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DEVELOPMENT OF COCAINE HYDROLASE FOR THERAPEUTIC
TREATMENT OF COCAINE ABUSE

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
Xiabin Chen
Lexington, KY
Director: Dr. Chang-Guo Zhan, Professor of Pharmaceutical Sciences
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ABSTRACT OF DISSERTATION

DEVELOPMENT OF COCAINE HYDROLASE FOR THERAPEUTIC TREATMENT OF COCAINE ABUSE

Cocaine abuse is a world-wide public health and social problem without a U.S. Food and Drug Administration (FDA)-approved medication. An ideal anti-cocaine medication would accelerate cocaine metabolism producing biologically inactive metabolites by administration of an efficient cocaine-specific exogenous enzyme. Recent studies in our lab have led to discovery of the desirable, highly efficient human cocaine hydrolases (hCocHs) that can efficiently detoxify and inactivate cocaine without affecting normal functions of central nervous system (CNS). Preclinical and clinical data have demonstrated that these hCocHs are safe for use in humans and effective for accelerating cocaine metabolism. However, the actual therapeutic use of a hCocH in cocaine addiction treatment is limited by the short biological half-life (*e.g.* 8 hours or shorter in rats) of the hCocH.

In the investigation described in this thesis, we have demonstrated that mCocH and hCocH have improved the catalytic efficiency of mBChE and hBChE against cocaine by ~8- and ~2000-fold, respectively, although the catalytic efficiencies of mCocH and hCocH against other substrates, including acetylcholine (ACh) and butyrylthiocholine (BTC), are close to those of the corresponding wild-type enzymes mBChE and hBChE. In addition, we have identified the first benzoylecgonine-metabolizing enzymes that can hydrolyze benzoylecgonine and accelerate its clearance in rats. The developed LC-MS/MS method has enabled us to simultaneously determine cocaine and nine cocaine-related metabolites in whole blood samples.

In development of the long-acting hCocHs, we have designed and discovered a novel hCocH form, catalytic antibody analog, which is an Fc-fused hCocH dimer (hCocH-Fc). The hCocH-Fc has not only a high catalytic efficiency against cocaine, but also a considerably longer biological half-life. A single dose of hCocH-Fc was able to accelerate cocaine metabolism in rats even after 20 days and, thus, block cocaine-induced hyperactivity for a long period of time. In consideration of the general observation that the biological half-life of a protein drug in humans is significantly longer than that in rodents, the hCocH-Fc could allow dosing once every 2-4 weeks, or longer for cocaine addiction treatment in humans.

KEYWORDS: Cocaine abuse; benzoylecgonine; enzyme therapy; protein engineering; butyrylcholinesterase; LC-MS/MS

Xiabin Chen

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07/01/2016

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DEVELOPMENT OF COCAINE HYDROLASE FOR THERAPEUTIC
TREATMENT OF COCAINE ABUSE

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This dissertation is dedicated to my dear parents and wife, whose love and support made this journey through graduate school possible.

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Chapter 1: Cocaine Abuse and Anti-Cocaine Medication

Cocaine (Figure 1.1) is a powerfully addictive drug which was originally extracted from the leaves of the *Erythroxylon coca*, a plant native to South America. Cocaine has been used as a local anesthetic starting from 1884, but currently is mostly used as a recreational drug due to its effects on the reward pathway in the brain ⁽¹⁾. The use of cocaine is associated with the risk of many medical problems, such as drug dependence and acute cardiovascular effects in case of cocaine overdose. In the United States (US), ~2.3% of population aged 16 to 64 uses cocaine, resulting in over 500,000 emergency department (ED) visits and many other problems each year ⁽²⁻⁵⁾. Unfortunately, there is no US Food and Drug Administration (FDA)-approved medication specific for cocaine abuse treatment. In this chapter, the molecular mechanisms of cocaine addiction and toxicity, and current development of potential treatments for cocaine abuse will be discussed.

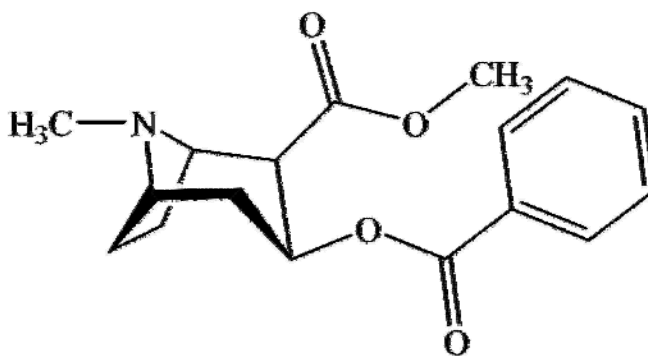


Figure 1.1 Molecular structure of (-)-cocaine free base.

1.1 Mechanism of cocaine addiction

Cocaine is one of the most reinforcing abused drugs, stimulating the reward

pathway of the brain and teaching the user to take it again ⁽⁶⁻⁸⁾. While cocaine produces its acute rewarding effects by binding mainly to dopamine and serotonin reuptake transporters and blocking them, here the focus is on just dopamine transporter (DAT) as a typical example to describe the mechanism of cocaine addiction. In the brain's communication system which is formed by trillions of neurons, dopamine functions as a neurotransmitter. Normally, dopamine is released by a transmitting neuron into the synapse, where it can bind to dopamine receptors on the receiving neuron. Then DAT terminates dopamine synaptic transmission by recycling dopamine back into the transmitting neuron ⁽⁹⁾. If cocaine is present, it can bind to the DAT and prevent the removal of dopamine from synapse through both blocking the initial DAT-dopamine binding and reducing the kinetic turnover of transporter after dopamine binds with DAT. Although the initial cocaine-binding site in the DAT does not overlap with the dopamine-binding site, dopamine cannot reach its binding site if cocaine is bound in the DAT as cocaine blocks the dopamine-entry tunnel. Even if dopamine binds with DAT before the DAT-cocaine binding (cocaine can always bind with DAT in the presence/absence of dopamine), cocaine may inhibit the conformational change of DAT which is necessary for the transport of dopamine ⁽¹⁰⁾. Blocking the recycling process of dopamine back to the transmitting neuron results in the increased level and prolonged presence of dopamine in the synapse, which contributes to the continuous response of the receiving neuron and therefore pleasurable effects of cocaine. Over time, in response to this activity, the receiving neuron begins to reduce the number of dopamine receptors on its cell surface. Meanwhile, the transmitting neuron expresses higher level of DAT ^(11, 12).

Gene expression dysregulations in the brain's communication system (e.g. dopaminergic pathways) are considered as the main reasons for drug seeking and craving. For example, the up-regulation of DAT results in less dopamine available in the synapse for neurotransmission, and the down-regulation of the dopamine receptor weakens the dopamine signal ⁽¹³⁻¹⁶⁾. Thus, for cocaine addiction treatment, it is

necessary to bring the brain's communication system back to normal to eliminate the drug craving. The problem is that gene expression (e.g. DAT and dopamine receptor) can be modulated rapidly in response to stimulant effects of cocaine, but its normalization is usually an extremely slow process, resulting in abnormal gene expression in the brain's dopaminergic signaling for at least one month ^(13, 16, 17). In addition, whenever cocaine is used during this long process of gene normalization, it can change the brain's communication system again for an additional month. Considering these reasons, successfully treating cocaine addiction requires the cocaine abuser to receive a long-term medicine to first effectively antagonize the stimulant effects of cocaine, and then bring the function of brain's communication system back to normal.

1.2 Mechanism of toxic effects of cocaine

The pharmacology of cocaine toxicity is complex with effects occurring simultaneously in several organ systems. However, the initial event of cocaine's toxicity for all of these systems is same: cocaine elicits its effects by binding with a variety of proteins, including neurotransmitter transporters, receptors, voltage-gate ion channels, and others ⁽¹⁸⁾. The effects triggered depend on the cocaine concentration at the sites of action. When cocaine is taken at a low dose, it first binds with proteins with a high affinity for cocaine. As the serum concentration of cocaine increases, target proteins with a high affinity for cocaine begin to be saturated, meanwhile target proteins with a low affinity for cocaine start to be occupied with cocaine and trigger cocaine's effect.

Cocaine elicits its toxic effects primarily by binding to noradrenergic transporters (indirect action, modulate calcium metabolism by β -adrenergic stimulation), leading to elevated central and peripheral norepinephrine levels, and therefore increased heart rate (HR), blood pressure and vasoconstriction seen in cocaine users ⁽¹⁹⁻²³⁾. The hypertensive and tachycardia effects of cocaine usually cause chest pain, which is the

most commonly reported reason for ED cases ⁽²⁴⁻²⁶⁾. The cardio-toxic effects of cocaine are also mediated directly by blocking several voltage-gated ion channels including sodium and potassium channels (direct action) ^(18, 27), which depresses myocardial contractility and ejection fraction ⁽²⁸⁾. Of these two primary cardio effects, indirect action predominates at low dose of cocaine, whereas the direct action is more prominent at high dose of cocaine ^(29, 30). In addition, cocaine stimulates the release of endothelin-1 from endothelial cells. Endothelin-1 acts as a vasoconstrictor by inhibiting the production of nitric oxide which is the principal vasodilator ⁽³⁰⁻³²⁾. The use of cocaine is also associated with increased thrombosis, severe oxidative stress, reactive oxygen species production, and apoptosis in the heart muscle ⁽³³⁻³⁸⁾. Overall, cocaine affects the cardiovascular system through the pathways described above and probably more pathways remain to be elucidated. Cocaine increases the demand of oxygen by increasing heart rate, blood pressure, and myocardial contractility. At the same time, cocaine decreases the myocardial oxygen supply by coronary vasoconstriction and enhancing thrombosis. Ischemia and infarction may occur when the myocardial oxygen demand exceed the myocardial oxygen supply ⁽³⁰⁾. What's more, cocaine causes systolic and diastolic dysfunctions, arrhythmias, atherosclerosis, and decreased myocardial contractility and ejection fraction ⁽³⁹⁾. The long-term use of cocaine is related to left ventricular hypertrophy ⁽⁴⁰⁾, prolonged deceleration time ⁽⁴¹⁾, and coronary endothelial dysfunction ⁽⁴²⁾.

In addition to cardiovascular effects of cocaine, cocaine abuse is linked to a variety of other medical problems including neurological effects, gastrointestinal and respiratory complications, AIDS, cancer, and other toxicities ^(3, 4). Cocaine abuse also causes drastic changes in the personality that can lead to aggressive, compulsive, criminal and/or erratic behaviors ⁽⁴³⁾. Thus, cocaine abuse is costly to both individuals and society.

1.3 Treatments for cocaine abuse

Disastrous medical and social consequences of cocaine make it a high priority to develop anti-cocaine medication for treatment-seeking users ^(44, 45). For emergency treatment, diazepam is usually used to decrease the increased blood pressure and heart rate, and physical cooling and paracetamol are used to treat hyperthermia; however, there is no officially approved drug specific for therapeutic treatment of cocaine overdose or addiction. Despite decades of effort, classical pharmacodynamic approaches of small molecules to block or counteract the drug's neuropharmacological actions have not proven successful for cocaine abuse, due in part to the extreme difficulty in antagonizing cocaine's physiological effects without affecting normal functions of the central nervous system (CNS) ⁽¹⁶⁾. In principle, pharmacological treatment for a drug of abuse can be pharmacodynamic or pharmacokinetic ⁽⁴⁶⁾. Most current medications for other drugs of abuse employ the classical pharmacodynamic approach of small molecules to block or counteract the drug's neuropharmacological actions at one or more neuronal binding sites. The inherent difficulties of antagonizing cocaine in the CNS led to the development of protein-based pharmacokinetic approaches with biologics like monoclonal antibodies, vaccines that produce antibodies in the body, and enzymes ^(16, 47). Compared to the small molecules used by the pharmacodynamics approach, both anti-cocaine antibodies and cocaine-metabolizing enzymes are too large to cross the blood brain barrier, thus reducing the off-target effects in the CNS ^(16, 47, 48).

1.3.1 Pharmacodynamic approach

According to the mechanisms implicated in cocaine toxicity and addiction, decades of effort have been made to yield small molecules to antagonize cocaine's effect at its sites of action. While several therapeutic candidates using small-molecule agents have been evaluated clinically (summarized in Table 1.1), none of them has been found to be safe and effective for treating cocaine overdose or addiction. The

failure in the pharmacodynamic approach may be due to several reasons. First, it would be extremely difficult to antagonize cocaine's physiological effects without affecting the normal functions of CNS ⁽¹⁶⁾. As described above, the cocaine-binding site in DAT is close to the dopamine-binding site, so a small-molecule agent that can block the cocaine-DAT binding usually affects the dopamine-DAT binding. In this way, DAT antagonist actually acts as a cocaine-like drug. Second, cocaine elicits its effects through binding with a variety of target proteins in several organs and system. Pharmacodynamic treatments that focus on only one or several proteins may not be able to completely eliminate the effects of cocaine ⁽⁴⁹⁾. To make the matters worse, patients may just take more cocaine in order to achieve the expected pleasurable effects of cocaine, meanwhile increase the toxic effects of cocaine. Finally, the pharmacological mechanisms mediating the effects of cocaine have not been fully understood. It is difficult to find the key target protein or system for the design and discovery of anti-cocaine small-molecule agents. Thus, treatments that do not focus on the physiological effects of cocaine may prove more promising as medications.

Table 1.1 Pros and cons of various pharmacodynamics approaches for cocaine overdose and addiction treatment

Approaches	Purpose	Pros	Cons
Dopaminergic agents			
-Bupropion ⁽⁵⁰⁾	Addiction		Negative effects
-Buspirone ⁽⁵¹⁾	Addiction		Negative effects
-Modafinil ⁽⁵²⁻⁵⁴⁾	Addiction	Reduced cocaine use	Limited effects
-Aripiprazole ⁽⁵⁵⁻⁵⁷⁾	Addiction		Limited effects
GABAergic agents			
-Vigabatrin ⁽⁵⁸⁾	Addiction		Negative effects
-Topiramate ⁽⁵⁹⁻⁶¹⁾	Addiction	Safe and well-tolerated	Negative or limited effects.
-Benzodiazepines ⁽⁶²⁾	Overdose	Decreased cardiovascular effects of cocaine	Limited effects
Adrenergic agents			
-Citicoline ⁽⁶³⁾	Addiction	Safe. Reduced cocaine use	Effects diminished over time
-Guanfacine ⁽⁶⁴⁾	Addiction		Negative effects
-Doxazosin ^(65, 66)	Addiction	Reduced cocaine use	Distinct adverse effects
-Lofexidine ⁽⁶⁷⁾	Overdose	Decrease cardiovascular effects of cocaine	Cardiovascular adverse effects
Amphetamine analogs			
-Lisdexamfetamine ⁽⁶⁸⁾	Addiction	Reduced cocaine use	Has abuse potential
Antipsychotic medications ⁽⁶⁹⁾	Addiction		Negative effects
Opioid antagonist			
-Naltrexone ⁽⁷⁰⁾	Addiction	Reduced cocaine craving	Limited effects

1.3.2 Pharmacokinetic approach

The pharmacokinetic approach acts on cocaine itself, aiming to lower the concentration of free cocaine at sites of action, thereby modulating the physiological effects of cocaine. Long-term reduction of (free) cocaine concentration in the brain might prevent patients from receiving any reinforcing effects of cocaine, leading to the

extinction of cocaine-seeking and cocaine-taking behaviors. In addition, acutely reducing the peripheral concentration of free cocaine could minimize the toxic effects of cocaine (e.g. the cardiovascular effects) ⁽⁷¹⁾. To reduce the concentration of free cocaine in both blood and brain, either anti-cocaine antibody or cocaine-metabolizing enzyme can be used to sequester cocaine in the blood or hydrolyze it, respectively, before cocaine reaches its site of action. Binding or hydrolyzing all cocaine molecules is not necessarily required, because for humans, “at least 47% of the transporters had to be blocked for subjects to perceive cocaine’s effects” ⁽⁷²⁾; however, the capability of the pharmacokinetic approach to keep the cocaine below its minimally effective concentration should not be easily overcome by taking more cocaine. Thus, to the success of long-term treatment of cocaine addiction, there are three key factors: (1) Sufficient pharmacokinetic capability to keep the cocaine concentration in the brain lower than the threshold required to produce detectable physiological effects; (2) Making it impractical for cocaine abusers to overcome the treatment effect by increasing drug intake; (3) Long-term effect to avoid frequent administrations for the treatment.

1.3.2.1 Immunological strategy

The immunological strategy aims to bind cocaine with an anti-cocaine antibody in the periphery, which blocks its access to the CNS to exert physiological effects, meanwhile making cocaine not available to activate receptors or ion channels in peripheral organs to generate the toxic effects ⁽⁷¹⁾. To completely eliminate cocaine’s effects, it is necessary for the immunological strategy to generate sufficient anti-cocaine antibodies in the body, with not only a high binding affinity but also a great specificity for cocaine. The anti-cocaine antibody should not bind with any other compounds, such as neurotransmitters and endogenous cocaine-metabolizing enzymes, to avoid any interference with normal physiological functions ⁽⁷¹⁾.

The immunological strategy can be administered by either passive monoclonal

antibody or active vaccine ⁽⁷³⁻⁷⁵⁾. Each approach has its own advantages and disadvantages. For the vaccine-approach, it requires weeks to months to generate active immunity. However, once the immunity is generated, a certain level of anti-cocaine antibody can persist for a long period of time. An anti-cocaine vaccine, therefore, might be useful for the long-term treatment of cocaine addiction, but not for treating acute cocaine overdose. In contrast, injected anti-cocaine antibody can immediately block cocaine's access to sites of action, thus eliminating the physiological effects and acute toxic effects of cocaine ^(76, 77). One main potential disadvantage of the antibody-approach is the need of large doses of the therapeutic agent ⁽⁷¹⁾.

The drug-protein conjugate vaccines for cocaine abuse treatment have been explored in clinical trials. For example, in a double-blind, placebo-controlled clinical trial of a cocaine vaccine which was made by covalently linking succinylnorcocaine to cholera B (SNC-rCTB), subjects received five vaccinations of vaccine or placebo. The treatment was safe and there were no serious side effects observed ⁽⁷⁸⁾. Compared to the subjects received vaccinations of placebo and subjects with low levels (< 43 µg/ml) of serum anti-cocaine antibody, those attained high levels of anti-cocaine antibody (> 43 µg/ml) had significantly more cocaine-free urines (one of main outcome measures). The proportion of subjects having considerably reduced cocaine use was significantly greater in the subjects with high IgG anti-cocaine antibody levels than that in subjects with low IgG anti-cocaine antibody levels. However, only 38% of patients developed relatively high levels of anti-cocaine antibody following multiple vaccinations ⁽⁷⁸⁾. The results of a Phase III clinical trial of this vaccine also demonstrated that those more vaccinated than placebo subjects attained abstinence for at least two weeks, and the high IgG group had the most cocaine-free urines for the last two weeks of treatment, but neither were significant ⁽⁷⁹⁾. Current effort is focused on new vaccines (e.g. produced by linking hapten to a new carrier-agent ⁽⁸⁰⁾) that can provide a higher concentration of anti-cocaine antibodies ⁽⁷⁷⁾. Recent work has yield effective

monoclonal antibodies with a very high affinity for cocaine. Preclinical studies have demonstrated that an anti-cocaine antibody can modify the drug-seeking and taking behaviors in animal models; however, a large amount of antibody is required ^(77, 81, 82). However, there is no anti-cocaine monoclonal antibody has been studied in human.

While immunotherapy provides some promise for treating cocaine abuse and can significantly reduce the drug seeking and drug-intake, its chief problem is obvious: one binding site of antibody binds stoichiometrically with cocaine ^(16, 48). There is no guarantee that a vaccine or antibody approach can completely eliminate drug reward, as the effects of any given level of anti-cocaine antibodies can be overcome with either repeated cocaine dosing or increasing the dose. The immunological strategy might work when the plasma concentration of cocaine is low ⁽¹⁶⁾. However, once the antibody is saturated by cocaine, unbound cocaine molecules are still free to either cross the blood-brain barrier to elicit their physiological effects, or activate other targeting proteins to produce toxic effects ⁽¹⁶⁾.

1.3.2.2 Cocaine-metabolizing enzyme

Unlike the stoichiometric binding of an antibody with drug, a cocaine-metabolizing enzyme can not only bind with cocaine, but also degrade cocaine to free itself for further binding and reaction. Each enzyme molecule can degrade multiple drug molecules, depending on the turnover number (catalytic rate constant k_{cat}) and Michaelis-Menten constant (K_M). Administration of an efficient cocaine-metabolizing enzyme can dramatically accelerate the elimination of cocaine in both brain and blood, effects that are attributable to a rapid hydrolysis of cocaine catalyzed by the cocaine-metabolizing enzyme ^(83, 84), and therefore leading to the significantly reduced toxicological and behavioral effects of cocaine ⁽⁸⁵⁻⁸⁷⁾. Two cocaine-metabolizing enzymes designed in Dr. Zhan's lab, including a thermally stable mutant of bacterial cocaine esterase (CocE) ⁽⁸⁸⁾ and a highly efficient mutant of human butyrylcholinesterase (BChE) ⁽⁸⁹⁾, are currently under clinical development for

the treatment of cocaine overdose and addiction, respectively. Both enzymes have been proven to be safe and effective as anti-cocaine agents ⁽⁹⁰⁻⁹²⁾.

1.4 Bacterial cocaine esterase

CocE cloned from the *Rhodococcus* strain of bacteria, which grows in the soil of cocaine-producing plants, was recognized as the most efficient natural enzyme for hydrolyzing cocaine ^(93, 94). It catalyzes the hydrolysis of cocaine at the benzoyl ester bond to produce biological inactive compounds ecgonine methyl ester (EME) and benzoic acid. There is no other natural esterase with a catalytic activity against (-)-cocaine (which is the naturally occurring enantiomer of cocaine) comparable to that of CocE ⁽⁹⁵⁾. However, a major obstacle to developing CocE into a therapeutic agent for cocaine abuse is the thermoinstability of wild-type CocE with a half-life of only ~12 min at physiological temperature (37°C) ^(88, 96). Based on a rational computational approach, CocE mutants were successfully engineered with synergistically enhanced thermal stability at 37°C ^(88, 95, 97). For example, double mutations (T172R/G173Q) of CocE significantly increased the half-life at physiological temperature by more than 20-fold compared to that of the native CocE, making the mutated CocE suitable as a potential medical agent for cocaine abuse ⁽⁸⁸⁾. This double mutated CocE, known as drug RBP-8000, is currently in a randomized, double-blind, placebo-controlled trial for treatment of cocaine overdose. In the presence of RBP-8000, the plasma concentration of cocaine was reduced by 90% within 2 min; cocaine-induced cardiovascular effects were significantly decreased compared to placebo ⁽⁹⁰⁾. Preclinical studies also have been carried out to evaluate the effects of CocE mutants in reducing the use of cocaine. Injection of a CocE mutant produced rightward shifts in the dose-response curves for cocaine self-administration and discrimination ⁽⁸⁵⁾. However, such effects cannot last long due to the short biological half-life of the CocE mutant. The improvement of thermal stability actually did not significantly prolong the *in vivo* half-life of CocE, suggesting that factors

beyond thermal stability of protein, such as protease degradation and glomerular filtration, shorten the biological half-life of CocE ^(23, 97, 98). Although polyethylene glycol conjugation (PEGylation) further improved the *in vivo* lifetime of CocE, the duration of effects of PEGylated CocE mutant (high dosage of CocE) on reducing the reinforcing effect and toxic effects of cocaine was still short ^(85, 95). In addition, no other effort has been reported to extend the biological half-life of CocE longer than that of PEGylated CocE.

As a whole, the newly designed CocE mutant can efficiently reduce the toxic effects of cocaine by quickly hydrolyzing cocaine into biological inactive compounds. It is a promising drug candidate for cocaine overdose treatment, which was strongly supported by the results of clinical studies ⁽⁹⁰⁾. The genes of CocE and thermostable CocE mutants have been cloned and proteins can be expressed in *Escherichia coli* cells in large quantities. The rapid elimination of CocE should reduce the likelihood of an immune response in humans, and prevent the accumulation of large aggregates, which may cause damage to capillary beds or organs ⁽⁹⁸⁾. Thus, the thermostable CocE mutant is a safe and effective therapeutic agent for cocaine overdose treatment, but not a suitable medicine for long-term treatment of cocaine addiction.

1.5 Human butyrylcholinesterase

BChE, previously known as pseudocholinesterase or serum cholinesterase, can be found in plasma and many tissues, such as liver and brain ^(99, 100). Purified human plasma BChE has a long history of clinical application, without any adverse events reported ⁽¹⁰⁰⁾. Two clinical trials (NCT00333515 and NCT00333528) of hBChE derived from plasma were performed by Baxter Healthcare Corporation, showing that human BChE is safe. BChE is the principal metabolic enzyme that catalyzes the hydrolysis of cocaine to produce biologically inactive metabolites. Unfortunately, the catalytic efficiency (k_{cat}/K_M) of wild-type BChE against naturally occurring (-)-cocaine is too low ($k_{cat} = 4.1 \text{ min}^{-1}$ and $K_M = 4.5 \text{ }\mu\text{M}$) ⁽¹⁰¹⁾ to be effective for cocaine metabolism.

It is highly desired to develop a mutant of human BChE with a considerably improved catalytic efficiency against (-)-cocaine.

1.5.1 Rational protein engineering of BChE

Extensive studies have been performed to increase the catalytic activity of BChE against (-)-cocaine by accelerating the rate-determining step of the entire catalytic reaction process without slowing down the other steps. Through structure and mechanism based computational design and wet experimental tests, Dr. Zhan's group has successfully designed and discovered a series of human BChE mutants, recognized as *true* cocaine hydrolases (CocHs) in literature ⁽⁸⁶⁾ when they have at least 1,000-fold improved catalytic efficiency against (-)-cocaine compared to wild-type human BChE ^(89, 102-104). The most recently designed BChE mutants, such as CocH3 (the A199S/F227A/S287G/A328W/Y332G mutant of BChE) and CocH4 (the A199S/F227A/P285A/S287G/A328W/Y332G mutant of BChE), are significantly more active against (-)-cocaine than the native CocE which is the fastest natural enzyme that can hydrolyze cocaine ^(102, 104).

1.5.2 CocHs for cocaine overdose treatment

As mentioned above, an efficient cocaine-metabolizing enzyme can eliminate cocaine's toxicity quickly, relies on the rapid hydrolysis of cocaine catalyzed by the enzyme. The dose of enzyme required to completely remove the toxic effects of cocaine is negatively related to the catalytic efficiency of enzyme against cocaine. The higher catalytic activity of enzyme, the lower dose of enzyme required for the treatment and, thus, the lower the costs of medicine. In the cocaine lethality studies using a thermostable CocE, at least 3.2 mg/kg (IV) of CocE was required to protect 100% of the animals from a lethal dose of cocaine (180 mg/kg, i.p.). Pretreatment of 1.0 mg/kg (IV) of CocE right before the injection of cocaine protected only 40% of the animals from a lethal dose of cocaine ⁽¹⁰⁵⁾. In comparison with CocE, recently designed CocHs are

more effective in protecting animals from cocaine overdose. Approximately 0.3 mg/kg of CocH3 or 0.15 mg/kg CocH4 can produce full protection in animals from cocaine overdose induced by a lethal dose of cocaine ^(102, 104). Thus, compared to the CocE, highly efficient CocHs might be more promising in treatment of cocaine overdose.

1.5.3 CocHs for cocaine addiction treatment

Using a pharmacokinetic agent, the key to successful cocaine addiction treatment is that the treatment can continuously keep the cocaine in the body below its minimally effective concentration. It requires the enzyme used for cocaine addiction treatment to have not only a high catalytic efficiency against cocaine, but also a long *in vivo* half-life. The human serum BChE displays high bioavailability and is long-lasting in human circulation ⁽¹⁰⁶⁾; however, the recombinant human BChE expressed in mammalian cells, goat milk of transgenic goats, insect cells, or tobacco plants displays a short biological half-life in the circulation of animals or human being ^(103, 107-109). The short *in vivo* half-life of the recombinant BChE in animals or humans can be explained by incomplete post-translational modification, mainly in glycosylation ⁽¹⁰⁰⁾. Glycosylation can vary from one expression system to another system; and from lot to lot. Although unwanted glycosylation residues can be deleted and necessary residues can be coexpressed ⁽¹⁰⁹⁾, for now it is impossible to produce recombinant BChE with exactly the same post-translational modification as that of plasma-derived BChE, no matter what expression system used. The heterogeneity in post-translational modification, mainly in glycosylation, makes the recombinant BChE foreign (may not be immunogenic) to the test animals or human being, accelerating the clearance of the enzyme and shortening its biological half-life ⁽¹¹⁰⁾.

To prolong the half-life of recombinant BChE, some efforts have been made in some research groups. Recombinant human BChE modified chemically with polyethyleneglycol (PEG) displayed a residence time which is 2-fold longer than that of unmodified recombinant human BChE, suggesting that PEG-modification can

extend the biological half-life of recombinant human BChE ⁽¹¹¹⁾. The first one of designed CocHs, known as CocH1 or E14-3 (the A199S/S287G/A328W/Y332G mutant of human BChE) ^(89, 112), truncated after amino acid #529 was fused with human serum albumin (HSA) to prolong the biological half-life ⁽⁸⁶⁾. This HSA-fused BChE mutant is also known as Albu-CocH, Albu-CocH1, AlbuBChE, or TV-1380 in literature ^(86, 91, 92, 113), TV-1380 has been proven safe and effective for use in animals and humans ^(91, 92), but its actual therapeutic value for cocaine addiction treatment is still limited by the moderate biological half-life which is ~8 hr in rats ⁽⁸⁶⁾ or 43-77 hr in humans ⁽⁹¹⁾; in general, the biological half-life of a therapeutic protein in humans is significantly longer than that in rats ⁽⁸⁶⁾. A biological half-life of 43-77 hr in humans might be good enough for a twice-weekly therapy, depending on the dose of the enzyme used. In addition, more recently designed and discovered CocHs ⁽¹⁰²⁻¹⁰⁴⁾ are significantly more active against (-)-cocaine compared to TV-1380. It is highly desired to further engineer a more active CocH with a biological half-life longer than TV-1380.

1.6 Summary of development of treatment for cocaine abuse and specific aims of the investigation described in this dissertation

In summary, cocaine abuse is a global problem with direct and indirect adverse impact on human health and social welfare. Unfortunately, there is no FDA-approved effective treatment specific for cocaine abuse. Despite decades of efforts, none of the pharmacodynamic agents tested so far has been proven effective for treatment of cocaine addiction or overdose. The inherent difficulties of antagonizing cocaine in the central nervous system have led to exploring protein-based pharmacokinetic approaches with biologics like monoclonal antibodies, vaccines that produce antibodies in the body, and enzymes ^(16, 47). The pharmacokinetic approach using an efficient metabolic enzyme is recognized as a truly promising treatment strategy for cocaine overdose and addiction ^(16, 86, 91, 92). Clinical studies show that the CocE mutant is safe and efficacious for accelerating the cocaine conversion to EME and benzoic acid in

humans ⁽⁹⁰⁾, but is not able to hydrolyze benzoylecgonine (a major, biologically active metabolite of cocaine, will be discussed in more detail below) at all. TV-1380 has been proven safe and effective for use in animals and humans ^(91, 92), but its actual therapeutic value for cocaine addiction treatment is still limited by the moderate biological half-life ^(86, 91). The investigation described in this dissertation is mainly focused on addressing some possible concerns in the further development of promising CocHs for cocaine abuse treatment. We have carried out kinetic characterization of an hCocH and a mCocH for their catalytic activities against (-)-cocaine and other substrates, identified enzymes that can hydrolyze not only cocaine, but also its major biologically active metabolite benzoylecgonine developed a LC-MS/MS method for determining pharmacokinetic profiles of cocaine and metabolites, and developed a long-acting form of hCocH.

Chapter 2: Kinetic Characterization of a Cocaine Hydrolase Engineered from Mouse Butyrylcholinesterase

Summary: In the investigation described in this chapter, mouse BChE (mBChE) and an mBChE-based cocaine hydrolase (mCocH, i.e. the A199S/S227A/S287G/A328W/Y332G mutant) have been characterized for their catalytic activities against cocaine, i.e. naturally occurring (-)-cocaine, in comparison with the corresponding human BChE (hBChE) and an hBChE-based cocaine hydrolase (hCocH, i.e. the A199S/F227A/S287G/A328W/Y332G mutant). It has been demonstrated that mCocH and hCocH have improved the catalytic efficiency of mBChE and hBChE against (-)-cocaine by ~8- and ~2000-fold, respectively, although the catalytic efficiencies of mCocH and hCocH against other substrates, including acetylcholine (ACh) and butyrylthiocholine (BTC), are close to those of the corresponding wild-type enzymes mBChE and hBChE. According to the kinetic data, the catalytic efficiency of mBChE against (-)-cocaine is comparable to that of hBChE, but the catalytic efficiency of mCocH against (-)-cocaine is remarkably lower than that of hCocH by ~250-fold. The remarkable difference in the catalytic activity between mCocH and hCocH is consistent with the difference between the enzyme-(-)-cocaine binding modes obtained from molecular modeling. Further, both mBChE and hBChE demonstrated substrate activation for all of the examined substrates ((-)-cocaine, ACh, and BTC) at high concentrations, whereas both mCocH and hCocH showed substrate inhibition for all three substrates at high concentrations. The amino-acid mutations have remarkably converted substrate activation of the enzymes into substrate inhibition, implying that the rate-determining step of the reaction in mCocH and hCocH might be different from that in mBChE and hBChE. The main results described in this chapter have been published ⁽¹¹⁴⁾.

2.1 Why a cocaine hydrolase engineered from mouse butyrylcholinesterase is interesting

Previous efforts of Dr. Zhan's group were focused on improving the catalytic activity of hBChE against (-)-cocaine, leading to discovery of various hBChE mutants ^(89, 101-104, 115-117) with a considerably improved catalytic efficiency towards that drug. The highly efficient hBChE mutants, such as A199S/S287G/A328W/Y332G ($k_{\text{cat}} = 3060 \text{ min}^{-1}$ and $K_M = 3.1 \text{ }\mu\text{M}$) ^(89, 112) or A199S/F227A/S287G/A328W/Y332G ($k_{\text{cat}} = 5700 \text{ min}^{-1}$ and $K_M = 3.1 \text{ }\mu\text{M}$) ⁽¹⁰²⁾, can be described as CocH. Initial experiments in rats and mice showed that CocH is likely to be effective as an enzyme therapy or gene therapy for treating cocaine abuse by greatly reducing the reward value of a given drug dosage ^(83, 86, 102, 103, 118-125). In addition, we have not seen any acute toxicity of hCocH in mice or rats nor have other investigators found that wild-type hBChE elicited adverse effects in experimental animals ⁽¹²⁶⁻¹²⁸⁾. Two clinical trials (NCT00333515 and NCT00333528) of hBChE have been performed by Baxter Healthcare Corporation, although the clinical data have not been made available.

Not surprisingly, some mice and rats eventually develop antibodies against hBChE and hCocH, accelerating the clearance of these enzymes and lowering their plasma levels ⁽¹²²⁾, although no immune response is noted when mouse BChE (mBChE) is injected into mice ⁽¹²⁹⁾. This outcome was expected because hBChE shares only ~80% sequence identity with its rodent counterparts ⁽¹³⁰⁾. We deemed it unlikely that human beings would generate antibodies to hCocH as the mutated residues are not exposed on the surface but occupy a deep and narrow catalytic gorge. Nonetheless, the mouse response called for further experiments to test the hypothesis that mutations in the catalytic site are not antigenic. Therefore we developed a conspecific cocaine hydrolase with equivalent mutations in mBChE: A199S/S227A/S287G/A328W/Y332G, designated mouse CocH or "mCocH". The catalytic properties of mCocH were compared with those of mBChE, hBChE, and

hCocH, and it was incorporated into viral gene transfer vector for *in vivo* studies with the aim of avoiding complications from an immune response in the animals.

In fact, initial gene transfer experiments with mCocH showed that very high levels of enzyme protein could be generated, on the order of 1000-fold above the native mBChE background level ⁽¹²²⁾. However, the levels of cocaine hydrolysis did not increase to the extent achieved with hCocH. This outcome suggested that although mCocH and hCocH contain similar mutations, their catalytic efficiencies with (-)-cocaine are different, and results from the gene transfer study were consistent with this interpretation ⁽¹²²⁾. To explore the reason, in the study described in this chapter, mBChE and mCocH proteins were compared with hBChE and hCocH in regard to catalytic properties against (-)-cocaine and various other substrates. In addition, homology modeling and molecular dynamics (MD) simulations (carried out by Dr. Xiaoqin Huang) were used to compare structural features of the two mutated enzymes. As will be shown in this chapter, the catalytic efficiency of mCocH against (-)-cocaine is indeed lower than that of hCocH, and computational modeling of the detailed three-dimensional (3D) structures provides some insight into the reasons for this conclusion, which in turn may facilitate future attempts at re-engineering enzymes for therapeutic purposes.

2.2 Materials and methods

2.2.1 Materials

The cDNA for mBChE containing N-terminal signal was kindly provided by Dr. Palmer Taylor (Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, CA). Cloned *pfu* DNA polymerase and *Dpn I* endonuclease were obtained from Stratagene (La Jolla, CA). Restriction enzyme, alkaline phosphatase (CIP), and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). All oligonucleotides were synthesized by Eurofins MWG Operon (Huntsville, AL). Vector pCMV-MCS was obtained from Agilent Technologies

(Santa Clara, CA). The QIAprep Spin Plasmid Miniprep Kit and QIAquick Gel Extraction Kit were obtained from QIAGEN (Valencia, CA). Chinese hamster ovary (CHO)-S cells and FreeStyle™ CHO Expression Medium were ordered from Invitrogen (Grand Island, NY). [³H](-)-Cocaine (50 Ci/mmol) and [³H]acetylcholine (ACh) were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Butyrylthiocholine (BTC) was obtained from Sigma-Aldrich (St Louis, MO).

2.2.2 Construction of eukaryotic expression plasmids

Site-directed mutagenesis for obtaining the mCocH cDNA was carried out using the QuikChange method ⁽¹³¹⁾. The cDNA for full-length mBChE or mCocH was constructed in a pCMV-MCS expression plasmid by using restriction enzyme EcoR I to digest the original vector and cDNA. Before the ligation, alkaline phosphatase (CIP) was used to dephosphorylate the 5' end of vector. Gel-purified cDNA was ligated with pCMV-MCS vector using T4 DNA ligase. Plasmids encoding hBChE and hCocH were obtained as previously described ⁽¹⁰²⁾.

2.2.3 Protein expression and purification

All proteins (m/hBChE and m/hCocH) were expressed in CHO-S cells separately. Cells were incubated at 37°C in a humidified atmosphere containing 8% CO₂, and transfected with plasmids encoding various proteins using TransIT-PRO® transfection kit once cells had grown to a density of $\sim 1.0 \times 10^6$ cells/ml. The culture medium (Gibco® FreeStyle™ CHO expression medium with 8 mM glutamine) was harvested 7 days after transfection. Secreted enzyme in the culture medium was purified by a two-step approach described previously ⁽⁸³⁾, including ion exchange chromatography using QFF anion exchanger and affinity chromatography using procainamide-sepharose. Pre-equilibrated procainamide-sepharose was added into protein sample purified by ion exchange chromatography, and incubated for 3 h with occasional stirring. After washing the column with 20 mM potassium phosphate, 1

mM EDTA, pH 7.0 until OD280 < 0.02, enzyme was eluted by buffer containing 0.3 M NaCl and 0.1 M procainamide-HCl. The eluate was dialyzed in phosphate buffer, pH 7.4 by Millipore centrifugal filter device. The entire purification process was carried out in a cold-room at 4°C. Concentration of the active enzyme was determined through active site titration with diisopropylfluorophosphate (DFP) as described previously ⁽¹²²⁾. Purified enzymes were stored at 4°C before enzyme activity assays.

2.2.4 Enzyme activity assays

The catalytic activities of enzymes against (-)-cocaine were determined with a radiometric assay based on toluene extraction of [³H](-)-cocaine labeled on its benzene ring. 150 µl enzyme solution (100 mM phosphate buffer, pH 7.4) was added to 50 µl [³H](-)-cocaine solution with varying concentration. The reactions were stopped by adding 200 µl of 0.1 M HCl which neutralized the liberated benzoic acid while ensuring a positive charge on the residual (-)-cocaine. [³H]Benzoic acid was extracted by 1 ml of toluene and measured by scintillation counting. The assays to determine catalytic activity with [³H]ACh differed only in that the reaction was stopped with 200 µl of 0.2 M HCl containing 2 M NaCl. To determine catalytic activities of enzymes against BTC, UV-Vis spectrophotometric assays were carried out in a GENios Pro Microplate Reader (TECAN, Research Triangle Park, NC) with XFluor software. 100 µl enzyme solution was mixed with 50 µl of 25 mM dithiobisnitrobenzoic acid and 50 µl of BTC in varying concentrations. Reaction rates were measured by recording the time-dependent absorption at 450 nm. All measurements were performed at 25°C. Kinetic data were analyzed by performing non-linear, least-squares fitting to Eq.(2.1) (which accounts for the potential secondary binding site of the enzyme, *i.e.* a peripheral anionic binding site around D70) ^(132, 133).

$$V = \frac{V_{\max}(1 + bS/K_{SS})}{(1 + K_M/S)(1 + S/K_{SS})} \quad (2.1)$$

In Eq.(2.1), S represents the concentration of the substrate, $V_{\max} = k_{\text{cat}}[E]$ in which $[E]$ is the enzyme concentration, K_{ss} is a binding constant for substrate at the secondary binding site, and b is a factor reflecting whether or not there is a substrate activation/inhibition. When $b = 1$, there is no substrate activation or inhibition, and the enzymatic reaction follows Michaelis-Menten kinetics. There is substrate activation when $b > 1$, and substrate inhibition when $b < 1$. Kinetic data were analyzed with Microsoft Excel, coding Eq.(2.1) for non-linear fitting .

2.2.5 Homology modeling

The 3D structure of mCocH was modeled based on our previously refined 3D structure ^(102, 134) of hCocH as a template. The hCocH structure was refined through MD simulations and hybrid quantum mechanics/molecular mechanics (QM/MM) calculations ^(102, 134) starting from the X-ray crystal structure (PDB entry code: 1P0P) ⁽¹³⁵⁾ available for hBChE. With the refined hCocH structure as a template, a 3D structure of mCocH was constructed and refined using the Protein Modeling module of Discovery Studio (Version 2.5.5, Accelrys, San diego, CA). The amino-acid sequence of mBChE was directly extracted from the PubMed website (NCBI access No. AAH99977), with the sequence changes necessary to generate the sequence of mCocH. The sequence alignment was generated by using ClusterW with the Blosum scoring function ^(136, 137). The best alignment was selected according to both the alignment score and the reciprocal positions of the conserved residues between human and mouse proteins, particularly the residues forming the catalytic triad (S198-H438-E325) and the oxyanion hole (G116-G117-A/S199). The sequence identity between mCocH and hCocH reached 80%. The coordinates of the conserved regions were directly transformed from the template structure, whereas the non-equivalent residues were mutated from the template to the corresponding ones of mCocH. The side chains of those non-conserved residues were relaxed during the process of homology modeling in order to remove the possible steric overlap or

hindrance with the neighboring conserved residues. The initial structure of mCocH was subject to energy minimization by using the Sander module of the Amber program ⁽¹³⁸⁾ with a conjugate gradient energy-minimization method and a non-bonded cutoff of 10 Å. First, the structure of mCocH was solvated in an orthorhombic box of TIP3P water molecules ⁽¹³⁹⁾ with a minimum solute-wall distance of 10 Å. Standard protonation states at physiological environment (pH ~7.4) were used for all ionizable residues of the proteins, and the proton positions were set properly on the Nδ1 atom of histidine residues. Additional Cl⁻ ions were added to the solvent as counter ions to neutralize the system. The final system size was about 94 Å × 91 Å × 87 Å, composed of 62,489 atoms, including 18,555 water molecules. The first 2,000 steps of the energy minimization were carried out for the backbone while the side chains were fixed, and then the next 60,000 steps for the side chains and water molecules. Finally, the system (mCocH) was energy-minimized for 6,000 steps for all atoms, and a convergence criterion of 0.001 kcal mol⁻¹ Å⁻¹ was achieved.

2.2.6 Molecular dynamics (MD) simulation

Using the homology model of mCocH, we further examined how mCocH binds with (-)-cocaine. First, (-)-cocaine was docked into the binding site, giving a binding mode similar to that for the corresponding hCocH binding with (-)-cocaine through the superposition. The atomic charges for (-)-cocaine were the restrained electrostatic potential (RESP) charges determined and used in our previous studies on hBChE and hCocH interacting with (-)-cocaine ^(89, 102). MD simulations were carried out on the mCocH-(-)-cocaine binding complex by using the Sander module of the Amber program. Each system was slowly heated to 300 K by the weak-coupling method ⁽¹⁴⁰⁾ and then equilibrated for 50 ps. During the MD simulations, a 10 Å non-bonded interaction cutoff was used and the non-bonded list was updated every 1,000 steps. The particle-mesh Ewald (PME) method ⁽¹⁴¹⁾ was applied to treat long-range electrostatic interactions. The lengths of covalent bonds involving hydrogen atoms

were fixed with the SHAKE algorithm ⁽¹⁴²⁾, enabling the use of a 2-fs time step to numerically integrate the equations of motion. Finally, the production MD was kept running for 4.0 ns with a periodic boundary condition in the NTP (constant temperature and pressure) ensemble at T = 300 K with Berendsen temperature coupling and at P = 1 atm with anisotropic molecule-based scaling ⁽¹⁴³⁾.

2.3 Catalytic parameters k_{cat} and K_M

The kinetic data are depicted in Figures 2.1 to 2.3, and the obtained kinetic parameters are summarized in Table 2.1. As seen in Table 2.1, compared to hBChE, mBChE has a smaller k_{cat} value (1.4 min^{-1} vs 4.1 min^{-1}) and a smaller K_M value ($1.6 \text{ }\mu\text{M}$ vs $4.5 \text{ }\mu\text{M}$) against (-)-cocaine. Overall, the catalytic efficiency of mBChE against (-)-cocaine ($k_{\text{cat}}/K_M = 8.8 \times 10^5 \text{ min}^{-1} \text{ M}^{-1}$) is comparable to that of hBChE ($k_{\text{cat}}/K_M = 9.1 \times 10^5 \text{ min}^{-1} \text{ M}^{-1}$). Concerning the effects of the mutations, hCocH has a ~2000-fold improved catalytic efficiency against (-)-cocaine compared to hBChE. From that standpoint, one might expect that mCocH would also have considerably greater catalytic efficiency against (-)-cocaine than mBChE. In fact, as seen in Table 2.1, the k_{cat} of mCocH against (-)-cocaine is ~180-fold larger than that of mBChE against (-)-cocaine, but the K_M of mCocH against (-)-cocaine is also larger than that of mBChE against (-)-cocaine (~22-fold). So, the improvement in k_{cat} is compromised by the significant increase of K_M , resulting in only ~8-fold improved catalytic efficiency over mBChE ($k_{\text{cat}}/K_M = 7.1 \times 10^6 \text{ min}^{-1} \text{ M}^{-1}$). As a result, compared to hCocH, mCocH has ~250-fold lower catalytic efficiency against (-)-cocaine.

According to the kinetic parameters in Table 2.1, against substrate ACh, mBChE has a slightly smaller k_{cat} value (38400 min^{-1} vs 61200 min^{-1}) and a slightly larger K_M value ($400 \text{ }\mu\text{M}$ vs $148 \text{ }\mu\text{M}$) compared to hBChE. Therefore, the catalytic efficiency of mBChE against ACh is ~4-fold lower than that of hBChE. Concerning the mutational effects on hydrolysis of ACh, both mCocH and hCocH exhibit catalytic efficiencies

only slightly lower than those of the wild-type enzymes (mBChE and hBChE). In other words, the mutations caused no substantial effect.

Against substrate BTC, mBChE has a slightly larger k_{cat} value than hBChE (35600 min^{-1} vs 29500 min^{-1}) but a significantly larger K_{M} value (72 μM vs 17 μM). Overall, the catalytic efficiency of mBChE against BTC is ~3-fold lower than that of hBChE. Concerning the mutational effects on enzyme activity against BTC, the catalytic efficiency of mCoch is only slightly lower than that of mBChE, whereas the catalytic efficiency of hCoch is only slightly higher than that of hBChE. Overall changes in the catalytic efficiency against BTC are probably not physiologically significant in either mutated enzyme.

Table 2.1 Kinetic parameters determined for (-)-cocaine, ACh, and BTC hydrolyses catalyzed by mBChE, mCocH, hBChE, and hCocH. This table came from ref. ⁽¹¹⁴⁾.

Enzyme	Substrate	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M ($\text{min}^{-1} \text{M}^{-1}$)	RCE ^a	K_{ss} (μM)	b
mBChE	(-)-cocaine	1.4	1.6	8.8×10^5	1	1300	3.13
mCocH	(-)-cocaine	250	35	7.1×10^6	8.2	1500	0.88
hBChE ^b	(-)-cocaine	4.1	4.5	9.1×10^5	1	216	1.80
hCocH ^c	(-)-cocaine	5700	3.1	1.8×10^9	2020	137	0.40
mBChE	ACh	38400	400	9.6×10^7	1	15200	1.79
mCocH	ACh	19000	210	9.0×10^7	0.94	25800	0.30
hBChE ^d	ACh	61200	148	4.1×10^8	1	31600	2.58
hCocH ^e	ACh	11900	37	3.2×10^8	0.78	30000	0.68
mBChE	BTC	35600	72	4.9×10^8	1	5000	2.77
mCocH	BTC	28200	99	2.8×10^8	0.58	254	0.19
hBChE ^f	BTC	29500	17	1.7×10^9	1	3010	3.36
hCocH ^e	BTC	28000	13	2.2×10^9	1.24	243	0.49

^aRCE refers to the relative catalytic efficiency (k_{cat}/K_M), *i.e.* the ratio of the k_{cat}/K_M value of a mutant (mCocH or hCocH) to that of the corresponding wild-type enzyme (mBChE or hBChE) against the same substrate.

^bThe k_{cat} and K_M for hBChE against (-)-cocaine were reported in ref.⁽¹⁰¹⁾.

^cThe k_{cat} and K_M for hCocH against (-)-cocaine were reported in ref.⁽¹⁰²⁾.

^dThe k_{cat} and K_M for hBChE against ACh were reported in ref.⁽⁸⁶⁾.

^eThe k_{cat} and K_M for hCocH against ACh and BTC were reported in ref.⁽¹⁴⁴⁾.

^fThe k_{cat} and K_M for hBChE against BTC were reported in ref.⁽¹¹⁸⁾.

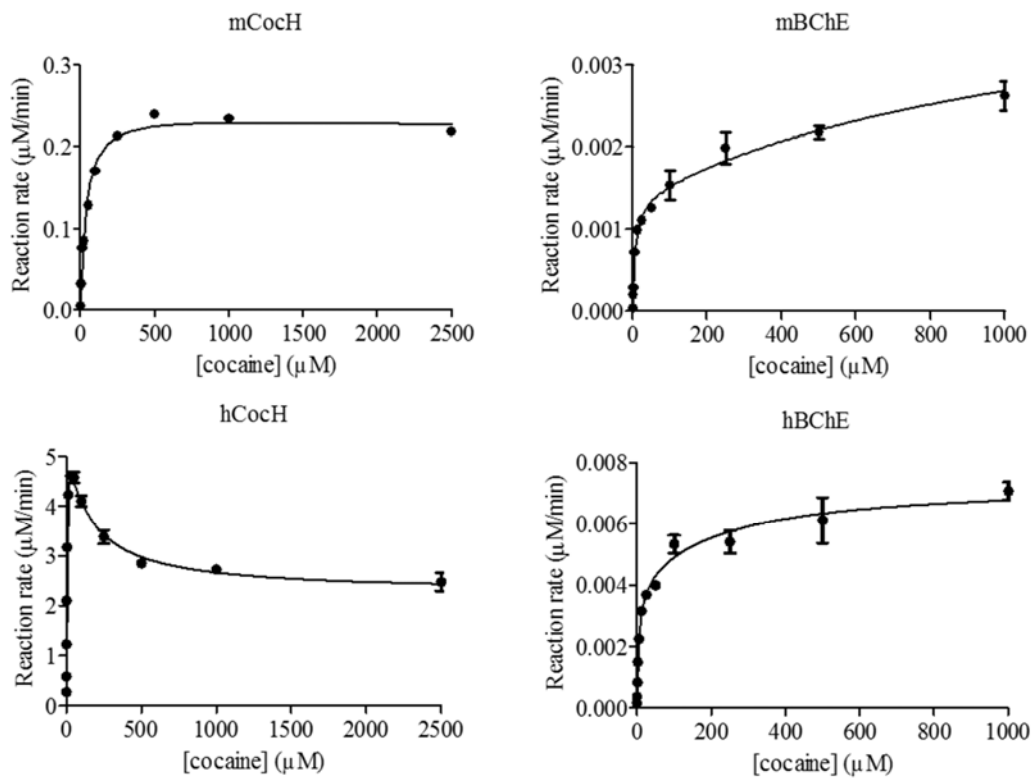


Figure 2.1 Kinetic data obtained in vitro for (-)-cocaine hydrolysis catalyzed by mCocH, mBChE, hCocH, and hBChE. The reaction rate is represented in $\mu\text{M min}^{-1}$ per nM enzyme. This figure came from ref.⁽¹¹⁴⁾.

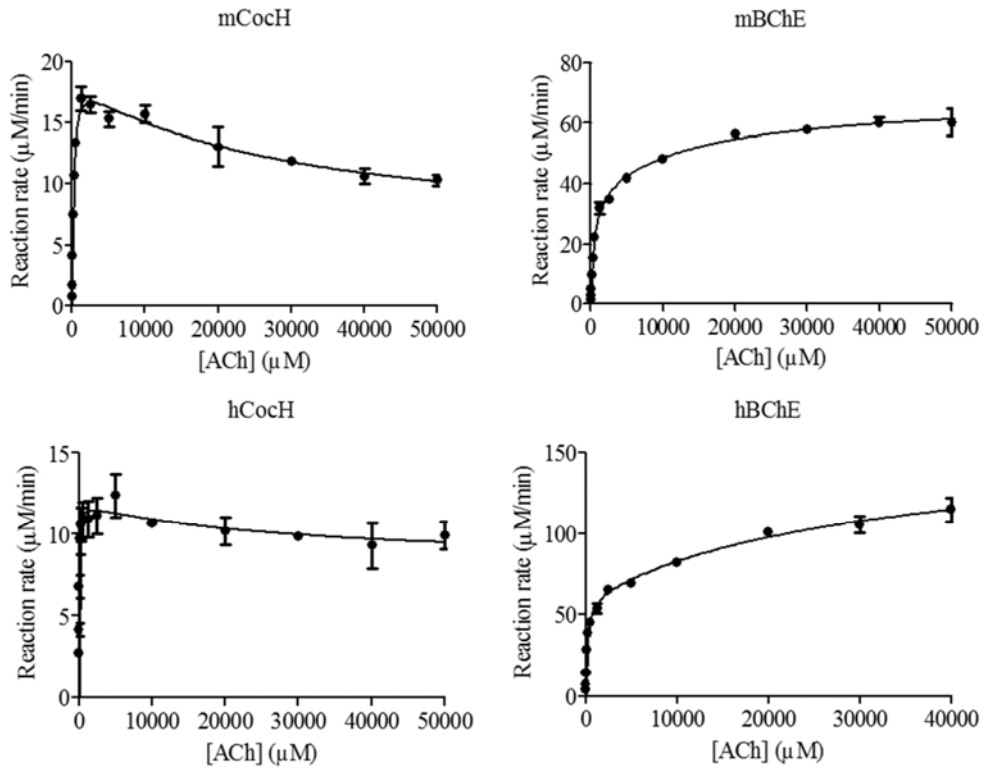


Figure 2.2 Kinetic data obtained in vitro for ACh hydrolysis catalyzed by mCocH, mBChE, hCocH, and hBChE. The reaction rate is represented in $\mu\text{M min}^{-1}$ per nM enzyme. This figure came from ref.⁽¹¹⁴⁾.

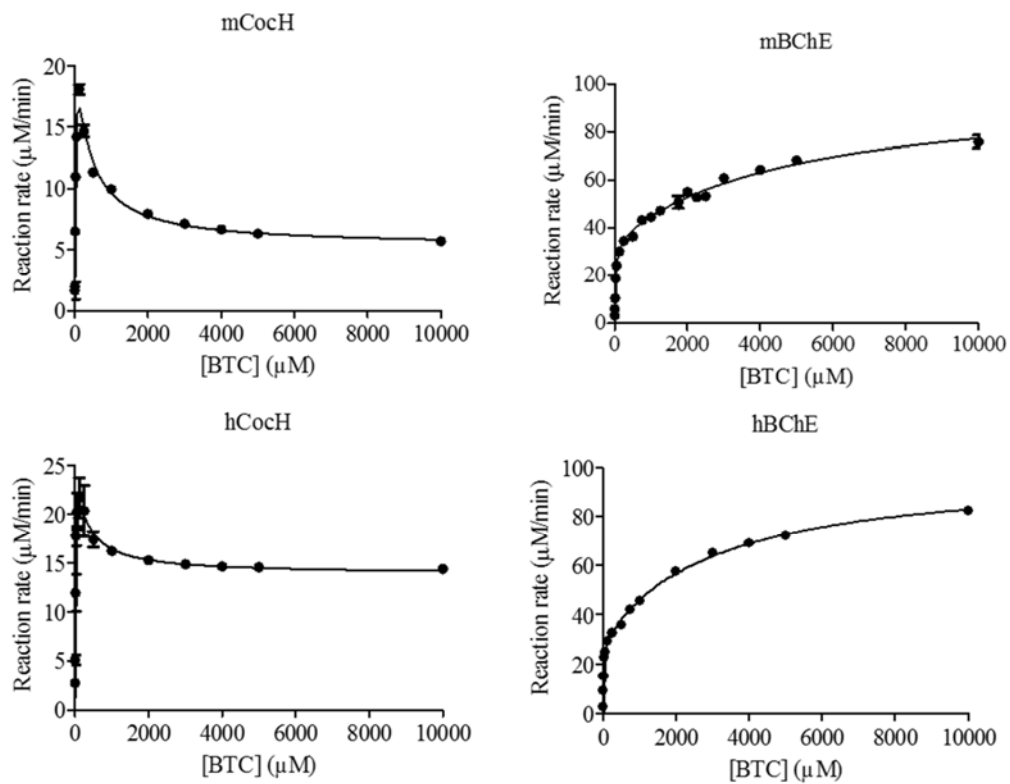


Figure 2.3 Kinetic data obtained in vitro for BTC hydrolysis catalyzed by mCocH, mBChE, hCocH, and hBChE. The reaction rate is represented in $\mu\text{M min}^{-1}$ per nM enzyme. This figure came from ref.⁽¹¹⁴⁾.

2.4 Insights from molecular modeling

To understand why mCocH has much lower catalytic efficiency against (-)-cocaine compared to hCocH, we modeled the 3D structure of mCocH binding with (-)-cocaine for comparison with the corresponding hCocH-(-)-cocaine binding. Depicted in Figure 2.4 are the aligned sequences of mCocH and hCocH, showing that the overall sequence identity between these two enzymes is as high as 80%. As shown in Figure 2.4, mCocH and hCocH share the same residues for the catalytic triad that reacts with (-)-cocaine (S198, H438, and E325), and the same oxyanion hole residues (G116, G117, and S199) that form hydrogen bonds with the carbonyl oxygen atom on the benzoyl group of (-)-cocaine. Another common feature of cocaine binding with hCocH and mCocH is that the cationic head of (-)-cocaine has a similar cation- π interaction with the side chain of W82.

The main difference between hCocH and mCocH is that the cationic head of (-)-cocaine interacts more favorably with the protein environment including side chains of F73 and W328 in hCocH, compared to the corresponding interactions in mCocH. This appears to be due to a difference in the detailed shape of the binding pockets in the two enzymes. For example, residue #72 is an alanine in mCocH and serine in hCocH. In hCocH, the hydroxyl group of S72 side chain forms a strong hydrogen bond with an oxygen atom in the carboxylate moiety of the D70 side chain (Figure 2.5C and D). This hydrogen bond apparently influences the orientation of the aromatic ring in F73 such that the cationic head of (-)-cocaine aligns nearly parallel to the vector normal to the plane of the aromatic ring of F73 side chain. As a result, the MD-simulated average distance between the positively charged N atom of (-)-cocaine and the center of the hCocH F73 side chain aromatic ring was 7.06 Å, and the MD-simulated average distance between the positively charged N atom of (-)-cocaine and the center of aromatic ring of W328 side chain was 5.99 Å.

In mCocH, with no hydrogen bond between the side chains of A72 and D70, the side chain of altered residue #72 is farther away from D70 than it is in hCocH. This

causes the orientation of the aromatic ring of the F73 side chain in mCocH to differ substantially from that in hCocH. Due to this alteration, the hydrogen atoms on the aromatic ring of F73 side chain in the initial model of mCocH-(-)-cocaine binding structure seemed too close to the (-)-cocaine atoms. After further simulation, the F73 side chain pushed away from the (-)-cocaine atoms and the MD-simulated average distance between the positively charged N atom of (-)-cocaine and the center of aromatic ring of F73 side chain grew to 8.58 Å (1.52 Å longer than that in hCocH). The difference in residue #72 also indirectly affected the interaction of (-)-cocaine with W328. The MD-simulated average distance between the positively charged N atom of (-)-cocaine and the center of the W328 side-chain aromatic ring was 6.90 Å in mCocH (0.91 Å longer than that in hCocH). Due to the less favorable interactions of the cationic head of (-)-cocaine with F73 and W328 side chains in mCocH, the overall binding of (-)-cocaine with mCocH can be expected to be weaker, which is consistent with the experimental observation that, compared to hCocH, mCocH has a significantly larger K_M value and a significantly smaller k_{cat} value against (-)-cocaine.

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mCocH EEDFIITTKTRVRLSMPVLLGGTVTAFILGIPYAQPPLGLRFRFKKQPLNKWPD IHNATQYANS CYQNIDQAFPGFGSEMWNPNFD 87
hCocH EDDIIATKNGKVRGMNLTVFGGTVTAFILGIPYAQPPLGLRFRFKKQPLTKWSD IWNATKYANSCCQNIDQSFPGFHHGSEMWNPNFD 87
*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*
mCocH LSEDCLYLNVWIPVPKPKNATVMVWIYGGGFQGTGTS SLPVYD GKFLARVERVIVVSMNYRVGALGFALPFGNPDAPGNMGLFDQQLA 174
hCocH LSEDCLYLNVWIPAPKPKNATVLIWIYGGGFQGTGTS SLHVYD GKFLARVERVIVVSMNYRVGALGFALPGNPEAPGNMGLFDQQLA 174
*****:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*
mCocH LQWVQRNIAAFGGNPKSITIFGES SGAASVSLHLLCPQSYPLFTRAILLES GSANAPWAVKHPEEARNRTLTLAKFTGCPKENEMEMI 261
hCocH LQWVQRNIAAFGGNPKSVTLFGES SGAASVSLHLLSPGSHSLFTRAILQSGSANAPWAVTSLYEARNRTLNLAKLTGCSRENETEI I 261
*****:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*
mCocH KCLRSKDPQEILRNERFVLPDSILGINFGPTVDGD FLTDMPTLLQLGKVKKAQILVGVNKDEGTWFLVGGAPGFSKDNDSLITRK 348
hCocH KCLRNKDPQEILNEAFVVPYGTPLGVNFGPTVDGD FLTDMPTLLELGQFKKTQILVGVNKDEGTWFLVGGAPGFSKDNNIITRK 348
****,*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*
mCocH EFQEGLNMYFPGVSR LGKEAVLFYVVDWLGEQPP EVYRDALDDVI GDYNIICPALEFTKKFAELENNAFFYFFEHRSSKLPWPEWMG 435
hCocH EFQEGLKIFFPGVSEFGKESILFHYTDWDDQRPENYREALGDVVGDYNIICPALEFTKKFSEWGNAFFYYFFEHRSSKLPWPEWMG 435
*****:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*
mCocH VMHGYEIEFVFGPLGRVNYTRAEIIFSR SIMKTWANFAKYGHPNGTQGNSTMWPFVFTSTEQKYLTLNTEKSKIYSKLRAPQCQFW 522
hCocH VMHGYEIEFVFGPLERRDNYTKAEIILSR SIVKRWANFAKYGNPNETQNNSTSWPVFKSTEQKYLTLNTESTRIMTKLRAQQCRFW 522
*****:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*
mCocH RLFPPKLEMTGDIDETE QEWKAGFHRWSNYMMDWQNFNDYTSKKECTAL 574
hCocH TSFFPPKLEMTGNIDEAEWEWKAGFHRWNNYMDWKNQFNDYTSKKECVGL 574
*****:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*

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Figure 2.4 Sequence alignment between mCocH and hCocH. Stars refer to identical residues, whereas filled period and double filled period refer to the conservative substitutions. This figure came from ref.⁽¹¹⁴⁾.

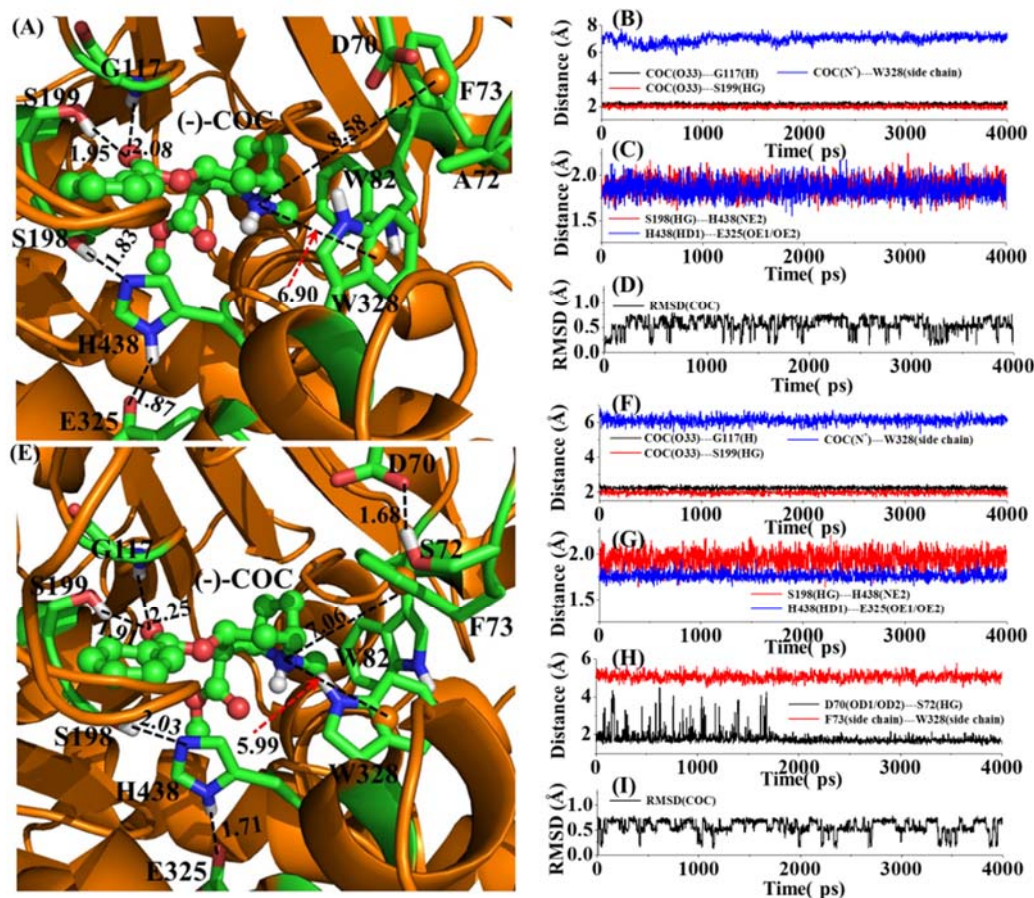


Figure 2.5 The MD-simulated structures of mCocH and hCocH binding with (-)-cocaine. (A) mCocH(-)-cocaine binding structure; (B) and (C) plots of key distances in the mCocH(-)-cocaine complex *versus* the simulation time; (D) plot of root-mean-squares deviation (RMSD) of the atomic positions of (-)-cocaine in the mCocH(-)-cocaine complex *versus* the simulation time; (E) hCocH(-)-cocaine binding structure; (F) to (H) plots of key distances in the hCocH(-)-cocaine complex *versus* the simulation time; (I) plot of the RMSD of the atomic positions of (-)-cocaine in the hCocH(-)-cocaine complex *versus* the simulation time. COC refers to (-)-cocaine. COC(O33)---G117(H) represents the distance between the carbonyl oxygen on the benzoyl group of (-)-cocaine and the backbone hydrogen atom of G117; COC(O33)---S199(HG) the distance between the carbonyl oxygen on the benzoyl group of (-)-cocaine and the hydroxyl hydrogen of S199 side chain;

COC(N⁺)---W328(side chain) the distance between the positively charged nitrogen of (-)-cocaine and the center of aromatic side chain of W328; S198(HG)---H438(NE2) the distance between the hydroxyl hydrogen of S199 side chain and the nitrogen atom (NE2) of H438 side chain; H438(HD1)---E325(OE1/OE2) the distance between the hydrogen atom (HD1) on the nitrogen atom of H438 side chain; COC(C32)---S198(OG) the distance between the carbonyl carbon on the benzoyl group of (-)-cocaine and the hydroxyl oxygen of S198 side chain; D70(OD1/OD2)---S72(HG) the shortest distance between the oxygen atoms of D70 side chain and the hydroxyl hydrogen of S72 side chain; and F73(side chain)---W328(side chain) the distance between the positively charged N atom of (-)-cocaine and the center of aromatic ring of W328 side chain. All distances and RMSD are given in Å. This figure came from ref.⁽¹¹⁴⁾.

2.5 Main insights obtained in from this investigation

Kinetic analysis reveals that the catalytic efficiencies (k_{cat}/K_M) of mBChE against ACh, BTC, and (-)-cocaine resemble those of hBChE. After comparable substitutions at five homologous sites in the catalytic gorge, the corresponding mutant forms mCocH and hCocH both retain similar activities against ACh and BTC and both show enhanced hydrolysis of (-)-cocaine. However, the magnitude of enhancement differs radically between the two enzymes: ~8-fold with mCocH and ~2000-fold with hCocH, leaving the mouse protein ~250-fold less efficient with (-)-cocaine than its human counterpart. A second surprise was that ACh, BTC, and (-)-cocaine all showed substrate activation in wild-type mouse and human BChE, but uniformly caused substrate inhibition in both of the mutated enzymes. That result implies that the rate-determining step of the reactions in mCocH and hCocH may differ from that in mBChE and hBChE. These unexpected outcomes posed an interesting challenge to rational, structure and mechanism based enzyme mutation. However, homology modeling and molecular dynamics simulations shed light on the underlying causes. In other words, the observed behavior was consistent with the enzyme-(-)-cocaine binding structures obtained from molecular modeling.

Chapter 3: Metabolic Enzymes of Cocaine Biomarker Benzoyllecgonine

Summary: Enzyme therapy using an efficient cocaine-metabolizing enzyme is recognized as the most promising approach to cocaine overdose treatment. The actual enzyme, known as RBP-8000, under current clinical development for cocaine overdose treatment is previously designed T172R/G173Q mutant of bacterial cocaine esterase (CocE). The T172R/G173Q mutant is effective in hydrolyzing cocaine, but inactive against benzoyllecgonine (a major, biologically active metabolite of cocaine) at all. Unlike cocaine itself, benzoyllecgonine has an unusually stable zwitterion structure resistant to further hydrolysis in the body and environment. In fact, benzoyllecgonine can last in the body for a very long time (a few days) and, thus, is responsible for the long-term toxicity of cocaine and a commonly used biomarker for drug abuse diagnosis in pre-employment drug tests ^(145, 146). Because CocE and its mutants are all active against cocaine and inactive against benzoyllecgonine, one might simply assume that other enzymes that are active against cocaine are also inactive against benzoyllecgonine. In the study described in this chapter, through combined computational modeling and experimental studies, we demonstrate for the first time that human BChE is actually active against benzoyllecgonine, and that a rationally designed BChE mutant can not only more efficiently accelerate cocaine hydrolysis, but also significantly hydrolyze benzoyllecgonine *in vitro* and *in vivo*. This sets the stage for advanced studies to design more efficient mutant enzymes valuable for development of an ideal cocaine overdose enzyme therapy and for benzoyllecgonine detoxification in environment. The main results discussed in the chapter have been published ⁽¹⁴⁷⁾.

3.1 Cocaine overdose and benzoyllecgonine

Efficient and thermally stable cocaine-metabolizing enzymes have been designed and developed recently as potential therapeutic candidates for treatment of cocaine overdose and addiction ^(88, 89, 102-104, 148). These computationally designed enzymes, that

are mutants of human BChE or bacterial CocE, can rapidly convert cocaine to physiologically inactive EME and benzoic acid. In particular, a thermally stable CocE mutant (T172R/G173Q) ⁽⁸⁸⁾, known as RBP-8000 ⁽⁹⁰⁾, is currently in clinical development for cocaine overdose treatment; the human clinical trial Phase IIa has been completed, showing that the CocE mutant is safe and efficacious for accelerating the cocaine conversion to EME and benzoic acid in humans ⁽⁹⁰⁾.

A potential concern for practical clinical use of the enzyme therapy for cocaine overdose is that cocaine is also converted to benzoylecgonine (BE) by endogenous liver carboxylesterase-1 (hCE-1) in the body (see Figure 3.1). BE is actually a more potent vasoconstrictor than cocaine, norepinephrine, and norcocaine ⁽¹⁴⁵⁾, and cerebral artery segments are significantly more sensitive to BE than to cocaine and the other cocaine metabolites ⁽¹⁴⁶⁾. More importantly, compared to cocaine itself, BE can exist in the body for a considerably longer period of time. For this reason, BE is a commonly used cocaine biomarker for cocaine abuse diagnosis in human drug tests. For cocaine overdose treatment in an ED, a patient intakes cocaine before going to the ED and, thus, a lot of cocaine molecules have already been converted to BE in the body prior to administration of an exogenous enzyme ⁽¹⁴⁵⁾. Hence, an ideal enzyme therapy for cocaine overdose should detoxify not only cocaine itself (which is mainly responsible for the acute toxicity and lethality), but also its long-lasting metabolite BE (which is mainly responsible for the long-term toxicity of cocaine). However, unlike cocaine, BE is a zwitterion extremely difficult to hydrolyze in not only human body, but also environment ⁽¹⁴⁹⁾. It has been extremely challenging to efficiently hydrolyze BE for both cocaine overdose treatment and BE detoxification in environment. In fact, CocE and its T172R/G173Q mutant (RBP-8000) ⁽⁸⁸⁾ are highly efficient against cocaine and norcocaine, but inactive against BE ⁽⁴⁾. To the best of our knowledge, no metabolic enzyme of BE has ever been reported in literature. Here we report the first observation of metabolic enzymes of BE, providing a promising starting point for future

development of an ideal enzyme therapy for cocaine overdose and for BE detoxification in environment.

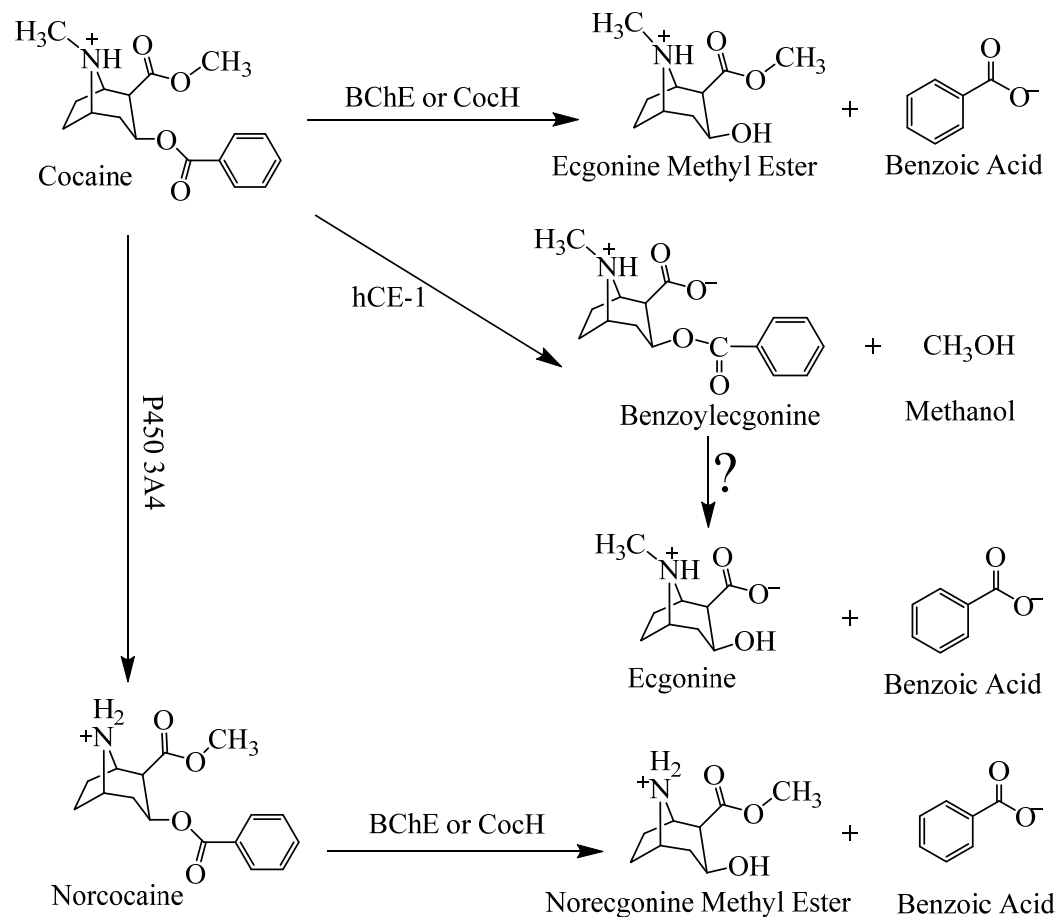


Figure 3.1 Metabolic pathways of cocaine in humans and animals with metabolic enzymes including BChE, hCE-1, and oxidation by cytochrome P450 (CYP) 3A4. This figure came from ref.⁽¹⁴⁷⁾.

3.2 Materials and methods

3.2.1 Molecular modeling

BE binding with human BChE and mutants was modeled by using our previously modeled structures of the same enzymes ^(89, 102, 103, 116, 125, 150-152). Our previous molecular dynamics (MD) simulations ⁽¹⁰³⁾ on the structures of enzyme-cocaine complexes ⁽¹²⁵⁾ started from the X-ray crystal structure deposited in the Protein Data Bank (pdb code: 1POP). For each enzyme (human BChE or mutant), BE was docked into the active site of the enzyme by using the AutoDock 4.2 program ⁽¹⁵³⁾, as we previously did for the enzyme binding with (-)-cocaine, norcocaine, and cocaethylene ⁽¹²⁵⁾. During the docking process, the Solis and Wets local search method ⁽¹⁵⁴⁾ was used for the conformational search and the Lamarkian genetic algorithm (LGA) ⁽¹⁵³⁾ was employed to deal with the enzyme-ligand interactions. The grid size was set to be 120 × 120 × 120. The finally obtained enzyme-BE binding structures were the ones with the lowest binding free energies.

3.2.2 *In vitro* enzyme activity assays

All enzymes (wild-type human BChE, E14-3, and E12-7) were expressed and purified as described previously ⁽¹⁵⁵⁾, and the catalytic activities of the purified enzymes against BE (and (-)-cocaine for comparison) were determined by performing a UV-Vis spectrophotometric assay. Using the UV-Vis spectrophotometric assay, the catalytic activity of the enzymes against BE and (-)-cocaine were determined at the same time under the same experimental conditions except the concentration of the enzymes and substrates, to make sure that the known enzyme activity against (-)-cocaine can be reproduced. The enzymatic reaction was initiated by adding 180 μl of a substrate (BE or (-)-cocaine) solution to 20 μl of an enzyme solution. The final reaction systems of BE have the initial BE concentrations of 500, 300, 200, 100, 50, 20, and 10 μM, whereas the final reaction systems of (-)-cocaine have initial (-)-cocaine concentrations

of 50, 30, 20, 10, 5, 2, and 1 μM . The reaction temperature was 25°C , and the buffer used was 0.1 M potassium phosphate (pH 7.4). The initial rates of the enzymatic hydrolysis of BE/(-)-cocaine in various initial substrate concentrations were estimated by following the change in the intrinsic absorbance peak of BE/(-)-cocaine at 230 nm with time using a GENios Pro Microplate Reader (TECAN, Research Triangle Park, NC) with the XFluor software. The initial reaction rates were calculated from the linear portions of the progress curves. All assays were carried out in triplicate. The Michaelis-Menten kinetic analysis was performed by using Prism 5 (GraphPad Software Inc., San Diego, CA) to determine the V_{max} and K_{M} values.

3.2.3 Kinetic modeling

Kinetic modeling of (-)-cocaine in humans was performed by use of a MatLab program (developed in house) ^(125, 156, 157) in a way similar to that of our recently developed pharmacokinetic modeling of (-)-cocaine in the presence of a cocaine-metabolizing enzyme ^(125, 158). The previously used kinetic model ⁽¹⁵⁵⁾ did not involve BE. By using a one-compartment model, the present kinetic modeling also accounted for the transformation of (-)-cocaine to BE and the subsequent BE hydrolysis in the presence of wide-type BChE or a highly efficient cocaine-metabolizing enzyme E12-7.

3.2.4 Subjects for *in vivo* studies

Male Sprague-Darley rats (200-250 g) were ordered from Harlan (Harlan, Indianapolis, IN) and were housed initially in 2 to 4 rats per cage. All rats 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°C . Experiments were performed in a 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 animal protocol was approved by the Institutional Animal Care and Use

Committee (IACUC) at the University of Kentucky.

3.2.5 *In vivo* tests of BE, ecgonine, and E12-7 in rats

BE and ecgonine were provided by the National Institute on Drug Abuse (NIDA) Drug Supply Program (Bethesda, MD). General anesthetic isoflurane was utilized with nose cone during the administration of BE and E12-7 (or saline). Rats were injected with saline, 0.15 or 5 mg/kg of E12-7 through tail vein 1 min before IV injection of 2 mg/kg BE (~6.9 $\mu\text{mole/kg}$). Four rats were used for each set of experiments ($n=4$). About 50 to 75 μl of blood was collected from saphenous veins into capillary tubes and immediately diluted in 100 μl of 250 μM paraoxon at 2, 5, 10, 30, 60, 90, 120, 150, and 180 min after the IV injection of BE or ecgonine. Paraoxon is an irreversible BChE inhibitor that can stop the enzymatic hydrolysis of BE between blood sampling and analysis. The diluted blood samples were stored at -70°C and assayed by using a high performance liquid chromatography tandem mass spectrometry (LC-MS/MS) method (details described in chapter four). For determination of the active E12-7 concentration, the collected blood samples were not diluted with paraoxon, but centrifuged to obtain the plasma for the enzyme activity assays.

3.3 Identification of enzymes hydrolyzing BE

Our search for a metabolic enzyme of BE started from molecular docking (using a protocol described previously ⁽¹⁵⁵⁾) which enabled us to predict whether BE may bind with various serine esterases, including AChE, BChE and various mutants available in our lab, in a way suitable for the enzymatic hydrolysis of cocaine benzoyl ester group. Potential BE-hydrolyzing enzymes predicted by molecular docking were assayed *in vitro* for their catalytic activity against BE (see Methods section). Because it has been known that CocE and the T172R/G173Q mutant are active against cocaine and inactive against BE, one might simply assume that BChE and its mutants that are active against cocaine are also inactive against BE. Surprisingly, unlike CocE and its mutants, both

molecular docking and *in vitro* activity assays consistently revealed that BChE and our rationally designed mutant enzymes, *i.e.* the A199S/S287G/A328W/Y332G mutant (E14-3)⁽⁸⁹⁾ and A199S/F227A/S287G/A328W/Y332G mutant (E12-7)⁽¹⁰²⁾ of human BChE, are all active against BE. E12-7 is also known as Coch3, and E14-3 is also known as Coch1, in literature^(48, 125).

According to the enzyme-substrate binding structures obtained from molecular docking, BChE and the two mutants all can bind with BE in a similar way as their binding with (-)-cocaine, suitable for the desired enzymatic hydrolysis of (-)-cocaine benzoyl ester group. Depicted in Figure 3.2A and B are the obtained structures of wild-type BChE binding with (-)-cocaine and BE, showing that the backbone NH groups of G117 and A199 residues (within the oxyanion hole) are all close to the common carbonyl oxygen of the substrates such that they may form two favorable hydrogen bonds with the carbonyl oxygen of the substrate in the anticipated transition state⁽⁸⁹⁾ during the enzymatic reaction process for both (-)-cocaine and BE. Further, according to the E12-7-BE binding structure depicted in Figure 3.2C, the carbonyl oxygen of BE is also close to the hydroxyl group of S199 side chain; similar structure (not shown) was also obtained for the E14-3-BE binding. Thus, the carbonyl oxygen of BE may form three hydrogen bonds with G117 and S199 residues of the enzyme (E12-7 or E14-3) within the oxyanion hole in the anticipated transition state during the enzymatic reaction process, suggesting that E12-7 (and E14-3) may have significantly improved catalytic activity against BE compared to wild-type BChE. Based on the modeling insights, we decided to carry out *in vitro* enzyme activity assays on human BChE, E14-3, and E12-7 against BE. Depicted in Figure 3.2D to F are data obtained from *in vitro* kinetic analysis on human BChE, E14-3, and E12-7 against BE. According to the kinetic data in Figure 3.2D to F, wild-type human BChE has a k_{cat} of 3.6 min^{-1} against BE comparable to its k_{cat} against (-)-cocaine ($k_{\text{cat}} = 4.1 \text{ min}^{-1}$). The main difference is that it has a relatively larger Michaelis-Menten constant value ($K_M = 83 \text{ }\mu\text{M}$) against BE compared to its K_M against (-)-cocaine ($K_M = 4.5 \text{ }\mu\text{M}$). Notably,

despite of the relatively lower catalytic efficiency against BE compared to its own efficiency against (-)-cocaine, the catalytic efficiency of human BChE against BE is actually higher than that of the known most active anti-cocaine catalytic antibody 15A10 ($k_{\text{cat}} = 2.3 \text{ min}^{-1}$ and $K_{\text{M}} = 220 \text{ }\mu\text{M}$) against (-)-cocaine ^(159, 160).

Interestingly, compared to wild-type human BChE, both enzymes E14-3 and E12-7 that have considerably improved catalytic activities against (-)-cocaine and norcocaine also have significantly improved catalytic activity against BE. As seen in Figure 3.2, $k_{\text{cat}} = 23 \text{ min}^{-1}$ for E14-3, $k_{\text{cat}} = 65 \text{ min}^{-1}$ for E12-7, and $k_{\text{cat}} = 3.6 \text{ min}^{-1}$ for the wild-type BChE against BE. So, E14-3 and E12-7 have improved the k_{cat} of the wild-type BChE against BE by ~6-fold and ~18-fold, respectively. Meanwhile, E14-3 and E12-7 have slightly larger K_{M} values (133 μM for E14-3 and 207 μM for E12-7). The improvement in k_{cat} against BE is significant, although not as much as that (>1000-fold improvement) against (-)-cocaine ⁽¹⁵⁵⁾.

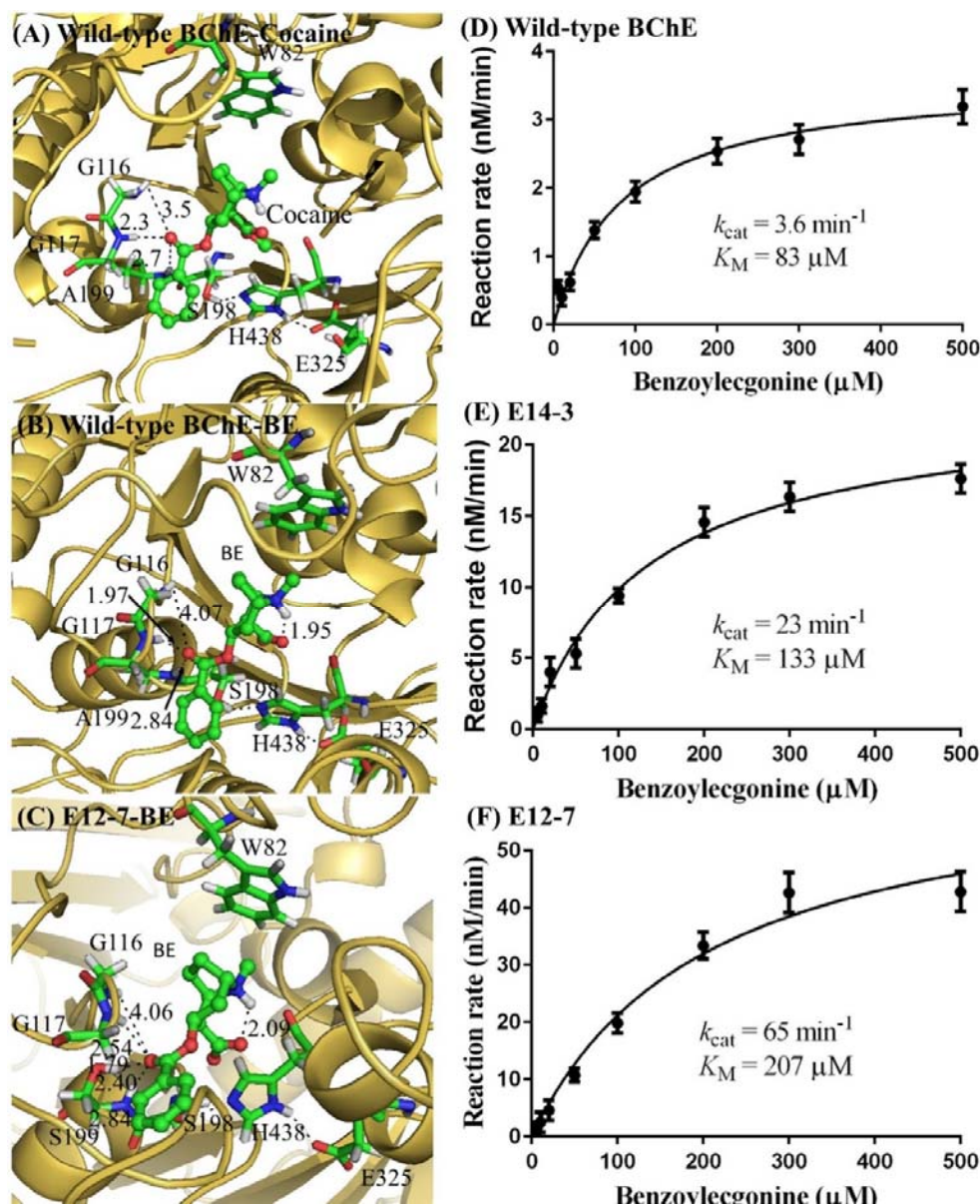


Figure 3.2 Data obtained from molecular docking and in vitro kinetic analysis. (A) Wild-type BChE binding with (-)-cocaine; (B) Wild-type BChE binding with BE; (C) E12-7 binding with BE; (D) Kinetic data for wild-type BChE against BE; (E) Kinetic data for E14-3 against BE; (F) Kinetic data for E12-7 against BE. Indicated in the docked binding structures are the key internuclear distances in Å. For the kinetic data, the reaction rate (represented in nM min^{-1} per nM enzyme) was determined by measuring the rate of the change of the absorbance at 230 nm. This figure came from ref.⁽¹⁴⁷⁾.

as that of endogenous BChE in human plasma ($[E] = 0.07 \mu\text{M}$)⁽¹⁵⁵⁾ or higher. Briefly, kinetic modeling of (-)-cocaine metabolism was carried out by using the kinetic equations shown in Figure 3.3 in the presence of three enzymes: BChE or CocH (which refers to either wild-type human BChE or E12-7) in human plasma; liver carboxylesterase; and CYP 3A4. Concerning CocH, a typical adult has a blood volume of $\sim 5 \text{ L}$ ⁽¹⁵⁸⁾. Previously reported concentrations of endogenous BChE protein in human plasma ranged from 4 to 7 mg/L⁽¹⁶¹⁻¹⁶³⁾, giving an average value of $\sim 6 \text{ mg/L}$ or $\sim 0.07 \mu\text{M}$ in terms of the total BChE protein concentration (denoted as $[E]$), assuming that a tetramer of human BChE has four active sites^(164, 165). According to the known kinetic data reported previously⁽¹⁵⁵⁾ and obtained in the present study, we should have $V_{\text{max}} = 0.29 \mu\text{M min}^{-1}$ and $K_{\text{M}} = 4.5 \mu\text{M}$ for the wild-type BChE against (-)-cocaine, $V''_{\text{max}} = 0.20 \mu\text{M min}^{-1}$ and $K''_{\text{M}} = 15 \mu\text{M}$ for the wild-type BChE against norcocaine, and $V'''_{\text{max}} = 0.25 \mu\text{M min}^{-1}$ and $K'''_{\text{M}} = 83 \mu\text{M}$ for the wild-type BChE against BE when $[E] = 0.07 \mu\text{M}$. These kinetic parameters were used in our modeling with the wild-type BChE. Similarly, for E12-7, according to the kinetic data reported previously⁽¹⁵⁵⁾ and obtained in the present study, we should have $V_{\text{max}} = 400 \mu\text{M min}^{-1}$ and $K_{\text{M}} = 3.1 \mu\text{M}$ against (-)-cocaine, $V''_{\text{max}} = 180 \mu\text{M min}^{-1}$ and $K''_{\text{M}} = 13 \mu\text{M}$ against norcocaine, and $V'''_{\text{max}} = 4.55 \mu\text{M min}^{-1}$ and $K'''_{\text{M}} = 207 \mu\text{M}$ against BE when $[E] = 0.07 \mu\text{M}$. It was reported that non-specific carboxylesterase in humans and rodents is responsible for catalyzing the hydrolysis of the methyl ester group of (-)-cocaine to BE, and the reaction follows the simple Michaelis-Menten kinetics with $K_{\text{M}} = 116 \mu\text{M}$ ⁽¹⁶⁶⁾. The human carboxylesterase mainly exists in liver, as well as other tissues. It is known that (-)-cocaine can diffuse in the body very rapidly to reach the equilibrium⁽¹⁵⁸⁾. Thus, it is reasonable to assume that (-)-cocaine, BE, and norcocaine distributions in the blood and other tissues can rapidly reach the equilibrium during the metabolic reactions. It was roughly estimated that $F_3 = 4.5 \mu\text{M min}^{-1}$ and $k_3 = 116 \mu\text{M}$ for (-)-cocaine hydrolysis to BE, according to the available experimental data including the enzyme activity⁽¹⁶⁷⁾ and the enzyme distribution in the body⁽¹⁶⁸⁾. In our previous study, it has been estimated that $F_2 = 14.4$

$\mu\text{M min}^{-1}$ and $k_2 = 2.7 \text{ mM}$ for the enzymatic oxidation of (-)-cocaine to norcocaine ⁽¹²⁵⁾. These roughly estimated kinetic parameters were used in our kinetic modeling with various initial concentrations; our additional modeling tests revealed that kinetic modeling using different values of the catalytic parameters would lead to the same qualitative conclusions mentioned below.

For possible cocaine overdose treatment using an exogenous cocaine-metabolizing enzyme, the cocaine abusers have already taken (-)-cocaine, and converted some (-)-cocaine to BE and norcocaine before the enzyme administration. In order to know whether E12-7 is also efficacious in hydrolysis of BE, in addition to (-)-cocaine and norcocaine, for the cocaine overdose treatment, we performed an additional, simplified kinetic modeling by assuming that 45% (-)-cocaine has been converted to BE, 5% (-)-cocaine has been converted to norcocaine, and only 50% (-)-cocaine remains as (-)-cocaine when $t = 0$. Depicted in Figure 3.4 are data obtained from the simplified kinetic modeling when $A(0)$ (the initial concentration of (-)-cocaine) = $50 \mu\text{M}$, $B(0)$ (the initial concentration of BE) = $45 \mu\text{M}$, and $F(0)$ (the initial concentration of norcocaine) = $5 \mu\text{M}$. As seen in Figure 3.4A to C, in the absence of E12-7, (-)-cocaine has a half-life ($t_{1/2}$) of 98 min ($t_{1/2} = 98 \text{ min}$), and $t_{1/2} = 65 \text{ min}$ for norcocaine and $t_{1/2} = 318 \text{ min}$ for BE. As seen in Figure 3.4D to F, in the presence of $0.07 \mu\text{M}$ E12-7, both (-)-cocaine and norcocaine are eliminated completely in less than 1 min. However, there was still a significant amount of BE left (AUC of BE = $2270 \mu\text{M}\cdot\text{min}$), but the half-life of BE is reduced significantly from 318 min to 36 min. These data qualitatively suggest that E12-7 can be effective for cocaine overdose treatment as it can completely break down (-)-cocaine and norcocaine, prevent (-)-cocaine from converting to BE, and significantly decrease the half-life and AUC of BE. So, E12-7 can lower the BE concentration in plasma through accelerating not only (-)-cocaine benzoyl ester hydrolysis, but also BE hydrolysis.

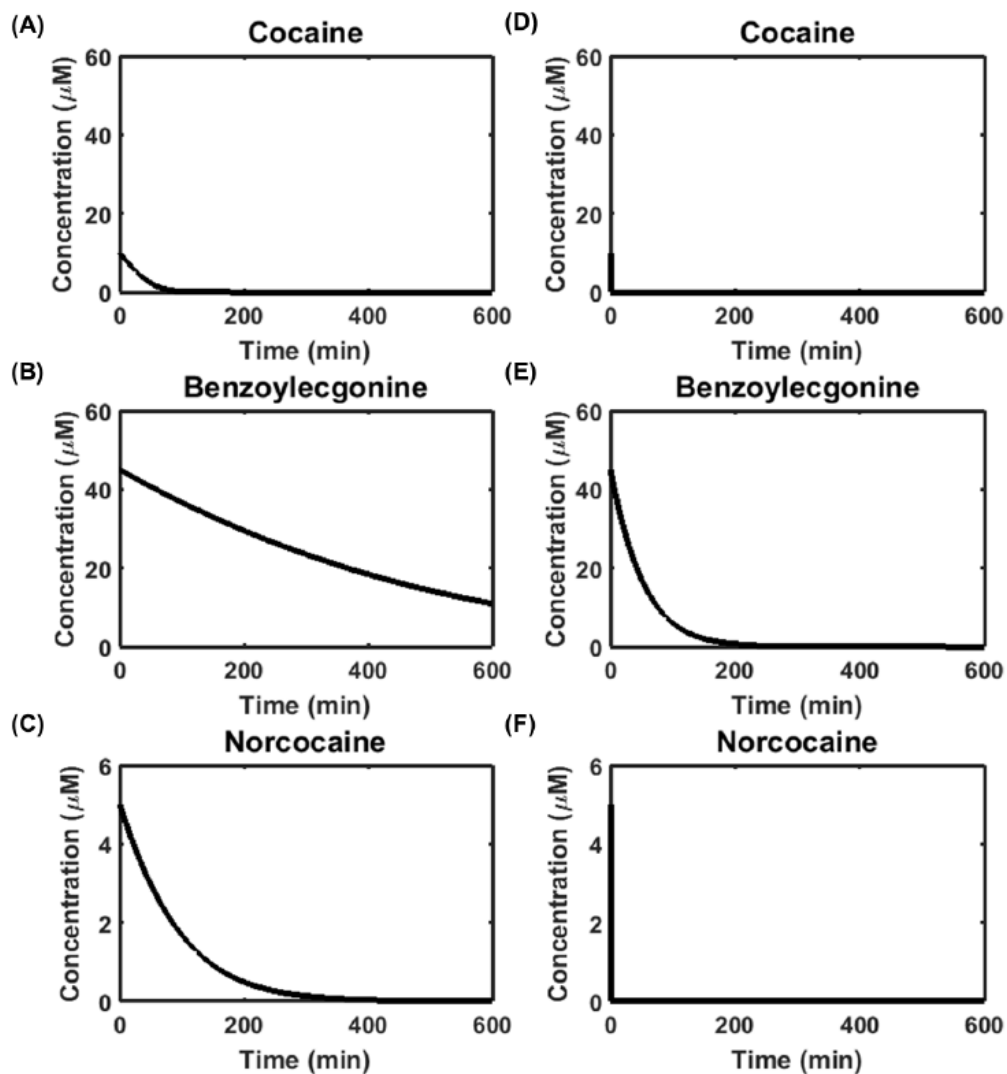


Figure 3.4 The modeled concentrations of (-)-cocaine, BE, and norcocaine in human blood when the initial concentrations of (-)-cocaine, BE, and norcocaine are 50, 45, and 5 μM , respectively. (A) to (C) refer to the time-dependent concentrations in the presence of wild-type human BChE (without E12-7), whereas (D) to (F) refer to the time-dependent concentrations in the presence of 0.07 μM E12-7. This figure came from ref.⁽¹⁴⁷⁾.

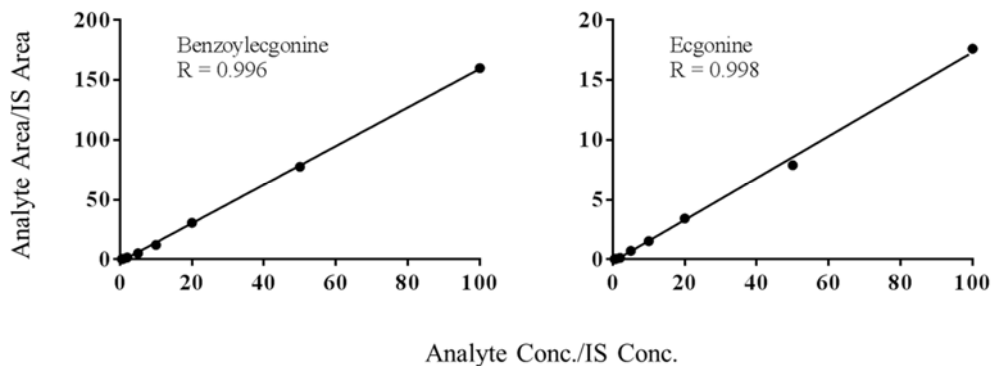


Figure 3.5 LC-MS/MS calibration curves for BE and ecgonine. Calibration curves were established by calculating the ratios of the peak area for analyte to that for the internal standard and plotting the ratio as a function of the ratio of the analyte concentration to the internal standard. X-axis (Analyte Conc. / IS Conc.) refers to the ratio of the analyte concentration to the corresponding internal standard concentration (IS Conc. = 0.1 μ M for each internal standard compound). Y-axis (Analyte Area / IS Area) represents the ratio of the measured area for the analyte to that for the corresponding internal standard. For each compound (BE or ecgonine), data were fitted to a linear curve using the least-squares analysis with 1/x weighting. This figure came from ref.⁽¹⁴⁷⁾.

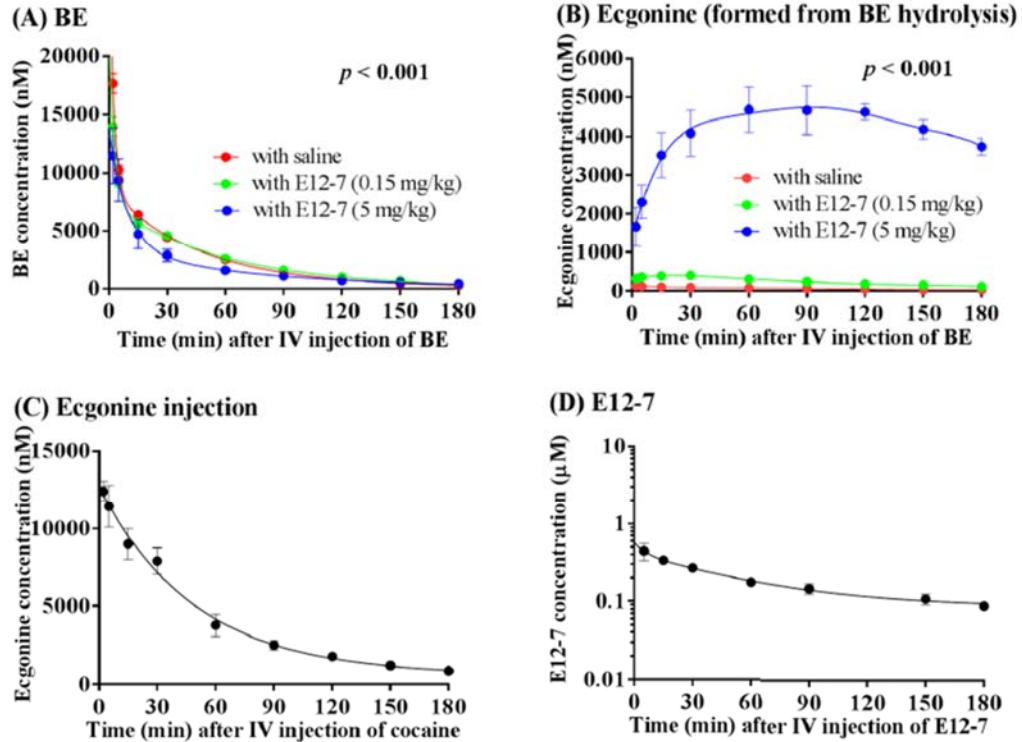


Figure 3.6 Metabolic profiles of BE in the presence and absence of an exogenous enzyme E12-7 (0.15 or 5 mg/kg) in rats ($n=4$ for each group). (A) Time course of BE concentration in rat blood. (B) Time course of ecgonine concentration formed from BE in rat blood. (C) Time course of ecgonine in rat blood after IV injection of 2 mg/kg ecgonine. (D) Time course of plasma E12-7 concentration in rat plasma after IV injection of 5 mg/kg E12-7. $p < 0.001$ represents that there were statistical differences in blood concentrations of BE and ecgonine between different experimental groups (treated with saline or 0.15 mg/kg E12-7 or 5 mg/kg E12-7) as determined by the two-way analysis of variance (ANOVA) tests using the SigmaPlot software (Systat Software, San Jose, CA). This figure came from ref.⁽¹⁴⁷⁾.

To validate the effectiveness of E12-7 in accelerating BE hydrolysis *in vivo*, four groups of rats (n=4 for each group) were used in this study. The obtained calibration curves for detecting BE and its hydrolysis product ecgonine are depicted in Figure 3.5, and the obtained *in vivo* data are shown in Figure 3.6. The first three groups of rats were injected intravenously (IV) with 0.15 mg/kg E12-7 or 5 mg/kg E12-7 or saline, followed by IV injection of 2 mg/kg BE one minute after the enzyme/saline injection. The dose of 0.15 mg/kg E12-7 led to an E12-7 concentration of ~3 mg/L (which is about a half of the average concentration of the endogenous BChE in human ⁽¹⁵⁵⁾) in plasma at ~2 min after IV injection of E12-7 according to our previous study ⁽⁸³⁾. The dose of 5 mg/kg resulted in a plasma E12-7 concentration between 0.45 μ M at 5 min and ~0.1 μ M at 180 min (Figure 3.6D). The final group of rats were injected with 2 mg/kg ecgonine (metabolite of BE), instead of 2 mg/kg BE, in order to know the elimination profile of ecgonine. For each rat, blood samples were collected at 2, 5, 10, 30, 60, 90, 120, 150, and 180 min after the BE or ecgonine injection. The collected blood samples were analyzed by using a sensitive LC-MS/MS method to determine the blood concentrations of BE and ecgonine. In addition, blood samples collected from the rats injected with E12-7 were also analyzed for the E12-7 concentrations.

Statistical analysis using the two-way analysis of variance (ANOVA) method implemented in the SigmaPlot software (Systat Software, San Jose, CA) revealed that $p < 0.001$, i.e. significant differences exist in the blood concentrations of BE and ecgonine between different experimental groups (treated with saline or 0.15 mg/kg E12-7 or 5 mg/kg E12-7). Further, according to the two-way ANOVA with post hoc Dunnett's testing, the differences in the mean BE concentrations between the high-dose group (treated with 5 mg/kg E12-7) and the control group (treated with saline) were significant ($p < 0.001$), although the differences between the low-dose group (0.15 mg/kg E12-7) and the control group were insignificant ($p = 0.117$). So, according to the *in vivo* data depicted in Figure 3.6, E12-7 dose-dependently accelerated BE hydrolysis to ecgonine. The enzyme-accelerated BE clearance (Figure 3.6A) is consistent with the

increased production of BE metabolite ecgonine (Figure 3.6B). In comparison with the ecgonine elimination profile shown in Figure 3.6C, the data depicted in Figure 3.6B revealed that ecgonine was formed continuously until BE was consumed.

3.5 Perspectives in further enzyme development for cocaine overdose treatment

This is the first identification of metabolic enzymes of BE, demonstrating that plasma enzyme BChE is the endogenous metabolic enzyme of BE and the BChE mutant E12-7 with significantly improved catalytic efficiency against BE compared to wild-type BChE may be used as an exogenous enzyme to effectively accelerate BE hydrolysis in the body for cocaine overdose treatment. Compared to RBP-8000 (the T172R/G173Q mutant of CocE) ^(88, 90) under current clinical development for cocaine overdose treatment, E12-7 is not only more efficient against cocaine ⁽¹⁰²⁾, but also effective in hydrolyzing BE. Hence, E12-7 should be a more promising therapeutic candidate for cocaine overdose treatment in comparison with the T172R/G173Q mutant of CocE, although one would like to further improve the catalytic efficiency against BE for cocaine overdose treatment.

In addition, our most recently reported study ⁽¹⁶⁹⁾ demonstrated a novel protein form of E12-7 (or Coch3) (see Chapter 5), denoted as Coch3-Fc or catalytic antibody analog, which is an Fc-fused Coch3 dimer (Coch3-Fc) constructed by using Coch3 to replace the Fab region of human immunoglobulin G1. A single dose of Coch3-Fc was able to accelerate cocaine metabolism in rats even after 20 days and, thus, block cocaine-induced hyperactivity and toxicity for a long period of time ⁽⁴⁸⁾. Thus, taking all of these together, E12-7 (or Coch3) in its Fc-fusion protein form may be used as a truly promising therapeutic candidate for treatment of both cocaine addiction and overdose.

Further, the findings in this study provide an exciting starting point for future efforts to rationally design and discover new BChE mutants with further improved catalytic efficiency against BE, in addition to cocaine itself and other toxic metabolites ^(124, 155). A highly efficient enzyme which can efficiently convert BE to biologically

inactive metabolites is valuable for not only cocaine overdose treatment, but also BE detoxification in environment because BE widely exists in environment worldwide ⁽¹⁴⁹⁾ including drinking water due to cocaine abuse.

Chapter 4: A Quantitative LC-MS/MS Method for Simultaneous Determination of Cocaine and Its Metabolites in Whole Blood

Summary: As new metabolic pathways of cocaine were recently identified, a LC-MS/MS method was developed to simultaneously determine cocaine and nine cocaine-related metabolites in whole blood samples. One-step solid phase extraction was used to extract all of the ten compounds and corresponding internal standards from blood samples. All compounds and internal standards extracted were separated on an Atlantis T3 (100Å, 3 µm, 2.1 mm X 150 mm I.D) column and detected in positive ion and high sensitivity mode with multiple reaction monitoring. This method was validated for its sensitivity, linearity, specificity, accuracy, precision, recovery, and stability. All of the ten compounds were quantifiable ranging from the lower limit of quantification (LLOQs) of ~10 nM (1.9-3.2 ng/ml) to ~1000 nM (190-320 ng/ml) without any interfering substance. Accuracy and precision were determined; and both of them were within the acceptance criteria of the US FDA and European Medicines Agency (EMA) guidelines. The recovery was above 66.7% for all compounds. Stability tests demonstrated the stability of compounds under different storage conditions in whole blood samples. The method was successfully applied to a pharmacokinetic study with co-administration of cocaine and alcohol to rats.

4.1 The need for development of a LC-MS/MS method

Cocaine is one of the most reinforcing and hepatotoxic drugs, accounting for majority of illicit drug-related ED visits in the United State ⁽¹⁷⁰⁾. In humans, approximately 40% of cocaine is hydrolyzed to biologically inactive metabolite EME by plasma enzyme BChE and liver CE-2, but more cocaine is biotransformed to BE *via* hydrolysis catalyzed by CE-1, and to norcocaine *via* oxidization catalyzed by liver microsomal cytochrome P450 (CYP) 3A4 ^(46, 171, 172). This metabolic profile of cocaine is highly affected by alcohol co-consumption which commonly happens

among cocaine users ⁽¹⁷³⁾. Especially, alcohol decreases the hydrolysis of cocaine to BE by inhibiting the catalytic activity of CE-1 ^(26, 174, 175). Meanwhile, ethanol reacts with cocaine *via* catalysis by CE-1 to produce cocaethylene, which is further oxidized to norcocaethylene by CYP 3A4 ^(26, 46, 171, 172). With alcohol co-administration, ~34% (oral), ~24% (IV), or ~18% (smoked) of cocaine is converted to cocaethylene through transesterification ⁽¹⁷³⁾.

Among the cocaine metabolites described above, BE, norcocaine, cocaethylene, and norcocaethylene are all physiologically/biologically active. These active metabolites have strong physiological effects such as vasoconstrictive effects, causing the hemodynamic changes, and producing lethality ^(171, 176-180). Recently, the metabolic pathways of those toxic compounds were identified during the process of developing therapeutic treatment for cocaine abuse. Briefly, BChE can catalyze the hydrolysis of BE to ecgonine (ECG), norcocaine to norecgonine methyl ester (NEME), cocaethylene to ecgonine ethyl ester (EEE), and norcocaethylene to norecgonine ethyl ester (NEEE) ^(124, 125, 147, 181).

Disastrous medical and social consequences of cocaine make it a high priority to develop an anti-cocaine medication for treatment-seeking users ^(44, 45). There have been extensive efforts in development of therapeutic agents for cocaine abuse ^(16, 47, 48, 77, 83, 91, 92, 102, 104, 172, 182-186). Although target-based, traditional pharmacodynamics approaches using a small-molecule compound to treat cocaine have not yielded a truly effective drug ⁽⁷⁷⁾, alternative approaches using biologics (vaccines, monoclonal antibodies, and cocaine-metabolizing enzymes) have been shown some promise. Preclinical and clinical studies indicate that biologic approaches are effective in both blocking cocaine toxicity and reducing drug craving ^{(48, 83, 102, 104, 172, 182, 183) (91, 92)}. Biologics block cocaine's access to the central nervous system (CNS) or other acting sites by either binding with cocaine or hydrolyzing it ^(16, 47, 48), possibly resulting in a significant change of cocaine's metabolic profile. There are extensive ongoing studies in developing highly effective medicines and, meanwhile, investigating their effects

on the altering metabolic profiles of cocaine in the presence/absence of other substances (*e.g.* alcohol) co-administrated. Therefore, it is highly desirable to develop a thoroughly validated bio-analytical method to simultaneously determine the blood concentrations of cocaine and related compounds.

A HPLC method with a UV detector was established in our previous studies to characterize pharmacokinetic profiles of cocaine and cocaine-related compounds in rats ^(83, 124, 125). However, the UV method cannot be used to determine EME, ECG, NEME, EEE, and NEEE due to the lack of a conjugated system in these compounds. Several GC-MS and LC-MS/MS methods have been reported for simultaneous determination of cocaine and its metabolites including BE, norcocaine, cocaethylene, EME and ECG ⁽¹⁸⁷⁻¹⁹²⁾. These methods provided great separation, sensitivity and selectivity. However, none of them was developed to determine NEME, EEE, or NEEE in whole blood. We hereby present a LC-MS/MS method combined with one-step solid phase extraction, allowing simultaneous determination of cocaine and all of the nine cocaine-related compounds. The method was applied to the analysis of whole blood samples for pharmacokinetic study in rats with co-administration of cocaine and alcohol.

4.2 Materials and methods

4.2.1 Materials

Cocaine, EME, BE, ECG, norcocaine, cocaethylene, EEE, and norcocaethylene were provided by the National Institute of Drug Abuse (NIDA) Drug Supply Program (Bethesda, MD). NEME and NEEE were synthesized in this study with the purity of >99%. The isotopic corresponding internal standards (IS) including cocaine-D₃, EME-D₃, BE-D₃, ECG-D₃, and cocaethylene-D₃ were ordered from Cerilliant (Round Rock, TX). Ethanol, paraoxon, heparin, HPLC-grade methanol, and acetonitrile were purchased from Thermo Fisher Scientific (Waltham, MA). Formic acid was from Sigma-Aldrich (St. Louis, MO). Drug-free blank rat whole blood was sampled from

male Sprague–Darley rats that were ordered from Harlan (Indianapolis, IN). Mixed cation exchange model solid phase extraction cartridges (Oasis MCX 1 cc Vac Cartridge, 10 mg) were obtained from Waters (Milford, MA).

4.2.2 Liquid chromatographic and mass spectrometric conditions

A Shimadzu HPLC system (Shimadzu, Kyoto, Japan), consisting of a DGU-20A/3R degasser, LC-20AD binary pumps, CBM-20A controller, and SIL-20A/HT auto sampler, was used in this study. The chromatographic analysis was carried out on an Atlantis T3 (100Å, 3 µm, 2.1 mm X 150 mm I.D) column (Waters, Milford, MA). The mobile phase A consisted of 0.1% formic acid and mobile phase B consisted of mobile phase A: acetonitrile (10:90, v/v). The flow rate was set at 0.2 ml/min. A 5 µl injection of each sample was loaded on to the column, separated and eluted using the following gradient: 0% B at 0 min, hold 0% B for 5 min, then B increased to 20% at 6 min, 40% B at 16 min, and 90% B at 18 min, hold 90% B for 4 min, 0% B at 23 min, and re-equilibrate at 0% B for 5 min. The total run time was 28 min. The column temperature was maintained at room temperature (~21°C). The auto sampler temperature was maintained at 15°C, and the auto sampler injection needle was washed with 200 µl mobile phase A: methanol (50:50, v/v) after each sample injection to reduce the carryover.

The mass spectrometer, AB SCIEX tripleTOF™ 5600 (AB SCIEX, Redwood City, CA), was run in positive ion and high sensitivity mode under the following conditions and settings: positive ions were generated in the source using nitrogen as the source gases. Ion source gas 1 (GS1) and 2 (GS2) were set at 25 and 35 respectively, and curtain gas (CUR) was set at 40. Source gas temperature was set at 500°C. Ion spray voltage floating (ISVF) was 3000 V. Compound-specific ionization parameters, ion transition, declustering potential (DP), collision energy (CE), collision energy spread (CES), ion release delay (IRD), and ion release width (IRW) were optimized and summarized in Table 4.1. Analyst® TF 1.7 software (AB SCIEX, Redwood City, CA)

was used for instrument control and data acquisition. MultiQuant™ 3.0 software (AB SCIEX, Redwood City, CA) was used for quantitative analysis.

Table 4.1 Optimized compound-specific source parameters for electrospray ionization

Drugs	Ion transition	DP (V)	CE (eV)	CES (V)	IRD	IRW
Cocaine	304.1→182.1	100	30	3.0	45	17
EME	200.1→182.1	80	31	3.0	45	17
BE	290.1→168.1	60	28	3.0	45	17
ECG	186.1→168.1	40	27	3.0	45	17
Norcocaine	290.1→168.1	50	25	3.0	45	17
NEME	186.1→136.1	90	36	3.0	45	17
Cocaethylene	318.2→196.1	50	29	3.0	45	17
EEE	214.1→196.1	40	28	3.0	45	17
Norcocaethylene	304.2→182.1	80	24	3.0	45	17
NEEE	200.1→182.1	60	22	3.0	45	17
Cocaine-D3	307.2→185.1	50	30	3.0	45	17
EME-D3	203.1→185.1	60	30	3.0	45	17
BE-D3	293.2→171.1	60	30	3.0	45	17
ECG-D3	189.1→171.1	30	25	3.0	45	17
Cocaethylene-D3	321.2→199.2	50	28	3.0	45	17

4.2.3 Treatment of blood samples for mass spectrometry

The sample extraction method used in this study was established in our previously studied ^(147, 169, 181). Briefly, internal standard (IS) solution (0.1 µM for each IS) with a volume equal to that of the whole blood was added to each blood sample. The mixture was vortexed and then centrifuged for 15 min at 13,000 rpm; and the supernatant was collected and mixed with 500 µl 4% formic acid. Before being loaded onto solid-phase extraction column, Oasis MCX 1 cc Vac Cartridge, conditioned by 1 ml methanol followed by 1 ml water, the sample was centrifuged at 13,000 rpm for 15 min. Loaded cartridge was washed twice with 1 ml methanol, and the contents were eluted twice with 500 µl methanol/water solution (95:5, v/v, with 7.5% ammonium hydroxide). Eluate was evaporated to dryness at 25°C using a vacuum concentrator, reconstituted in

74 μ l 0.1% formic acid, and centrifuged at 13,000 rpm for 15 min. Supernatant was transferred to a vial and stored refrigerated until analysis by LC-MS/MS.

4.2.4 Preparation of stock, calibration standards and quality control samples

Combined stock solution was prepared by mixing solutions of cocaine and its metabolites with those of the corresponding deuterium-labeled IS. The final concentration is 10 μ M for each analyte, and 0.1 μ M for each IS. Combined standard solutions, prepared by diluting the combined stock solution using the IS solution (0.1 μ M for each IS), with the various concentrations for each analyte and 0.1 μ M for each corresponding IS. Calibration standards were prepared by adding 74 μ l different concentrations of combined standard solutions into 174 μ l blood mixture (74 μ l whole blood + 100 μ l paraoxon solution) from untreated Sprague-Dawley rats. Quality control samples were prepared in the same manner. The same method as described above was used to extract cocaine, nine cocaine-related compounds, and all ISs.

4.2.5 Method validation

Linearity and sensitivity. The validation was performed according to the United States (US) Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines ^(193, 194). A calibration curve was established by calculating the ratio of the peak area for analyte to that for the internal standard and plotting the ratio as a function of the ratio of the analyte concentration to the internal standard. The limit of detection (LOD) was considered the lowest concentration of analyte where the signal-noise ratio was greater than 3. The lower limit of quantification (LLQC) was defined as the lowest concentration of analyte with accuracy < 20% of the coefficient of variation (CV) and a precision within 20% of the nominal concentration.

Selectivity and carryover. Six sources of blank rat blood were individually analyzed and evaluated to determine if any interfering components were more than 20% of the LLQC for the analytes and 5% for the internal standards. Carryover was assessed

by injection of a drug-free blank sample after a high concentration sample. Carryover in the blank sample should be less than 20% of the LLOQ for the analytes and 5% for the internal standards.

Extraction recovery and matrix effect. Matrix effect and extraction recovery were determined in six replicates, at LLOQ, medium, and high concentration for all analytes. Samples were prepared in three ways as described by Liu et al. [2]: (a) prepared in the absence of blank matrix, (b) prepared by adding analyte to blank matrix extract, and (c), prepared by adding analyte to blank matrix prior to solid phase extraction. The matrix effect was calculated by dividing peak area of sample b by sample a; and the extraction recovery was calculated by dividing peak area of sample c by sample b.

Precision and accuracy. Accuracy was expressed as the percentage of the determined concentration to the nominal concentration of the analyte; and precision was expressed as % CV. Intra-day accuracy and precision of this method were assessed by analyzing in a single run of six QC samples at three levels including LLOQ, medium QC, and high QC. For inter-day accuracy and precision, LLOQ, medium QC, and high QC from three runs on three different days were evaluated.

Stability. The stabilities of cocaine and cocaine-related compounds in whole blood were evaluated using low, medium, and high QC samples (n = 6 for each level). The analyte was considered to be stable if the mean concentration at each level was within $\pm 15\%$ of the nominal concentration except for the LLOQ which should be within $\pm 20\%$ of the nominal value. The freeze and thaw stability samples were analyzed following four freeze-thaw cycles. The short-term stability samples were stored at room temperature for 4 hours, and the long-term stability samples were stored at -70°C for 4 months.

4.2.6 Animal tests and sample collection

Male Sprague–Darley rats (wt) were ordered from Harlan (Indianapolis, IN), and

housed initially as one or two rats per cage. All rats were allowed ad libitum access to food and water and maintained on a 12 h light/12 h dark cycle, with the lights on at 8:00 a.m. at a room temperature of 21–22°C. 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 animal protocol was approved by the IACUC (Institutional Animal Care and Use Committee) at the University of Kentucky. Rats (n=10) received IP injection of 1 g/kg alcohol followed by cocaine administration (60 mg/kg, IP) 30 min after the injection of alcohol. Blood samples (50–75 µl) were collected into a heparin-treated capillary tube at 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240, 300, 360, 420, 480, and 600 min after the cocaine administration, and mixed with 100 µl paraoxon solution (250 µM paraoxon, 10 U/ml heparin) immediately. Blood samples were stored at –80°C until sample extraction.

4.3 Method development

The optimization of sample preparation was performed with rat whole blood. Both perchloric acid and formic acid were tested for the sample acidification. With the treatment of perchloric acid, majority of protein in blood samples was precipitated, but the extraction recovery rates of analytes were significantly lower than those with the treatment of formic acid. Thus, the formic acid was chosen to acidify the sample. Various concentrations of formic acid with different volumes were also tested to clean up the blood samples before being loaded on the SPE column. Eventually, 4% formic acid with a volume 500 µl was selected.

A great challenge of this LC-MS/MS method is to separate three pairs of compounds: cocaine and norcocaehtylene, norcocaine and BE, and ECG and NEME. Both compounds within each pair have exactly the same m/z , and very similar physicochemical properties and ion transition. Unqualified separation within the pair makes it extremely difficult to avoid the interference from each other. To solve this problem, the liquid chromatography method was modified according to the

well-established method by Dr. Imbert ⁽¹⁹⁵⁾. The aqueous phase (phase A) of 2 mM ammonium formate, pH 3.0, was replaced by 0.1% formic acid. The gradient program was also adjusted with 0% B for the first 5 min separation, and longer holding of the initial condition before the next injection.

Although the liquid chromatography method was optimized to get the good separation, the retention time of ECG (3.27 min) is still close to that of NEME (3.04 min). In order to further avoid the interference between ECG and NEME, compound-specific source parameters for electrospray ionization were optimized to generate fragment ion of ECG mainly at m/z 168.1 and fragment ion of NEME mainly at m/z 136.1.

4.4 Method validation

Selectivity and carryover. Six blank matrix samples from different sources were analyzed to access the selectivity. There was no endogenous interference observed. The auto sampler injection needle was washed with 200 μ l mobile phase A: methanol (50:50, v/v) after each sample injection to reduce carryover. Only slight carryover effects (less than 20% of the LLQCs for the analytes and 5% for the internal standards) in the blank samples were observed. To further avoid the carryover, a 0.1% formic acid sample was always injected following the samples.

Linearity and sensitivity. LODs, LLOQs, and regression diagnostics of all analytes were reported in Table 4.2. The LODs and LLOQs for this method ranged from 0.2 to 0.9 ng/ml and 1.9 to 3.2 ng/ml, respectively, for all the substrates in whole blood samples. The calibration curve was linear over the concentration range of LLOQ to 200/300 ng/ml, with the correlation coefficients (r) > 0.99.

Table 4.2 Linearity, regression diagnostics, LODs, and LLOQs

Compound	Internal standard	Slope	Intercept	<i>r</i>	LOD (ng/ml)	LLOQ (ng/ml)
Cocaine	Cocaine-D3	0.7279	0.0319	0.9973	0.9	3.0
EME	EME-D3	0.8994	0.0352	0.9984	0.6	2.0
BE	BE-D3	0.6905	0.0562	0.9969	0.9	2.9
ECG	ECG-D3	0.6526	0.0263	0.9965	0.6	1.9
Norcocaine	Coc-D3	0.3274	0.0054	0.9974	0.3	2.9
NEME	Coc-D3	0.0816	0.0044	0.9942	0.2	1.9
Cocaethylene	Cocaethylene-D3	0.8920	0.0178	0.9986	0.3	3.2
EEE	Coc-D3	0.5001	0.0030	0.9972	0.2	2.1
Norcocaethylene	Coc-D3	0.4590	0.0061	0.9974	0.3	3.0
NEEE	Coc-D3	0.1131	0.0026	0.9966	0.2	2.0

Extraction recovery and matrix effect. Extraction recovery and matrix effect values at low, medium, and high concentrations are reported in Table 4.3. Significant ion suppression was observed for EME at low, medium, and high concentrations, with matrix effects of 23.2%, 31.2%, and 34.6% respectively. Although the similar ion suppression for ECG in the blood matrix was reported ⁽¹⁹²⁾, there was no such strong ion suppression for ECG observed in this study, with the matrix effects of ECG 67.6%, 70.2 %, and 71.8% at low, medium, and high concentrations, respectively. The matrix effects of other eight substrates ranged from 52.1% to 104.1% at low concentration, from 57.7% to 88.3% at medium concentration, and from 59% to 84.7% at high concentration. The one-step solid phase extraction yielded good recoveries for all of the ten analytes, with the extraction recovery rates ranging from 66.7% to 96.2 at low concentration, from 82.9% to 113.9% at medium concentration, and from 76.8% to 96.7 at high concentration.

Precision and accuracy. Intra- and inter-day precision and accuracy were evaluated by analyzing QC samples (n = 6) at the low, medium, and high concentrations. The accuracy and precision data are summarized in Table 4.4. The intra-day precision (%CV) was 12.5% or less, and the inter-day precision was 13.6% or less. The intra-day accuracy was within ± 13.6 , and the inter-day accuracy was within ± 15.0 . According to

the US FDA and EMA guidelines ^(193, 194), the bias values for intra- and inter-accuracy should be within 15% of the nominal values for QC samples, except for the LLOQ which should be within 20% of nominal concentrations, and the CV values for intra- and inter-precision should not be larger than 15% for QC samples, except for the LLOQ which should not exceed 20%. Therefore, the accuracy and precision values for the intra- and inter-studies of all cocaine and the nine related compounds are acceptable.

Stability. The stability testing is very important to evaluate the stability of all substrates during the sample collection and handling. The stability of analytes was evaluated at three concentrations after long-term and short-term storage, and after four freeze-thaw cycles. Results of stability testing are shown in Table 4.5. The mean concentration of each compound (cocaine or metabolite) at each level was always within 15% of the nominal concentration for all stability tests. These results indicate that all compounds were stable at room temperature for at least four hours and at -80°C for at least four months; and the QC samples of all compounds were not affected by the four freeze-thaw cycles.

Table 4.3 Matrix effect (ME) and extraction recovery at low, medium, and high concentrations in rat whole blood

Compound	Low concentration		Medium concentration		High concentration		IS	
	ME (%)	ER (%)	ME (%)	ER (%)	ME (%)	ER (%)	ME (%)	ER (%)
Cocaine	62.3 ± 8.3	78.8 ± 19.2	82.2 ± 3.8	97.1 ± 7.4	81.8 ± 3.2	77.8 ± 8.2	84.2 ± 4.9	73.9 ± 3.0
EME	23.2 ± 3.4	87 ± 8.6	31.2 ± 1.8	82.9 ± 5.6	34.6 ± 2.3	87.9 ± 3.4	23.6 ± 1.4	79.4 ± 5.6
BE	104.1 ± 7.4	96.2 ± 6.8	82.8 ± 4.5	112.5 ± 9.3	65.5 ± 2.8	87.9 ± 3.4	87.8 ± 4.8	81.4 ± 5.2
ECG	67.6 ± 6.5	95.1 ± 18.9	70.2 ± 4.7	113.9 ± 3.8	71.8 ± 3.7	90.8 ± 3.5	91.2 ± 5.6	84.2 ± 7.5
Norcocaine	82.1 ± 6.1	73.1 ± 3.1	88.3 ± 4.8	92.2 ± 8.3	83.8 ± 4.4	83.9 ± 8.9	84.2 ± 4.9	73.9 ± 3.0
NEME	52.1 ± 5.2	66.7 ± 11.3	57.7 ± 5.2	98 ± 6.9	59 ± 4.3	91.5 ± 5.0	84.2 ± 4.9	73.9 ± 3.0
Cocaethylene	58.5 ± 4.8	73.9 ± 4.9	72.3 ± 2.6	90.3 ± 2.9	75.3 ± 4.1	76.8 ± 6.3	59.7 ± 4.8	72.9 ± 4.3
EEE	75.6 ± 7.7	81.3 ± 10.2	82.6 ± 6.7	103.7 ± 8.2	84.7 ± 4.2	86.4 ± 4.5	84.2 ± 4.9	73.9 ± 3.0
Norcocaethylene	79.9 ± 10.6	69.1 ± 4.7	77.7 ± 5.7	92.8 ± 2.8	75.9 ± 4.3	76.8 ± 8.4	84.2 ± 4.9	73.9 ± 3.0
NEEE	84.3 ± 13.2	86.5 ± 11.5	81.6 ± 10.1	100.3 ± 13.8	81.8 ± 10.9	96.7 ± 9.1	84.2 ± 4.9	73.9 ± 3.0

Table 4.4 The intra- and inter-day precision and accuracy of the LC-MS/MS method used to quantify cocaine and its related compound in whole blood samples

Drug	Intra-day						Inter-day					
	L		M		H		L		M		H	
	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)
Cocaine	10.3	-11.1	7.1	4.5	4.0	0.3	10.8	-13.3	2.0	2.2	0.6	0.9
EME	8.0	-2.9	4.0	-0.4	3.5	-3.2	7.8	-8.8	3.2	3.3	2.7	-0.2
BE	11.1	-13.6	5.9	-0.7	7.1	-4.4	0.7	-14.8	1.1	0.6	1.9	-3.2
ECG	12.5	9.7	10.9	-3.7	5.2	1.2	6.9	3.5	2.9	-0.5	4.3	1.7
Norcocaine	2.5	11.8	2.9	2.3	7.9	9.9	0.9	12.8	1.1	3.4	1.6	11.5
NEME	9.8	11.9	4.2	-6.4	5.9	10.2	13.6	8.5	7.0	-11.1	2.3	12.6
Cocaethylene	4.1	-11.6	2.0	1.7	5.8	-1.1	1.9	-10.4	0.5	1.2	1.1	-2.3
EEE	8.2	5.7	3.2	1.1	3.4	5.6	7.7	-1.1	6.4	-5.9	4.3	10.1
Norcocaethylene	2.2	13.5	4.7	3.3	7.4	1.2	1.2	15.0	1.2	1.8	1.4	2.9
NEEE	5.9	12.5	3.5	-4.1	6.0	7.2	11.9	11.4	1.6	-5.8	0.6	7.8

Table 4.5 Stability of analytes in whole blood samples

Drug	Stability	Low concentration		Medium concentration		High concentration	
		CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)
Cocaine	Freeze-thaw (4 cycles)	15.0	2.8	9.8	-0.7	4.5	-4.7
	Bench top (4 hours)	12.4	2.3	6.7	0.3	4.8	-6.7
	Long-term (-70°C, 4 months)	17.7	-0.4	9.9	3.1	5.7	-3.1
EME	Freeze-thaw (4 cycles)	19.8	2.1	9.8	4.2	5.9	4.0
	Bench top (4 hours)	15.4	-9.6	4.7	5.9	6.9	0.3
	Long-term (-70°C, 4 months)	6.9	-3.1	8.6	6.3	4.6	3.5
BE	Freeze-thaw (4 cycles)	10.7	-0.9	7.2	5.5	7.9	3.4
	Bench top (4 hours)	13.6	-0.5	8.6	0.7	6.1	1.7
	Long-term (-70°C, 4 months)	9.3	-1.4	5.1	2.2	6.3	3.8
ECG	Freeze-thaw (4 cycles)	14.9	3.5	9.4	10.2	6.9	2.6
	Bench top (4 hours)	9.5	-1.5	4.8	7.6	10.4	3.6
	Long-term (-70°C, 4 months)	12.7	-4.9	9.7	10.7	6.5	3.8
Norcocaine	Freeze-thaw (4 cycles)	8.2	-1.0	11.7	-0.2	3.2	-1.2
	Bench top (4 hours)	8.4	-1.1	4.6	3.5	7.2	0.6
	Long-term (-70°C, 4 months)	3.3	-2.9	6.4	2.2	4.2	-1.4
NEME	Freeze-thaw (4 cycles)	12.4	-12.8	6.6	-5.4	9.4	0.1
	Bench top (4 hours)	11.3	-14.3	5.7	-1.5	10.6	-2.2
	Long-term (-70°C, 4 months)	10.0	-13.0	4.0	-1.7	10.8	-8.7

Table 4.5 (continued) Stability of analytes in whole blood samples

Drug	Stability	Low concentration		Medium concentration		High concentration	
		CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)
Cocaethylene	Freeze-thaw (4 cycles)	4.7	0.7	4.8	-3.7	3.2	-4.1
	Bench top (4 hours)	4.2	1.7	4.5	-2.1	7.6	0.2
	Long-term (-70°C, 4 months)	5.4	3.7	3.9	-1.8	1.4	-0.6
EEE	Freeze-thaw (4 cycles)	7.0	-3.2	9.4	-9.3	7.7	9.4
	Bench top (4 hours)	8.5	1.2	4.4	-4.4	8.3	6.8
	Long-term (-70°C, 4 months)	6.2	-2.2	8.8	-5.2	13.5	-5.2
Norcoxaethylene	Freeze-thaw (4 cycles)	5.9	0.1	7.2	-4.4	5.4	-6.4
	Bench top (4 hours)	3.2	-0.2	7.2	-0.9	4.1	-2.2
	Long-term (-70°C, 4 months)	5.1	-1.0	7.7	-1.3	3.6	2.8
NEEE	Freeze-thaw (4 cycles)	4.9	1.2	13.3	3.3	4.7	6.5
	Bench top (4 hours)	8.6	1.4	5.1	2.4	12.1	3.7
	Long-term (-70°C, 4 months)	10.1	-1.3	8.1	-0.2	9.0	-11.9

4.5 Application of the method

The validated method was used to quantify the concentrations of cocaine and the nine related compounds in blood samples collected in a pharmacokinetic study with co-administration of cocaine and alcohol to rats. The mean blood concentration time-course profiles for cocaine and its metabolites after the IP injection of 60 mg/kg cocaine (30 min after the injection of 1 g/kg alcohol) to rats are depicted in Figure 4.1. Cocaine reached its highest concentration of 2400 ng/ml 20 min after the injection of cocaine, and then slowly fell over the 600-min period. Although part of cocaine was hydrolyzed to biological inactive product EME, majority of cocaine was converted to physiologically/biologically active metabolites, including BE, norcocaine, cocaethylene, and norcocaethylene. These active metabolites were further metabolized to compounds with no or little physiological effects ^(125, 147, 196) such as ECG and EEE by the endogenous enzymes, but their concentrations fell very slowly.

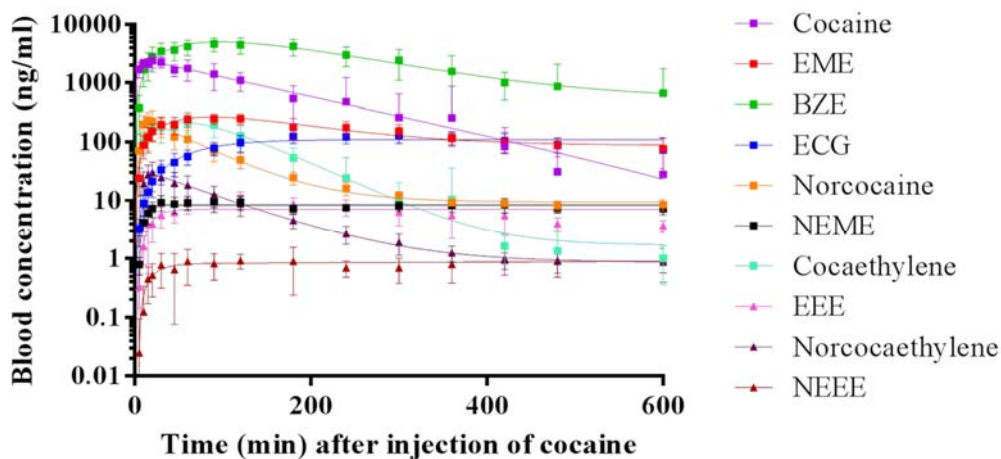


Figure 4.1 Metabolic profiles of cocaine in the presence of alcohol in rats. Rats (n=10) were injected (IP) with 1 g/kg alcohol 30 min before the IP injection of 60 mg/kg cocaine.

4.6 Potential applications of the developed method to future studies

We have successfully developed a simple and rapid LC-MS/MS method combined with one-step solid phase extraction to simultaneously determine the concentrations of cocaine, EME, BE, ECG, norcocaine, NEME, cocaethylene, EEE, norcocaethylene, and NEEE in the rat blood samples. Great specificity, sensitivity, linearity, recovery, accuracy, precision, and stability of the method have been well demonstrated. With a successful application to a pharmacokinetic study in rats with co-administration of cocaine and alcohol, this method will be valuable for further studies that aim to characterize anti-cocaine therapeutic agents and their effects on metabolic profiles of cocaine in the presence/absence of alcohol.

Chapter 5: Long-acting Cocaine Hydrolase for Addiction Therapy

Summary: In the investigation described in this chapter, we demonstrate a novel CocH form, catalytic antibody analog, which is an Fc-fused CocH dimer (CocH-Fc) constructed by using CocH to replace the Fab region of human immunoglobulin G1. The CocH-Fc has not only a high catalytic efficiency against cocaine, but also a considerably longer biological half-life (e.g. ~107 hours in rats) like an antibody. A single dose of CocH-Fc was able to accelerate cocaine metabolism in rats even after 20 days and, thus, block cocaine-induced hyperactivity and toxicity for a long period of time. In consideration of the general observation that the biological half-life of a protein drug in humans is significantly longer than that in rodents, the CocH-Fc reported in this study could allow dosing once every 2-4 weeks, or longer for cocaine addiction treatment in humans. The main results described in this chapter have been published ⁽⁴⁸⁾.

5.1 Cocaine hydrolase for cocaine addiction and its biological half-life

Preclinical and clinical data have demonstrated that these CocHs are safe for use in humans and effective for accelerating cocaine metabolism. However, the actual therapeutic use of a CocH in cocaine addiction treatment is limited by the short biological half-life (e.g. 8 hours or shorter in rats) of the CocH. While TV-1380 (Figure 5.1 B) has been proven safe and effective for use in animals and humans ^(91, 92), its actual therapeutic value for cocaine addiction treatment is still limited by the moderate biological half-life which is ~8 hr in rats ⁽⁸⁶⁾ or 43-77 hr in humans ⁽⁹¹⁾. In addition, our more recently designed and discovered CocHs ⁽¹⁰²⁻¹⁰⁴⁾ are significantly more active against (-)-cocaine compared to TV-1380. It is highly desired to further engineer a more active CocH with a biological half-life longer than TV-1380.

Our current enzyme engineering design strategy is based on the observation that an antibody (Ab), like human immunoglobulin G (IgG), has a very long biological

half-life, because the Fc region of IgG can bind with neonatal Fc receptor (FcRn) in the acidic environment of the endosome, and is later transported to the cell surface where upon exposure to a neutral pH IgG is released back to the main bloodstream ⁽¹⁹⁷⁾. In comparison, a recombinant enzyme such as BChE usually has a very short biological half-life, and an antibody usually has no catalytic activity. Using a stable analog of transition state for cocaine hydrolysis, Landry *et al.* ⁽¹⁹⁸⁾ successfully generated the first anti-cocaine catalytic antibody (CAb). Further effort generated the most active anti-cocaine CAb (Figure 5.1C), known as 15A10 ($k_{\text{cat}} = 2.3 \text{ min}^{-1}$ and $K_M = 220 \text{ }\mu\text{M}$) ^(159, 160), whose catalytic activity is still significantly lower than that of wild-type BChE against (-)-cocaine. It has been difficult to further improve the CAb activity because a CAb, unlike an enzyme, can only help to stabilize the transition state for the non-enzymatic cocaine hydrolysis; there is no covalent bond formation or breaking between the substrate and CAb during the reaction process in light of a mechanistic study ⁽¹⁵⁰⁾.

We aimed to design a long-acting CocH form which has both the long biological half-life of an antibody and the high catalytic activity of a CocH against (-)-cocaine. For this purpose, starting from human IgG1 (dimer) which has both the Fc region (constant) and Fab region (variable) as seen in Figure 5.1C, we may replace the Fab region by a CocH for each subunit of the dimeric IgG1 to construct a catalytic antibody analog. Technically, the C-terminus of CocH3 (the A199S/F227A/S287G/A328W/Y332G mutant ⁽¹⁰²⁾ of human BChE, full-length (FL) or truncated after amino acid #529 to delete the tetramerization domain) was fused with the N-terminus of the hinge region linked with Fc. CocH3 has a significantly higher catalytic efficiency ⁽¹⁰²⁾ than CocH1 or TV-1380 against (-)-cocaine. The obtained dimeric CocH3-Fc fusion enzyme depicted in Figure 5.1D is highly efficient for cocaine hydrolysis. Obviously, the CocH3-Fc is not, but structurally similar to, antibody IgG1, containing Fc which can bind with FcRn in the acidic environment to prolong the biological half-life. Further, possible mutations on the Fc region of CocH3-Fc were also examined in order for the CocH3-Fc to have

the longest possible biological half-life. Various Coch3-Fc forms including Fc mutants were proposed, prepared, and tested for their actual *in vitro* and *in vivo* profiles. In addition, Albu-Coch1 or TV-1380 was also prepared and tested for comparison. As shown below, the Coch3-Fc optimized, indeed, has not only a significantly higher catalytic activity against cocaine, but also a much longer biological half-life compared to TV-1380.

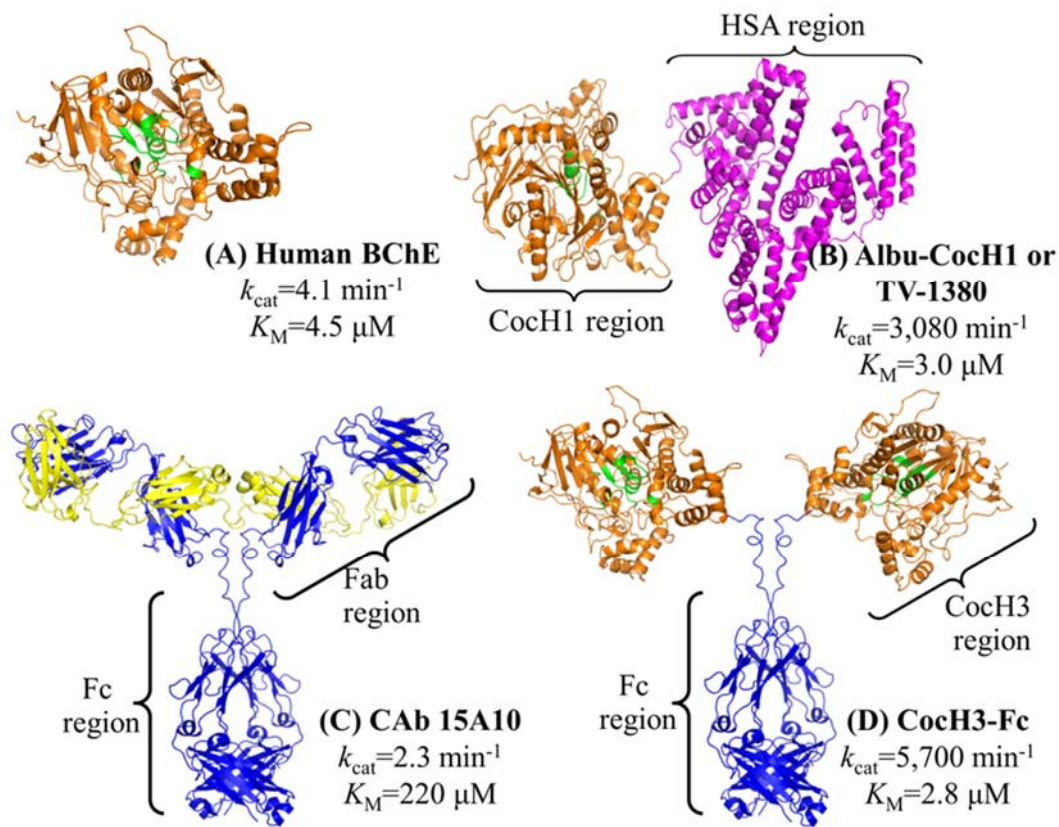


Figure 5.1 Protein structures and their catalytic parameters for cocaine hydrolysis. (A) Human BChE; (B) Albu-Coch1 (or TV-1380); (C) CAb 15A10; and (D) Coch3-Fc. This figure came from ref.⁽⁴⁸⁾.

5.2 Materials and methods

5.2.1 Materials

The cDNAs for CocH1 (the A199S/S287G/A328W/Y332G mutant of human BChE) and CocH3 (the A199S/F227A/S287G/A328W/Y332G mutant of human BChE) containing N-terminal signal were generated in our previous studies ^(89, 102). pFUSE-hIgG1-Fc2 plasmid was purchased from InvivoGen (San Diego, CA). The cDNA (Clone ID: HsCD00005810) for human serum albumin (HSA) was obtained from DF/HCC DNA Resource Core (Boston, MA). Protein expression vector pCMV-MCS was ordered from Agilent (Santa Clara, CA), and pCSC-SP-PW lentiviral vector (plasmid 12335), pMDLg/pRRE (plasmid 12251), pRSV-Rev (plasmid 12253), and pCMV-VSV-G (plasmids 8454) were obtained from Addgene (Cambridge, MA). Phusion DNA polymerase, restriction endonucleases, T4 DNA ligase were ordered from New England Biolabs (Ipswich, MA). DpnI endonuclease was obtained from Thermo Fisher Scientific (Waltham, MA). All oligonucleotides were synthesized by Eurofins MWG Operon (Huntsville, AL). Chinese Hamster Ovary-suspension (CHO-S) cells, Human Embryonic Kidney (HEK)-293FT, FreeStyle™ CHO Expression Medium, HT Supplement, L-glutamine, Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), 4-12% Tris-Glycine Mini Protein Gel, and SimpleBlue SafeStain were from Invitrogen (Grand Island, NY). TransIT-PRO® Transfection Kit was obtained from Mirus (Madison, WI). The rmp Protein A Sepharose Fast Flow was purchased from GE Healthcare Life Sciences (Pittsburgh, PA). Centrifugal Filter Units were from Millipore (Billerica, MA). (-)-Cocaine was provided by the National Institute on Drug Abuse (NIDA) Drug Supply Program (Bethesda, MD); and [³H](-)-Cocaine (50 Ci/mmol) was ordered from PerkinElmer (Waltham, Massachusetts).

Sprague-Darley rats (200–250 g) and CD-1 mice (25–30 g) were ordered from Harlan (Harlan, Indianapolis, IN). All other chemicals were purchased from Thermo

Fisher Scientific (Waltham, MA) or Sigma-Aldrich (St. Louis, MO).

5.2.2 Expression plasmids construction

With specific base-pair alterations, site-directed mutagenesis was performed to obtain the cDNAs for Fc(WT) (*i.e.* wild-type Fc), Fc(M1) (*i.e.* the A1Q/C6S/C12S/C15S/P24S mutant Fc), Fc(M2) (*i.e.* the A1V/E58Q/E69Q/E80Q/D98N/N101D/D142E/L144M mutant Fc), and Fc(M3) of human IgG1 using pFUSE-hIgG1-Fc2 plasmid as the template ⁽¹⁹⁹⁾. Five expression plasmids, pCMV-CocH3-Fc(WT), pCMV-CocH3-Fc(M1), pCMV-CocH3-Fc(M2), pCMV-CocH3-Fc(M3), and pCMV-CocH3(FL)-Fc(M3), were constructed. Each plasmid contains a sequence encoding native BChE signal peptide followed by CocH3 (truncated or full length) linked to the N-terminal of Fc (WT or mutant). To fuse CocH3 to Fc, overlap extension polymerase chain reaction (PCR) with Phusion DNA polymerase was used. Primers (4 primers for each construction) used for plasmid construction were listed in Table 5.1. Primers 1 and 2 were used to amplify CocH3 with N-terminal signal peptide using pRc/CMV-CocH3 as template; primers 3 and 4 were used to amplify Fc using corresponding Fc cDNA as template. Once two DNA fragments were obtained, they were fused together by using another PCR reaction with primers for the far ends (primers 1 and 4). Then the PCR product was digested with restrict endonucleases, Hind III and Bgl II, and was ligated to pCMV-MCS expression vector using T4 DNA ligase. The same method was used to construct plasmids pCSC-CocH3-Fc(M3) and pCSC-Albu-CocH1 for large-scale protein production as well.

Table 5.1 Primers for plasmids construction. This table came from ref. (48).

Construction	Primer name	Primer sequence
pCMV-CocH3(FL)-Fc(M3)	Primer 1	CCCAAGCTTGGGAAGCCACCATGGATAGC AAAGTCACAAT
	Primer 2	GCAGGACTTAGGCTCCACGAGACCCACAC AACTTTCTTTCTTGCTAG
	Primer 3	TCTCGTGGAGCCTAAGTCCTGCGACAAAA CTCACACATGCCACCGTGCC
	Primer 4	GGAAGATCTTCATTTACCCGGAGACAGGG AGAG
pCMV-CocH3-Fc(WT) pCMV-CocH3-Fc(M1) pCMV-CocH3-Fc(M2) pCMV-CocH3-Fc(M3)	Primer 1	CCCAAGCTTGGGAAGCCACCATGGATAGC AAAGTCACAAT
	Primer 2	GCAGGACTTAGGCTCCACGACTTTTGAA AAAATGATGTCCAGAAT
	Primer 3	AGTCGTGGAGCCTAAGTCCTGCGACAAA ACTCACACATGCCACCGTGCC
	Primer 4	GGAAGATCTTCATTTACCCGGAGACAGGG AGAG
pCSC-CocH3-Fc(M3)	Primer 1	CCCGGATCCGGGAAGCCACCATGGATAGC AAAGTCACAAT
	Primer 2	ACTCTTGTGTGCATCGACTTTTGAAAA ATGATGTCCAGAA
	Primer 3	CATTTTTTCCAAAAGTCGATGCACACAAG AGTGAGGTTGCTC
	Primer 4	GGAGGGCCCTCATTTACCCGGAGACAGGG AGAG
pCSC-Albu-CocH1	Primer 1	CCCGGATCCGGGAAGCCACCATGGATAGC AAAGTCACAAT
	Primer 2	ACTCTTGTGTGCATCGACTTTTGAAAA ATGATGTCCAGAA
	Primer 3	CATTTTTTCCAAAAGTCGATGCACACAAG AGTGAGGTTGCTC
	Primer 4	GGAAGATCTTCATTTACCCGGAGACAGGG AGAG

5.2.3 Protein expression and purification in small-scale

CHO-S cells, incubated at 37°C in a humidified atmosphere containing 8% CO₂, were transfected with plasmids encoding the proteins using TransIT-PRO® Transfection Kit once cells had grown to a density of 1.0×10^6 cells/ml. The culture medium (FreeStyle™ CHO Expression Medium with 8 mM L-glutamine) was harvested 7 days after transfection. Secreted enzyme in the culture medium was purified by using protein A affinity chromatography. Pre-equilibrated rmp Protein A Sepharose Fast Flow was added into cell-free medium, and incubated overnight with occasional stirring; then packed the suspension in a column. After washing the column with 20 mM Tris-HCl, pH 7.4 until OD₂₈₀ < 0.02, protein was eluted by pH and salt concentration adjustment. The eluate was dialyzed in storage buffer (50 mM HPEPS, 20% Sorbitol, 1 M Glycine, pH 7.4) by Millipore Centrifugal Filter Units. This whole purification process was carried out in cold room at 6°C. The purified protein was stored at -80°C before enzyme activity tests and *in vivo* studies.

5.2.4 Protein expression and purification in large-scale

A lentivirus-based method described in our previous report⁽⁸³⁾ was performed to generate a high-efficient stable cell line expressing Coch3-Fc(M3). Briefly, to package the lentivirus particles carrying the gene of Coch3-Fc(M3), lentivirus was produced by co-transfecting pCSC-Coch3-Fc(M3) plasmid with the packaging vectors (pMDLg/pRRE and pRSV-Rev) and envelope plasmid (pCMV-VSV-G) into HEK-293FT cells by lipofection. The packaged lentivirus particles were transfected to CHO-S cells. After lentiviral transductions, infected cells were allowed to recover from the infection for 2 or more days, and transferred to a shake flask for further culture. The obtained stable CHO-S cell pool was kept frozen before being used for large-scale protein production.

The large-scale protein production was performed in an agitated bioreactor BioFlo/CelliGen 115 (Eppendorf, Hauppauge, NY). Before being transferred into the

bioreactor, cells grew at 37°C in shake flasks till to designated volume and density. On the day of transferring, cells in shake flasks were centrifuged at 1500 rpm for 5 minutes at room temperature, resuspended in fresh culture medium, and transferred into the bioreactor. CO₂/air gas overlay was set such that the pH of cell culture medium was maintained at 7.0-8.0. The bioreactor was run in a batch model with a temperature of 32°C. After 9 days, the culture medium was harvested and the protein was purified. Coch3-Fc(M3) was purified by using the aforementioned protein A affinity chromatography which was performed on ÄKTA avant 150 system (GE Healthcare Life Sciences, Pittsburgh, PA). The purified protein was dialyzed in storage buffer, and stored at -80°C.

5.2.5 Electrophoresis

Purified Coch3-Fc(M3) was analyzed by SDS-PAGE electrophoresis. Two protein samples, protein in its native state and protein in a denatured state, were loaded in a 4-12% Tris-Glycine Mini Protein Gel. In order to break up quaternary protein structure, protein sample was mixed with SDS-loading buffer, heated at 95°C for 10 minutes in the presence of a reducing agent, dithiothreitol (DTT). Electrophoresis was run at 100 volt for 2 hours. The protein gel was stained with SimpleBlue SafeStain.

5.2.6 *In vitro* activity test against (-)-cocaine

A radiometric assay which was used in our previous studies^(102, 104) was used to test catalytic activities of enzymes against (-)-cocaine. Briefly, 150 µl enzyme solution (100 mM phosphate buffer, pH 7.4) was added to 50 µl varying concentrations of [³H]-labeled (-)-cocaine, denoted as [³H](-)-cocaine (with ³H labeled on the benzene ring). The reaction was stopped by adding 200 µl 0.1 M HCl, which neutralized the liberated benzoic acid while ensuring a positive charge on the residual (-)-cocaine. [³H]Benzoic acid was extracted by 1 ml of toluene and measured by scintillation counting. All measurements were performed at 25°C. Substrate

concentration-dependent data were analyzed using standard Michaelis-Menten kinetics so that the catalytic parameters (k_{cat} and K_M) were determined.

5.2.7 Determination of biological half-lives in rats

Rats were used with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. The experimental protocols were approved by the IACUC (Institutional Animal Care and Use Committee) at University of Kentucky. Rats were injected with purified protein *via* tail vein (0.06, 0.2 or 0.6 mg/kg for Coch3-Fc(M3); 0.06 or 5 mg/kg for Albu-Coch1; and 0.06 mg/kg for all other Coch3-Fc protein forms). Blood samples were taken from saphenous vein puncture using a needle. Approximately 50-100 μl blood was collected into a heparin-treated capillary tube at various time points after enzyme administration. Collected blood samples were centrifuged for 15 min at a speed of 5000 g to separate the plasma, which was kept at 4°C before analysis. Radiometric assay using 100 μM (–)-cocaine was used to measure the enzyme concentration in plasma. The time-dependent enzyme concentrations ($[E]_t$) were fitted to a well-known double-exponential equation⁽²⁰⁰⁾ by GraphPad Prism 5: ($[E]_t = Ae^{-k_1t} + Be^{-k_2t}$) 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 ($t_{1/2}$) associated with the enzyme elimination rate constant k_2 is known as the elimination half-life or biological half-life.

5.2.8 Characterization of cocaine clearance accelerated by Coch3-Fc(M3)

Four rats received saline (IV) before injection of (–)-cocaine (5 mg/kg, IV), and other four rats received enzyme Coch3-Fc(M3) (0.2 mg/kg, IV) on Day 0, followed by (–)-cocaine administration (5 mg/kg, IV) on Days 8, 11, 14, and 20 after the Coch3-Fc(M3) injection. Blood samples (50-100 μl) were collected from saphenous vein into a heparin-treated capillary tube at 2, 5, 10, 15, 30, and 60 minutes after the

(-)-cocaine administration, and mixed with 100 μ l of 25 μ M paraoxon (which inactivates the enzymes) immediately. Blood samples were stored at -80°C until analysis by using an HPLC. To assay the (-)-cocaine and benzoic acid (the product of cocaine hydrolysis by the enzyme) concentrations in the blood samples, frozen blood samples were thawed on ice for more than 3 hours. Then 150 μ l mobile phase (76% 0.1% trifluoroacetic acid and 24% acetonitrile) and 50 μ l 7% HClO_4 were added to each sample. The extraction mixture was vortex mixed for 1 minute, then centrifuged at 4°C for 15 minutes at 13,200 rpm. The supernatant was decanted into a 1.5 mL tube, labeled and stored at -80°C until analysis by HPLC. The chromatographic analysis was carried out on a Gemini[®] 5 μ m C18 column (Phenomenex, Torrance, CA), using a mobile phase at a flow rate of 1 ml/min. (-)-cocaine fluorescence was monitored at 315 nm while exciting at 230 nm^(83, 201), and benzoic acid absorbance was monitored at 230 nm. Chromatographic system used is composed a Waters 1525 binary HPLC pump, a 717 plus autosampler, a 2487 dual λ absorbance detector, and a 2475 multi λ fluorescence detector (Waters, Milford, MA).

5.2.9 Locomotor activity assay

The effects of Coch3-Fc(M3) on cocaine-induced hyperactivity was evaluated by using a video-tracking system at the University of Kentucky's Rodent Behavior Core. Mice received a single dose of Coch3-Fc(M3) (0.2 or 2 mg/kg, IV) or saline, followed by multiple doses of cocaine (15 mg/kg, IV) or saline after 1 hour, 1, 3, 5, 7, 9, 11, 13 and 15 days. Before cocaine or saline administration, mice were allowed to acclimate to the test chambers for 1 h. The total distance traveled during the last half hour, which was collected in 5-min bins, was used as the basal level. After cocaine or saline administration, mice were immediately returned to the test chamber for the remaining 1 hour of the session for activity monitoring. The locomotor tests were performed in high density, non-porous plastic chambers measuring 50 cm (L) \times 50 cm (W) \times 38 cm (H) in a light- and sound-attenuating behavioral test enclosure (San Diego Instruments, San

Diego, CA). Cumulative distance traveled was recorded by EthoVision XT video tracking system (Noldus Information Technology, Wageningen, Netherlands) to represent the locomotor activity.

5.2.10 Protection study in mice

Cocaine-induced acute toxicity was characterized in this study by the occurrence of convulsions. Cocaine-induced convulsion was defined as loss of righting posture for at least 5 seconds with the simultaneous presence of clonic limb movements ⁽²⁰²⁾. Mice received a single dose of Coch3-Fc(M3) (2 mg/kg, IV) or saline (IV) on Day 0, followed by daily dosing of cocaine (100 mg/kg, IP) starting on Day 4 until the occurrence of convulsion for a given mouse. Following cocaine administration, mice were immediately placed in containers for observation. The presence or absence of a convulsion was recorded for 60 min following cocaine administration ⁽¹⁰²⁾.

5.3 Optimization of the Coch3-Fc entity

Key *in vivo* data obtained are depicted in Figures 5.2, 5.4, 5.7, 5.8, 5.9, 5.10, and 5.11. All pharmacokinetic (PK) data depicted in Figures 5.2, 5.4, 5.7, and 5.8 were based on intravenous (IV) injection of the enzymes in rats. The PK data reveal that a Coch3-Fc form with the truncated Coch3 (without the tetramerization domain) has a much longer biological half-life (with $t_{1/2}$ up to ~107 hours) compared to that ($t_{1/2} = \sim 18$ hours) of the corresponding Coch3-Fc containing the full-length Coch3, denoted as Coch3(FL) in Figure 5.2, with a given Fc region. Notably, the only difference between Coch3(FL) and Coch3 is that Coch3(FL) has the extra 45 amino-acid residues (#530 to #574, an α -helix) ⁽²⁰³⁾ on the C-terminus. Compared to the Coch3-Fc structure, Coch3(FL)-Fc has an extra 45 amino-acid residues between Coch3 and Fc. These 45 residues increase the length of the linker between Coch3 and Fc. Due to the existence of the longer linker, the Coch3 region is far away from the Fc region in Coch3(FL)-Fc as shown in Figure 5.3. The longer linker may be proteolyzed more easily because it is

flexible and, thus, Coch3(FL)-Fc has a shorter biological half-life than Coch3-Fc. The Coch3-Fc forms to be discussed below will be only those with the same Coch3 region, *i.e.* the truncated Coch3.

To optimize the Fc region of the Coch3-Fc, we tested the use of wild-type Fc, denoted as Fc(WT), and a triple mutant (*i.e.* A1V/D142E/L144M) denoted as Fc(M3). In addition, we also tested the use of known Fc mutants, denoted as Fc(M1) ⁽²⁰⁴⁾ and Fc(M2) ⁽²⁰⁵⁾ here for convenience (see Methods section for the detail), that have been used to significantly prolong the half-lives of other types of therapeutic proteins (abatacept ⁽²⁰⁴⁾ and alefacept ⁽²⁰⁵⁾). As seen in Figure 5.4, Coch3-Fc(M3) showed the longest biological half-life ($t_{1/2} = \sim 107$ hours) in rats within the Coch3-Fc forms examined. The A1V/D142E/L144M mutations on the Fc region prolonged the biological half-life of the Coch3-Fc by ~ 21 hours. The other two Coch3-Fc forms, *i.e.* Coch3-Fc(M1) and Coch3-Fc(M2), as the Fc region actually shortened the biological half-life of Coch3-Fc compared to Fc(WT), although Fc(M1) and Fc(M2) successfully prolonged the biological half-lives of other fusion proteins ^(204, 205) compared to Fc(WT).

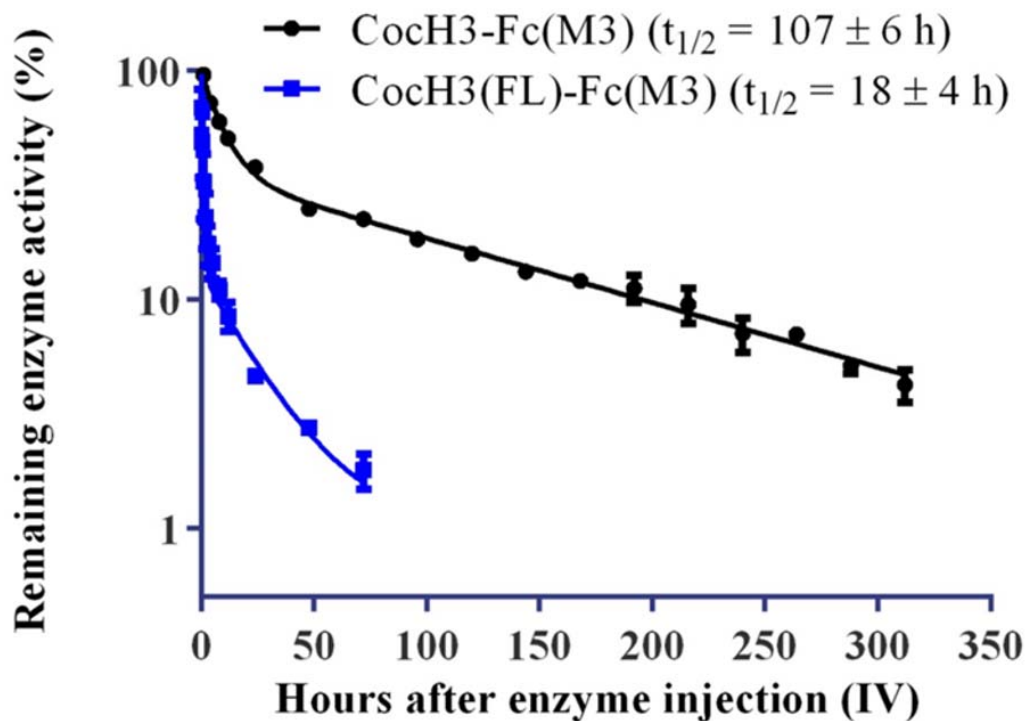


Figure 5.2 Time-dependent normalized CocH activity of various CocH3-Fc forms. For convenience, CocH3(FL) represents the full-length (FL) CocH3, whereas CocH3 (without indicating “FL”) refers to the truncated CocH3 (amino-acid residues 1-529). Rats ($n=3$ for each dose condition) were injected (IV) with the enzyme (CocH3(FL)-Fc(M3) or CocH3-Fc(M3)) at a dose of 0.06 mg/kg body weight. Blood samples were collected at various time points, and the separated plasma samples were analyzed by using a sensitive radiometric assay using [^3H]($-$)-cocaine. This figure came from ref.⁽⁴⁸⁾.

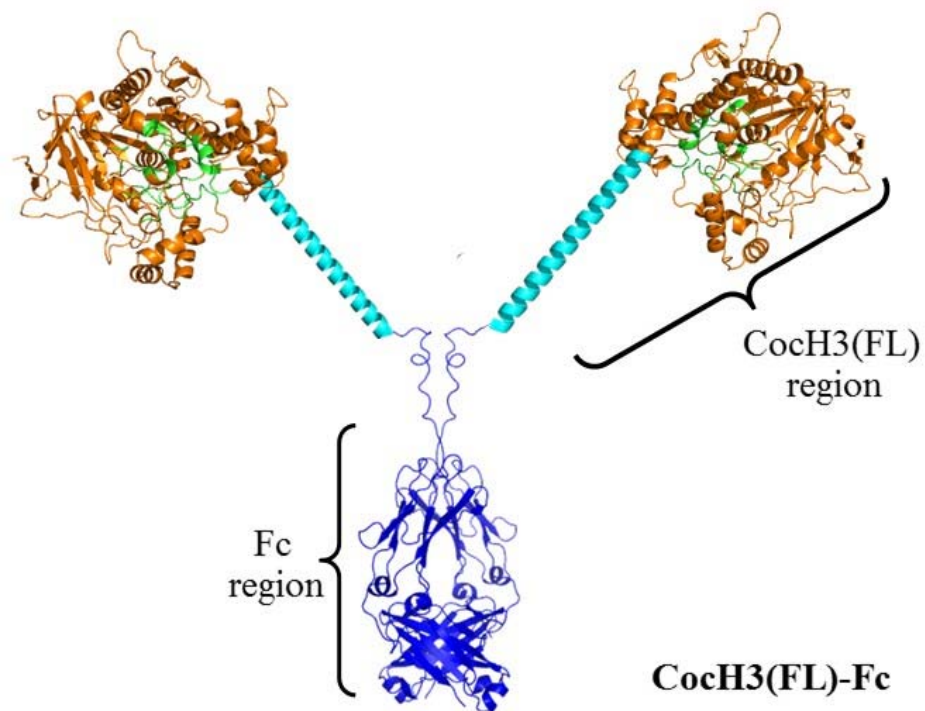


Figure 5.3 Molecular structure of Coch3(FL)-Fc. Compared to the Coch3-Fc structure depicted in Figure 5.1D, the only difference is that Coch3(FL)-Fc shown in this figure has extra 45 amino-acid residues (forming an α -helix) existing between the Coch3 and Fc regions. Here, Coch3 refers to the truncated Coch3 consisting of the first 529 amino-acid residues the enzyme, whereas Coch3(FL) represents the full-length enzyme consisting of 574 amino-acid residues. This figure came from ref.⁽⁴⁸⁾.

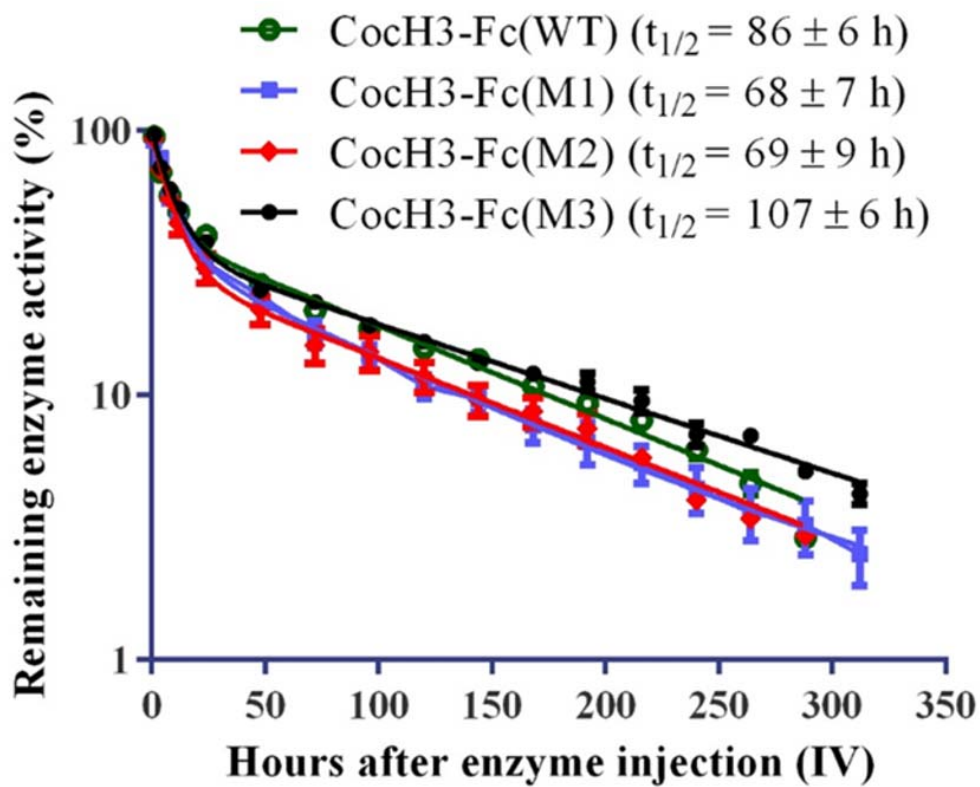


Figure 5.4 Time-dependent normalized CocH activity of various CocH3-Fc forms. Rats ($n=3$ for each dose condition) were injected (IV) with the enzyme at a dose of 0.06 mg/kg body weight. Blood samples were collected at various time points, and the separated plasma samples were analyzed by using a sensitive radiometric assay using [^3H]($-$)-cocaine. This figure came from ref.⁽⁴⁸⁾.

5.4 Dose dependence of the enzyme activity in comparison with TV-1380

To further characterize the most promising Coch3-Fc form, Coch3-Fc(M3), we developed a stable cell line using a lentiviral vector ⁽⁸³⁾ to produce Coch3-Fc(M3) in large-scale. The purified Coch3-Fc(M3) is indeed a dimer as expected (see Figure 5.5). The *in vitro* kinetic parameters of the fused CochHs are essentially the same as the corresponding unfused CochHs (see Figure 5.1B and D and Figure 5.6 for the determined k_{cat} and K_M values).

As seen in Figure 5.7, the biological half-life of Coch3-Fc(M3) (injected IV) in rats is independent of the dose and, therefore, the Coch3-Fc(M3) concentration at a given time point is linearly proportional to the dose. Depicted in Figure 5.9 are the time-dependent maximum Coch activity against cocaine: $V_{max} \equiv k_{cat}[E]$ where $[E]$ represents the concentration of the enzyme (Coch3-Fc(M3) or Albu-CochH1) in rat plasma. $V_{max} = 1$ U/L means that the enzyme is capable of hydrolyzing up to 1 μ mole cocaine in 1 liter of plasma per minute (*i.e.* 1 μ M min^{-1}). The data in Figure 5.8 indicate that Coch3-Fc(M3) is far superior to Albu-CochH1 (TV-1380). In particular, after injection of 5 mg/kg Albu-CochH1 (TV-1380), the rat plasma had $V_{max} \geq 50$ U/L within ~ 48 hours. In comparison, $V_{max} \geq 50$ U/L within ~ 100 hours after 0.2 mg/kg Coch3-Fc(M3) injection and ~ 275 hours after 0.6 mg/kg Coch3-Fc(M3) injection. Even a tiny dose (0.06 mg/kg) of Coch3-Fc(M3) produced a larger V_{max} value in the plasma compared to that produced by 5 mg/kg Albu-CochH1 (TV-1380) starting at ~ 80 hours after the Coch (Coch3-Fc(M3) or Albu-CochH1) injection; 0.2 mg/kg Coch3-Fc(M3) generated a larger V_{max} value in the plasma compared to that generated by 5 mg/kg Albu-CochH1 (TV-1380) starting at ~ 38 hours after the Coch injection; and 0.6 mg/kg Coch3-Fc(M3) led to a larger V_{max} value in the plasma compared to that generated by 5 mg/kg Albu-CochH1 (TV-1380) starting at ~ 18 hours after the Coch injection.

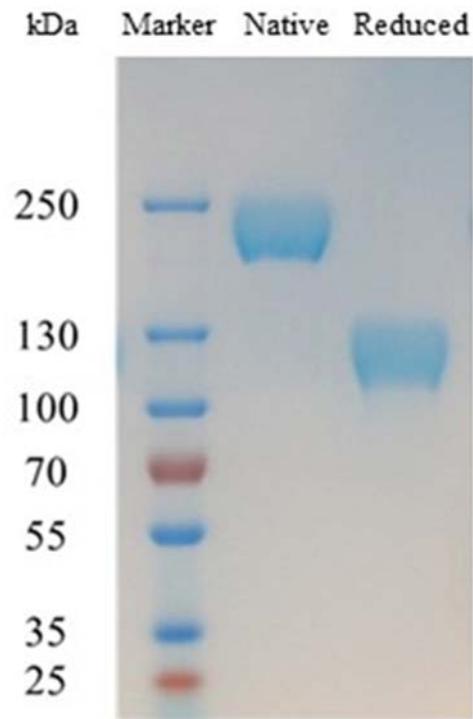


Figure 5.5 Coomassie-Blue stained SDS electrophoresis gel of the purified CocH3-Fc(M3) (Marker – protein ladder; Native – native state; Reduced: under reducing conditions), supporting that the native structure of the purified CocH3-Fc(M3) was a dimer. This figure came from ref.⁽⁴⁸⁾.

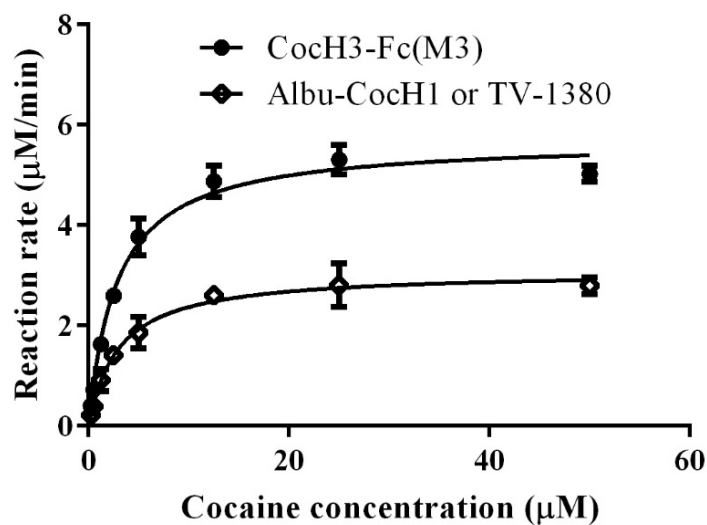


Figure 5.6 Kinetic data obtained in vitro for (-)-cocaine hydrolysis catalyzed by CocH3-Fc(M3) and Albu-CocH1 (TV-1380). All measurements were in triplicate (n=3). The reaction rate is represented in $\mu\text{M min}^{-1}$ per nM enzyme. Based on the Michaelis-Menten analysis, $k_{\text{cat}} = 5,700 \text{ min}^{-1}$ and $K_M = 2.8 \mu\text{M}$ for CocH3-Fc(M3) against (-)-cocaine, and $k_{\text{cat}} = 3,080 \text{ min}^{-1}$ and $K_M = 3.0 \mu\text{M}$ for Albu-CocH1 against (-)-cocaine. This figure came from ref.⁽⁴⁸⁾.

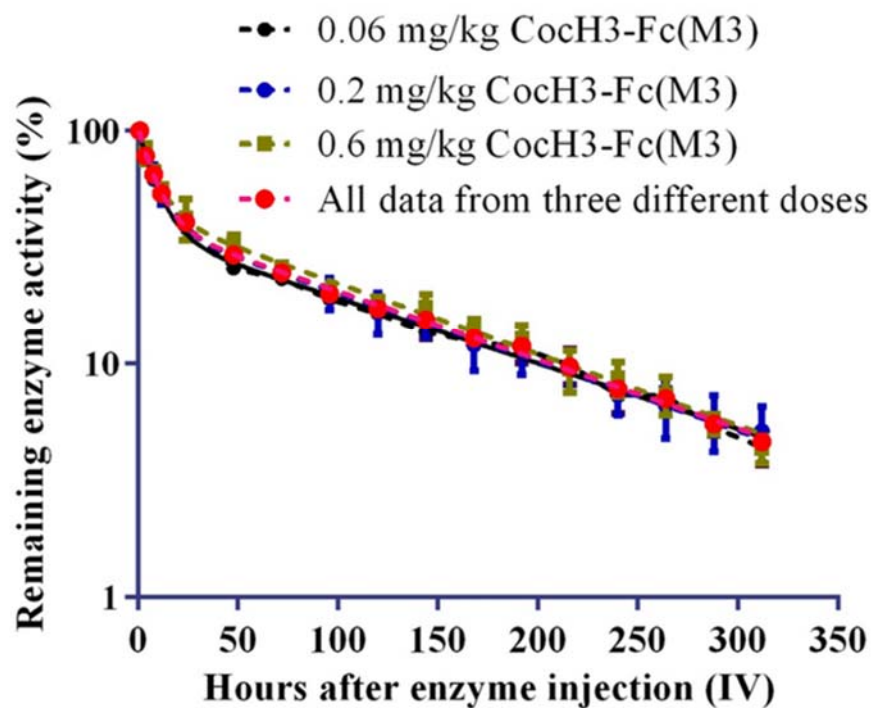


Figure 5.7 Time-dependent normalized CocH activity of CocH3-Fc(M3). Rats (n=3 for each dose condition) were injected (IV) with the enzyme at a dose of 0.06, 0.2, and 0.6 mg/kg body weight. Blood samples were collected at various time points, and the separated plasma samples were analyzed by using a sensitive radiometric assay using [³H](–)-cocaine. This figure came from ref.⁽⁴⁸⁾.

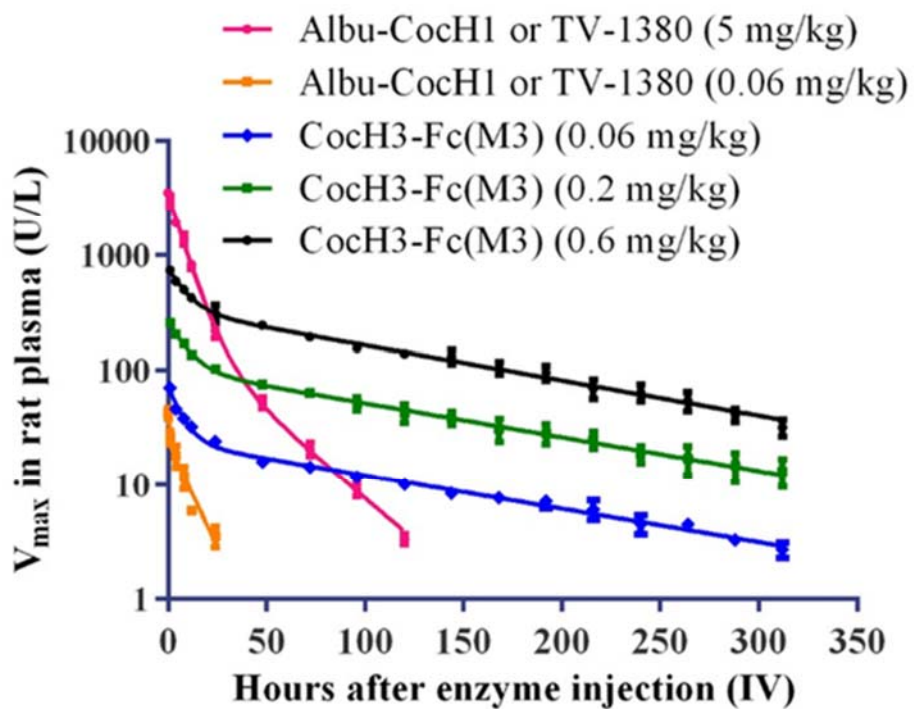


Figure 5.8 Time-dependent normalized CocH activity or V_{max} (U/L) of CocH3-Fc(M3) and Albu-CocH1 (or TV-1380). Rats (n=3 for each dose condition) were injected (IV) with the enzyme (CocH3-Fc(M3) or Albu-CocH1) at a dose of 0.06 mg/kg body weight unless indicated explicitly otherwise. Blood samples were collected at various time points, and the separated plasma samples were analyzed by using a sensitive radiometric assay using [³H](-)-cocaine. This figure came from ref.⁽⁴⁸⁾.

5.5 Cocaine clearance accelerated by CochH3-Fc(M3)

In a further *in vivo* test, rats were injected with a single dose of CochH3-Fc(M3) (0.2 mg/kg, IV on Day 0), followed by IV injection of cocaine (5 mg/kg) on Days 8, 11, 14, and 20. After each cocaine injection, blood samples were collected at 2, 5, 10, 15, 30, and 60 min and analyzed for the concentrations of cocaine and benzoic acid (cocaine metabolite). The control curves in Figure 5.9 reflect the overall effects of all possible cocaine elimination pathways^(83, 124, 206). 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 was ~0.2 μM . In the presence of CochH3-Fc(M3) on Day 8 after CochH3-Fc(M3) injection, the average concentration of cocaine at ~2 min in the blood sample was below 1 μM (~0.9 μM , see Figure 5.9A), while the average concentration of benzoic acid at the first time point (2 min) was ~11 μM (Figure 5.9B). So, CochH3-Fc(M3) hydrolyzed nearly all of the cocaine molecules within ~2 min after the cocaine injection on Day 8. According to the data in Figure 5.9B, the overall enzyme activity in rats gradually decreased from Day 8 to Days 11, 14, and 20 as expected. But the CochH3-Fc(M3) activity in rats at even such a low dose (0.2 mg/kg) was still significant after 20 days.

It should be pointed out that CochH3-Fc(M3) is constructed from human protein sequences with mutations on only a few residues. As the human protein sequences associated with CochH3-Fc(M3) are different from the corresponding rodent protein sequences, we did not try to examine the potential antigenicity/immunogenicity of CochH3-Fc(M3) in animals in this study. However, according to clinical data reported for TV-1380⁽⁹¹⁾, one can reasonably assume that CochH3-Fc(M3) would induce none, a weak, or merely a transient immune response in humans. The fact that the CochH3-Fc(M3) was effective in these animals for 20 days suggests that this assumption might be true.

5.6 Effectiveness of CocH3-Fc(M3) in blocking the physiological effects of cocaine

In animal behavior studies, mice were injected (IV) with a single dose (2 or 0.2 or 0 mg/kg) of CocH3-Fc(M3) on Day 0, followed by multiple sessions of intraperitoneal (IP) injection of cocaine (15 mg/kg) and hyperactivity (measured by increased horizontal distance traveled) recording after 1 hour and 24 hours (Day 1) and then every other day (Days 3, 5, 7, 9, 11, 13, and 15). For each session, the hyperactivity recording started 30 min prior to cocaine injection. Data recorded for these sessions are depicted in Figure 5.10A to H. The data show that CocH3-Fc(M3) completely blocked cocaine-induced hyperactivity one day (24 hours) after the 0.2 mg/kg CocH3-Fc(M3) injection, and still significantly attenuated cocaine-induced hyperactivity even after 11 days. At a dose of 2 mg/kg, CocH3-Fc(M3) completely blocked cocaine-induced hyperactivity within 9 days, and still significantly attenuated cocaine-induced hyperactivity even after 15 days.

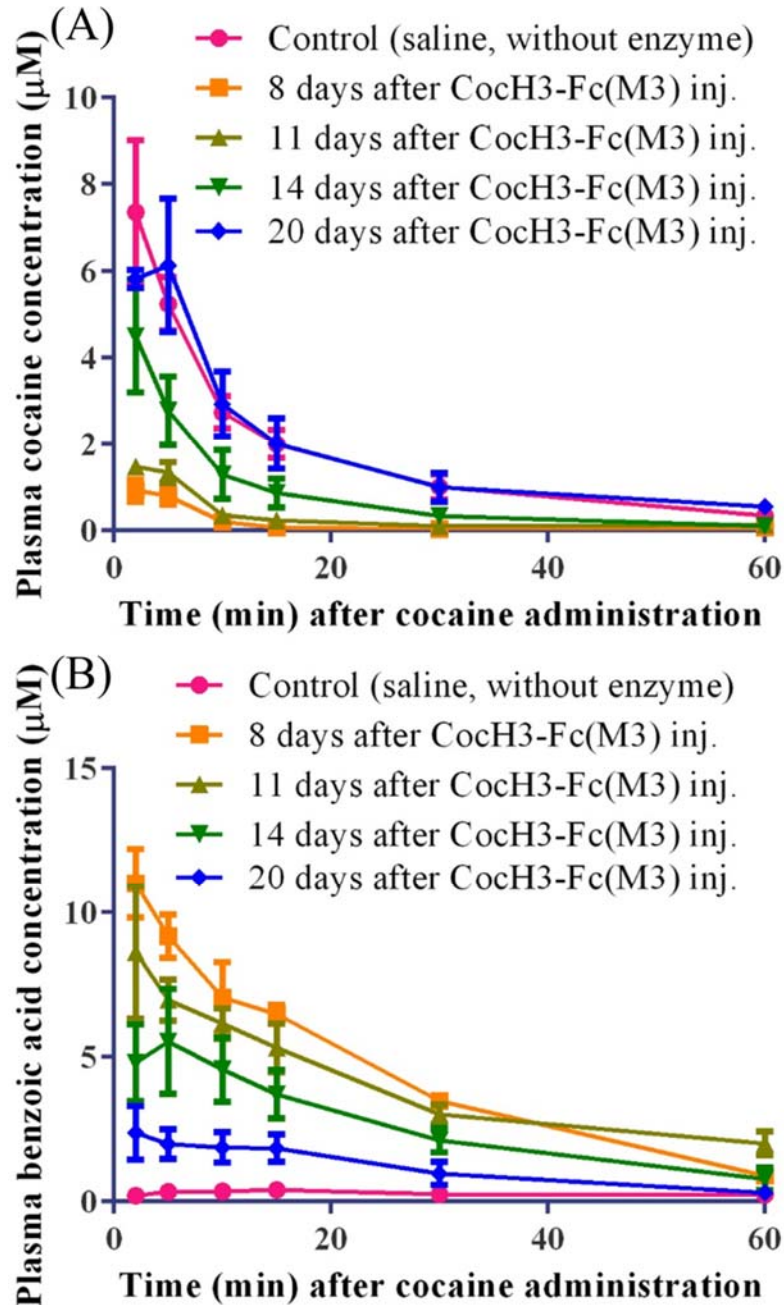


Figure 5.9 Cocaine clearance accelerated by CochH3-Fc(M3). Saline or 0.2 mg/kg CochH3-Fc(M3) was injected IV in rats (n=4), followed by IV injection of 5 mg/kg cocaine after 8, 11, 14, and 20 days. After each IV injection of 5 mg/kg cocaine, blood samples were collected at 2, 5, 10, 15, 30, and 60 min, and analyzed for the concentrations of (A) cocaine and (B) benzoic acid (a metabolite of cocaine). This figure came from ref.⁽⁴⁸⁾.

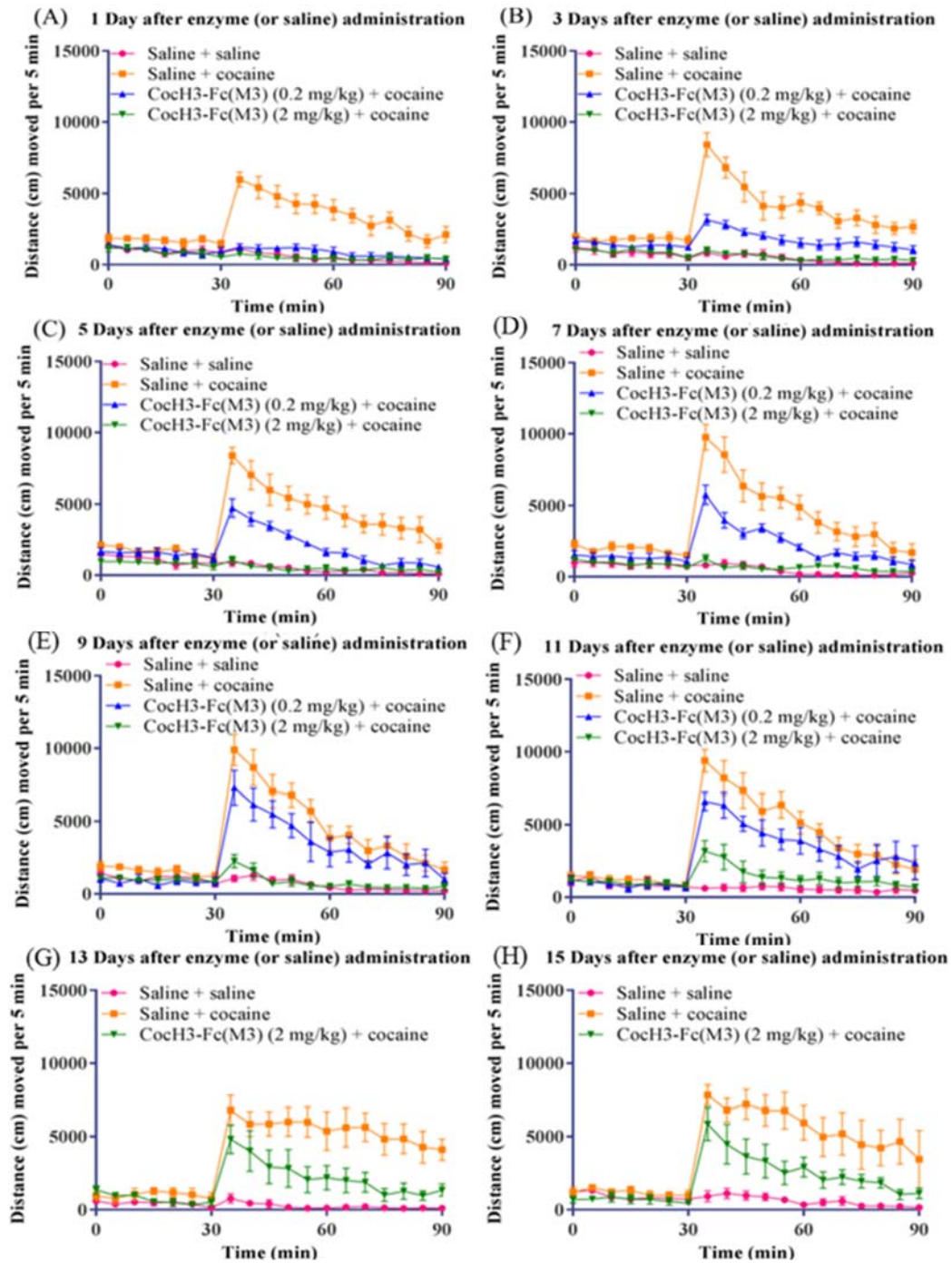


Figure 5.10 Effects of CocH3-Fc(M3) on cocaine-induced hyperactivity in mice. Hyperactivity test (n=8 for each dose condition): Saline or CocH3-Fc(M3) (enzyme) was injected IV, followed by multiple sessions of locomotor activity tests with IP injection of 15 mg/kg cocaine or saline after 1 hour and 24 hours (one day) and then every other day. Panels (A) to (H) respectively refer to the locomotor activity sessions on Days 1, 3, 5, 7, 9, 11, 13, and 15 after the CocH3-Fc(M3) injection. For each

locomotor activity session, the locomotor activity recording started at 30 min prior to the cocaine injection. This figure came from ref.⁽⁴⁸⁾.

5.7 Effectiveness of CocH3-Fc(M3) in blocking the lethal effects of cocaine

It is well-known that cocaine is lethal when the dose is high, with LD₅₀ (the lethal dose which causes the death of 50% of a group of test animals) = 14 mg/kg (IV)⁽²⁰⁷⁾ or 95.1 mg/kg (IP)⁽²⁰⁸⁾ for mouse. To examine how long CocH3-Fc(M3) can protect mice from the acute toxicity of a lethal dose of cocaine, mice were injected with a single dose of CocH3-Fc(M3) (2 mg/kg, IV) on Day 0, followed by a lethal dose of cocaine (100 mg/kg, IP) every day (starting on Day 4) until convulsion occurred (end point of the toxicity test) for a given mouse. For the control group (without CocH3-Fc(M3) injection), all mice had convulsions within minutes after the first cocaine challenge, and 60% of the mice died soon after convulsion. For the test group receiving 2 mg/kg CocH3-Fc(M3), convulsions occurred only during the final cocaine challenge after 10.2 days in average; convulsions did not occur during the daily cocaine challenge within 9.2 days in average. The average protection time $\langle t_p \rangle$ provided by 2 mg/kg CocH3-Fc(M3) is 9.7 ± 1.7 days. Thus, injection of 2 mg/kg CocH3-Fc(M3) can effectively protect mice from the acute toxicity of the lethal dose of cocaine (100 mg/kg, IP) for nearly 10 days.

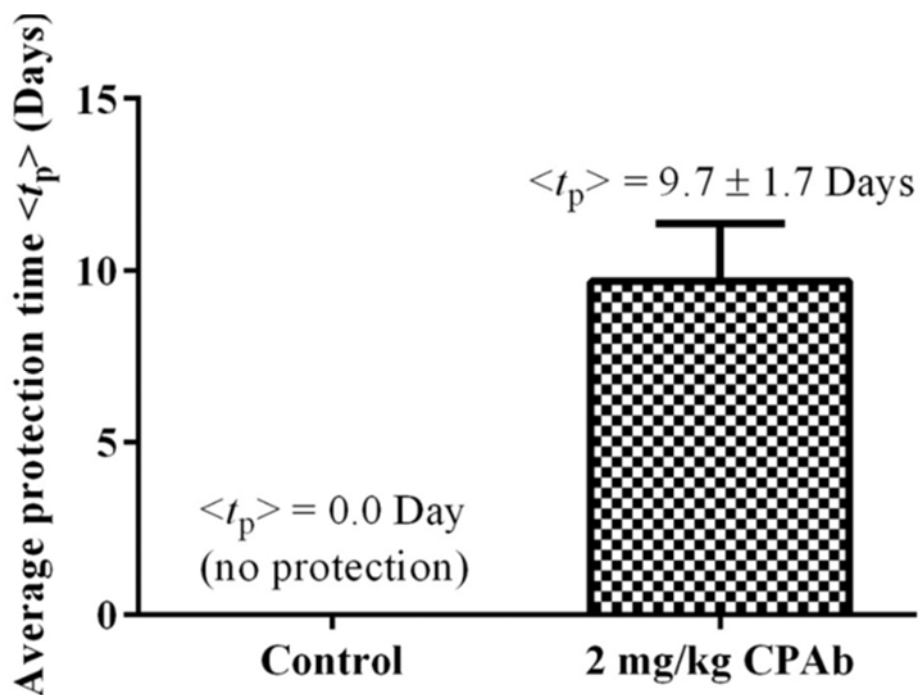


Figure 5.11 Effects of CochH3-Fc(M3) on cocaine-induced toxicity in mice. Toxicity test (n=5): A single dose of CochH3-Fc(M3) (2 mg/kg, IV) was followed by a daily dosing of cocaine (100 mg/kg, IP) starting on Day 4 until convulsions occurred (end point of the toxicity test) for a given mouse. For a given mouse, if convulsions occurred during the final cocaine challenge on Day m , but did not occur during the cocaine challenge on and before Day $m-1$, then the protection time (t_p) for this mouse was considered as between $m-1$ and m days: $m-1 < t_p < m$ or $t_p = m-1/2$ days. $\langle t_p \rangle$ represents the average t_p value for all mice in the group. This figure came from ref.⁽⁴⁸⁾.

5.8 Perspectives in clinical development of cocaine hydrolase for cocaine addiction treatment

We have successfully designed a novel long-acting cocaine-metabolizing enzyme form, CocH3-Fc. The optimized CocH3-Fc entity, CocH3-Fc(M3), indeed has a long biological half-life like an antibody, but with an unprecedentedly high catalytic efficiency compared to all catalytic antibodies known to date. Compared to the most active anti-cocaine catalytic antibody 15A10 ($k_{\text{cat}} = 2.3 \text{ min}^{-1}$, $K_M = 220 \text{ }\mu\text{M}$, and $t_{1/2} < 24$ hours in rats) ⁽²⁰⁹⁾, the CocH3-Fc(M3) has an even significantly longer biological half-life ($k_{\text{cat}} = 5,700 \text{ min}^{-1}$, $K_M = 2.8 \text{ }\mu\text{M}$, and $t_{1/2} = \sim 107$ hours in rats) and $\sim 200,000$ -fold improved catalytic efficiency against cocaine. The long-acting CocH3-Fc(M3) is highly efficient in blocking the physiological and toxic effects of cocaine for a long period of time. Further, it is well known that a protein drug generally has a significantly longer biological half-life in humans compared to that in rats, *e.g.* $t_{1/2} = 3$ -6 days in rats and 12-23 days in humans for abatacept, Fc(M1)-fused human cytotoxic lymphocyte associated antigen ⁽²⁰⁴⁾. Thus, one may reasonably expect that an appropriately chosen dose of CocH3-Fc(M3) may effectively prevent both relapse and overdose for 2-4 weeks or longer during cocaine addiction treatment for humans.

The general strategy of developing a long-acting drug-metabolizing enzyme for the treatment of cocaine abuse may also be useful in the development of long-acting forms of other therapeutic enzymes. To develop a truly long-acting enzyme form fused with Fc, one must structurally optimize both the enzyme and Fc portions of the Fc-fused enzyme. Further, even if the protein fusion with Fc can really prolong the biological half-life of a therapeutic protein, the data obtained in this investigation demonstrate that certain amino-acid mutations on the Fc portion that can prolong biological half-life of an Fc-fused therapeutic protein may not necessarily prolong the biological half-life of another Fc-fused therapeutic protein.

Chapter 6: Concluding Remarks and Future Plan

Enzyme therapy using an efficient cocaine-metabolizing enzyme is recognized as the most promising treatment strategy for cocaine overdose and addiction ^(16, 86, 91, 92). While the thermal instability and low catalytic activity against cocaine limit the clinical use of wild-type bacterial CocE and human BChE, site-directed mutagenesis based on molecular modeling have solved these problems and created efficient and thermally stable cocaine-metabolizing enzymes, that are mutants of CocE and BChE, as promising anti-cocaine medications ^(88, 89, 102-104, 148). A thermally stable CocE mutant (T172R/G173Q) ⁽⁸⁸⁾, known as RBP-8000 ⁽⁹⁰⁾, is currently in clinical development for cocaine overdose treatment; the human clinical trial Phase IIa has been completed, showing that the CocE mutant is safe and effective in hydrolyzing cocaine ⁽⁹⁰⁾, but inactive against BE (a major, biologically active metabolite of cocaine) ⁽⁴⁾. Theoretically speaking, CocHs should be more promising candidates for the treatment of not only cocaine overdose, but also cocaine addiction, due to a relatively higher catalytic activity against cocaine, and longer biological half-life. However, whether CocHs can degrade BE into biological inactive metabolites remain to be one of the main concerns for cocaine overdose treatment as no metabolic enzyme of BE has ever been reported in literature. In addition, while TV1380, an HSA-fused CocH, showed some promise for cocaine addiction treatment, its actual therapeutic value for cocaine addiction treatment is still limited by the moderate biological half-life which is ~8 hr in rats ⁽⁸⁶⁾ or 43-77 hr in humans ⁽⁹¹⁾. Thus, to address these two problems and some other concerns in further development of CocHs for the treatment of cocaine overdose and addiction, in the investigation described in this thesis, we have carried out kinetic characterization of an hCocH and a mCocH for their catalytic activities against cocaine and other substrates, identified enzymes that can hydrolyze not only cocaine, but also its long-lasting metabolite benzoylecgonine, developed a LC-MS/MS method for determining pharmacokinetic profiles of cocaine and metabolites, and developed a

long-acting form of hCocH.

6.1 Summary of the major conclusions obtained from this investigation

1) Enzymes mCocH and hCocH have improved the catalytic efficiency of mBChE and hBChE against (-)-cocaine by ~8- and ~2000-fold, respectively, although the catalytic efficiencies of mCocH and hCocH against other substrates, including ACh and BTC, are close to those of the corresponding wild-type enzymes mBChE and hBChE. According to the kinetic data, the catalytic efficiency of mBChE against (-)-cocaine is comparable to that of hBChE, but the catalytic efficiency of mCocH against (-)-cocaine is remarkably lower than that of hCocH by ~250-fold. The remarkable difference in the catalytic activity between mCocH and hCocH is consistent with the difference between the enzyme-(-)-cocaine binding modes obtained from molecular modeling.

2) We have demonstrated for the first time that plasma enzyme BChE is the endogenous metabolic enzyme of BE and the BChE mutant E12-7 with significantly improved catalytic efficiency against BE compared to wild-type BChE may be used as an exogenous enzyme to effectively accelerate BE hydrolysis in the body for cocaine overdose treatment. Compared to RBP-8000 (the T172R/G173Q mutant of CocE) ^(88, 90) under current clinical development for cocaine overdose treatment, E12-7 is not only more efficient against cocaine ⁽¹⁰²⁾, but also effective in hydrolyzing BE. Hence, E12-7 should be a more promising therapeutic candidate for cocaine overdose treatment in comparison with the T172R/G173Q mutant of CocE, although one would like to further improve the catalytic efficiency against BE for cocaine overdose treatment.

3) We have successfully developed a simple and rapid LC-MS/MS method combined with one-step solid phase extraction to simultaneously determine the concentrations of cocaine, EME, BE, ECG, norcocaine, NEME, cocaethylene, EEE, norcocaethylene, and NEEE in the rat blood samples. Great specificity, sensitivity, linearity, recovery, accuracy, precision, and stability of the method have been well demonstrated. With a successful application to a pharmacokinetic study in rats with

co-administration of cocaine and alcohol, this method will be valuable for further studies that aim to characterize anti-cocaine therapeutic agents and their effects on metabolic profiles of cocaine in the presence/absence of alcohol.

4) We have developed a novel CocH form, catalytic antibody analog, which is an Fc-fused CocH dimer (CocH-Fc) constructed by using CocH to replace the Fab region of human immunoglobulin G1. The CocH-Fc has not only a high catalytic efficiency against cocaine, but also a considerably longer biological half-life (e.g. ~107 hours in rats) like an antibody. A single dose of CocH-Fc was able to accelerate cocaine metabolism in rats even after 20 days and, thus, block cocaine-induced hyperactivity and toxicity for a long period of time. In consideration of the general observation that the biological half-life of a protein drug in humans is significantly longer than that in rodents, the CocH-Fc reported in this study could allow dosing once every 2-4 weeks, or longer for cocaine addiction treatment in humans. The long-acting enzyme approach may provide a novel, truly promising therapy capable of effectively blocking the physiological and toxic effects of cocaine without affecting normal functions of the brain and other critical organs and prevent relapse during abstinence.

6.2 Future plan concerning rational design of CocHs for cocaine abuse

1) To further improve the catalytic efficiency of CocH against BE for cocaine overdose treatment;

2) To further characterize the detailed toxicological and pharmacological profiles of CocH-Fc, such as the effectiveness, selectivity, and duration of CocH-Fc's actions in cocaine self-administration and discrimination;

3) To optimize a promising CocH form which has not only a higher catalytic activity against cocaine, but also a longer in vivo half-life compared to CocH-Fc;

4) The most promising CocH form optimized in the further investigation will possibly be ready for cGMP (current Good Manufacturing Practices) protein manufacturing and subsequent clinical trials.

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Awards & Honors

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