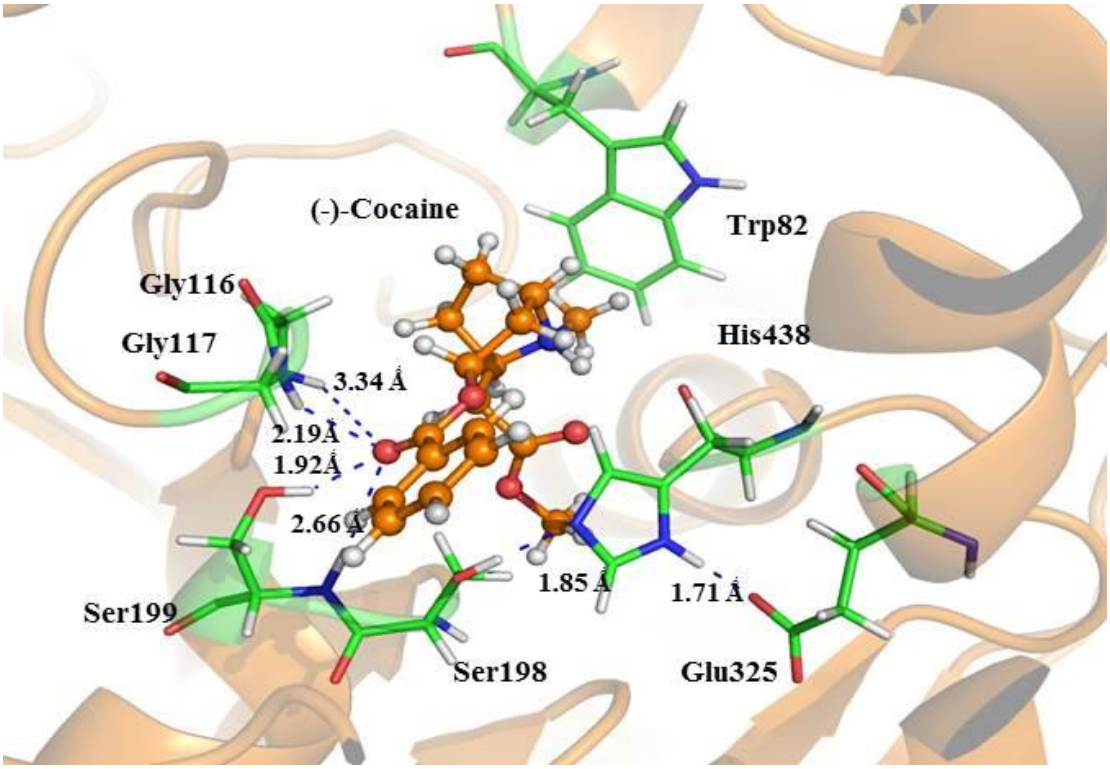

Figure 2-4. Demonstration of (-)-cocaine binding with CocH2 in the prereactive ES complex.

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 2-5. 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 2-6 is the modeled structure of ACh binding with the CocH2 as an example; the modeled structures of ACh binding with the other three mutants are similar to this one. These modeling results suggest that the mutations of CocH1, CocH2, CocH3, and E13-1 should not significantly increase the catalytic activity of BChE against ACh, while significantly improving the catalytic activity against (-)-cocaine.

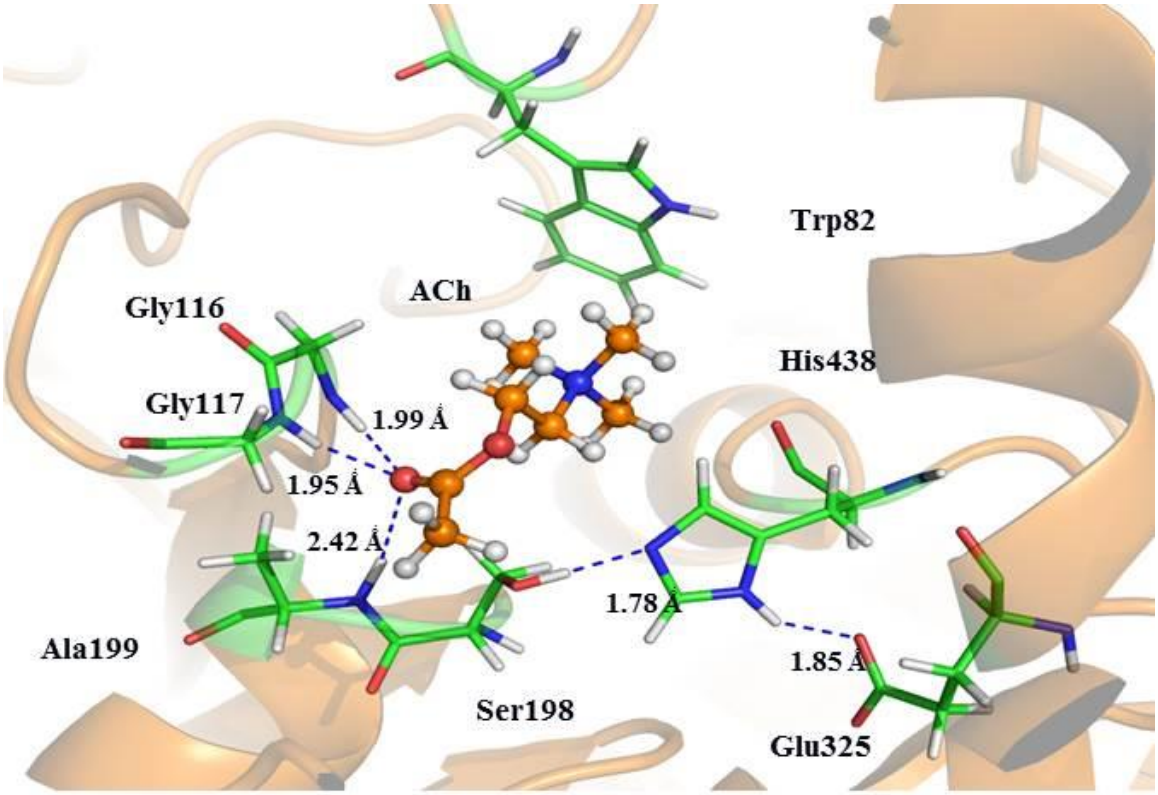

Figure 2-5. Demonstration of acetylcholine binding with wild-type BChE in the prereactive ES complex.

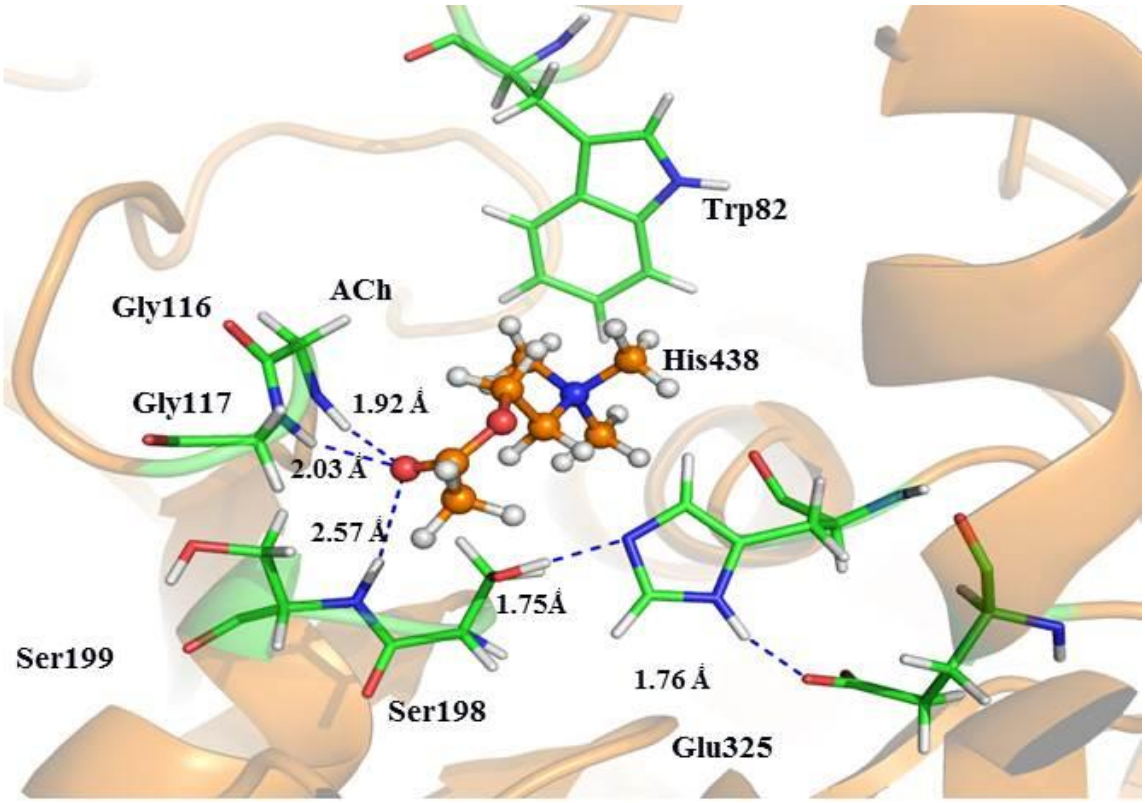

Figure 2-6. Demonstration of acetylcholine binding with CocH2 in the prereactive ES complex.

2.4 *In vitro* activity of the BChE mutants against (-)-cocaine

Based on the above computational insights, we carried out *in vitro* experimental tests, including site-directed mutagenesis, protein expression, and *in vitro* enzyme activity assays, on CocH1, CocH2, CocH3 and E13-1 mutants of human BChE. To minimize the possible systematic experimental errors of the *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 [^{3}H](-)-cocaine (labeled on its benzene ring) or [^{3}H]ACh (labeled on its acetyl group) with varying concentrations of substrate. Depicted in Figures 2-7 to 2-10 are the measured kinetic data. Summarized in Table 2-1 are the determined kinetic parameters for hydrolysis of (-)-cociane.

The four mutants of human BChE collected in Table 2-1 all have a considerably improved catalytic efficiency against (-)-cocaine. CocH1 has a 1,080-fold improved catalytic efficiency against (-)-cocaine (k_{cat}/K_M = 9.9 × 10^8 M^{-1} min^{-1}) compared to the wild-type enzyme (k_{cat}/K_M = 9.1 × 10^5 M^{-1} min^{-1}). Both k_{cat} and K_M values of CocH2 against (-)-cocaine are significantly lower than the corresponding values of CocH1. Overall, CocH2 has a 1,730-fold improved catalytic efficiency against (-)-cocaine

(k_{cat}/K_M = 1.6 × 10^9 M^{-1} min^{-1}) compared to the wild-type enzyme. Starting from CocH2, the additional Y332G mutation produces E13-1. The additional Y332G mutation significantly increases both the k_{cat} and K_M values of CocH2 against (-)-cocaine. Overall, the catalytic efficiency (k_{cat}/K_M = 1.3 × 10^9 M^{-1} min^{-1}) of E13-1 is slightly lower than that of CocH2.

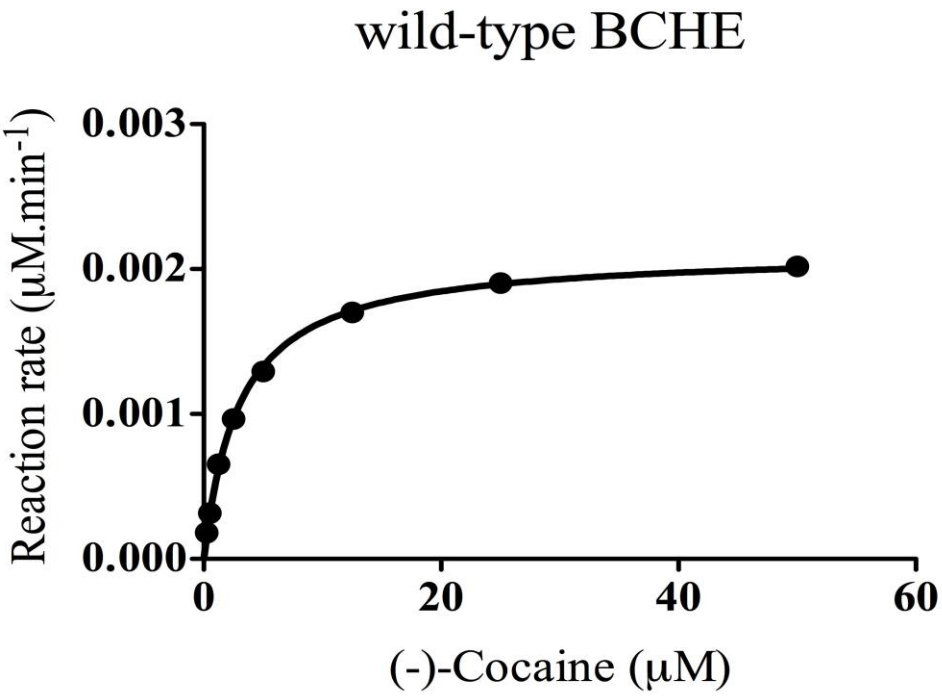

Figure 2-7. Plot of measured reaction rates (with error bars) *versus* the concentration of (-)-cocaine for cocaine hydrolysis catalyzed by wild-type BChE.

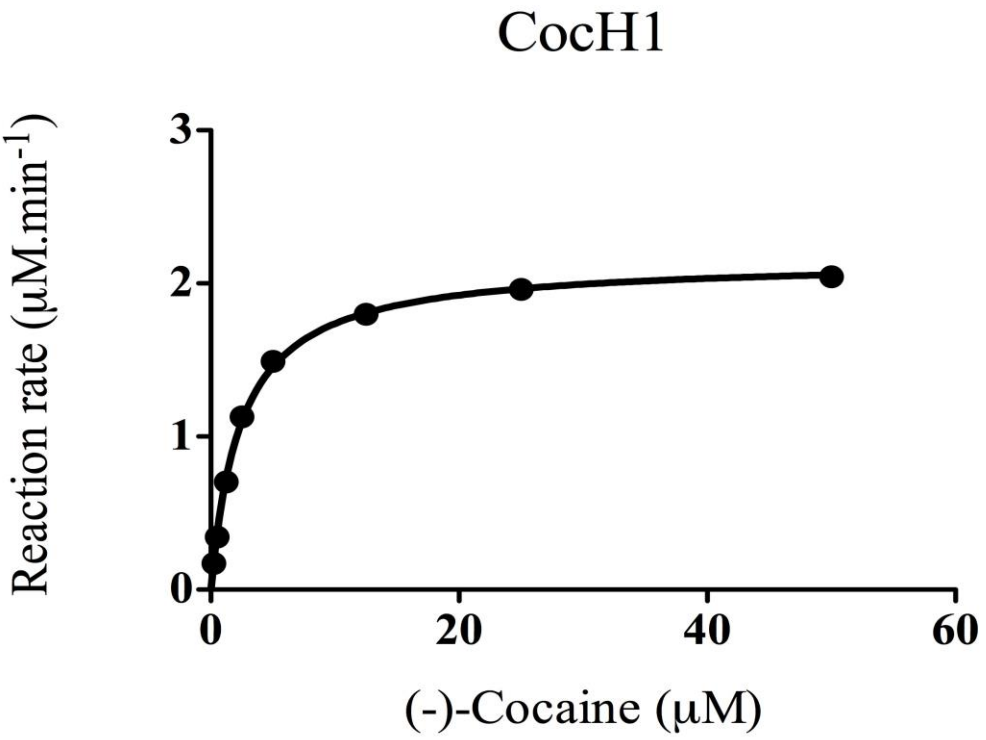

Figure 2-8. Plot of measured reaction rates (with error bars) *versus* the concentration of (-)-cocaine for cocaine hydrolysis catalyzed by CocH1

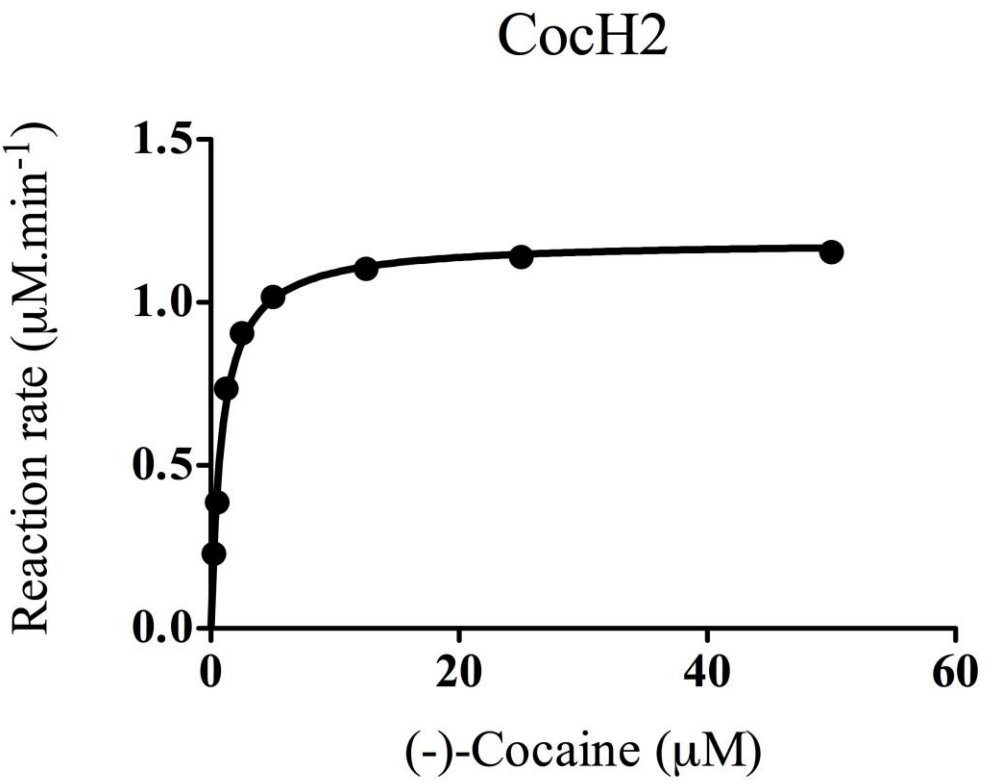

Figure 2-9. Plot of measured reaction rates (with error bars) *versus* the concentration of (-)-cocaine for cocaine hydrolysis catalyzed by CocH2

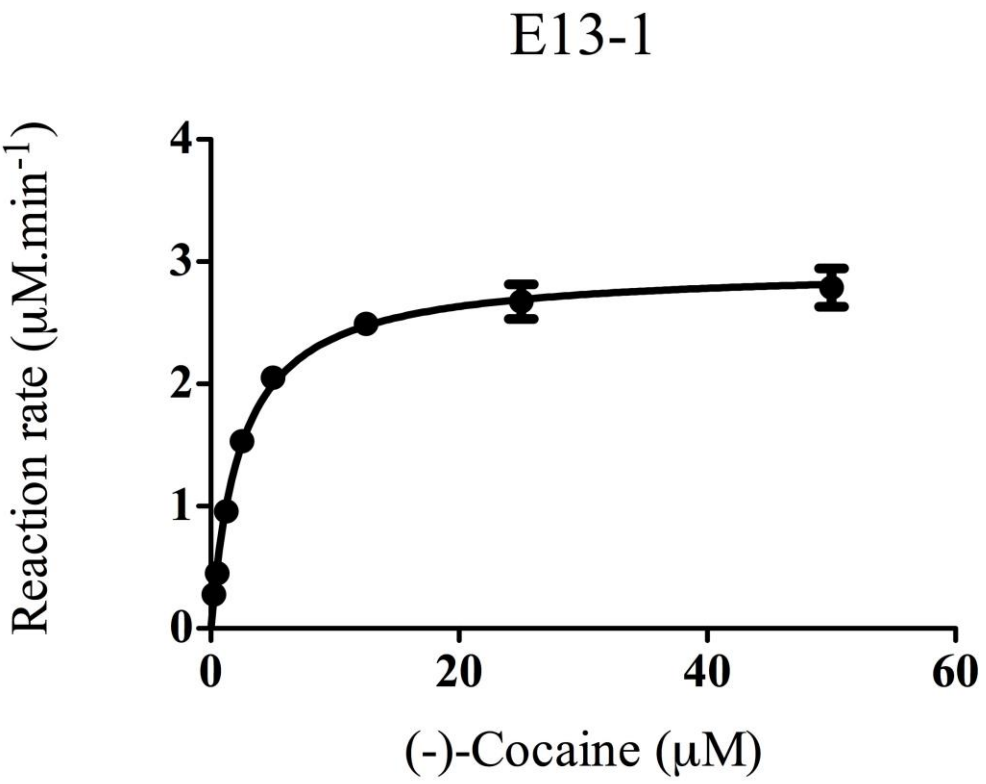

Figure 2-10. Plot of measured reaction rates (with error bars) *versus* the concentration of (-)-cocaine for cocaine hydrolysis catalyzed by E13-1.

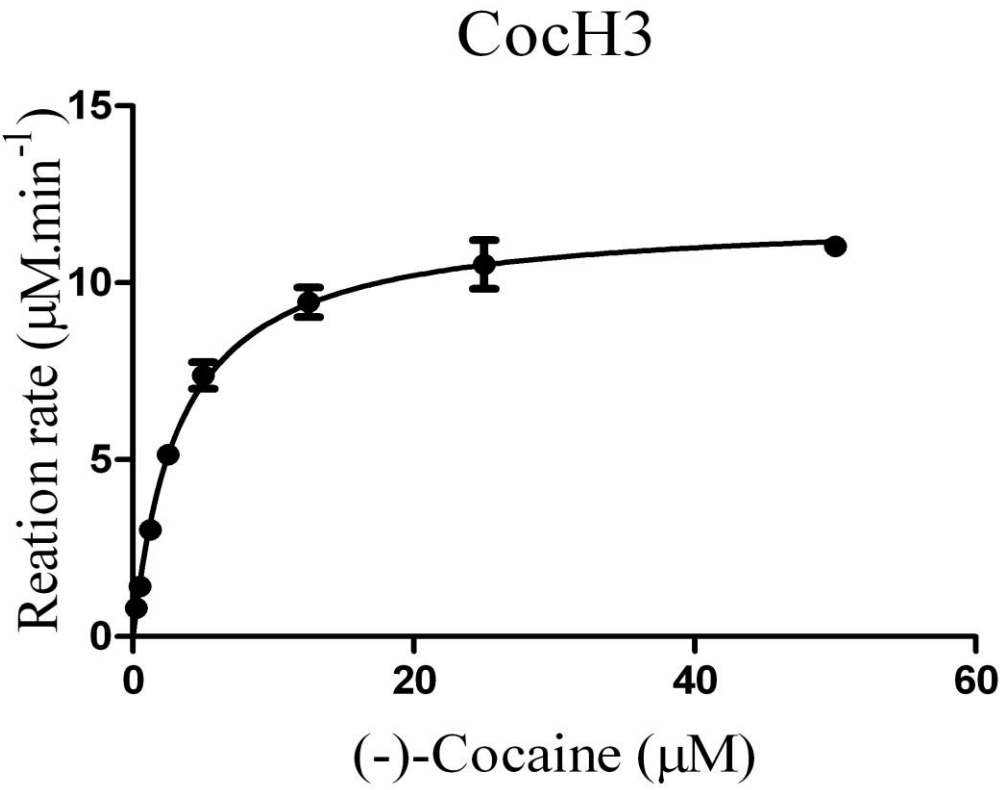

Figure 2-11. Plot of measured reaction rates (with error bars) *versus* the concentration of (-)-cocaine for cocaine hydrolysis catalyzed by CocH3.

Table 2-1. Kinetic parameters determined for (-)-cocaine hydrolysis by wild-type BChE and its mutants

Enzyme	**K_M (μM)**	**k_{cat} (min^{-1})**	**k_{cat}/K_M ($M^{-1}min^{-1}$)**	**RCE[a]**
Wild-type BChE[b]	4.5	4.1	9.1×10^5	1
CocH1	3.1	3,060	9.9×10^8	1,080
CocH2	1.1	1,730	1.6×10^9	1,730
CocH3	3.1	5,700	1.8×10^9	2,020
E13-1	3.5	4,430	1.3×10^9	1,390

[a] RCE refers to the relative catalytic efficiency (k_{cat}/K_M)

[b] Data for wild-type BChE from reference (Sun et al., 2002a).

2.5 *In vitro* activity of the BChE mutants against acetylcholine

Regarding the catalytic activity of wild-type BChE against ACh (shown in Figures 2-12 to 2-16), we obtained k_{cat} = 86,000 min^{-1} and K_M = 148 μM. The K_M value of 148 μM obtained from our study is nearly identical to the K_M value of 150 μM reported by Gao *et al*. [55]. Our determined k_{cat} value of 86,000 min^{-1} is slightly larger than, but reasonably close to, the k_{cat} value of 61,200 min^{-1} reported by Gao *et al*. [55]. It is remarkably notable in Tables 2-1 and 2-2 and Figures 2-12 to 2-16 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_{cat}/K_M = 5.8 × 10^8 M^{-1} min^{-1}), all of the mutants listed in Table 2-2 have significantly lower k_{cat} and K_M values against ACh. Overall, the catalytic efficiency (k_{cat}/K_M = 5.4 × 10^8 M^{-1} min^{-1}) of CocH2 against ACh is slightly lower than that of the wild-type enzyme (~0.93-fold), whereas the other three mutants even have a significantly lower catalytic efficiency against ACh (about one third).

Further, as seen in Table 2-2, the catalytic efficiency of wild-type BChE against ACh (k_{cat}/K_M = 5.8 × 10^8 M^{-1} min^{-1}) is ~640-fold higher than that of the same enzyme against (-)-cocaine (k_{cat}/K_M = 9.1 × 10^5 M^{-1} min^{-1}). However, it is interesting to note that, for each of the four BChE mutants, the catalytic efficiency against (-)-cocaine (k_{cat}/K_M = 9.9 × 10^8 to 1.6 × 10^9 M^{-1} min^{-1}) becomes significantly higher than that of any enzyme

(wild-type BChE or mutant) in Table 2-2 against ACh (k_{cat}/K_M = 1.8–5.8 × 10^8 M^{-1} 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 summary, 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 new BChE mutants that not only have a considerably improved catalytic efficiency against (-)-cocaine, but also have the desirable selectivity for (-)-cocaine over ACh.

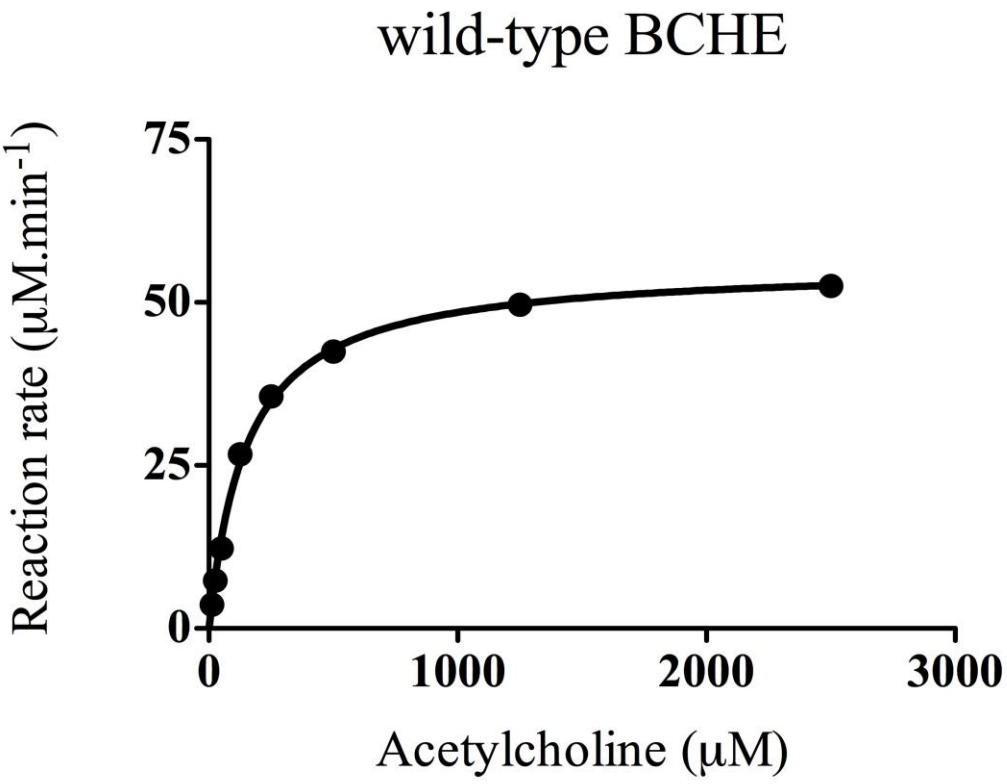

Figure 2-12. Plot of measured reaction rates (with error bars) *versus* the concentration of acetylcholine for acetylcholine hydrolysis catalyzed by wild-type BChE.

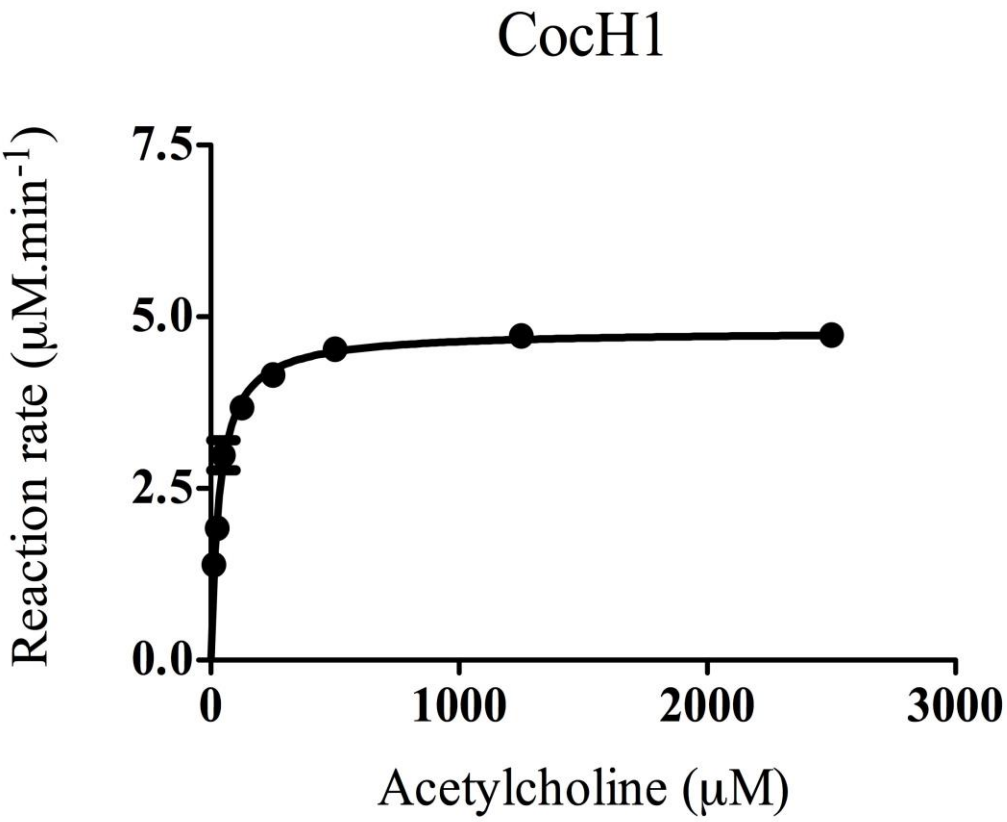

Figure 2-13. Plot of measured reaction rates (with error bars) *versus* the concentration of acetylcholine for acetylcholine hydrolysis catalyzed by CocH1.

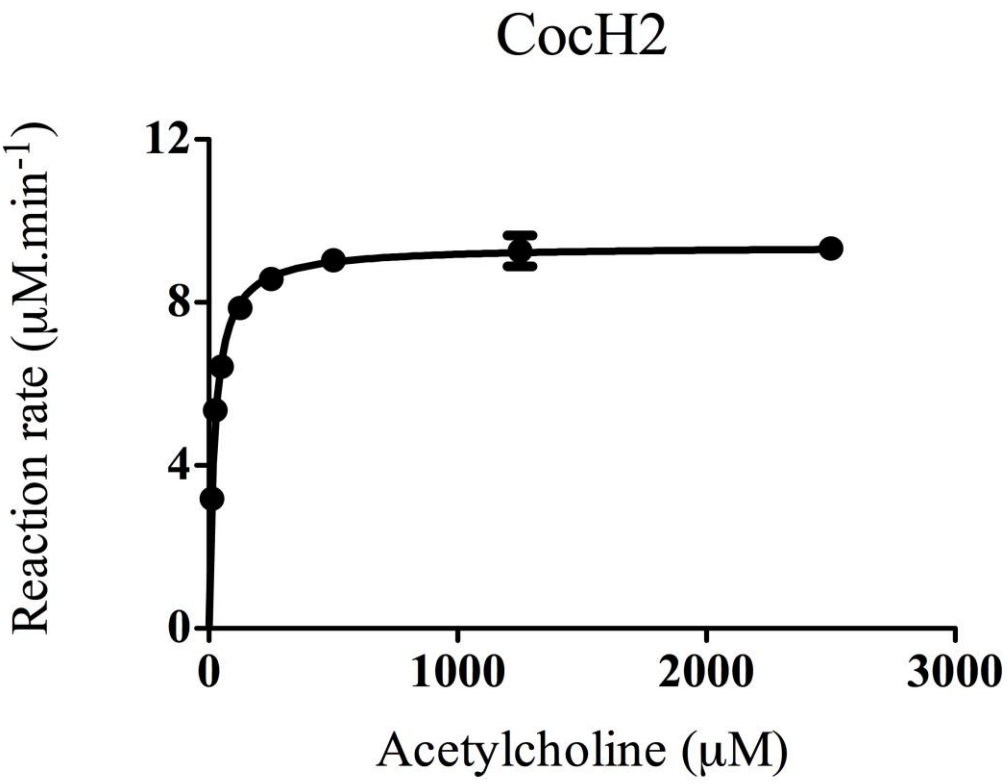

Figure 2-14. Plot of measured reaction rates (with error bars) *versus* the concentration of acetylcholine for acetylcholine hydrolysis catalyzed by CocH2.

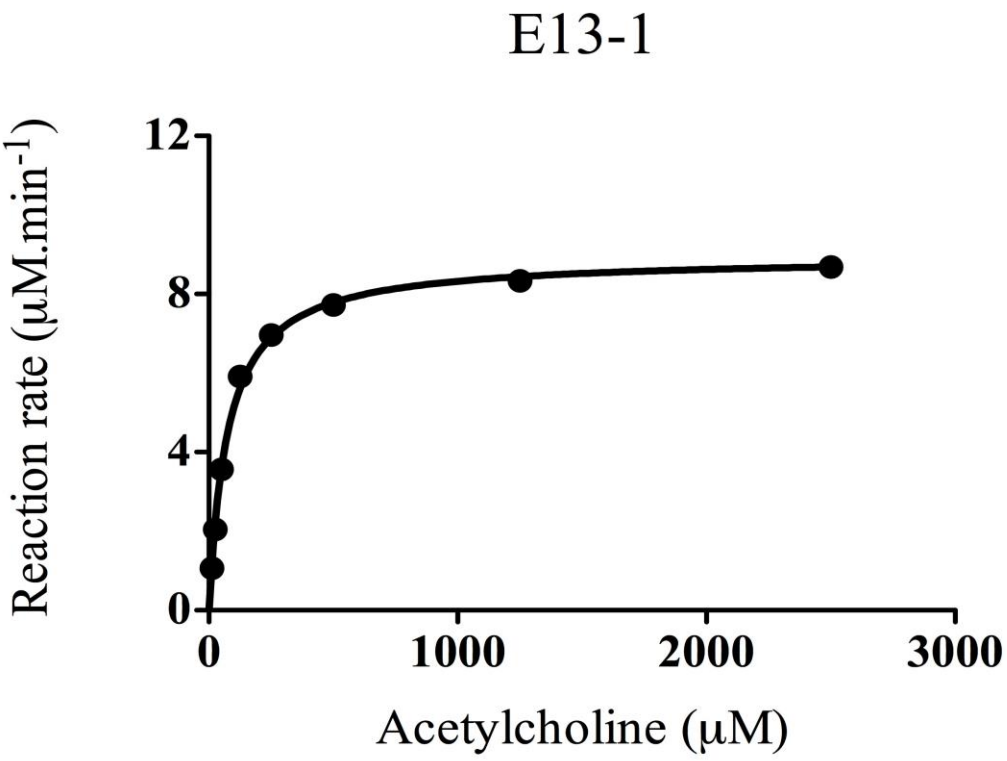

Figure 2-15. Plot of measured reaction rates (with error bars) *versus* the concentration of acetylcholine for acetylcholine hydrolysis catalyzed by E13-1.

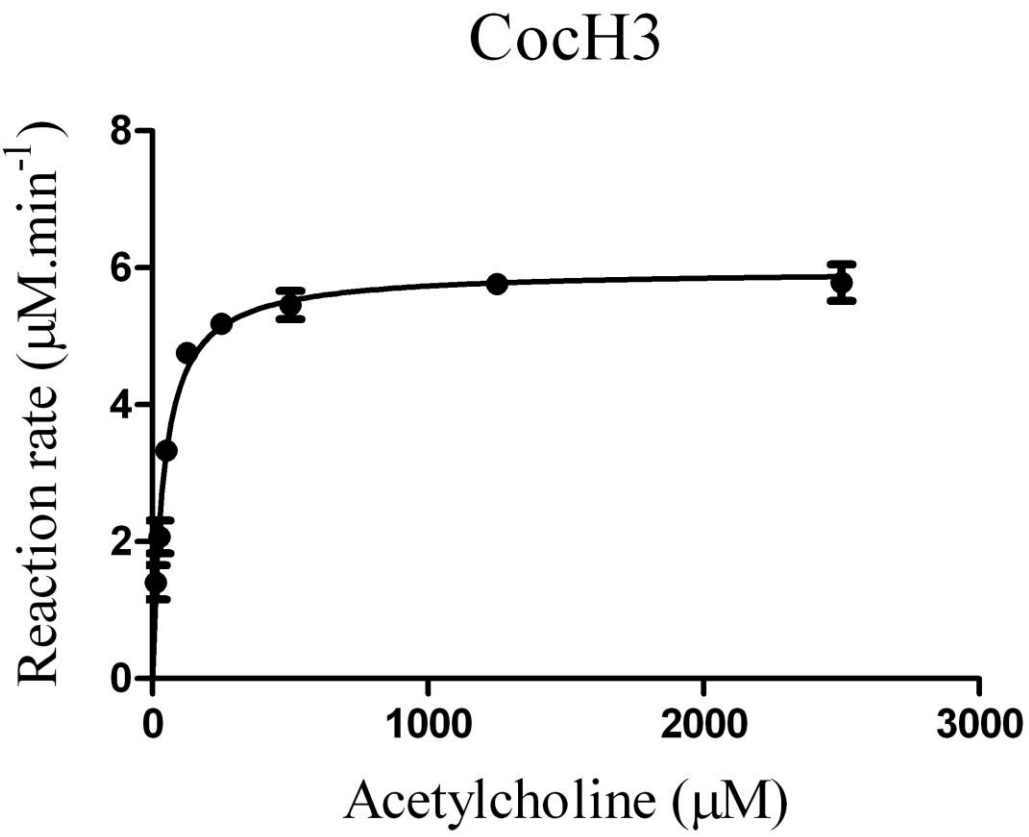

Figure 2-16. Plot of measured reaction rates (with error bars) *versus* the concentration of acetylcholine for acetylcholine hydrolysis catalyzed by CocH3.

Table 2-2. Kinetic parameters determined for acetylcholine hydrolysis by wild-type BChE and its mutants

Enzyme	K_M **(μM)**	k_{cat} **(min^{-1})**	k_{cat}/K_M **($M^{-1} min^{-1}$)**	**RCE**[a]
Wild-type BChE[b]	148	86,000	5.8×10^8	1
CocH1	36	7,500	2.1×10^8	0.36
CocH2	27	14,600	5.4×10^8	0.93
CocH3	45	8,600	1.9×10^8	0.30
E13-1	75	13,800	1.8×10^8	0.32

[a] RCE refers to the relative catalytic efficiency (k_{cat}/K_M)

[b] Data for wild-type BChE from reference (Sun et al., 2002a)

CHAPTER 3 Development of Stable Cell Lines for Large-scale Production of Cocaine Hydrolases

3.1 General consideration of the stable cell line development

As summarized in Chapter 2, unique computational strategies and protocols (based on virtual screening of rate-determining transition states of the enzymatic reaction) developed in our lab have been employed to design and discover new BChE mutants with a considerably improved catalytic activity against (-)-cocaine.[2, 43-45, 56] It is interesting to characterize these high-activity mutants of human BChE in animal models of cocaine overdose and addiction. The desired characterization of each new enzyme in animal models requires a stable expression cell line efficiently producing the enzyme in large quantity.

Traditionally, generation of a stable cell line for therapeutic protein production begins with construction of an expression vector. A suitable vector (plasmid or virus) usually carries the gene for target protein and a metabolic selectable marker or an antibiotics selectable marker for the cell line of choice. After transfected with the vector, the cells are then grown under the selection pressure and screened for the expression level

of the target protein. The high-productivity clones are selected and amplified for scale-up production. Here the integration position and plasmid copy number affect the productivity of the cells. One of the more recently developed strategies is site-specific integration. Since only 0.1~1% of genome region are actively transcribed, integration of the target gene into specific loci would ensure high and stable productivity.[57-59] Several recombinases, such as Cre and Flp, were utilized for this purpose because of their capability to identify specific sequences and to mediate the integration of the foreign gene for a therapeutic protein.[57, 58] In all the approaches mentioned above, the selection and screening for highly productive single clones are a time-consuming process which may take months.

Currently, Chinese Hamster Ovary (CHO) cells are used for production of about 70% of the therapeutic proteins.[59] They have been proven to be safe and able to provide efficient, proper post-translational modification for proteins. However, CHO cells are also difficult to transfect and unstable for the foreign genes. So the commonly used forms of CHO cells are genetically modified, such as dihydrofolate reductase (DHFR)-deficit CHO cell lines DUXB11 and DG44.[58, 59] The expression vector will carry both the gene encoding the lacked enzyme (such as DHFR) with an impaired promoter and the gene for expression of the target protein. After the transfection and metabolic selection, the target gene could be stabilized and amplified by addition of increasing dose of an inhibitor for the lacked enzyme (such as DHFR). Recombinase aided site-specific integration is also

applied alone or in combination with the gene amplification system to achieve establishment of highly efficient stable cell lines.[57, 58]

There have been many efforts in developing high-efficiency stable cell lines to produce BChE in a relatively larger scale. Traditional transfection-selection method has been used to generate stable CHO cell lines that yield about 3 to 5 mg/L pure BChE or mutant.[60] Insect cells, which can produce monoclonal antibody with the yield of 52 mg/L/day in batch culture,[61] are generally considered as a highly efficient expression system to produce recombinant proteins. However, in expression of a truncated human BChE mutant, insect cells can only achieve 4 mg/L of the production level.[62] It is difficult to generate cell lines that can stably express BChE or its mutant at a high level. Alternatively, transgenic animals/plants strategies[30, 63] have succeeded in yielding recombinant BChE and mutants at high production levels. But the produced proteins have other problems. For example, recombinant BChE produced in transgenic goats has a short *in vivo* half-life.[30] In addition, it requires a long period of time to generate the transgenic animals or plants. It is challenging to develop an effective method for scale-up production of BChE or its mutant.

Here we report the use of a lentivirus-based repeated transduction approach which transduces the prepared lentivirus into CHO cells repeatedly for stable cell line generation. This approach has led us to successfully generate stable cell lines which can efficiently produce several highly efficient cocaine hydrolases (CocHs), including CocH3 (*i.e.* the A199S/F227A/S287G/A328W/Y332G mutant). In addition, a stable human

embryonic kidney (HEK) 293F cell line was also developed to produce CocH3 for comparison. The discussion below will be first focused on the CocH3-expressing CHO cell line in order to illustrate our approach to the stable cell line generation.

3.2 Materials and methods used for the investigation in this chapter

3.2.1 Preparation of lentivirus encoding the gene of the BChE mutant

The BChE mutant (CocH3) cDNA in lentivirus plasmid was constructed into pCSC-SP-PW vector at ApaI and XhoI sites. The A199S/F227A/S287G/A328W/Y332G mutations were generated on cDNA of full-length human BChE (access number: P06276 in the Swiss Protein Database) on pRC/CMV-BChE by using site-directed mutagenesis. The CocH3 gene was amplified by PCR with pRC/CMV–CocH3 as a template. The forward primer is gagggcccaaggtgcacggcccacgt (ApaI), and backward primer is ccgctcgagttagagacccacacaactttct (XhoI). Then the CocH3 cDNA was ligated with pCSC-SP-PW fragment that was double digested by ApaI and XhoI. The sequence of construct was confirmed by DNA sequencing. To package the lentivirus particles carrying the gene of CocH3, HEK293FT cells were cultured in DMEM-10% Fetal Bovine Serum (FBS) (Life Technologies, Grand Island, NY) with 250 ng/ml G418 (Life Technologies, Grand Island, NY). Cells were transfected at approximately 70% confluence by lentivirus plasmid encoding CocH3 (CocH3/pCSC-SP-PW) along with three other packaging plasmids, pMDL-pg.RRE, pRSV.rev, and pVSVG, at a mass ratio of 10:6.5:2.5:3.5.

Transfection was achieved by lipofection. In brief, for a 10 cm dish of cells, total DNAs about 22.5 μg were mixed first, and then diluted in 1.5 ml of Opti-MEM® I Reduced Serum Medium (Invitrogen) without serum. 60 μl of Lipofetamine™ 2000 (Invitrogen) was then mixed with 1.5 ml of Opti-MEM® I Reduced Serum Medium and incubated at room temperature for 5 min. The diluted DNAs and Lipofetamine™ 2000 were mixed and incubated at room temperature for 20 min before added dropwise onto the cell culture. The cells were cultured at 3% CO_2 at 37 ℃. Culture medium was changed 12 to 16 h after transfection. The medium was collected three times at a 24 h-interval beginning 24 h after the post-transfection change of medium. The medium was filtered through a 0.45-μm cellulose acetate filter and spun in Beckman SW28 rotor at 19,400 rpm for 2 h at 4°C to pellet the virus particles. Lentivirus was then suspended in Hank's balanced salt solution and aliquoted to be stored at -80 ℃. The physical concentration of lentivirus was determined by using QuickTiter™ lentivirus rapid quantitation kit (Cell Biolabs, San Diego, CA).

3.2.2 Generation of stable cell line by lentivirus infection

Scale-up preparation of enzyme was first achieved by infecting CHO-S cells with lentivirus followed by resuspending attached CHO-S cells in protein free suspension culture in Gibco® FreeStyle™ CHO expression medium (Life Technologies, Grand Island, NY) with 8 mM L-glutamine. The day before infection, the cells were loaded at 1 $\times 10^5$ cells/ml in 12-well plate and cultured steadily in freestyle CHO expression medium

with 8 mM L-glutamine and 1% FBS. Cells began to attach to the plate soon in the presence of FBS after the change of culture condition. Lentivirus was then added to infect the cells for one day. The cross linker polybrene (Santa Cruz Biotechnology, Santa Cruz, CA) was also added to cell culture to increase the infection efficiency by neutralizing the charge repulsion between the virus and cell culture. The infection was stopped by change of medium. The infected cells were allowed to recover from the infection for one to two days (or more days, depending on the status of the cells), then were suspended by 0.05% trypsin-EDTA and split into two halves. One half was cultured in a 6-well plate, and then transferred to a 10 cm plate in 1% FBS freestyle CHO expression medium. Then the cells were changed back to suspension culture, and the culture volume increased from 6-well plate to 125-ml shake flask for yield determination. The other half was seeded in a 12-well plate for the next round of infection. After each infection, the yield was determined for a 9-day fermentation of 30 ml cell culture. The pool with the highest yield was selected and amplified for scale-up production of the enzyme. Culture medium was changed every two to three days, and collected to store at 4℃. The scalability and stability of the production was also evaluated during the process. The stable cell line in HEK293F was also generated by using the same method. For the difference, the HEK293F cells were cultured in Gibco® FreeStyle™ 293 medium (Life technologies, Grand Island, NY), with addition of 1 μM poly-L-proline (Sigma Aldrich, St. Louis, MO) to facilitate the formation of the CocH3 tetramer.

To compare the productivity of the pool with that of single clones, the cells from the pool were seeded at 2-10 cells/well in 96-well plates in free-style CHO expression medium with 1% FBS to select the single clones. The cells were cultured for another 14 to 21 days without changing medium and shaking until single clones clearly appeared. Single clone cell lines were chosen to culture in 48-well plates, then 12-well plates and 6-well plates in 1% FBS-freestyle CHO expression medium. High-expression single clones were selected by determining the enzyme activity in the medium. Selected cell-line cells were then changed to suspension culture and the culture volume was changed from 6-well plate to 125-ml flask. The yields of single clones were determined for a 9-day fermentation of 30 ml cell culture.

3.2.3 Enzyme purification

Scale-up purification of the enzyme in the medium was achieved by a two-step purification using ion exchange chromatography followed by affinity chromatography. In brief, the crude medium 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℃ with occasional stirring for 1 h. More than 95% enzyme activity was found to bind to the resin after the incubation. The suspension was then packed in a column and the medium was allowed to flow through rapidly with the aid of suction of (50-100 ml /min). The QFF resin was repacked again in a washing buffer (20 mM Tris-HCl) after the entire medium was excluded. After washing the column with 20 mM Tris-

HCl, pH 7.0, overnight at 4 ℃, the enzyme was eluded by 20 mM Tris-HCl, 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 hydroxyapatite column (Clarkson Chem. Co., Williamsport, PA) (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 then the enzyme was eluted by 10 mM sodium phosphate buffer, pH 7.0, containing 0.3 M NaCl. The purified enzyme was dialyzed against phosphate-buffered saline and stored at 4 ℃ or -80 ℃. The purified enzyme had a catalytic activity of 34 U/mg against (-)-cocaine.

3.2.4 Nondenaturing gel electrophoresis

Activity-stained nondenaturing polyacrylamide gel was ultilized to estimate the relative amount of tetramers, dimers, and monomers. 4% polyacrylamide stacking gel and 8 % separating gel were prepared in a Biorad gel apparatus. Electrophoresis was at 8 mA constant current for 6 h at 4 ℃. The gel was stained for BChE activity against 2 mM butyrylthiocholine iodide by using the method described by Karnovsky and Roots.[(64)]

3.3 Productivity of the stable cell line expressing CocH3

We first wanted to explore an efficient method to generate a high-productivity cell line stably expressing a BChE mutant. The BChE mutant cDNA in lentivirus plasmid was

constructed into pCSC-SP-PW vector. The lentivirus was packed in 293FT cells by transfection of pCSC-SP-PW-BChE along with three other packaging plasmids and then purified by centrifugation. The CHO-S cells were loaded in 12-well plate and transduced by adding purified lentivirus repeatedly. After each infection, the yield of transduced cells was determined in a 9-day fermentation of 30 ml cell culture. The use of the lentivirus-based repeated transduction method resulted in a stable CHO-S cell line efficiently expressing CocH3 (BChE mutant). The production level is related to the number of times of transduction performed, as seen in Figures 3-1 and 3-2. The productivity of the cell pool was increased after each additional round of transduction-recovery cycle until after the 7th time (Figure 3-2). The data suggested that, during the process, more copies of the target gene were integrated into the genome for enhanced target protein expression. After the 7th time, further transduction no longer increased the yield of the 9-day fermentation. In fact, after the 7th transduction, further transduction decreased the yield gradually. The decrease in the yield might be due to the possibility that the hot-spots of chromosomal loci were already saturated by the foreign genes after the 7th transduction and the random insert into other regions of genome might harm the health of the cells. The amount of produced enzyme was proportional to the incubation time during the first a few days when the nutrient was sufficient (Figure 3-1). Then the cells started to die due to lack of nutrients and accumulation of toxins like lactate. The production rate was gradually slowing down until the production level hit the plateau when all the cells died.

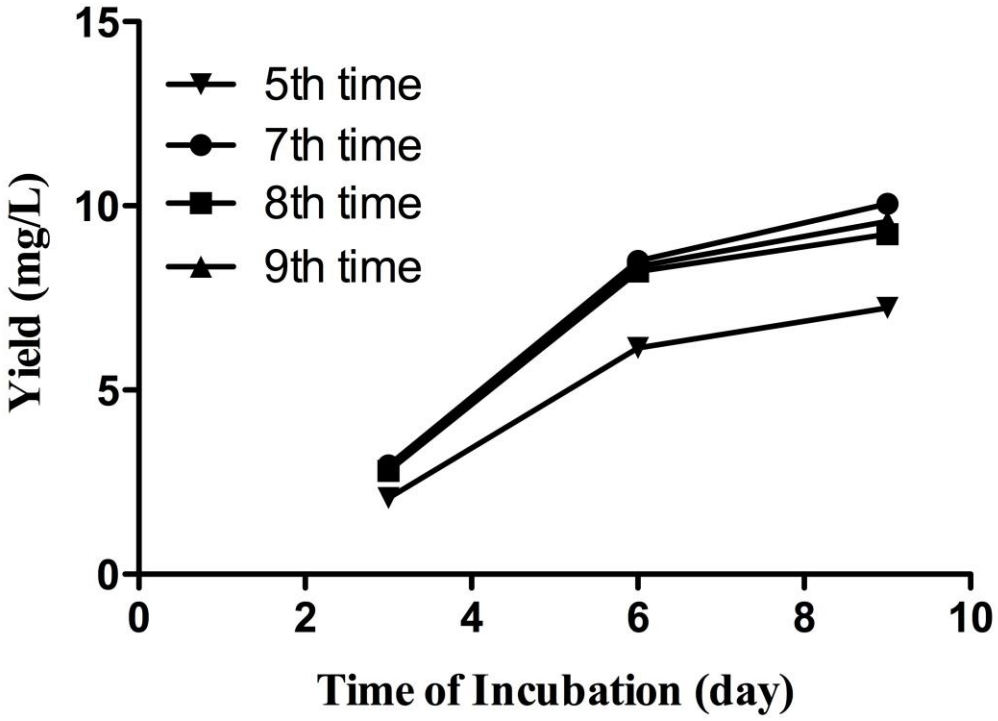

Figure 3-1. Time course of CocH3 production in transduced CHO-S cells.

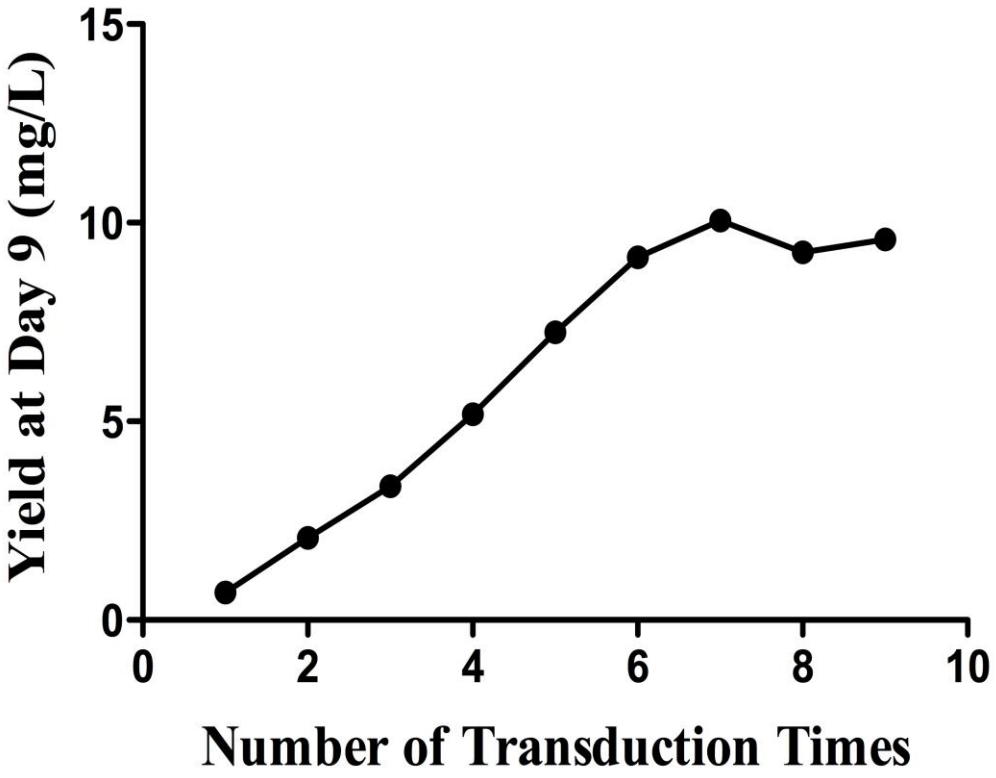

Figure 3-2. CocH3 production rate in a 9-day fermentation *versus* the number of rounds of transduction cycles.

We also selected single clones from the pool and determined the yields associated with the single clones (Figure 3-3). The single clones did not display significantly higher or lower yields in the 9-day fermentation test compared to the pool, suggesting that the cells were transduced rather homogenously such that almost all of the cells had a similar productivity. Based on this observation, in the future work, we may simply skip the step of the selection of single clones so as to save a lot of time during the development of stable cell lines for other BChE mutants.

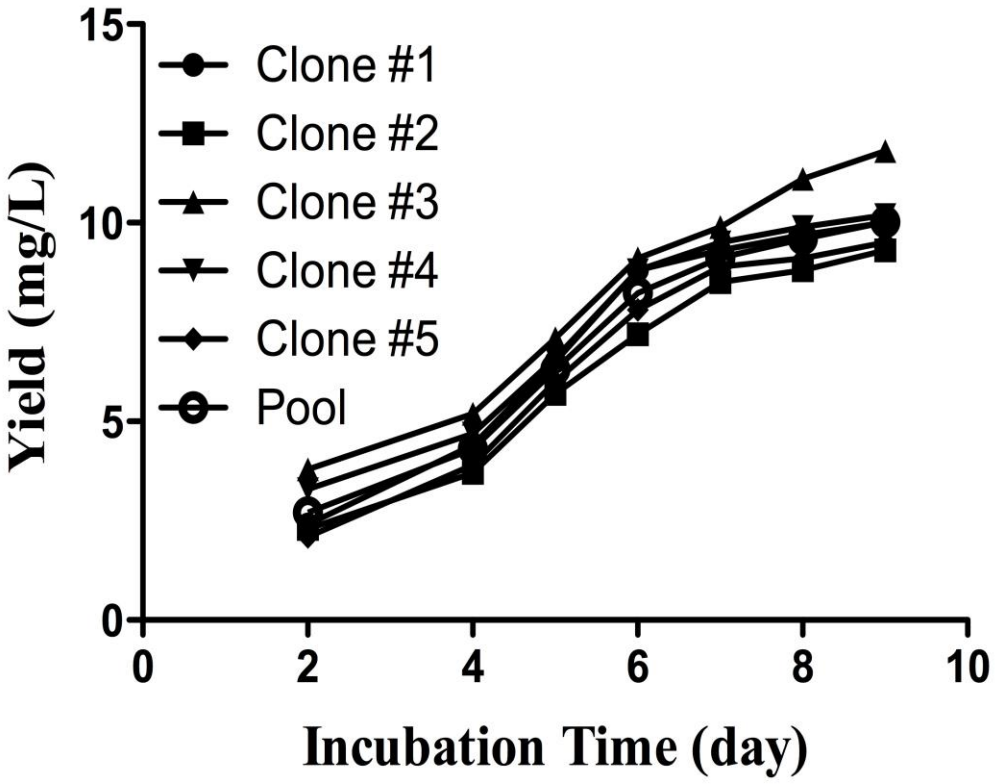

Figure 3-3. CocH3 production rates in a 9-day fermentation using 7-time transduced cells and the single clones selected from the pool.

Since CHO cell is known to be difficult to sustain the expression level of foreign genes, we also evaluated the scalability and stability of the established CocH3-expressing cell line. For the stability test, the cells were seeded at 6×10^5 cells/ml after each passage and cultured for three days. Then, one eighth of them was passed while the rest were cultured continuously for 6 days to determine the yield of the 9-day fermentation. As seen in Figure 3-4, after several times of passages, the cells did not significantly decrease the productivity. In the scalability test, the yield rate did not significantly change by increasing the volume of the cell culture from milliliters to liters. So, the cells were sustainable and scalable for the protein production.

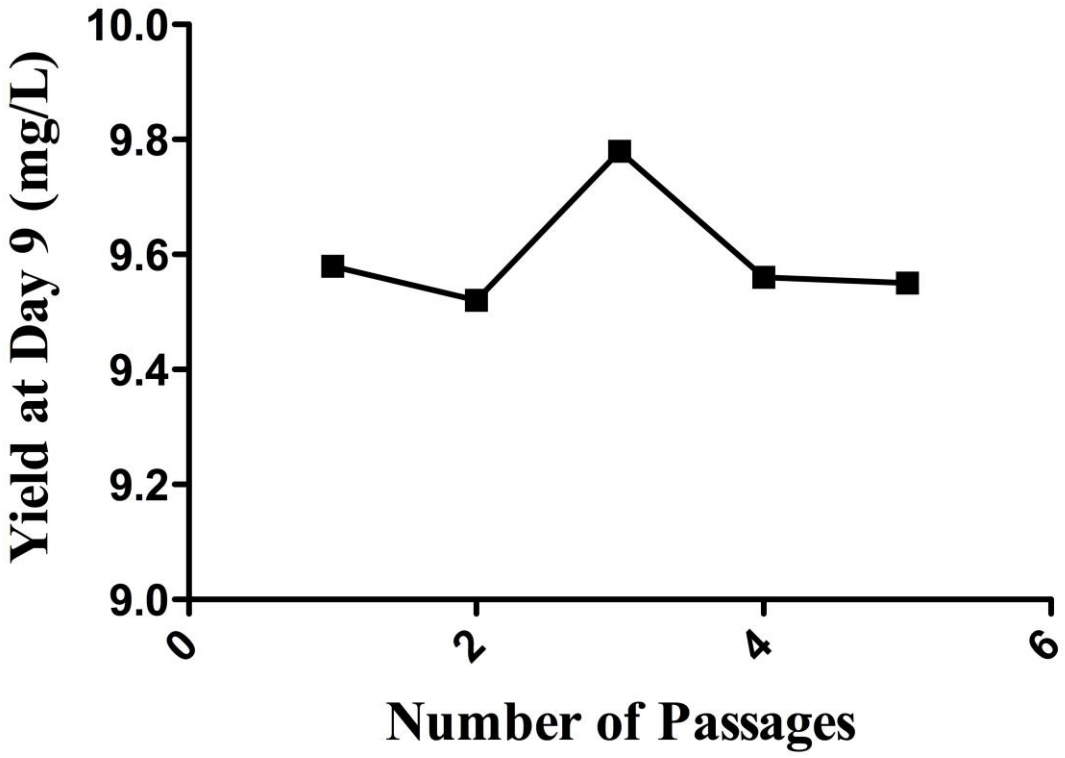

Figure 3-4. Stability of the CocH3 production using the stable CHO-S cells obtained from the 7-time transduction. The cells were passed several times. The yield was determined after each passage.

Depicted in Figure 3-5 is nondenaturing gel (8%) stained for the BChE activity of the CocH3 materials expressed in the HEK293F cells (with 1 μM poly-L-proline in the medium) and the CHO-S cells. According to the data in Figure 3-5A, CocH3 materials expressed in the HEK293 cells (with 1 μM poly-L-proline in the medium) and the CHO-S cells all predominantly existed in tetramer. For CocH3 expressed in the HEK293F cells (with 1 μM poly-L-proline in the medium), there was no band observed for the monomer or dimer. For CocH3 expressed in the CHO-S cells, a very weak (wide) band was noted for the monomer (with a negligible amount), but no dimer was noted. In comparison, weak (wide) bands were noted for both the monomer and dimer in CocH3 expressed in the transiently transfected CHO-S cells (with 1 μM poly-L-proline in the medium).

Concerning the catalytic activity of the BChE mutant, according to our previously reported *in vitro* assay,[(2)] we determined that k_{cat} = 5700 min^{-1} and K_M = 3.1 μM for CocH3 expressed in HEK293F cells. The same *in vitro* assay (the sensitive radiometric assay) was employed to determine the enzymatic activity of CocH3 expressed in the stable CHO-S cells, showing no significant change in the catalytic activity (data not shown).

As the nondenaturing gel staining (Figure 3-5) revealed that the CocH3 proteins expressed in the HEK293F and CHO-S cells were predominantly tetramer, there was no significant difference in oligomerization between the two protein forms.

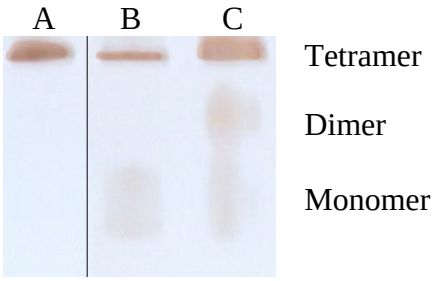

Figure 3-5. Nondenaturing gel (8%) stained for the BChE activity of the CocH3 mutant expressed (A) the stable HEK293F cells (with 1 μM poly-L-proline in the medium), (B) the stable CHO-S cells, (C) the transiently transfected CHO-S cells (with 1 μM poly-L-proline in the medium). The gel was run with the constant current of 8 mA at 4℃ overnight. The gel was stained for the BChE activity with butyrylthiocholine iodide as substrate at room temperature for 1 to 4 hours until the protein bands with the enzyme activity were clearly identified.

3.4 Stable cell lines expressing other CocH forms

By using the repeated transduction protocol described above, stable cell lines for production of other BChE mutants were established, including the CHO-S stable cell line expressing the most recently designed A199S/F227A/P285A/S287G/A328W/Y332G mutant, denoted as E30-6 (k_{cat} = 29,200 min^{-1}, K_M = 3.65 μM, and k_{cat}/K_M = 8.0 × 10^9 min^{-1} M^{-1} at 37 ℃, pH 8.0).[65] Not surprisingly, the yield of the E30-6 production was similar to that of the CocH3 production. The large-scale production of E30-6 using the stable cell line allowed us to characterize the enzyme in animal models that will be discussed in Chapter 4.

In addition, the same protocol was also employed to generate a stable cell line expressing CocH3 fused with Fc fragment of IgG1, a new enzyme form of CocH (denoted as Fc-CocH3 for convenience) developed recently in our lab to prolong the circulation time of the enzyme. As seen in Figure 3-6, the developed Fc-CocH3-expressing stable cell line had a higher yield (~30 mg/L) in a 10-day fermentation, compared to that of the CocH3-expressing stable cell line. The large-scale production of Fc-CocH3 using the stable cell line allowed us to perform extensive *in vivo* studies that will be reported elsewhere later.

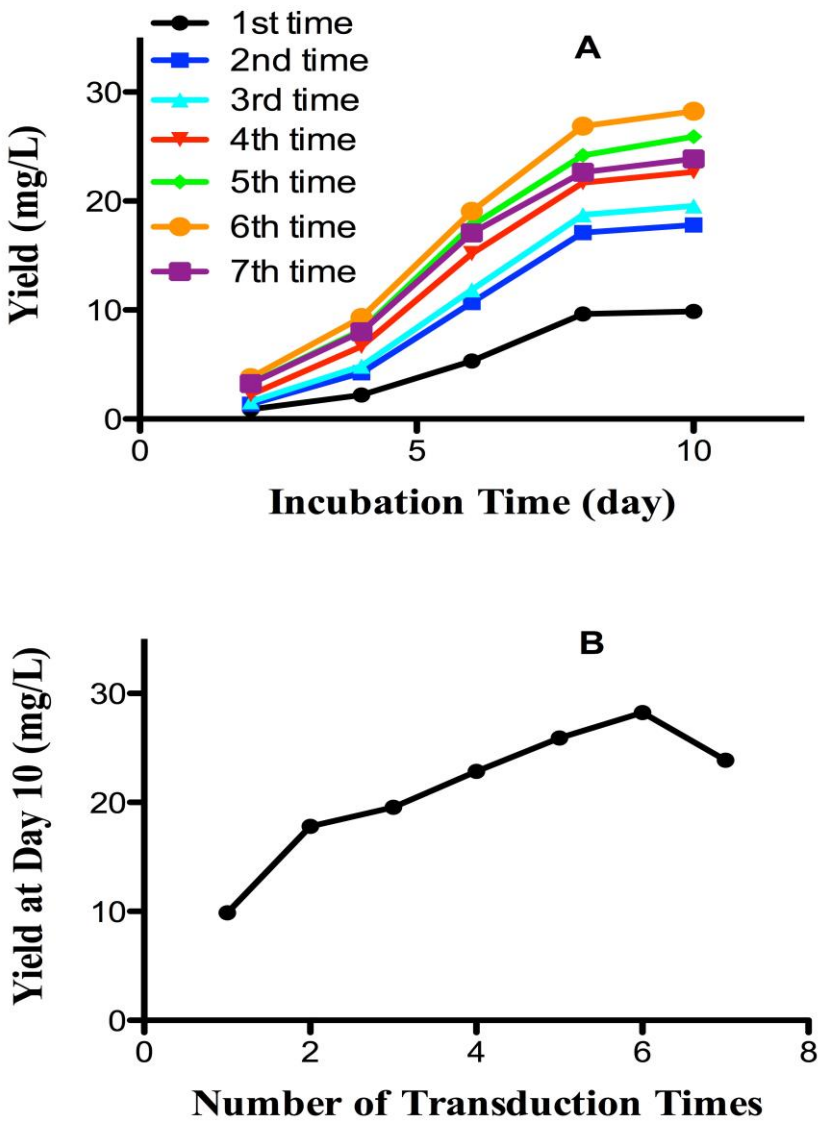

Figure 3-6. Production of Fc-fused CocH3 (Fc-CocH3) in the transduced CHO-S cells. (A) Time course of the enzyme production. (B) The yield of the Fc-CocH3 production in 10-day fermentation is related to the number of transductions.

CHAPTER 4 *In vivo* characterization of cocaine hydrolases for their potency in metabolizing cocaine

4.1 Subjects for *in vivo* studies

Sprague-Darley (male or female) rats (200-250 g), male CD-1mice and Swiss Webster male mice (27-30 g) were ordered from Harlan (Harlan, Indianapolis, IN). The rats were housed initially in 2 to 4 rats per cage. The mice were housed in groups of 2 to 5 mice per page. All the animals were allowed ad libitum access to food and water and were maintained on a 12-hour light and dark cycle with lights on at 8 AM in a room kept at a temperature of 21 to 22℃. Each animal was only used once. 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 procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Kentucky.

In addition to the *in vivo* assays at the University of Kentucky, some *in vivo* studies (including the protection experiments with three BChE mutants and rescue experiments) were carried out at the University of Michigan. For the studies at University of Michigan, 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 21–22ºC. Each mouse was used only once and each dosing condition contained 6 or 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 procedures were approved by the University Committee on the Use and Care of Animals at the University of Michigan.

4.2 Pharmacokinetic profiles of CocH3

4.2.1 Determination of *in vivo* half-life in rats

Rats were injected intravenously (i.v., *via* tail vein) with 0.15 mg/kg of the purified enzyme expressed in the stable CHO-S or HEK293F cells. The saphenous veins were punctured with a needle. About 50 to 75 μl of blood was collected into a capillary tube at various time points after the enzyme injection. The blood samples were centrifuged at 5000 g for 15 min. The serum was tested for the enzyme activity using a radiometric method as described in our previous reports.[2, 44, 50] The data for elimination of the enzyme from the circulation were fitted to a double-exponential equation which was described by Kronman.[66]

4.2.2 Biological half-lives determined

The CocH3 protein material expressed in the stable CHO-S cells was tested for its pharmacokinetic (PK) profile in rats. Rats (n=5) were administered intravenously (i.v., *via* tail vein) with 0.15 mg/kg of the purified enzyme. The blood was sampled at 2, 15, and 30 min, and 1, 2, 3, 5, 8, 12, 24, and 48 h after the enzyme injection. For comparison, the protein material expressed in the stable HEK293F cells was also tested in rats. Depicted in Figure 4-1 are the time courses of the active enzyme concentrations after the i.v. injection of the enzyme materials. The measured time-dependent concentrations of the active enzyme were fitted to a well-known double exponential equation ($[E]_t = Ae^{-k_1 t} + Be^{-k_2 t}$) which accounts for both the enzyme distribution process (the fast phase, associated with k_1) and elimination process (the slow phase, associated with k_2). The half-life associated with the enzyme elimination rate constant k_2 is called the biological half-life (the usually referred *in vivo* half-life).

The CHO-S cells-expressed CocH3 displayed a biological half-life of 7.3 hours, which is significantly longer than that (2.8 hours) of the same enzyme expressed in the HEK293F cells, in rats. The native human BChE (purified from human serum) has a half-life of 7~12 days in humans and 24 hours in rats.[67] Recombinant BChE has a much shorter biological half-life.[33] It should be noted that multiple factors could affect the biological half-life of a protein, including oligomerization and glycosylation. As the nondenaturing gel staining (Figure 3-5) revealed that the CocH3 proteins expressed in the HEK293F and CHO-S cells were predominantly tetramer, there was no significant difference in oligomerization between the two protein forms. Thus, the difference in the

biological half-life is mainly due to the difference in the post-translational modification, particularly glycosylation. The CHO-S cells-expressed CocH3 may have glycosylation closer to the native enzyme compared to that for the HEK293F cells-expressed CocH3.

Generally speaking, it is desirable to have a long *in vivo* half-life for the potential therapeutic protein in cocaine addiction treatment. The longer the enzyme can stay in the body, the longer it can protect the subjects, and the lower dosing and/or lower frequency the therapeutic protein would be needed for administration to the patients. It also lowered the potential risk of unexpected adverse effects, and increased the chances of full protection of patients against cocaine effects. Based on the data depicted in Figure 4-1, future CocH3 production should use the stable CHO-S cell line.

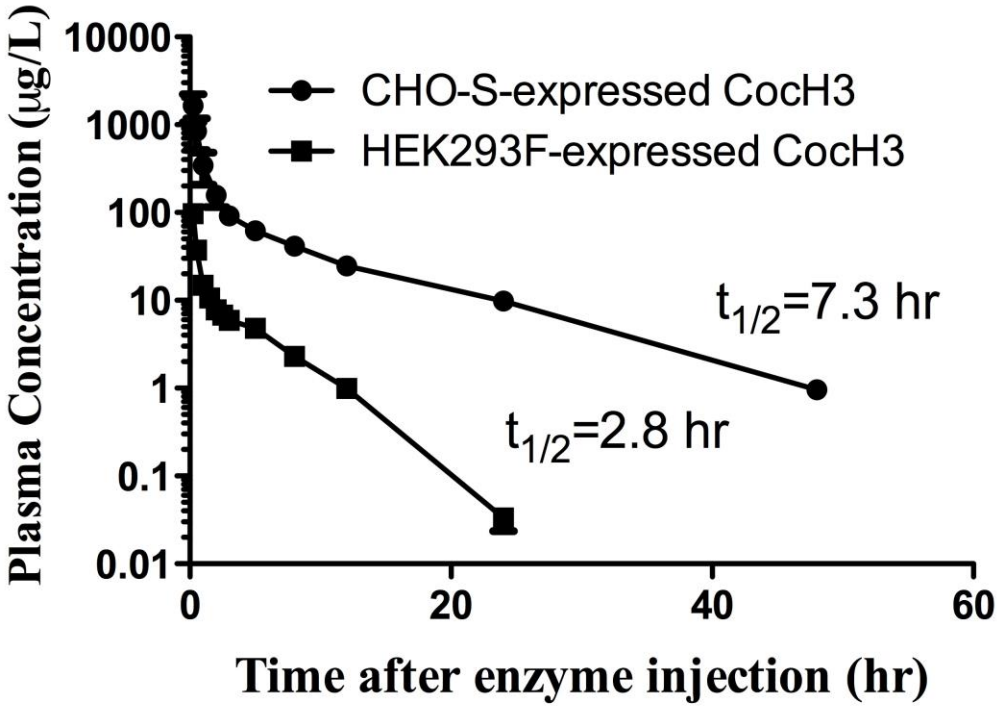

Figure 4-1. Time-dependent concentrations of the active enzyme CocH3 expressed in the CHO-S and HEK293F cells after the enzyme injection. 0.15 mg/kg of CHO-S-expressed or HEK293F-expressed CocH2 were injected to rats (n=5). Blood was sampled at 2, 15, and 30 min, 1, 2, 3, 5, 8, 12, and 24 hours and every 24 hours after the enzyme injection. The active CocH3 concentrations in the blood samples were determined in duplicate by using a sensitive radiometric assay.

4.3 Characterization of cocaine clearance accelerated by cocaine hydrolases

4.3.1 Animal procedure

General anesthetic isoflurane was utilized with nose cone during the course of study. Four rats were injected with saline through tail vein 1 min before i.v. injection of 5 mg/kg cocaine, and other four rats were injected with the enzyme (cocaine hydrolase) followed by i.v. injection of the same dose of cocaine. About 50 to 75 μl of blood from saphenous veins was collected into capillary tubes and immediately diluted in 100 μl of 250 μM paraoxon at 2, 5, 10, 15, 30, and 60 min after the i.v. injection of cocaine. Paraoxon is an irreversible BChE inhibitor that can stop the enzymatic hydrolysis of cocaine between sampling and analysis. The diluted blood samples were stored at -70 ℃ and assayed by using a High-Performance Liquid Chromatographic (HPLC) method.

Benzoic acid is the product of cocaine hydrolysis catalyzed by the enzyme. The standard cocaine and benzoic acid were purchased through Sigma-Aldrich (St. Louis, MO). Cocaine and benzoic acid concentrations in the blood samples were assayed by the following method. The frozen whole blood samples were thawed on ice for 3 hours. Then 150 μl of mobile phase (74% acetonitrile and 0.26% TFA) was mixed with each sample. Then 50 μl of 7% $HClO_4$ was added to break the blood cell membrane. The mixture was vortexed for 1 min and then centrifuged at 25,000 g for 15 min, and the supernatant was transferred to an autosampler vial of which 200 μl was injected into the chromatographic system.

Chromatography was performed using a Waters 1525 binary HPLC pump (Waters Corporation, Milford, MA), a Waters 2487 dual λ absorbance detector, a Waters 2475 multi λ fluorescence detector, and a Waters 717 plus autosampler. The mobile phase is 74% acetonitrile and 0.26 % TFA. The flow rate was 1 ml/min. The eluent was monitored at 230 nm for absorbance of benzoic acid and at 465 nm for fluoresce of cocaine when exciting at 383 nm. The cocaine peaks appeared at 10.5 min, and the benzoic acid peaks occurred at 14.5 min. The quantification of cocaine and benzoic acid was performed by comparing the corresponding HPLC peak areas with those of authentic standards.

4.3.2 Cocaine clearance accelerated by CocH3

In order to examine the *in vivo* potency of CocH3 for metabolizing cocaine, we characterized the pharmacokinetic profiles of cocaine clearance with and without the presence of CocH3 in rats by using a chromatographic assay. The data are depicted in Figures 4-2 and 4-3. The rats (n=4) were injected with saline or 0.1 mg/kg CocH3, followed by i.v. injection of 5 mg/kg cocaine. CocH3 can hydrolyze cocaine to produce benzoic acid and ecgonine methyl ester, and greatly accelerate the clearance of cocaine from the body. It has been known that the endogenous BChE in rats is very inefficient in metabolizing cocaine and, for this reason, cocaine was mainly metabolized by carboxylesterase in the blood to produce benzoylecgonine and methanol in rats.[53] The control curves in Figure 4-2 reflect the overall effects of the all cocaine elimination pathways. As seen in Figure 4-2, in the control rats, the average concentration of cocaine

at the first time point (2 min) was 7.4 μM, while the average concentration of benzoic acid (metabolite) was 0.5 μM. In the presence of CocH3, the average concentration of cocaine at ~2 min in the blood sample was below the detectable level (Figure 4-2), while the average concentration of benzoic acid at the first time point (2 min) was 12.2 μM (Figure 4-3). Most of the cocaine was hydrolyzed by CocH3 between the i.v. cocaine injection and the first blood sampling at 2 min after the injection. The CocH3-caused dramatic changes in both the cocaine and benzoic acid concentrations clearly indicated that cocaine was metabolized rapidly to benzoic acid in the presence of CocH3.

It should be mentioned that the total plasma concentration of cocaine and benzoic acid (12.2 μM) in the presence of CocH3 (when the benzoic acid concentration was higher) was higher than that (7.4 μM) in the absence of CocH3 (when the cocaine concentration was higher). This observation might be associated with the potentially different distribution volumes of cocaine and benzoic acid in the body. As well known, cocaine is an amine drug which can readily cross cell membranes under physiological condition, while benzoic acid primarily exists in the benzoate ion under physiological condition. So, benzoic acid is expected to have a relatively smaller distribution volume compared to cocaine.

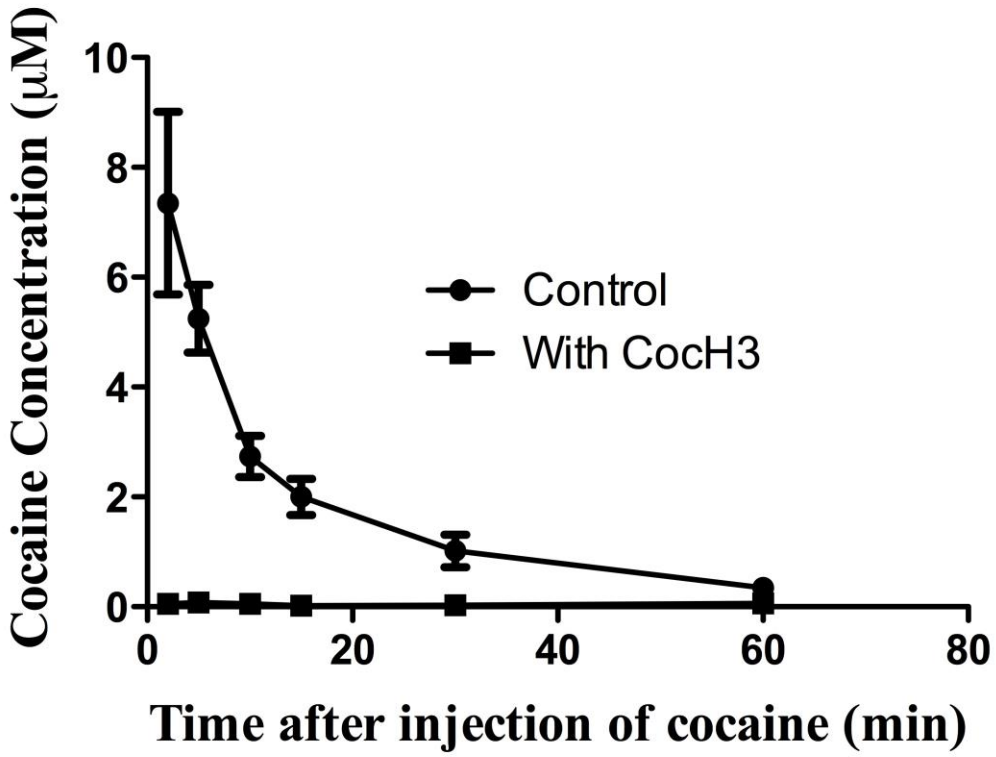

Figure 4-2. Time-dependent concentrations of cocaine in cocaine clearance accelerated by CocH3 expressed in stable CHO-S cells. 5 mg/kg cocaine was i.v. injected 1 min before i.v. injection of CocH3 or saline. The blood was sampled from 2 to 60 min after the injection of cocaine. The concentrations of cocaine in the blood samples were quantitated in duplicate by using an HPLC.

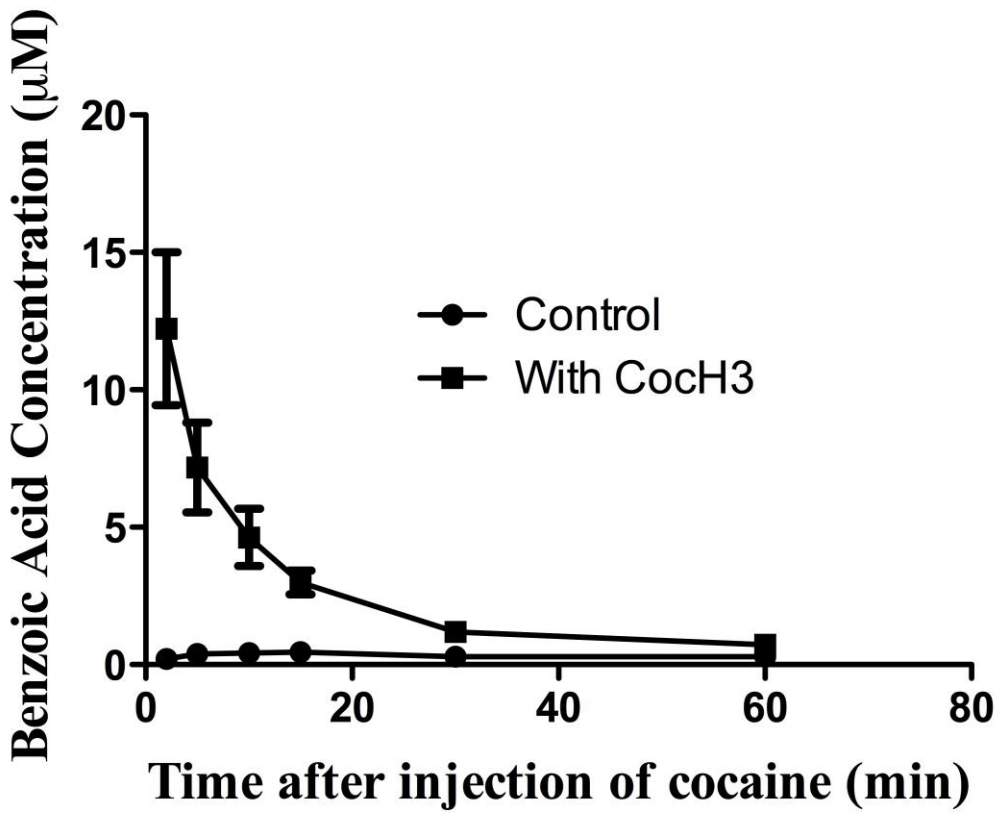

Figure 4-3. Time-dependent concentrations of benzoic acid in cocaine clearance acceleration characterized by time-dependent concentrations of benzoic acid. Benzoic acid is the product of BChE- or CocH3-catalyzed hydrolysis of cocaine. 5 mg/kg cocaine was i.v. injected 1 min after i.v. injection of CocH3 or saline. The blood was sampled from 2 to 60 min after the injection of cocaine. The concentrations of benzoic acid in the blood samples were quantitated in duplicate by using an HPLC.

4.3.3 Cocaine clearance accelerated by E30-6

The effect of E30-6 on acceleration of cocaine clearance was also evaluated in the similar experiment. In the experiment, E30-6 accelerated cocaine clearance dose-dependently as depicted in Figure 4-4. With a very low dose (0.005 mg/kg) of E30-6 pretreatment, the initial cocaine peak (5.7 μM) was slightly lowered than the control (7.5 μM). When the rats were given 0.02 mg/kg E30-6 prior to the cocaine challenge, the initial cocaine concentration at 2 min fell below the detection level. The corresponding initial benzoic acid peak was elevated, indicating that the accelerated clearance is due to the increased BChE activity in the plasma. The high potency of E30-6 is consistent with the high *in vitro* activity against cocaine.[65] This E30-6 dose (0.02 mg/kg) used is significantly lowered than the previously used dose of CocH3 (0.1 mg/kg) to produce the same effect.

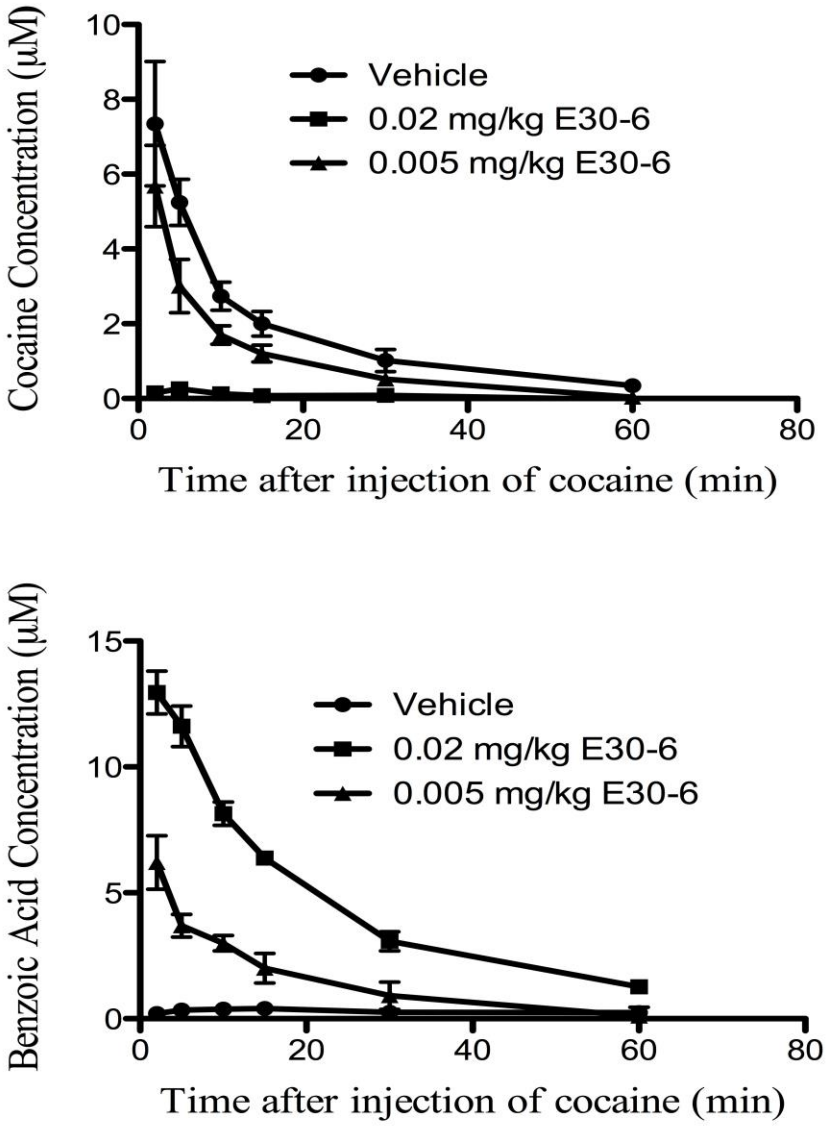

Figure 4-4. Cocaine clearance accelerated by E30-6 expressed in the stable CHO-S cells. Male rats (n=4) were i.v. injected with saline or 0.005 or 0.02 mg/kg E30-6 1 min before i.v. administration of 5 mg/kg cocaine. The concentrations of cocaine and benzoic acid in the blood samples were quantitated in duplicate by using an HPLC.

4.4 Protection of mice from acute toxicity of a lethal dose of cocaine

4.4.1 Animal procedure

(-)-Cocaine HCl (National Institute on Drug Abuse, Bethesda, MD) was dissolved in sterile water and was administered intraperitoneally at a volume of 0.01 mL/g. The purified enzyme was diluted to the required concentration in phosphate buffered saline and administered intravenously at a volume of 0.2 mL/mouse. Cocaine-induced acute toxicity was characterized by the occurrence of convulsion and lethality. Cocaine-induced convulsion was defined as loss of righting posture for at least 5 sec with the simultaneous presence of clonic limb movements [(68)]. Lethality was defined as cessation of observed movement and respiration.

The mouse was placed in a small restraint chamber that left the tail exposed. The tail was cleansed with an alcohol wipe and a 27G1/2 (University of Kentucky) or 30G1/2 (University of Michigan) precision glide needle (Fisher Scientific, Pittsburgh, PA) was inserted into one of the side veins for infusion. The intravenous injection volume of E30-6 or the pure phosphate buffered saline (the control set) was 0.2 mL per mouse and it was given 1 min before intraperitoneal administration of cocaine 180 mg/kg. To staunch the bleeding, sterile gauze and pressure were applied to the injection site. The protective effects of the enzyme were compared with those of the phosphate buffered saline.

4.4.2 *In vivo* potency

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 4-5 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, CocH1 at the dose of 0.03 mg produced full protection in mice after receiving a lethal dose of cocaine 180 mg/kg ($p<0.05$). CocH2 was more potent than CocH1. As seen in Figure 4-5, CocH2 at 0.02 mg also produced full protection in mice after receiving a lethal dose (180 mg/kg) of cocaine ($p<0.05$). 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 CocH1 and 0.02 mg for CocH2.

Data in Figure 4-6 demonstrated that pretreatment with CocH3 (*i.e*. 1 min prior to cocaine administration) dose-dependently protected mice against cocaine-induced convulsion and lethality. In particular, CocH3 at doses of 0.33 mg/kg and 1 mg/kg all produced full protection of mice from acute toxicity induced by a lethal dose of cocaine 180 mg/kg ($p < 0.05$).

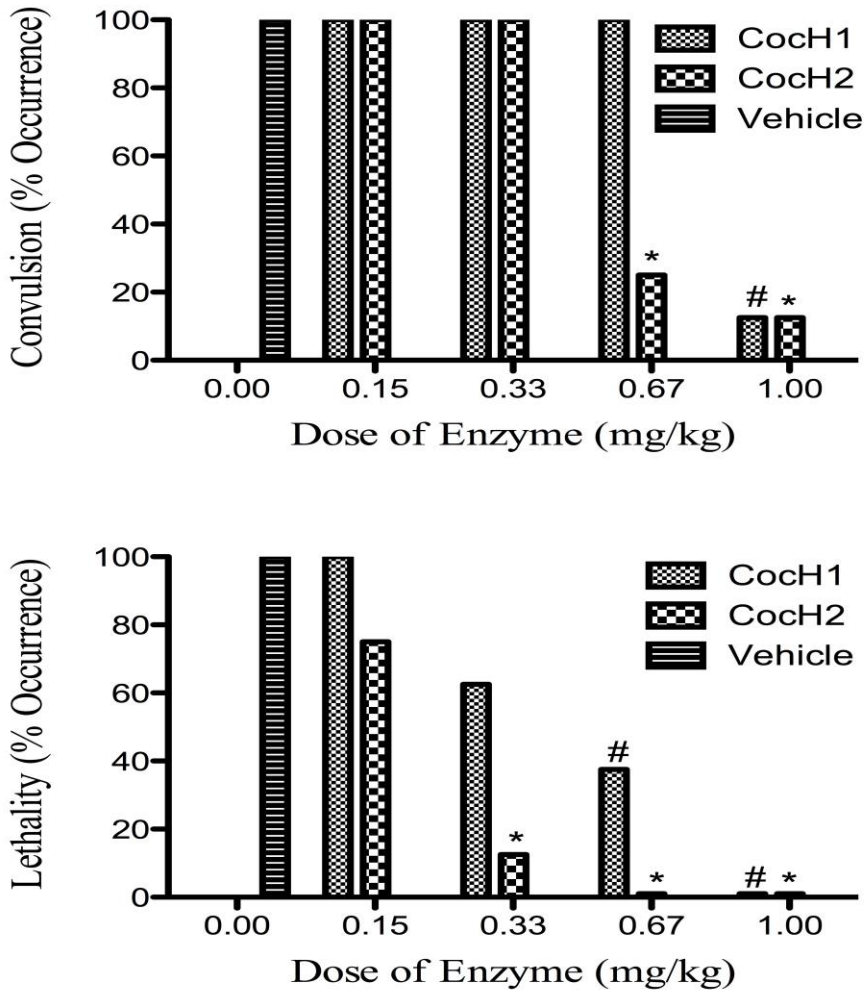

Figure 4-5. Potency of protective effects of CocH1 and CocH2 against acute toxicity of a lethal dose of cocaine (n=8). The BChE mutant (mg/mouse) was administered i.v. 1 min before i.p. administration of 180 mg/kg cocaine. * and # represent significant differences from the conditions of mice pretreated with phosphate buffered saline ($p<0.05$). This experiment was conducted by Drs. M.-C. Ko and J. H.Woods *et al.* at the University of Michigan

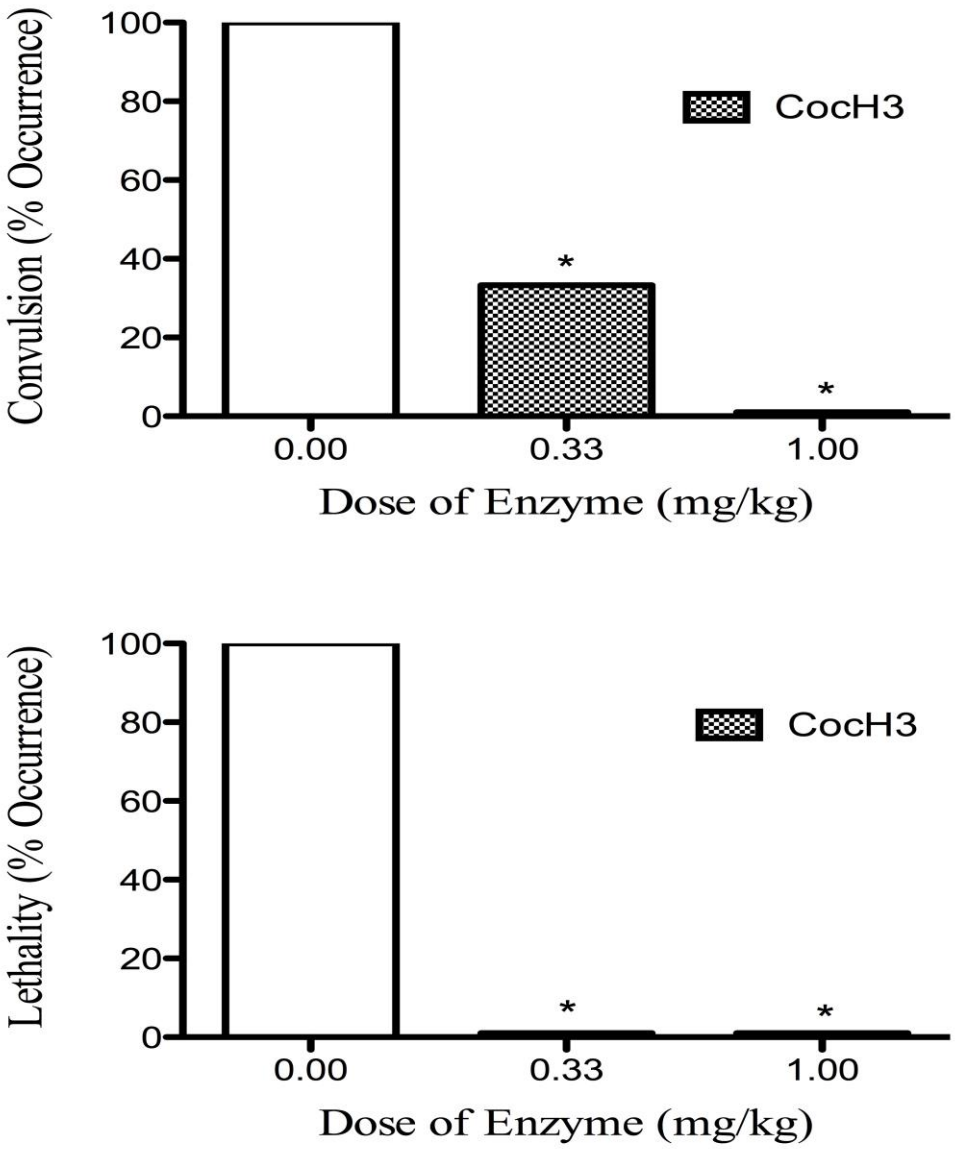

Figure 4-6. Potency of protective effects of CocH3 against acute toxicity of a lethal dose of cocaine (n=6). The BChE mutant (mg/mouse) was administered i.v. 1 min before i.p administration of 180 mg/kg cocaine. * and # represent significant differences from the conditions of mice pretreated with phosphate buffered saline ($p<0.05$).

E30-6 (our most recently designed mutant of human BChE) was also studied for its effectiveness in protecting CD-1 mice from the acute toxicity of a lethal dose of cocaine (180 mg/kg, LD_{100}) in our lab at the University of Kentucky. As shown in Figure 4-6, for the negative control experiments without administration of the enzyme, intraperitoneal (i.p.) administration of 180 mg/kg cocaine produced lethality in all tested mice (n=6). Pretreatment with E30-6 (*i.e*. 1 min prior to cocaine administration) dose-dependently protected mice against cocaine-induced lethality. In particular, E30-6 at a tiny dose of 0.15 mg/kg produced full protection in mice after receiving a lethal dose of cocaine (180 mg/kg). The *in vivo* data depicted in Figure 4-7 suggest that E30-6 is indeed promising for cocaine detoxification in therapeutic treatment of cocaine abuse.

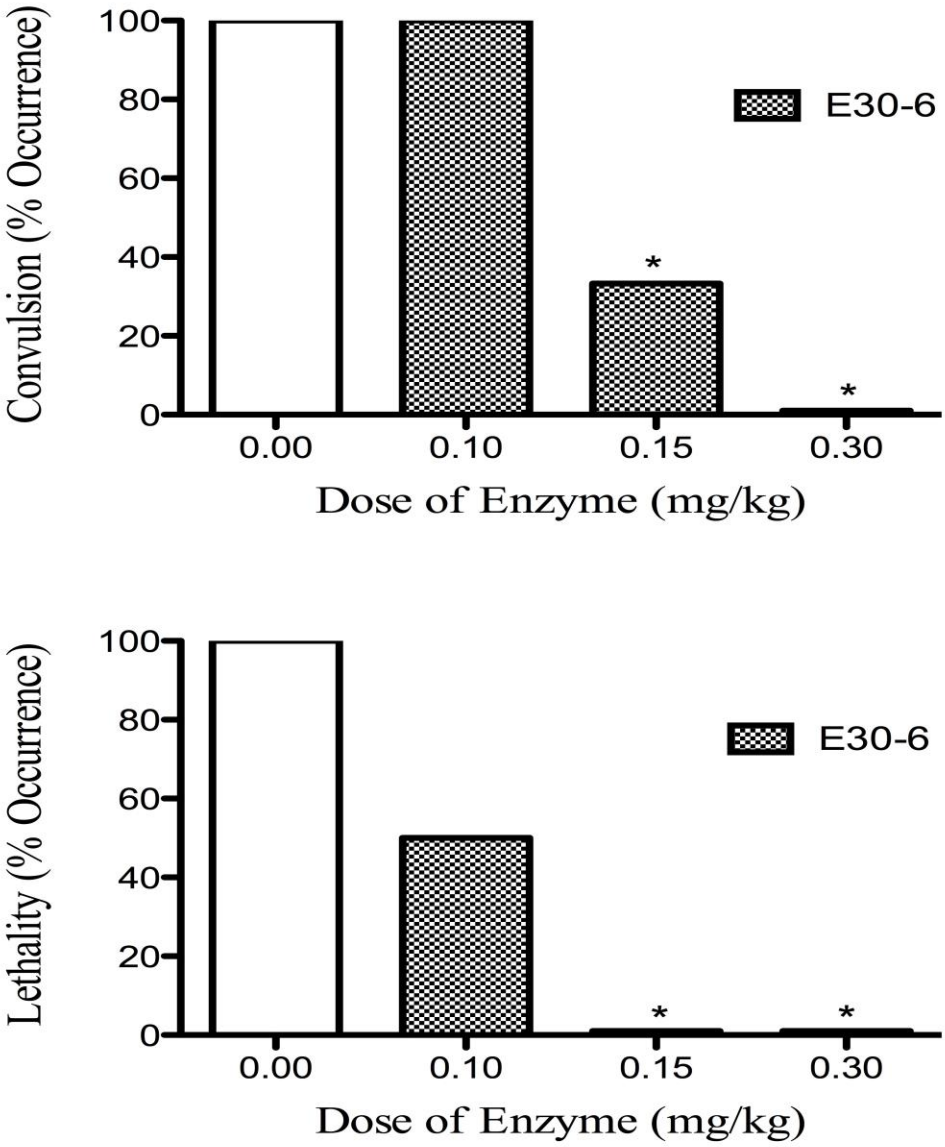

Figure 4-7. Potency of protective effects of E30-6 against acute toxicity of a lethal dose of cocaine (n=6). The BChE mutant (mg/kg) was administered i.v. 1 min before i.p. administration of 180 mg/kg cocaine. * and # represent significant differences from the conditions of mice pretreated with phosphate buffered saline ($p<0.05$).

4.5 Rescue effect of E30-6 against a lethal dose of cocaine

The rescue effect of E30-6 against cocaine-induced toxicity was investigated in NIH Swiss mice in Dr. James Woods' lab at the University of Michigan. In the study, different doses of E30-6 were administered i.v. within the 1st min after the occurrence of convulsions induced by i.p. cocaine (100 mg/kg). This specific dose of cocaine was chosen because it induced convulsions in 100% of mice and lethality in 40 to 60% of mice based on the previous study. At this dose of i.p. cocaine, there were 4 to 5 minutes between the onset of cocaine-induced convulsion and death. The enzyme injection was given during this time interval to rescue the mouse from cocaine induced convulsion and lethality. This is a simplified animal model to reflect the scenario of cocaine overdose treatment.

The results of this study were depicted in Figure 4-8. After the i.p. cocaine (100 mg/kg) administration, all four groups of mice showed convulsion, and the intervals between cocaine administration and the onset of the convulsion among groups were similar. Injection of E30-6 at dose of 0.10 mg/kg saved all mice from death (compared with 50% occurrence of lethality in the saline-treated group). Intravenous administration of E30-6 dose-dependently decreased the time to recovery from the convulsion. In the saline-treated group, although half of the mice survived, it took the survived mice for about 24 min to recover from cocaine-induced convulsion to walk normally. For the E30-6-treated groups, the recovery time (between the injection and recovery to normal walk)

were about 1.6 min for the treatment with 0.3 mg/kg E30-6 and about 4.9 min for the treatment with 0.1 mg/kg E30-6.

The ability of E30-6 to rescue the mice from cocaine-induced lethality and convulsion reveals the potential therapeutic value of E30-6 in cocaine overdose treatment.

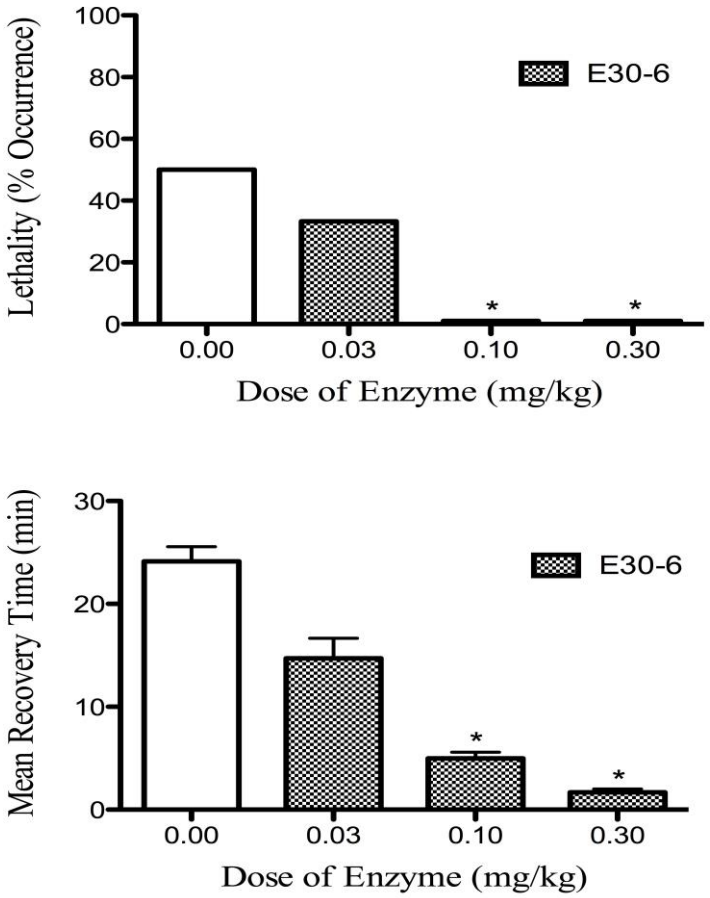

Figure 4-8. Potency of rescue effects of E30-6 (n=6) against acute toxicity of a lethal dose of cocaine (n=6). 100 mg/kg cocaine was injected i.p. to mice to induce convulsion.

Immediately after the onset of cocaine-induced convulsion (within 1 min), E30-6 was i.v. injected to rescue the mice. Recovery time mentioned here is the time interval from the enzyme injection to the recovery of normal walk. The asterisks represent significant differences from the conditions of mice pretreated with phosphate buffered saline ($p<0.05$). This experiment was conducted by our collaborators (Drs. M.-C. Ko and J. H.Woods *et al.*) at the University of Michigan.

4.6 Effects on the hyperactivity induced by cocaine

We have tested two BChE mutants in mice for their effects on the hyperactivity induced by cocaine: CocH3 (*i.e*. the A199S/F227A/S287G/A328W/Y332G mutant of human BChE) and E30-6 (*i.e*. A199S/F227A/P285A/S287G/A328W/Y332G mutant of human BChE).

4.6.1 Effects of CocH3

Development of stable cell lines for the scale-up protein production enabled us to characterize the potency of the enzyme in elimination of the physiological effects of cocaine. In this study, mice (n=6) were injected with cocaine alone, saline alone, or the BChE mutant (CocH3) 1 min before 25 or 90 mg/kg of i.p. cocaine. The mice were returned to the cages and recorded for their locomotor activity for the first 10 min after the cocaine injection. The first cocaine dose used for our locomotor activity tests in mice was 25 mg/kg (i.p.). As seen in Figure 4-9, without pretreatment of the enzyme, 25 mg/kg cocaine (i.p.) induced rather strong hyperactivity in mice. With pretreatment of 1.5 mg/kg CocH3 (i.v.), the mice had a slight hyperactivity between 2 and 5 min and then returned back to the baseline level of activity. With pretreatment of 2 mg/kg CocH3 (i.v.), 25 mg/kg cocaine (i.p.) did not induce any significant hyperactivity in mice, suggesting that the minimum dose of CocH3 required to completely block the hyperactivity induced by 25 mg/kg cocaine (i.p.) was 2 mg/kg.

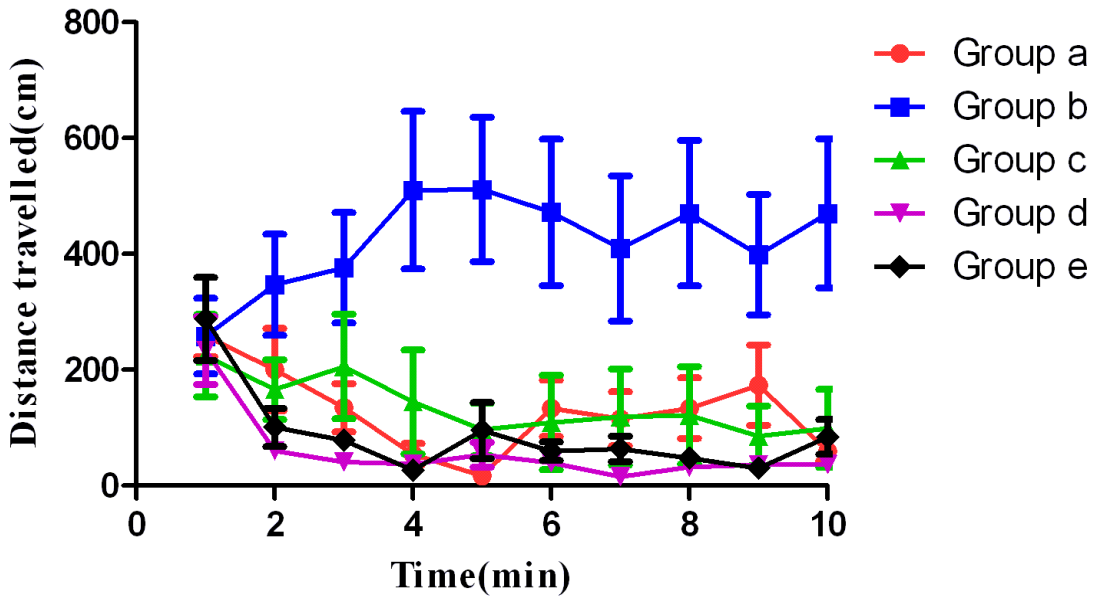

Figure 4-9. Effects of the exogenous enzyme (CocH3 expressed in the stable HEK293F cells) on cocaine-induced hyperactivity. Saline or enzyme was injected i.v. through tail veins of mice 1 min before i.p. injection of saline or cocaine. Five dose conditions were tested using five groups of mice, and each group had six mice (n=6). Group **a** were treated with i.v. saline and i.p. saline; Group **b** were treated with i.v. saline and i.p. 25 mg/kg cocaine; Group **c** were treated with i.v. 1.5 mg/kg CocH3 and i.p. 25 mg/kg cocaine; Group **d** were treated with i.v. 2 mg/kg CocH3 and i.p. 25 mg/kg cocaine; Group **e** were treated with i.v. 5 mg/kg and i.p. 90 mg/kg cocaine.

We further increased the dose of cocaine to 90 mg/kg (LD_{50} for i.p. cocaine), and noted that 90 mg/kg cocaine (i.p.) did not induce any significant hyperactivity (or any sign of toxicity in our further observation after the hyperactivity tests) in the mice pretreated with 5 mg/kg CocH3 as seen in Figure 4-9. The observation suggests that the physiological effect of such a lethal dose of cocaine can be blocked completely by pretreatment of 5 mg/kg CocH3.

The concept of a possible enzyme therapy for cocaine abuse treatment is based on a hypothesis that the effects of cocaine are dependent on how intensely and how quickly cocaine gets to the brain, and that the therapeutic enzyme can alter the pharmacokinetics of cocaine in a favorable manner by rapidly metabolizing cocaine. In this way, a therapeutic enzyme could reduce cocaine's entry into the brain to an amount (threshold) that is too low to produce detectable physiological effects. When the cocaine concentration in brain dose not reach the "threshold" value because of the presence of an efficient enzyme in plasma, one may consider that the enzyme has *effectively* blocked cocaine from reaching brain. Further, the threshold concentration of cocaine in brain is related to the degree of the DAT occupancy by cocaine. Volkow *et al.* demonstrated that, for humans, "*at least 47% of dopamine transporter has to be blocked for subjects to perceive cocaine's effects*".[(69)] The threshold concentration of cocaine in brain required to produce physiological effects was estimated to be 0.22±0.07 μM in light of a recently reported cocaine pharmacokinetic modeling.[(25)] The data in Figure 4-9 indicate that, in the presence of the exogenous cocaine hydrolase (5 mg/kg), cocaine can be degraded so

rapidly that even a lethal dose of cocaine (90 mg/kg, i.p.) did not produce a brain cocaine concentration being greater than the threshold (0.22±0.07 μM) required to induce detectable physiological effects; the animals did not show any detectable behavioral abnormality. The data indicate that it is possible to completely eliminate cocaine's physiological effects by administration of a highly efficient cocaine hydrolase, providing a proof of the principle for the desirable enzyme therapy for cocaine abuse treatment.

According to previous studies,[17] one-time use of cocaine could increase dopamine transporter (the primary target protein for cocaine) expression on the cell surface for months so that it takes a long time for the brain's communication system returning to normal. Repeated administration of cocaine would reinforce the effects on the expression levels of transporters/receptors and continuously change the circuits in the brain. Hence, for effective treatment of cocaine addiction, it is essentially important to completely protect the brain from the cocaine effect so that the brain can gradually recover to function normally. It would be interesting to further test the cocaine hydrolase in a well-established animal addiction model including cocaine self-administration in the near future.

4.6.2 Effects of E30-6

The effect of E30-6 on cocaine-induced hyperactivity was evaluated by using a video-tracking system at the University of Kentucky's Rodent Behavior Core (RBC). The locomotor activity test was performed in high density, non−porous plastic chambers

measuring 50 cm (L) × 50 cm (W) × 38 cm (H) in a light− and sound−attenuating behavioral test enclosure (San Diego Instruments, San Diego, CA). Cumulative distance traveled and speed was recorded by EthoVision XT video tracking system (Noldus Information Technology, Wageningen, Netherlands) to represent the locomotor activity. The test session was 60 min long and data was collected in 5−min bins. Male Swiss Webster mice were introduced to the test chambers for habituation (90 min long per session) on two consecutive days before the test day. On the test day, mice were allowed to acclimate to the test chambers for 60 minutes, and the total distance traveled during this period of time was used to determine the basal activity. Then E30-6 or saline was administered through intravenous (i.v.) injection, followed by intraperitoneal (i.p.) injection of 25 mg/kg cocaine or saline. After the cocaine/saline administration, mice were immediately returned to the test chamber for the remaining 60 minutes of the session for activity monitoring.

Based on the results shown in Figure 4-10, compared with the control group (treated with i.v. saline and i.p. saline), the group injected with 0.05 mg/kg E30-6 still displayed slight hyperactivity, but the extent and duration of hyperactivity were decreased considerably. Further, 0.1 mg/kg E30-6 completely suppressed the hyperactivity induced by 25 mg/kg cocaine, as seen in Figure 4-10.

We note that the minimum dose (0.1 mg/kg) of E30-6 required to completely suppress the hyperactivity induced by 25 mg/kg cocaine was much lower than that (2 mg/kg) of CocH3 required to completely suppress the hyperactivity induced by 25 mg/kg

cocaine. The remarkable difference is due to several factors. First, the catalytic efficiency of E30-6 against cocaine is significantly higher than that of CocH3. Second, the two enzymes used in the locomotor activity tests were produced in different stable cell lines: E30-6 was produced in the CHO-S cells, whereas CocH3 was produced in the HEK293F cells. As noted in Figure 4-1, the enzyme expressed in the CHO-S cells has a significantly longer biological half-life compared to that expressed in the HEK293F cells. Finally, different strains of mice were used in the studies on the two enzymes: CD-1 mice (for CocH3) and Swiss Webster mice (E30-6).

This study suggested that CHO-S-expressed E30-6 is a much more efficient cocaine hydrolase in eliminating cocaine-induced hyperactivity.

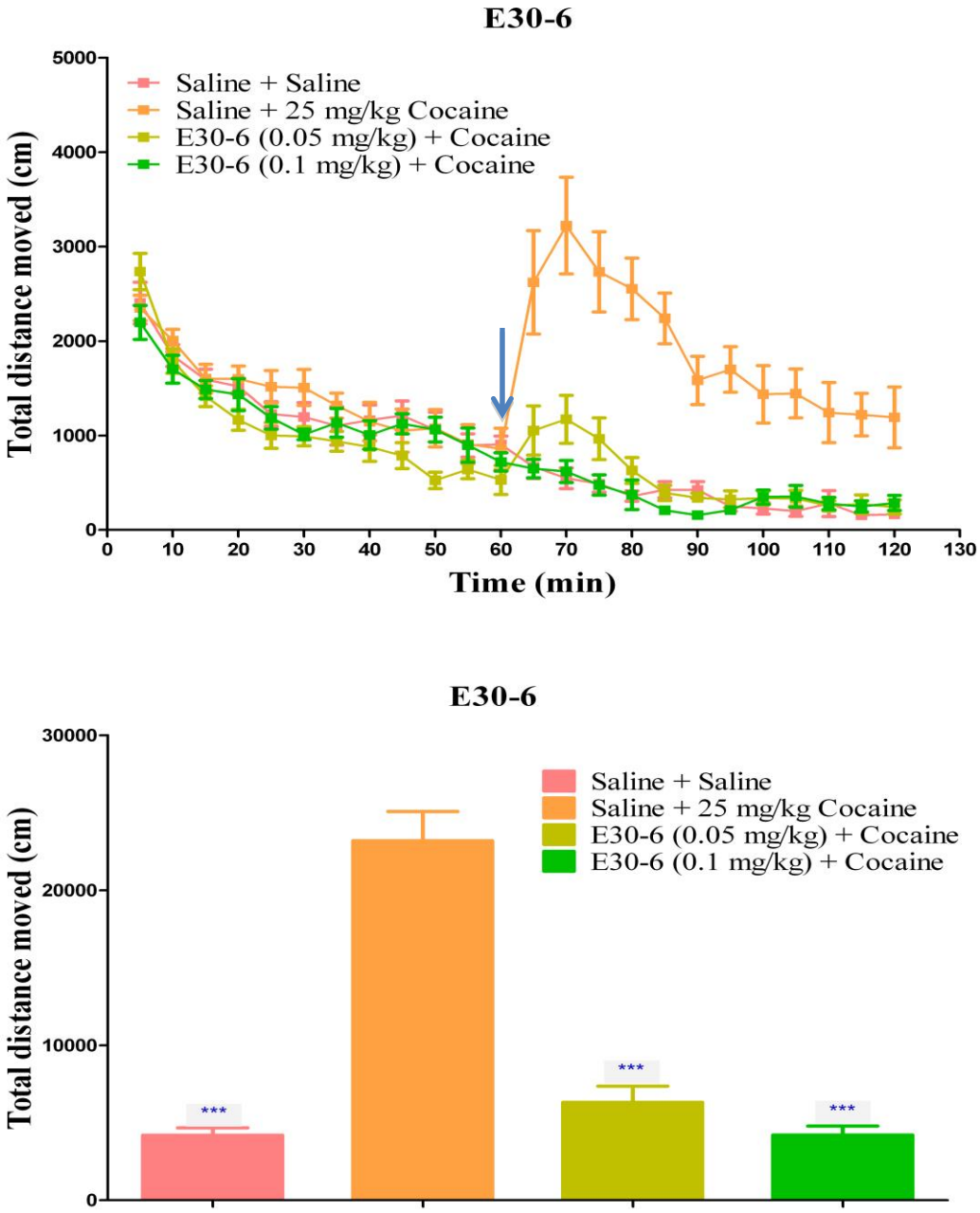

Figure 4-10. Effects of the exogenous enzyme (E30-6 expressed in the stable CHO-S cells) on cocaine-induced hyperactivity. After an hour of habitation, saline or enzyme

was injected i.v. through tail veins of mice 1 min before i.p. injection of saline or cocaine. Four dose conditions were tested using four groups of mice, and each group had eight mice (n=8). Group pink were treated with i.v. saline and i.p. saline; Group orange were treated with i.v. saline and i.p. 25 mg/kg cocaine; Group yellow were treated with i.v. 0.05 mg/kg E30-6 and i.p. 25 mg/kg cocaine; Group green were treated with i.v. 0.1 mg/kg E30-6 and i.p. 25 mg/kg cocaine. The arrow marks the time point of the injections.

CHAPTER 5 Perspectives Of High-Activity Mutants Of Human Butyrylcholinesterase As Anti-Cocaine Enzyme Therapeutics

Development of a protein drug for practical use in humans relies on large-scale production of the protein. This chapter is a brief discussion of the current state of the art in therapeutic protein production and future development of enzyme therapy for cocaine abuse.

5.1 Large-scale production in mammalian cells: Challenges and opportunities

In 1986, the first therapeutic protein, human tissue plasminogen activator (Genentech, USA), was approved by FDA, which symbolized the beginning of a new era in pharmaceutical industry.[58, 70] The number of new recombinant proteins and monoclonal antibodies (MAbs) entering preclinical and clinical studies is estimated to be about hundreds per year.[71] Compared with traditional small-molecule drugs, therapeutic proteins have higher specificity, less adverse effects, usually well tolerated, and faster clinical development.[72] Meanwhile, increasing productivity with proper quality control in large-scale production of therapeutic proteins is more complicated than that of small-molecule drugs. Therefore, development of large-scale production of those proteins has received increased attention. The aforementioned technology advances in large-scale

protein production and understanding of the effects of physical and biological parameters have opened the options to optimization of large-scale production process.

5.1.1 Expression systems

Microbial expression was first chosen for protein production because of its advantages at cost efficiency, scalability, and product uniformity. Soon it was found that the prokaryotic environment was not always able to provide proper protein folding, not alone post-translational modifications. The expression environment influences the properties of the protein produced, including solubility, stability, biological activity and circulation time in human.[73] Thus, mammalian cells are still the primary choice for production of majority protein drugs.

The selection of a cell line for production of certain protein drugs is based on the balance of high productivity and proper product quality. Even for mammalian cells, different cell lines offer different post-translation modifications, which impacts the pharmacokinetic and pharmacodynamic profiles of the protein therapeutics. 70% of currently marketed therapeutic proteins are expressed in CHO cells. NS0, PER.C6®, HEK293 or Baby hamster kidney (BHK) are also used for production of therapeutic proteins.[59]

5.1.2 Productivity

Date back to the year of 1986, cells were usually seeded at 1×10^5 cells/mL in the protein production of a 7-day batch process and the concentration can reach 2×10^6 cells/mL whereas the productivity was below 10 pg/cell/day.[70, 74] Nowadays, with advanced biotechnology, the cell density of 10×10^6 cells/mL can be achieved and accumulated yield of 3-week fermentation can reach as high as 4.7 g/L with a productivity of ~90 pg/cell/day.[70] A number of factors have been considered to optimize the cell culture processes for therapeutic protein production.

5.1.2.1 Delivery and integration of gene of interest

For traditional development of a stable cell line, the first step is to engineer on the level of vectors with a strong promoter/enhancer and some other *cis* acting elements (like scaffold/matrix attachment regions, antirepressor elements, and uquitous chromatin opening elements) to enhance stable production of proteins.[74] As discussed in Chapter 3, to overcome the negative position effect of random insertion of the foreign gene[70], site-specific integration into transcriptive "hot spots" of host genome is the currently favorable strategy for efficient stable expression of therapeutic proteins.[58, 59]

The traditional transfection-selection process is time and labor consuming. Techniques of therapeutic protein production by using transient transfection or stably transfected pools at a higher efficiency have been studied and developed by many companies like Genentech (San Francisco, CA, USA), Roche (Basel, Switzerland), and Bayer (Barkeley, CA, USA).[70, 71]

5.1.2.2 Host cell engineering

Researches on genetic modification of host cells aim to promote cell viability or modify metabolism for overall higher productivity. Anti-apoptosis engineering in the host cell line can down-regulate or prevent the apoptosis triggered by the nutrient deprivation and waste accumulation in the batching process and then improve viable cell density.[59, 75] Accumulation of cell growth by-products, such as lactate and ammonia, changes the pH and osmolality of the cell culture and inhibits cell growth. Studies suggested that metabolic engineering was able to reduce the toxic metabolites without influencing the proliferation rate of the cells.[75] In some studies, co-expression of molecular chaperones and the gene of interest led to several-fold increase in productivity.[75] Increasing attentions have been paid on host cell engineering for enhanced cell-specific growth rate and specific productivity.

5.1.2.3 Medium optimization and bioreactor conditions

In the old time, 2-20% of fetal bovine serum was added as essential nutrient in the culture medium for adhesive cells growth.[70, 71] Currently, the prevailing system for large-scale production is suspension culture. Removing serum from the culture medium lowers the cost and avoids the risks of transmitting adventitious agents[70, 71], and the suspension cells are easier to scale up. Therefore, chemically defined serum-free media have become the primary choice for industrial production.

The batch process was the traditional way of cell culture in industrial setting. During batch process, the viable cell density reaches the plateau of 2×10^6 cells/mL within a few days and began to drop mainly due to consumption of nutrients and accumulation of waste.[70] For higher yields, fed-batch or continuous perfusion processes are currently employed with addition of nutrient supplement frequently or continuously. Hydrolysates, which are protein digests, or chemically defined feeds[76] are added into medium as supplements in fed-batch process.[59]10-fold higher concentration of viable cells and significantly extended culture time can be achieved by using the feeding strategy.[76] Meanwhile, the concentrations of glucose and glutamine could be kept low in fed-batch and perfusion processes, which would decrease the accumulation of lactate and ammonia.[59]

Physical parameters, like temperature, gas flow, pH, dissolved oxygen (DO), osmolality, speed of agitation and hydrodynamic shear, could be controlled and optimized for productivity and product quality.[59]

5.1.3 Quality control

Product quality is another bottleneck of the marketing of therapeutic proteins. The quality of final products needs to meet the current standard good manufacturing standards (cGMP) regulations. Aggregation, glycosylation and charge heterogeneity may be lot-to-lot variants of protein drugs.[59] Because of the nature of proteins and cell culture expression system, the oligomerization forms and post-translational modification

of produced proteins may vary upon the change of cell culture conditions. Since proteins are delicate functional complexes, many factors may impact their stability, activity, pharmacokintic and pharmacodynamic profiles. Multiple analytical tools should be employed to assess the uniformity of the final products.[59]

In addition, it is a crucial step to scale-up protein purification process. The purification techniques have to meet the standards of high purity required for clinical application and low cost of the practical drug manufacture.[72] The produced therapeutic proteins should stay therapeutically active after purification and storage for a sufficiently long period of time.[72] These are still challenges to be faced and overcome in the future.

5.2 Clinical development of high-activity BChE mutants for cocaine addiction treatment

One of our designed and discovered mutants, CocH1 (A199S/S287G/A328W/Y332G), has been fused with human serum albumin (HSA) and studied in the clinical trial by Teva Pharmaceutical Industries, Ltd. (North Wales, PA) as a protein therapeutic for cocaine addiction treatment.[77] The HSA-fused CocH1 was known as Albu-CocH, TV-1380 or AlbuBChE in various reports.[78] A double-blind, placebo-controlled, single ascending dose of AlbuBChE followed by multiple doses of cocaine was performed. On Day 1, each one of 40 cocaine recreational volunteers received an intramuscular injection of AlbuBChE (50 to 300 mg) or placebo. On Day -1,

1, 4, 8 and 10, 40 mg i.v. cocaine was given to the subjects. The safety, immunogenicity, pharmacokinetic and pharmacodynamic parameters, and behavioral and psychological effects were evaluated in the study. It was demonstrated that AlbuBChE was safe to administrate to humans. In behavioral psychological test, the subjects were reported that administration of AlbuBChE decreased "cocaine liking" and "desire to take cocaine again".[77]

5.3 Perspectives in clinical use

Our combined computational and experimental protocols have lead to the design and discovery of a series of high-activity human BChE mutants as candidates for cocaine overdose and addiction treatment. In *in vitro* experiments, the designed human BChE mutants displayed high activity against cocaine and desired selectivity against cocaine over acetylcholine. For further characterization of the BChE mutants in animal models, we successfully developed high-efficiency stable cell lines efficiently expressing the BChE mutants by using a lentivirus-based repeated-transduction method. The large-scale protein production enabled us to further characterize the *in vivo* profiles of the BChE mutants concerning the biological half-life and potency in accelerating cocaine clearance. In particular, it has been demonstrated that the BChE mutants can rapidly metabolize cocaine and completely eliminate cocaine-induced hyperactivity in rodents.

One of high-activity BChE mutants designed and discovered in our lab has been developed in clinical trials and shown therapeutic potential for cocaine addiction treatment. Those high-activity cocaine hydrolases may also work as therapeutic agents for cocaine overdose patients in the Emergency Department. Our second-generation of high-activity BChE mutants will be in combination of higher activity and longer circulation time as promising therapeutic agents for cocaine abuse treatment.

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Vita

Liu Xue

EDUCATION

Ph.D candidate, Pharmaceutical Sciences — Expected 2013
University of Kentucky, Lexington, KY

B.S., Biology — May 2008
Purdue University, West Lafayette, IN

B.S., Life Sciences — June 2008
China Agricultural University, Beijing, China

RESEARCH EXPERIENCE

1/2007-5/2008 Microbiology lab of Dr. Louis Sherman, Department of Biological Science, College of Sciences, Purdue University, West Lafayette, IN

- Research on the nature of the exopolysaccharide (EPS) change with respect to growth phase of cyanobacterium *Cyanothece*.
- Investigation of the nature of photosystem II PsbA proteins.

6/2007-5/2008 Starch and gum lab of Dr. Yuan Yao, Department of Food Science, College of Agriculture, Purdue University, West Lafayette, IN

- Analysis of fine starch structure using flurophore assisted carbohydrate electrophoresis (FACE).
- Analysis of starch digestion ability.

10/2008-8/2013 Drug Discovery and Development Lab of Dr. Chang-Guo Zhan, Department of Pharmaceutical Sciences, University of Kentucky, Lexington, KY

- Preparation and *in vitro* validation of computationally-designed BChE mutants on their catalytic activities against (-)-cocaine and other substrates

- Establishment of stable cell lines for large-scale production of high-activity BChE mutants.
- Characterization of BChE mutants for their pharmacokinetic and pharmacodynamic profiles and their effectiveness in protecting against (-)-cocaine toxicity and modifying cocaine-induced hyperactivity in rodent models.

HONORS AND AWARDS

2011 AAPS Drug Design and Discovery (DDD) Graduate Student Symposium Award
2008-2010 Daniel R. Reedy Quality Achievement Fellowship Award
2007 and 2006 Semester Honors and Dean's list at Purdue University
2005 Outstanding Student Scholarship of China Agricultural University

PUBLICATIONS

1. Zheng F, Yang W, Xue L, Hou S, Liu J, Zhan CG. Design of high-activity mutants of human butyrylcholinesterase against (-)-cocaine: structural and energetic factors affecting the catalytic efficiency. *Biochemistry* 49, 9113–9119 (2010).

2. Yang W, Xue L, Fang L, Zhan CG. Characterization of a high-activity mutant of human butyrylcholinesterase against (-)-cocaine. *Chem. Biol. Interact.* 187, 148–152 (2010).

3. Xue L, Ko MC, Tong M, Yang W, Hou S, Fang L, Liu J, Zheng F, Woods JH, Tai HH, Zhan CG. Design, preparation and characterization of high-activity mutants of human butyrylcholinesterase specific for detoxification of cocaine. *Mol. Pharmacol.* 79, 290-297 (2011).

4. Xue L, Hou S, Yang W, Fang L, Zheng F, Zhan CG. Catalytic activities of a cocaine hydrolase engineered from human butyrylcholinesterase against (+)- and (-)-cocaine. *Chem. Biol. Interact.* 203(1):57-62 (2013).

5. Xue L, Hou S, Tong M, Fang L, Chen X, Jin Z, Tai H-H, Zheng F, Zhan CG. Preparation and in vivo characterization of a cocaine hydrolase engineered from human butyrylcholinesterase for metabolizing cocaine. *Biochem. J.* 453, 447-454 (2013).

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8. Fang Z, Xue L, Hou S, Yang W, Zhan CG. Design of human butyrylcholinesterase mutants with improved catalytic efficiency against (-)-cocaine, submitted for publication (2013).

9. Fang L, Hou S, Xue L, Fang Z, Zhan CG. Computational design of amino-acid mutations to prolong the biological half-life of a highly efficient cocaine hydrolase engineered from human butyrylcholinesterase, submitted for publication (2013).

MEETING PRESENTATIONS/ABSTRACTS

Podium presentation

Xue L, Ko MC, Tong M, Woods JH, Tai HH, and Zhan CG. Design and identfication of high-activity mutants of human butyrylcholinesterase specific for detoxification of cocaine. Podium presented on October 25th, 2011, during 2011 AAPS Annual Meeting and Exposition, Washington, DC, October 23-27, 2011.

Abstracts

1. Yang W, Xue L, Zheng F, Fang L, Pan Y, Zhan CG. Substrate selectivity of a highactivity mutant of human butyrylcholinesterase. Poster presented at 2009 AAPS Annual Meeting and Exposition, Los Angelis, November 8-12 2010.

2. Hou S, Yang W, Xue L, Zhan CG. Characterization of mutants of human butyrylcholinesterase against (-)-cocaine and (+)-cocaine. Poster presented at FIP Pharmaceutical Sciences 2010 World Congress, New Orleans, November 14-18, 2010

3. Xue L, Yang W, Hou S, Zheng F, Fang L, Chen X, Liu J, Zhan CG (2010) Rational engineering of human butyrylcholinesterase for selectively improving the catalytic efficiency against cocaine. Poster presented at FIP Pharmaceutical Sciences 2010 World Congress, New Orleans, November 14-18, 2010

4. Xue L, Ko MC, Tong M, Woods JH, Tai HH, and Zhan CG. Design and identfication of high-activity mutants of human butyrylcholinesterase specific for detoxification of cocaine. Poster presented during 2011 AAPS Annual Meeting and Exposition, Washington, D.C., October 23-27, 2011.

5. Xue L, Hou S, Fang L, Zheng F, and Zhan CG. Design, preparation, in vitro and in vivo characterization of a cocaine hydrolase engineered from human butyrylcholinerase. Poster presented during 2012 AAPS Annual Meeting and Exposition, Chicago, October 14-17, 2012.