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HUMAN BUTYRYLCHOLINESTERASE MUTANTS FOR COCAINE DETOXIFICATION

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HUMAN BUTYRYLCHOLINESTERASE MUTANTS FOR COCAINE DETOXIFICATION

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
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Lexington, Kentucky
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2014

ABSTRACT OF DISSERTATION

HUMAN BUTYRYLCHOLINESTERASE MUTANTS FOR COCAINE DETOXIFICATION

Cocaine is one of the most reinforcing drugs of abuse and has caused serious medical and social problems. There is no FDA-approved medication specific for cocaine. It is of a high priority to develop an effective therapeutic treatment for cocaine abuse. Human butyrylcholinesterase (BChE) has been recognized as a promising candidate of enzyme therapy to metabolize cocaine into biologically inactive metabolites and prevent it from reaching central nervous system (CNS). However, the catalytic activity of wide-type human BChE against cocaine is not sufficiently high for treatment of cocaine abuse. Dr. Zhan's lab has successfully designed and discovered a series of high-activity mutants of human BChE specific for cocaine metabolism.

This dissertation is mainly focused to address the possible concerns in further development of promising human BChE mutants for cocaine detoxification, including whether the administration of this exogenous enzyme will affect the cholinergic system, whether it can efficiently hydrolyze cocaine's toxic metabolites, and whether the commonly used therapeutic agents will significantly affect the catalytic activity of the BChE mutants against cocaine when they are co-administered. According to the results obtained, all of the examined BChE mutants have a considerably improved catalytic efficiency against (-)-cocaine, without significantly improving the catalytic efficiency against any of the other examined substrates, including neurotransmitter acetylcholine. Two representative mutants (including E12-7) also have a considerably improved catalytic activity against cocaethylene (formed from combined use of cocaine and alcohol) compared to wild-type BChE, and E12-7 can rapidly metabolize cocaethylene, in addition to cocaine, in rats. Further evaluation of possible drug-drug interactions between E12-7 and some other commonly used therapeutic agents revealed that all of the examined agents, except some tricyclic antidepressants, do not significantly inhibit E12-7. In addition, an effort to discover new mutants with further improved activity against cocaine led to the discovery of a new BChE mutant, denoted as E20-7, according to both the *in vitro* and *in vivo* assays. The encouraging outcomes of the present investigation suggest that it is possible to develop a more effective enzyme therapy for cocaine abuse treatment using one of the most promising BChE mutants, such as E12-7 or E20-7.

KEYWORDS: Protein drug, enzyme therapy, human butyrylcholinesterase, cocaine, and drug abuse treatment.

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1.1 Cocaine and its abuse

Cocaine is a powerfully addictive drug that primarily acts on the brain.(*1*) Pure cocaine was originally extracted from the leaves of coca plant which grows in South America, mainly in Peru, Bolivia and Colombia.(*2*) Coca leaves have been chewed and ingested for at least 8,000 years, while the purified cocaine has been an abused substance for over 100 years.(*2*) This alkaloid was first purified from coca leaves in 1855 by the German chemist Friedrich Gaedcke (*3*) and first characterized chemically in 1859 by Albert Niemann (*4, 5*). Cocaine was proposed as a "wonder drug", once becoming the cure for opium, morphine, and alcohol problems by the 1860s, and first brought into clinical use as a local anesthetic in 1884 by Karl Koller.(*6*) As a result of its medical use, cocaine gained great popularity by the end of 19th and the beginning of 20th century. It was one of the first alkaloids to be chemically synthesized. The total synthesis of cocaine was confirmed by Richard Willstätter and his colleagues in 1923, while the full stereochemistry of cocaine molecule was not clear until E. von Hardeggar and Hans Ott figured it out in 1955.(*2*) As cocaine gained popularity and its uncovered properties, society became alarmed over its addictive potential and other effects on humans. Because of this, its use was under strict regulations by the beginning of 20th century.(*6*)

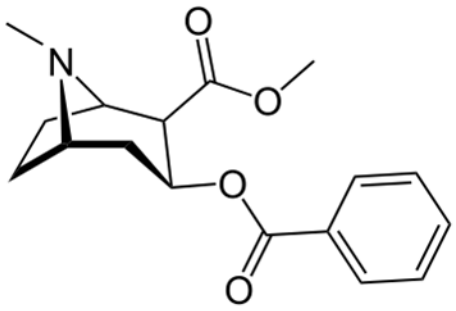


Figure 1.1 Molecular structure of (-)-cocaine free base.

Cocaine is abused in two forms: the water-soluble hydrochloride salt, and the water-insoluble cocaine free base. The salt can be taken orally, snorted (intranasal), or injected.(*7*) The high melting temperature (190 ℃) of the salt makes it unsuitable for

smoking because it will decompose before vaporization. The free base can be smoked or snorted. The relatively low melting temperature (98 ℃) makes it suitable for smoking.(*8*)

Onset of effects, peak effects, duration of euphoria, and plasma half-life of cocaine are affected by route of administration and dosage. Cocaine can take effect in from less than one minute to around 15 minutes and last for 15 minutes to one hour, depending on the actual dose.(*9*) The faster the drug is absorbed, the earlier and stronger the resulting euphoric effects, however, the shorter the effects last. Compared with oral and intranasal use of cocaine, the effects of intravenous and smoked cocaine are rapid and short-lived. The severity of cocaine dependence is also associated with the route of administration. Intravenous injection of cocaine correlates with the highest levels of dependence; intranasal use correlates with the lowest levels, and crack smoking is intermediate between the two.(*10*)

In general, after cocaine is taken, the users feel rapid and intense high. The high is relatively short and followed by a coming down period, which makes the users repeat the use for high and, leading to addiction. Besides the feeling of euphoria, effects of cocaine also include feeling of energetic, talkative, increased alertness, reduced appetite, and sensation of light, sound and touch.(*1*) Once addicted to cocaine, the risk of adverse psychological or physiological effects increases. It can lead to increased irritability, restlessness, panic attacks, and paranoia—even hallucinations. Long-term use of cocaine could cause serious physical problems, including cardiovascular failures, respiratory difficulties, and gastrointestinal complications, and mental problems, including depression, agitation, nervous, and tired but unable to sleep.(*11*) Cocaine overdose could be more serious and even lethal, due to the possible respiratory failure, heart-failure, stroke, or seizures induced by cocaine's toxicity.(*12*, *13*)

1.2 Mechanism of cocaine action and cocaine-induced toxicity

The pharmacology of cocaine is complicated and involves several organ systems, primarily complicates the brain, heart, and lung. Cocaine-protein interaction is the foundation that affects all these systems. Cocaine binds to several proteins, including monoamine transporters, neurotransmitter receptors, plasma proteins, and voltage gated ion channels in central and periphery nervous systems.(*14*)

1.2.1 Reinforcing effects and cocaine addiction

Cocaine is a powerful addictive drug, causing its reinforcing and toxic effects in CNS by binding with monoamine transporters, especially dopamine transporter (DAT).(*1, 14-17*) Cocaine acts primarily as a dopamine transporter blocker in the rewarding center of CNS.(*15, 17*) The brain's key areas involved in reward lies in a region of the midbrain called the ventral tegmental area (VTA) and extended to a region known as the nucleus accumbens. During the normal dopaminergic signaling cycle, neurotransmitter dopamine (DA) is released into synaptic cleft from the transmitting neuron and binds with dopamine receptors on the receiving neuron to conduct the signaling. Dopamine will be then recycled *via* DAT located on the transmitting neuron to end the signaling cycle. However, cocaine entering into the brain would interfere with this signaling cycle. It will bind with the DAT to form a complex that block the binding of dopamine.(*18*) This can result in accumulation of dopamine in the synaptic cleft and lead to the prolonged and enhanced effect of dopaminergic signaling, which causes the euphoria feeling as reported by cocaine users. The DAT is responsible for the major psychostimulant and reinforcing effects of cocaine.(*14*)

Moreover, chronic use of cocaine changes the structure and function of brain. It is reported that one-time use of cocaine could cause the change of the dopaminergic signaling, *i.e.* the down-regulation of dopamine receptors and up-regulation of dopamine transporter for at least months.(*1, 19, 20*) Therefore, cocaine users would not achieve the same "high" as they felt at the first time they used the drug, leading to the development of cocaine addiction. Once developed, it is very hard to get rid of due to the change is usually an extremely slow process to recover. During the addiction development, patients first feel impulsive to take cocaine (positive reinforcement), and soon become compulsive because of negative emotional state (negative reinforcement).(*21, 22*) They reported anxiety, dysphoria and irritability when cocaine is not available.

Besides DAT, Cocaine also interacts with other transporters, including the serotonin transporter (SERT), and the norepinephrine transporter (NET).(*14, 23, 24*) The GABAergic system and the NMDA-glutamate systems are also intimately involved in cocaine toxicity.(*25*) Although cocaine does not directly interact with the GABA receptor, GABA is the most important neurotransmitter which could be manipulated for treatment

of cocaine abuse.(*14*)

1.2.2 Acute toxicity and cocaine overdose

Besides three monoamine transporters in CNS, cocaine also binds with norepinephrine transporter, muscarinic acetylcholine and sigma receptors, and several voltage-gated ion channels in periphery nervous system.(*14*) The inhibition constant (K_i) of cocaine for proteins, including transporters, receptors, and ion channels, are summarized in Table 1.1. The cocaine concentration in human body in the case of cocaine overdose is much higher than it in the case of cocaine addiction. Therefore, as the cocaine concentration increases, proteins with high affinities for cocaine will be saturated, and cocaine will start to bind to proteins with lower affinities. Those interactions contribute to the acute toxicity of cocaine.

The classic signs of cocaine overdose include increased heart rate, increased body temperature, elevated blood pressure, and chest pain. Besides, it can also cause cardiovascular effects (heart attacks), neurological effects (seizures), and gastrointestinal complications (abdominal pain and nausea).(*26*) Cocaine-related deaths generally result from cardiac arrest or seizures, and the followed respiratory failure.

Most of the cardiac toxicity of cocaine can be attributed to two basic mechanisms: one is its ability to interact with all three ion channels, leading to a local anesthetic effect; the second is its ability to block reuptake of norepinephrine in the presynaptic neurons in the central and peripheral nervous systems, resulting in increased catecholamines and sympathetic output.(*27*)

There are some conflicting data concerning the effects of muscarinic acetylcholine and sigma receptors on the convulsion and lethality effects of cocaine.(*14*) Therefore, their contributions to cocaine-induced toxicity are still unclear.

Table 1.1 Inhibition constants of cocaine against interacting proteins.(*14*)

Protein	K_i (μM)
Dopamine transporter	0.64
Serotonin transporter	0.14
Norepinephrine transporter	1.6
Muscarinic acetylcholine receptor (rat)	19, 24, 40
Sigma receptor (rat)	6.7
Cardiac myocyte sodium channel	8
Cardiac potassium rectifier channel	5.6

1.3 Pharmacotherapy for cocaine abuse — pharmacodynamic approach

1.3.1 Cocaine addiction

Medication-assisted pharmacotherapy and behavioral therapy are commonly employed in drug addiction treatment. Pharmacotherapy for cocaine abuse includes pharmacodynamic and pharmacokinetic approach. Because the traditional pharmacodynamic approach has been successful in the treatment of opioid and nicotine dependence (*28*), it was also extensively studied for cocaine abuse treatment. Pharmacodynamic approach is to develop a small molecule targeting a specific transporter/receptor to manipulate various neurotransmitter systems in order to block cocaine's effects, such as dopaminergic, serotoninergic, noradrenergic, cholinergic, glutamatergic, GABAergic, and opioidergic pathways.(*29*) As the neurochemistry and neurobiology of drug dependence progress recently, various compounds have been tested in clinical studies to interfere with cocaine's action, including the drugs approved for other diseases. However, due to ineffectiveness and/or unwanted side effects and/or its potential addiction tendency, no ideal pharmacodynamic agent specific for cocaine abuse treatment has been identified yet.

1.3.1.1 Compounds targeting the dopamine system

Many studies have indicated that dopaminergic system is intimately involved in drug reward and addiction.(*30, 31*) Almost all the addictive drugs, including cocaine, heroin, nicotine, and alcohol, have been shown to directly stimulate the dopaminergic

system by increasing extracellular DA in the nucleus accumbens through different mechanisms.(*21, 30, 31*) The increase in DA level has been thought to result in the euphoria effects of abused drug.(*16, 32*) Based on this dopamine-based hypothesis, much effort has been focused on manipulation of DA transmission in the brain reward system by targeting DAT (agonist therapy), or DA receptors (antagonist therapy) in development of cocaine addiction treatment.

Compounds targeting the dopamine transporter

Agonist or substitution therapies have proven effective in the treatment of opioid and nicotine dependence.(*28*) Similarly, drugs that block the DAT, but are less addictive than cocaine, might be promising for treatment of cocaine addiction as well. In fact, this strategy has been in the leading place for more than a decade in the development of anti-cocaine medication. However, because the binding site of cocaine in DAT is overlapped with the binding site of dopamine (*18, 33, 34*), it is very challenging to find a small molecule that can block cocaine action on DAT without interfering the normal function of DAT and the downstream signaling. Many DAT inhibitors have been developed, and several of them have been tested on human, such as vanoxerine, methylphenidate and modafinil. However, none of them have shown to be successful due to their own significant abuse liability and/or adverse side-effects.(*35*)

Compounds targeting the dopamine receptor

Cocaine's action in brain reward center is primarily mediated by elevation of extracellular DA, followed by activation of postsynaptic DA receptors, and producing the enhanced effect of dopaminergic signaling. Thus, blockade of DA receptors is another therapeutic approach for cocaine addiction treatment. Among the five subtypes of DA receptor in the brain, both D1 and D2 subtypes have shown their importance in modulating actions of DA.(*32*) However, clinical trials using selective D1 or D2 receptor antagonists for cocaine dependence treatment have failed due to ineffectiveness and/or unwanted side-effects.(*28*) As a result, more efforts have switched to develop low selective D1/2 receptor antagonists or D3 receptor antagonists, such as BP-897, GSK598809, and ABT-925 for cocaine addiction treatment.(*28*)

1.3.1.2 Compounds targeting the serotonin system

As discussed earlier, besides its action on DAT, cocaine also binds with the norepinephrine transporter (NET) and serotonin transporter (SERT), causing the accumulation of those monoamines as well. Acute cocaine intoxication is characterized by enhanced dopamine transmission and increased release of serotonin (*36*) while cocaine withdrawal is characterized by decreased levels of serotonin in the nucleus accumbens.(*37*) These evidences suggest the involvement of serotonin in cocaine reward and reinforcement. Pharmacological manipulation of the serotonin system using selective serotonin reuptake inhibitors and serotonin receptor antagonist for the treatment of cocaine addiction showed mixed results, which needs further evaluation in clinical study.(*38*)

1.3.1.3 Compounds targeting the GABAergic system

GABA is the major inhibitory neurotransmitter in CNS, and GABAergic neurons can modulate brain dopaminergic reward functions. Significant evidences suggest that GABA can inhibit cocaine-induced release of dopamine and reduce the reinforcing effects of cocaine in animals, and increase of GABA activity in addicted brains leads to decrease in drug craving and relapse.(*15, 39*) Much efforts have focused on two approaches to increase GABAergic activity: (1) by inhibiting GABA transaminase, the primary enzyme involved in metabolism of GABA, such as the irreversible inhibitor vigabatrin; or (2) by directly stimulating GABA receptors with an agonist, including benzodiazepines, tiagabine, topiramate, baclofen, and valproic acid.(*29*)

Although there is no direct interaction between cocaine and GABAergic system, GABA is the most important target which could be manipulated for treatment of cocaine abuse, according to the outcomes of recent preclinical and clinical studies.(*14*) One proposed mechanism for the interaction between cocaine and GABA is that the activation of dopamine D2 receptors by cocaine may decrease GABA release, which will cause enhanced CNS excitation shown in cocaine-induced toxicity.(*14*) Benzodiazepines, are a class of drugs for cocaine overdose treatment in emergency room. Benzodiazepines as the agonists bind to a unique allosteric site on GABA receptor, and increase GABAergic neurotransmission and the frequency of GABA-mediated chloride channel opening,

producing the CNS depression to relieve cocaine-induced toxicity.

1.3.1.4 Compounds targeting the glutamate system

L-glutamate acts as the major excitatory neurotransmitter in CNS through two classes of glutamate receptors: ionotropic (iGluR) and metabotropic (mGluR) glutamate receptors. Although the exact role of glutamate in modulating cocaine's rewarding effects remains unclear, more and more studies indicate that glutamate modulates cocaine-related dopamine release and is intimately involved in relapse to drug-seeking behavior.(*40, 41*) Various types of mGluR antagonist/agonist have been examined in the preclinical study and also human clinical trials, such as N-acetylcysteine, a cystine prodrug to increase the basal levels of extracellular glutamate; fenobam and MPEP as mGlu5 antagonists; AZD8529 and LY404039 as mGlu2/3 agonists; memantine, and dizocilpine as NMDA receptor antagonists.(*28*)

1.3.1.5 Compounds targeting the opioid system

The endogenous opioid peptides regulate and modulate dopaminergic function within the brain reward center.(*42*) Three major classes of opioid receptors, μ, δ, and κ become the targets for further manipulation. Candidate compounds which differentially target opioid μ, δ, and κ receptors have been evaluated for possible anti-cocaine medications. Naltrexone and naloxone, opioid μ receptor antagonists, approved for opioid dependence and overdose treatment, are evaluated in preclinical study. Buprenorphine, a partial opioid μ receptor agonist and opioid κ receptor antagonist, and enadoline, an opioid κ receptor agonist, are evaluated on humans.(*29*)

1.3.2 Cocaine overdose

According to DAWN report released in 2012, cocaine misuse or abuse is the most common cause of illicit drug-related Emergency Department (ED) visits in the US, accounting for 488,101 ED visits in 2010.(*43*) Cocaine overdose could be potentially lethal. The typical symptoms of cocaine overdose are high blood pressure, rapid heart rate, and increased rate of breathing. The most frequent complaint by patients is cocaine-associated chest pain, accounting for 40% of cocaine-related ED visits.(*44*) Emergency

Department treatments are mainly to relieve the chest pain and possible heart attack. After the use of cocaine is confirmed by self-reports or by analysis of cocaine metabolite in the urine, the cocaine users should be provided with intravenous fast-onset benzodiazepines (diazepam and midazolam) as early management to counter agitation, irritability, seizures, and hyperexcitable state. When patients do not calm down, sodium nitroprusside, nitroglycerin, or phentolamine can be applied to relieve hypertension. If patients show hyperthermia, cold water, fans, cooling blankets, and acetaminophen could be given to the patients. If patients do not respond to benzodiazepines and vasodilators, calcium channel blockers may be considered. Besides, if patients have had a true heart attack (myocardial infarction), antiplatelet, such as aspirin and heparin could be given to patients, and aspirin is recommended to be routinely administrated.(*45*, *46*) Cocaine blocks norepinephrine reuptake and lead to accumulation of norepinephrine. Excess norepinephrine causes stimulation of both α- and β-adrenergic receptors. Clinically, the use of a β-adrenergic antagonist in cocaine-overdosed patient has resulted in death. Alternatively, α-adrenergic antagonist, such as phentolamine, can counter the vasoconstrictive effects of cocaine, suggesting that the mechanism is cocaine-induced elevation in α-adrenergic tone.(*45*)

To summarize, the existing ED protocol treat patients only by controlling symptoms according to their complaints and clinical presentations, i.e. it only simply relieves the cocaine-induced symptoms, instead of completely blocking cocaine's effects. "Recidivism is high among patients with cocaine-associated chest pain (60% admit to cocaine use in the next year)"(*46*), which may suggest that more effective medications need to be developed to quickly detoxify large amount of cocaine and prevent it from entering the brain.

1.4 Pharmacotherapy for cocaine abuse — pharmacokinetic approach

Most anti-addiction treatment has been using the traditional pharmacodynamic approach for decades, i.e. to develop a small molecule binding with DAT or another transporter/receptor to block cocaine's action.(*29*) However, despite decades of efforts, there is no FDA-approved medication specific for cocaine abuse so far.(*47*) Recently, the alternative protein-based pharmacokinetic approach has drawn more and more attention,

i.e. to develop an agent (an enzyme, antibody, or vaccine which producing an antibody in the body) that can accelerate the metabolism of cocaine in the body and/or prevent the delivery of cocaine from reaching CNS.(*17, 48, 49*) Protein-based agents would not cross the blood brain barrier and, thus, would not enter the brain. So, they are not expected to block the normal transporter and receptor functions. In particular, the enzyme therapy has the advantage of higher efficiency over antibody and vaccine approaches, which makes the enzyme therapy more appealing for cocaine abuse treatment. The feasibility of the pharmacokinetic approaches for the treatment of cocaine addiction and overdose has been discussed in detail in a recent review article (*17*).

1.4.1 Cocaine vaccine or antibody for treatment of cocaine dependence

It has been known that a small molecule (hapten), such as cocaine, will not evoke an immune response unless covalently attached to an immunogenic protein (carrier).(*50*) To be specific, it involves an antigen consisting of cocaine or cocaine-like molecular as the hapten, covalently attached to the carrier protein with the aid of a functional linker, and an adjuvant to stimulate the immune system's response to the antigen. The conjugate will be immunized to produce cocaine-specific antibody as well as the foreign protein-specific antibody. Therefore, the immune system can generate antibodies that bind to cocaine. When cocaine molecules are bound by anti-cocaine antibody in the bloodstream, cocaine distribution will be altered between brain and plasma. Because the antibody-cocaine complex is so large that it could not cross the blood/brain barrier, cocaine is prevented from producing its reinforcing effects in brain. Antibody-bound cocaine is released slowly, and can be metabolized by endogenous enzymes, especially human BChE, in the blood or in tissues.(*51*)

Both active and passive immunization approaches have been investigated for treatment of cocaine abuse.(*52*) Antibody in active immunization approach is naturally produced by the antigen in recipient and stays active for a longer period of time in months, whereas antibody in passive immunization is usually generated in another species or *in vitro* and transferred to the recipient for cocaine binding, and can only be stable for a period of time in weeks.

A cocaine vaccine/antibody shows two advantages over existing agonist approach

for treatment of cocaine dependence. First, it does not require daily administration. A cocaine vaccine could last for weeks or months. Second, because the antibodies do not act in the brain so that they are likely to have fewer adverse effects than drugs acting in the brain. It also has some potential disadvantages. The major disadvantage of the vaccine /antibody strategy is its low efficiency due to the fact that each antigen-binding site of the antibody can bind with only one cocaine molecule. It will not completely block the effects of cocaine in all circumstances. When cocaine is administered with a high dose, it would be extremely difficult to have a sufficiently high amount of antibodies for binding with all cocaine molecules. If antibodies could not completely suppress the cocaine reward in the brain, it may induce patient using a higher dose to override the cocaine vaccine effects. Another disadvantage is that there is so much variability in antibody responses to cocaine among the human subjects. TA-CD, a cocaine vaccine made from succinyl norcocaine conjugated to cholera toxin B, has finished three-phase clinical trials in 2009. This vaccine showed no serious adverse effects and patients who achieved sufficient antibody levels reduced their cocaine use. However, only 38% of the subjects in the trial attained the "high" amount of antibody (≥43 μg/mL).(*53-55*) Report also showed that antibody response of most subjects is 6 weeks later, and cannot last for longer than 12 weeks.(*55*) Furthermore, for the active immunization, it takes quite a long time to generate enough amounts of antibodies, which will limit its potential clinical use. To improve the efficiency of vaccine, the better vaccine and adjuvant, and/or vaccination strategies will be needed to produce a higher-titer and longer-lasting antibody in order to fully block the effects of commonly used dose of cocaine.

1.4.2 Cocaine-metabolizing enzyme for treatment of cocaine abuse

Compared with the above-mentioned immunological strategy, developing a highly efficient cocaine-metabolizing enzyme for treatment of cocaine abuse can be a more promising direction.(*17, 56-58*) Enzyme therapy has the advantage of higher efficiency over antibody and vaccine approaches. The desired therapeutic enzymes can not only bind with cocaine but also metabolize it in the serum before it reaches CNS. What is more, an enzyme molecule can free itself for further degradation of another cocaine molecule. Thus, one enzyme molecule can keep metabolizing cocaine molecules as long

as it retains its activity. Besides, it has potential advantages over active immunization since its administration would immediately enhance cocaine metabolism and would not require an immune response to be effective, which is important for patients with cocaine overdose in ED.(*17*)

In order to find out the possible enzyme candidate, it is necessary to understand the metabolism pathways of cocaine in human. Around 95% of cocaine is metabolized in human by enzymatic hydrolysis of the two ester groups.(*58, 59*) Hydrolysis of cocaine at the benzoyl ester generates ecgonine methyl ester (EME) and benzoic acid; whereas hydrolysis of cocaine at the methyl ester produces benzoylecgonine (BE) and methanol.(*60*) BChE is the primary cocaine hydrolase in human plasma capable of hydrolyzing cocaine at the benzoyl ester.(*61*) One of the liver carboxylesterases, known as hCE-1, is responsible for hydrolyzing cocaine at the methyl ester.(*58, 62*) The remaining 5% is metabolized through oxidation by the liver microsomal cytochrome P450 system, producing norcocaine.(*58, 60*) Among the metabolites of cocaine (EME, BE, and norcocaine), EME is known to be the least pharmacologically active and may even cause vasodilation (*63*), whereas both BE and norcocaine have similar physiological effects as cocaine, and cause vasoconstriction and lower the seizure threshold.(*58, 63-65*) Moreover, norcocaine is hepatotoxic due to the further oxidative metabolite of norcocaine.(*58, 66, 67*) Therefore, the metabolic pathway through BChE-catalyzed hydrolysis at the benzoyl ester is most promising for cocaine detoxification from the body.

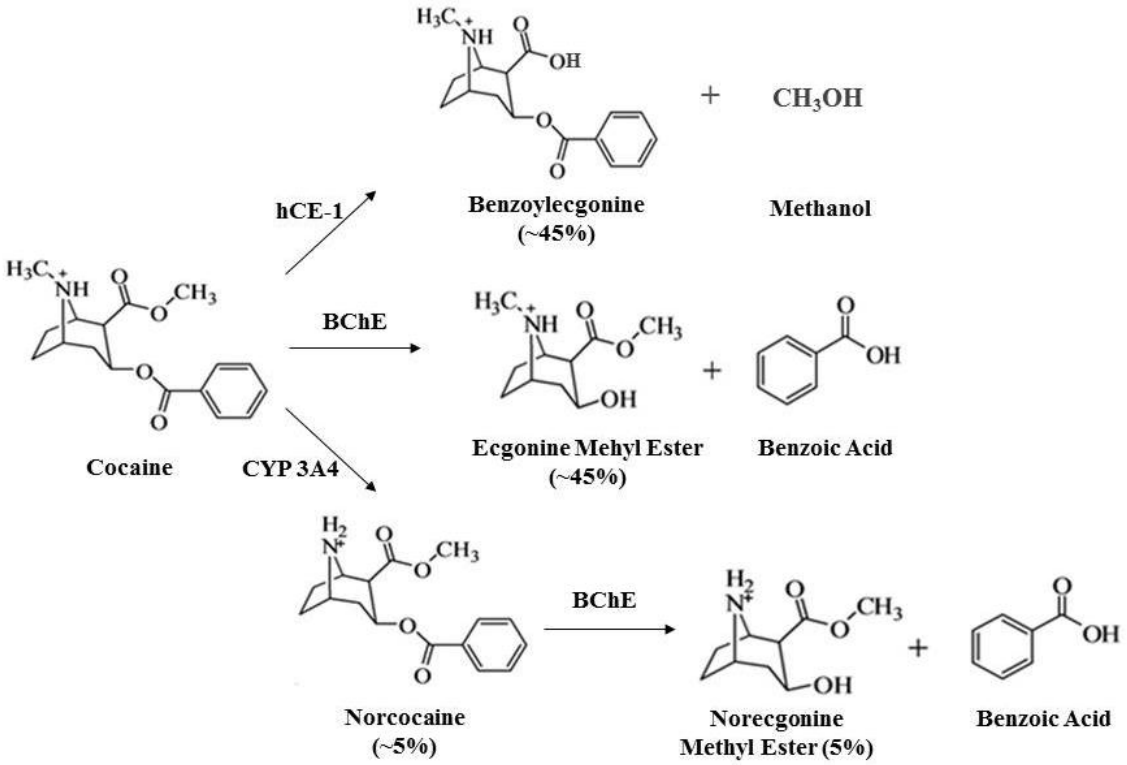


Figure 1.2 Cocaine metabolic pathways in human.

Cocaine, as a stimulant on brain, can cross the blood-brain barrier back and forth rapidly, and cocaine concentrations in the brain and plasma can quickly reach the equilibrium as demonstrated in the positron emission tomography (PET) imaging studies.(*68, 69*) Although exogenous enzymes administrated into the plasma do not enter the brain, if cocaine in the plasma is quickly metabolized, cocaine in the brain will move back to the plasma. Eventually, cocaine in CNS can be cleared by the exogenous enzyme administrated into the plasma. Therefore, a cocaine-metabolizing enzyme can be used for cocaine abuse treatment. For cocaine overdose treatment, it requires the enzyme to have a high catalytic efficiency in order to rapidly clear cocaine from the body.

As mentioned earlier, a single dose of cocaine would cause change in gene expression levels of dopamine transporter and receptor, which could persist for months after withdrawal. That is the reason why cocaine addiction is so hard to treat and so easy to relapse. For cocaine addiction treatment, it is necessary to completely block the stimulant effects of cocaine for quite a long time, which requires the enzyme to have a

high catalytic efficiency and long circulation time in the body. Under this condition, the patient can have a better chance to bring the function of the brain's communication system back to normal.

1.5 Human BChE as a potential therapeutic enzyme for treatment of cocaine abuse

Human BChE has been recognized as a promising candidate for metabolizing cocaine. BChE naturally exists in human body, mainly in plasma, liver, kidney, and lung. It was first called pseudocholinesterase because of its ability to hydrolyze acetylcholine (ACh). It has been known that complete inhibition of BChE activity has no effect on muscle contraction.(*70*) So it is not BChE's main function to hydrolyze ACh. Studies have suggested that BChE is involved in neurodegenerative disorders such as Alzheimer's disease (AD), and may be an important drug target for development of new treatments of AD.(*71, 72*) The exact biological function of BChE is not completely clear yet.(*60*) It has been proposed to be a housekeeping enzyme since it is responsible for detoxification of a wide range of toxins including succinylcholine, organophosphates, and cocaine.(*73-75*)

Human BChE has exhibited several advantages in clinical use as a potential anti-cocaine medication.(*17, 76*) First, BChE is the primary enzyme to hydrolyze cocaine in human body and produce biological inactive metabolites, and thus to help reducing the reinforcing and toxic effects of cocaine. Many studies have demonstrated that administration of exogenous human BChE (purified from donated blood) accelerates cocaine metabolism and decreases cocaine's half-life in rodents.(*60*) Second, human BChE is from a human source, and there are over 65 different naturally occurring variants of human BChE, and none of them is antigenic.(*77, 78*) So, the immune response, which is usually evoked by exogenous protein, may be avoided. Third, human BChE has a long history of clinical use to detoxify organophosphates, carbamate pesticide, and succinylcholine without adverse effects observed.

However, the potential use of human BChE in cocaine detoxification is limited by its low catalytic activity (k_{cat} = 4.1 min^{-1} and K_M = 4.5 μM) against (-)-cocaine (*17, 79*), which is not sufficient to rapidly clear cocaine from the body. Both cocaine addiction and overdose treatments require the enzyme to have a sufficiently high catalytic activity against (-)-cocaine to quickly clear cocaine from body and prevent it from entering the

brain. One interesting fact is that the activity of human BChE against the synthetic (+)-cocaine (k_{cat} = 6420 min^{-1} and K_M = 4.7 μM) is ~1500-fold higher than that against (-)-cocaine and (+)-cocaine is biological inactive (*80*), which may give us some insights in re-engineering of human BChE for cocaine abuse treatment. It is reported that a very low dose of 0.2 mg/kg (-)-cocaine has a half-life of 47 min, whereas (+)-cocaine at the same dose can be cleared from body by BChE within seconds.(*48*) PET mapping (+)- and (-)-cocaine binding in baboon indicates that (+)-cocaine has no uptake in CNS, whereas (-)-cocaine peaks in minutes primarily at striatum.(*68, 81*) The main reason why (+)-cocaine is biologically inactive could be that it is hydrolyzed so rapidly by BChE that it never reached the CNS for PET imaging.(*68, 81*) If BChE can be re-engineered to obtain an activity against (-)-cocaine as high as it against (+)-cocaine, it would be feasible for treatment of cocaine addiction and overdose.

1.6 Further development of the therapeutic enzymes

Cocaine, recognized as one of the most reinforcing drugs of abuse, has caused serious medical and social problems. Unfortunately, there is still no FDA-approved effective treatment for cocaine addiction or overdose. Due to ineffectiveness and/or unwanted side effects and/or its potential addiction tendency, no clinically useful pharmacodynamic agent specific for cocaine abuse treatment has been identified yet. However, enzyme therapy has been recognized as a promising approach to treat cocaine abuse. As a good candidate for this purpose, human BChE has several therapeutic potentials, but the application is limited by its low catalytic activity against (-)-cocaine. Dr. Zhan's lab has successfully designed and discovered a series of high-activity mutants of human BChE, using novel integrated computational-experimental approach. One of the BChE mutants designed in our group, *i.e*. A199S/S287G/A328W/Y332G BChE (denoted as E14-3), fused with human serum albumin (denoted as AlbuBChE) is under human clinical trial phase II by Teva Pharmaceutical Industries Ltd. When AlbuBChE was administered to human subjects by intramuscular injection, no significant increase in blood pressure was observed, nor was lethargy reported. The findings indicate that intramuscular administration of AlbuBChE to humans at the specified dosages (50 to 300 mg per subject) did not result in any unacceptable side effects and that the specified

dosages will allow for the successful treatment of cocaine exposure. Human subjects administrated with AlbuBChE did not report a significant increase in cocaine craving. In contrast, the desire to use cocaine again and the "overall drug liking" are significantly decreased following the AlbuBChE dosing.(*82*) Another promising BChE mutant designed in our lab, *i.e.* A199S/F227A/S287G/A328W/Y332G BChE (denoted as E12-7) which was known as the most active cocaine hydrolase reported, has a ~2000-fold improved catalytic efficiency against (-)-cocaine compared to the wild-type BChE, a ~2-fold improved catalytic efficiency against (-)-cocaine compared to E14-3.(*76*) The *in vivo* data in a mice model demonstrated that the minimum effective dose of the enzyme to protect mice from cocaine-induced lethality (180 mg/kg) is ~0.3 mg/kg.(*76*)

In summary, the rationally designed BChE mutants are valuable in development of anti-cocaine therapeutic agents. This dissertation is mainly focused to address the possible concerns in further development of the promising BChE mutants for cocaine abuse treatment, such as whether the administration of this exogenous enzyme will affect the cholinergic system, whether it can efficiently hydrolyze the cocaine's toxic metabolites, whether the commonly used therapeutic agents will significantly affect the catalytic activity of E12-7 against cocaine when they are co-administered. In addition, an effort to discover new mutants with further improved activity led to a more promising therapeutic BChE mutant A199S/F227A/P285Q/S287G/A328W/Y332G (denoted as E20-7). The identified mutants of human BChE are expected to be valuable candidates for development of a more efficient enzyme therapy for cocaine abuse.

Chapter Two: Substrate Selectivity of High-Activity Mutants of Human Butyrylcholinesterase

Summary

Cocaine is one of the most addictive drugs, and there is still no FDA-approved medication specific for cocaine. A promising therapeutic strategy is to accelerate cocaine metabolism producing biologically inactive metabolites *via* a route similar to the primary cocaine-metabolizing pathway, *i.e.* cocaine hydrolysis catalyzed by human BChE in plasma. However, the native BChE has a low catalytic efficiency against the abused cocaine, *i.e.* (-)-cocaine. Our recently designed and discovered mutant E12-7 (A199S/F227A/S287G/A328W/Y332G) and other mutants of human BChE have a considerably improved catalytic efficiency against (-)-cocaine. For the investigation described in this chapter, we carried out both computational modeling and experimental kinetic analysis on the catalytic activities of these promising new BChE mutants against other known substrates, including neurotransmitter acetylcholine (ACh), acetylthiocholine (ATC), butyrylthiocholine (BTC), and (+)-cocaine, in comparison with the corresponding catalytic activity against (-)-cocaine. Both the computational modeling and kinetic analysis have consistently revealed that all of the examined amino-acid mutations only considerably improve the catalytic efficiency of human BChE against (-)-cocaine, without significantly improving the catalytic efficiency of the enzyme against anyone of the other substrates examined. In particular, all of the examined BChE mutants have an even slightly lower catalytic efficiency against neurotransmitter ACh compared to the wild-type BChE. This observation gives us confidence in development of an anti-cocaine enzyme therapy by using one of these BChE mutants, particularly the E12-7 mutant. The studies described in this chapter have been published.(*83*)

2.1 Significance of substrate selectivity of the human BChE mutants

The integrated computational-experimental studies have led to discovery of a series of BChE mutants with a significantly improved catalytic efficiency against (-)-cocaine.(*76, 84-89*) The first one of our designed and discovered high-activity mutants of human BChE, *i.e.* the A199S/S287G/A328W/Y332G mutant (*84*), was validated by an

independent group of scientists who concluded that this mutant is "*a true CocH with a catalytic efficiency that is 1,000-fold greater than wild-type BChE*".(*90, 91*) This BChE mutant is currently in double-blind, placebo-controlled clinical trials in humans by Teva Pharmaceutical Industries Ltd for cocaine abuse treatment.(*17*) Our recently designed and discovered new mutants (*76, 89*) of human BChE are even more active against (-)-cocaine *in vitro* and *in vivo*, without knowing whether these mutations also considerably increase the catalytic efficiencies against other substrates.

An ideal, therapeutically valuable mutant of human BChE should have not only a high catalytic activity against (-)-cocaine, but also certain selectivity for (-)-cocaine over neurotransmitter ACh, the only known natural substrate of BChE in the body, such that one would not expect systemic administration of the BChE mutant to interrupt cholinergic transmission. The catalytic activities of wild-type BChE against neurotransmitter ACh, ATC, BTC, and (+)-cocaine are all much higher than that against (-)-cocaine. So, we wanted to know whether the same amino-acid mutations designed to considerably increase the catalytic activity of BChE against (-)-cocaine also considerably increase the catalytic activities of BChE against other substrates. In this chapter, both computational modeling and experimental kinetic analysis on the catalytic activities of these promising new BChE mutants against (-)-cocaine and other known substrates were performed, in order to evaluate the substrate selectivity of these designed high-activity mutants of human BChE.

2.2 Computational methods

Various substrates interacting with human BChE and its mutants were modeled for their enzyme-substrate binding complexes (denoted as ES's) and transition states for the initial reaction step (denoted as TS1's) by using the same modeling strategy and approach that we used to study (-)-cocaine interacting with the enzymes.(*76, 84-89*) The general strategy of performing an energy minimization or molecular dynamics (MD) simulation on a transition state structure of an enzymatic reaction using a classical force field (molecular mechanics) has been described and justified in our recent reports.(*76, 84, 88, 89, 92*) During the energy minimization or MD simulation on the TS1 structure, the lengths of transition bonds (*i.e.* the covalent bonds that are being broken or formed

gradually during the initial reaction step) were restrained while all other geometric parameters were allowed to move. The transition bond lengths used in our modeling of the TS1 structures for each pair of enzyme and substrate were based on our previously reported molecular modeling and reaction-coordinate calculations on BChE-catalyzed hydrolysis of (-)-cocaine or ACh or ATC.(*76, 88, 93*) Specifically, the transition-bond lengths used in our modeling of the TS1 structures with (−)-cocaine or (+)-cocaine were the same as those in the QM/MM-optimized TS1 geometry for BChE-catalyzed hydrolysis of (-)-cocaine.(*78)* The transition-bond lengths used in our modeling of the TS1 structures with ACh were the same as those in the QM/MM optimized TS1 geometry for BChE-catalyzed hydrolysis of Ach.(*106*) The transition-bond lengths used in our modeling of the TS1 structures with ATC or BTC were the same as those in the QM/MM-optimized TS1 geometry for BChE-catalyzed hydrolysis of ATC.(*93*) The amino acid mutations and the minor structural difference in the substrate were not expected to significantly change the transition-bond lengths based on our previous experience in the transition-state simulations.(*76, 84, 89, 92, 94, 95*) As discussed in our previous computational studies related to the transition-state modeling,(*76, 84, 89, 92, 94, 95*) the computational procedures for modeling a TS1 structure were the same as those for modeling the corresponding ES structure, except for keeping the transition bond lengths restrained during the energy minimization or MD simulation on the TS1 structure. Technically, each transition-bond length in a TS1 structure was restrained by defining a new type of covalent bond whose force constant was one half of the normal covalent bond between the two atoms. It should be pointed out that the sole purpose of performing this type of computational modeling on a transition state was to examine the hydrogen bonding interaction between the carbonyl group of the substrate and the oxyanion hole of the enzyme. We were only interested in the modeled structures, as the total energies calculated in this way would be meaningless. The modeled structures were used to estimate the hydrogen bonding energies (HBE) by using an HBE equation utilized in our earlier studies.(*84*)

The initial structures of BChE and the mutants used in the molecular modeling were prepared on the basis of our previous MD simulation on the enzyme-substrate complex for wild-type BChE binding with (-)-cocaine.(*84, 88, 96*) Our previous MD simulations

on the enzyme-substrate complexes started from the X-ray crystal structure deposited in the Protein Data Bank (pdb code: 1P0P).(*97*) The general procedure for carrying out the MD simulations on the enzyme-substrate interactions in water was essentially the same as that used in our previously reported computational studies on other complexes. Each starting structure was neutralized by adding counter ions (one chloride ion) and was solvated in an orthorhombic box of TIP3P water molecules with a minimum solute-wall distance of 10 Å (which means that the shortest distance between an atom of the enzyme–substrate complex and the boundary of the box is longer than 10 Å). The obtained box sizes of the solvated systems were about 99 Å × 92 Å × 87 Å. All the energy minimizations and MD simulations (using Newton's equations of motion) were performed by using the Sander module of the Amber11 package.(*98*) The solvated systems were carefully equilibrated and fully energy-minimized. First, the solvent molecules were minimized for 5000 steps (including 2500 steps using the steepest descent method and 2500 steps using the conjugate gradient method) with the ligand and enzyme restrained. Second, the solvent, ligand, and side chains of the enzyme were energy-minimized for 1000 steps (including 500 steps using the steepest descent method and 500 steps using the conjugate gradient method) with the backbone of enzyme restrained. Finally the whole system was energy-minimized for 5000 steps (including 2500 steps using the steepest descent method and 2500 steps using the conjugate gradient method).These systems were gradually heated from T = 10 K to T = 298.15 K in 30 ps before running the MD simulation at T = 298.15 K for 1 ns or longer, making sure that we obtained a stable MD trajectory for each of the simulated structures. The time step used for the MD simulations was 2 fs. Periodic boundary conditions in the NPT ensemble at T = 298.15 K with Berendsen temperature coupling and P = 1 atm with isotropic molecule-based scaling were applied. The SHAKE algorithm was used to fix all covalent bonds containing hydrogen atoms. The non-bonded pair list was updated every 10 steps. The particle mesh Ewald (PME) method was used to treat long-range electrostatic interactions. A residue-based cutoff of 10 Å was utilized to the non-covalent interactions. The final snapshot of the stable MD trajectory was fully energy-minimized using the steepest descent method for 5000 steps and then the conjugate gradient method until the convergence criterion for an energy gradient of 0.1 kcal mol^{-1} $Å^{-1}$ was achieved.

2.3 Experimental methods

2.3.1 Enzyme expression

Both wild-type and mutants of human BChE were expressed and their enzyme activities against various substrates were assayed at the same time under the same experimental conditions; the wild-type was used a standard reference and validated according to the catalytic parameters reported for the wild-type in literature. The proteins (wild-type and mutants of BChE) were expressed in human embryonic kidney 293F cells. Cells at the density of ~1 × 10^6 cells/ml were transfected by 293fectin reagent-DNA complexes at the ratio of 2 μl : 1 μg per ml of the cells. Cells were cultured for five more days. The culture medium was harvested for the BChE activity assays.

2.3.2 Kinetic activity of human BChE and its mutants against different substrates

For determining the catalytic activity of the enzymes against (-)-cocaine, we used a sensitive radiometric assay based on toluene extraction of [^{3}H](-)-cocaine labeled on its benzene ring.(*99*) In brief, to initiate the enzymatic reaction, 100 nCi of [^{3}H](-)-cocaine along with (-)-cocaine was mixed with culture medium. The enzymatic reactions proceeded at room temperature (25^{o}C) with varying concentrations of (-)-cocaine. 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. [^{3}H]benzoic acid (a product of (-)-cocaine hydrolysis) was extracted by 1 ml of toluene and measured by scintillation counting. Finally, the measured (-)-cocaine concentration-dependent radiometric data were analyzed by using the standard Michaelis-Menten kinetics so that the catalytic parameters were determined along with the use of a well-established standard enzyme-linked immunosorbent assay (ELISA) protocol.(*84*) The enzyme activity assay with [^{3}H]ACh was similar to the assay with [^{3}H](-)-cocaine. The primary difference is that the enzymatic reaction was stopped by addition of 200 μl of 0.2 M HCl containing 2 M NaCl and that the product was [^{3}H]acetic acid for the ACh hydrolysis. All measurements were performed at room temperature.

The catalytic activities of the enzymes against ATC, BTC, and (+)-cocaine were determined by UV-Vis spectrophotometric assays using a GENios Pro Microplate Reader (TECAN, Research Triangle Park, NC) with the XFluor software. (+)-Cocaine were

dissolved in water to make a 0.1 M stock containing 34 mg/ml. Aliquots were frozen at -20 ℃, thawed once, and discarded. The reaction rate of (+)-cocaine hydrolysis was measured at 25 ℃ by recording the time-dependent absorption at 230 nm. ATC and BTC stock solutions of 0.2 M were prepared in water and frozen at -20 ℃. The reaction rates were measured at 25 ℃ by recording the time-dependent absorption at 450 nm in the presence of 1 mM dithiobisnitrobenzoic acid, in 0.1 M potassium phosphate, pH 7.2.

2.4 Insights from molecular modeling

2.4.1 The interaction of (-)-cocaine with human BChE and its mutants

Molecular modeling enabled us to understand how human BChE and its mutants interact with ACh, ATC, BTC, (+)-cocaine, and (-)-cocaine in the ES and TS1 structures. According to the modeling, for (-)-cocaine interacting with wild-type BChE, there is only one hydrogen bond (H-bond) between the carbonyl oxygen of (-)-cocaine and the oxyanion hole (G116, G117, and A199) in the ES structure, and there are two H-bonds in the TS1 structure, as seen in Figure 2.1A and 2.1B. With the A199S/F227A/S287G/A328W/Y332G mutant (E12-7), there are two H-bonds between the carbonyl oxygen of (-)-cocaine and the oxyanion hole (G116, G117, and S199) in the ES structure and three H-bonds in the TS1 structure, as seen in Figure 2.1C and 2.1D. The extra H-bond in both the ES and TS1 structures is with the hydroxyl group of S199 after the A199S mutation. The modeled ES and TS1 structures suggest that this mutant should have a significantly higher catalytic activity against (-)-cocaine compared to the wild-type BChE.

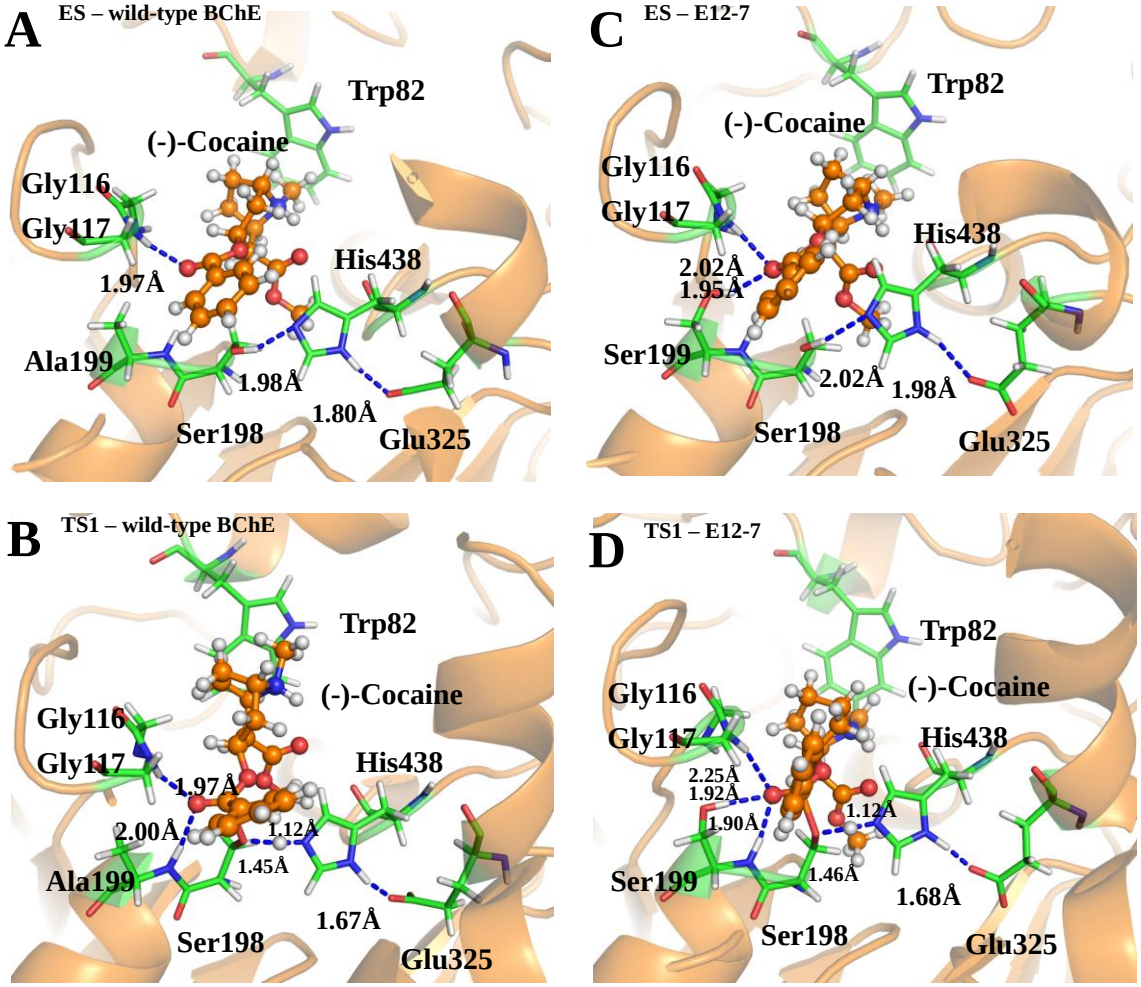


Figure 2.1 The energy-minimized ES and TS1 structures for (-)-cocaine interacting with wild-type human BChE and E12-7.

For (-)-cocaine interacting with other mutants examined, *i.e.* the A199S/A328W/Y332G (E14-1), A199S/F227A/A328W/Y332G (E14-2), A199S/S287G/A328W/Y332G (E14-3), and A199S/F227A/S287G/A328W/E441D (E12-4) mutants, the modeled ES and TS1 structures are all qualitatively similar to the corresponding ES and TS1 structures with the E12-7 in terms of the number of H-bonds. Thus, we only depict the ES and TS1 structures with the E12-7 in Figure 2.1 as a typical example of the five mutants examined. The modeled ES and TS1 structures qualitatively suggest that each of these mutants should have a considerably higher catalytic activity against (-)-cocaine compared to the wild-type BChE.

2.4.2 The interaction of other substrates with human BChE and its mutants

For other substrates (including ACh, ATC, BTC, and (+)-cocaine) interacting with the wild-type BChE or anyone of the aforementioned mutants, there are always two H-bonds between the carbonyl oxygen of the substrate and the oxyanion hole (G116, G117, and A/S199) in the ES structure and three H-bonds in the TS1 structure. Depicted in Figures 2.2 to 2.5 are the modeled ES and TS1 structures with wild-type BChE and a representative mutant E12-7. The hydroxyl group of S199 in the mutant does not form an extra H-bond in the ES or TS1 structure for the mutant interacting with any substrate other than (-)-cocaine, suggesting that these BChE mutants should not have dramatically improved catalytic activities against ACh, ATC, BTC, and (+)-cocaine compared to the wild-type BChE.

Based on the modeling results, the computationally examined five sets of mutations are all expected to considerably improve the catalytic efficiency of human BChE against (-)-cocaine, without considerably improving the catalytic efficiency of human BChE against ACh, ATC, BTC, or (+)-cocaine.

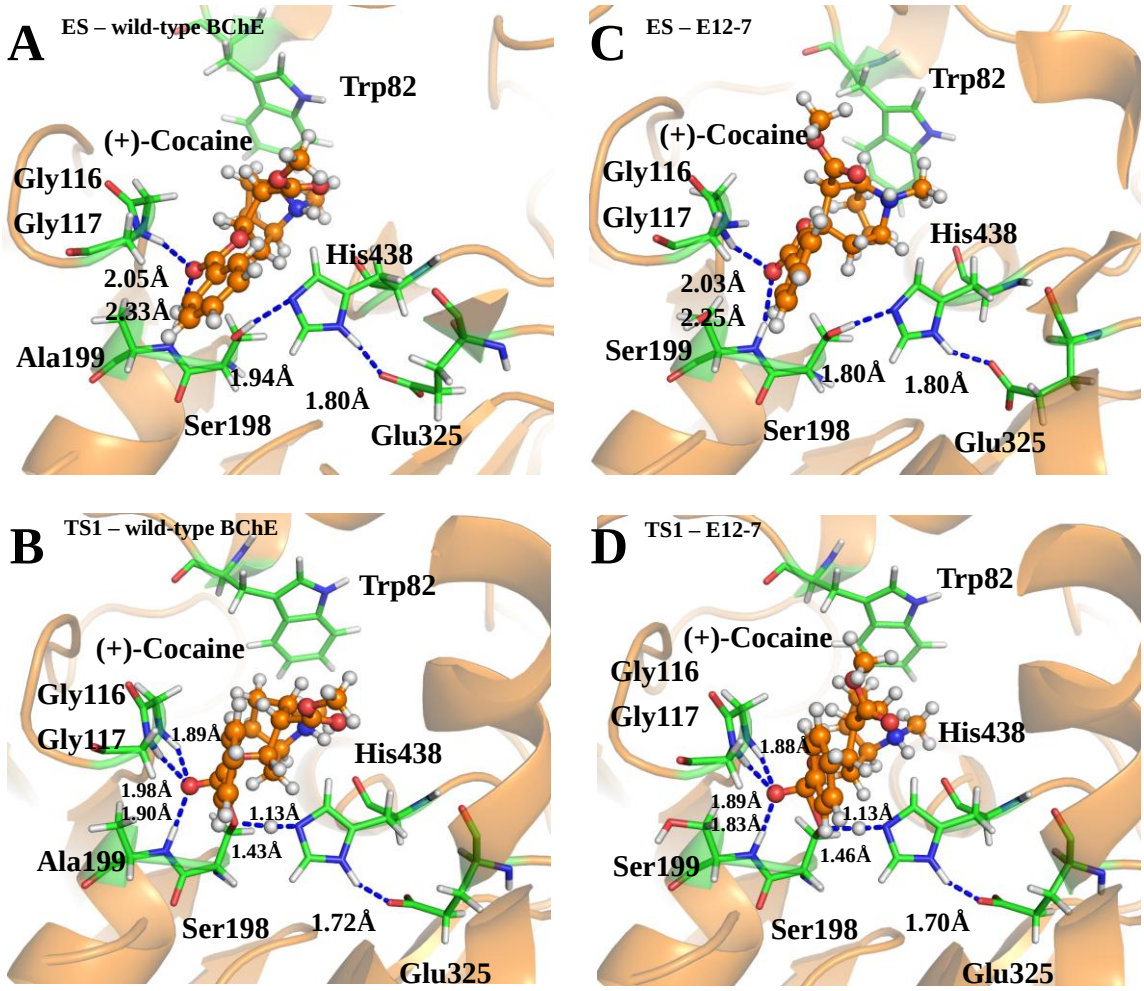


Figure 2.2 The energy-minimized ES and TS1 structures for (+)-cocaine interacting with wild-type human BChE and E12-7.

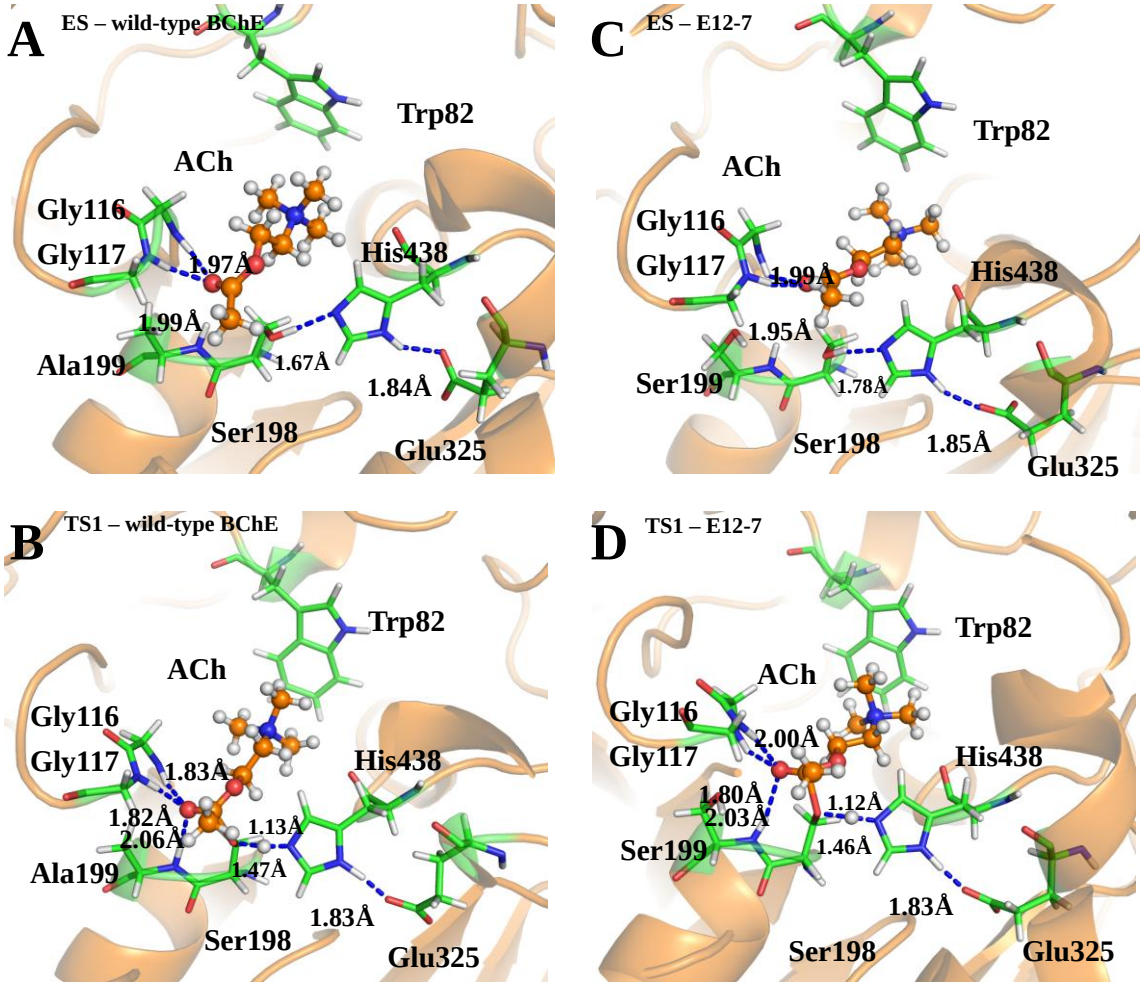


Figure 2.3 The energy-minimized ES and TS1 structures for ACh interacting with wild-type human BChE and E12-7.

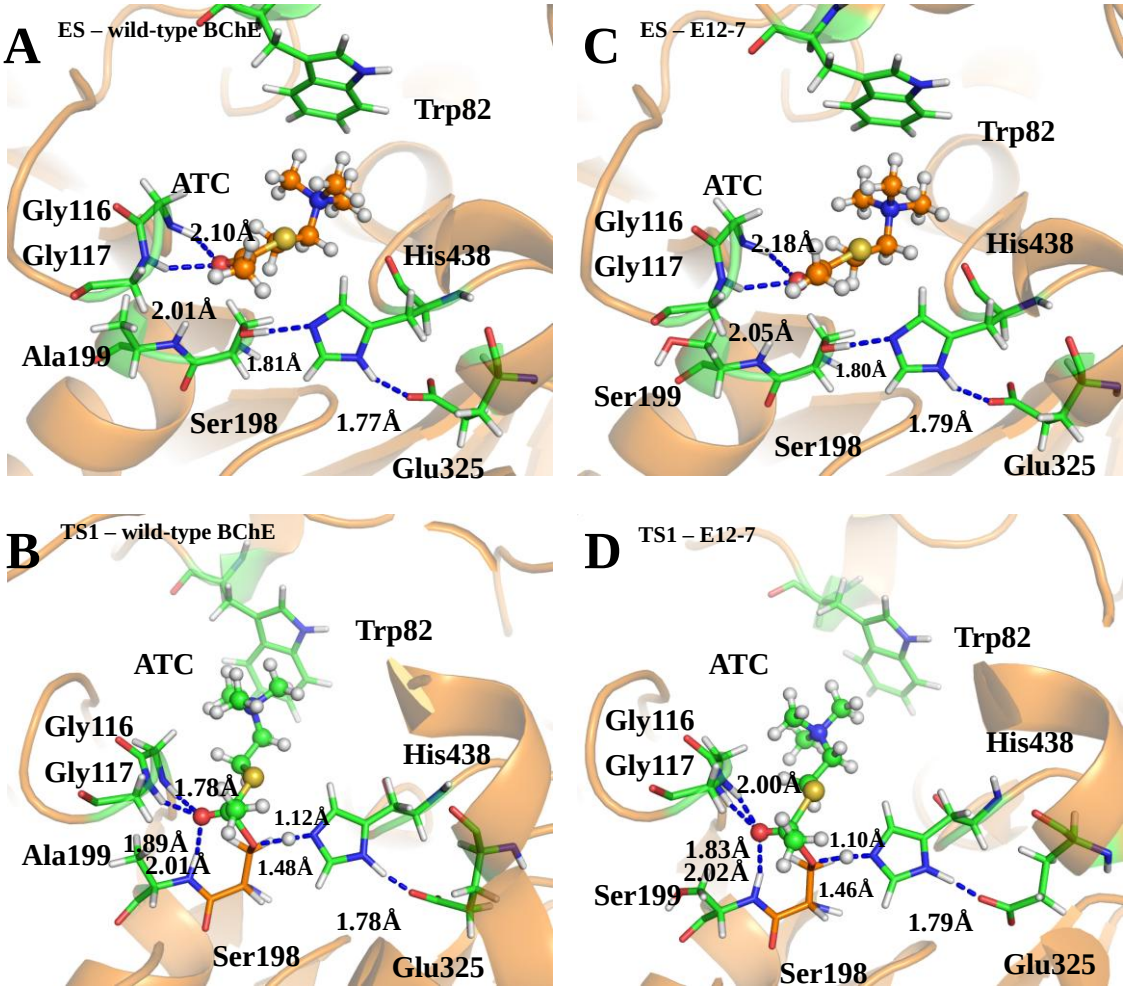


Figure 2.4 The energy-minimized ES and TS1 structures for ATC interacting with wild-type human BChE and E12-7.

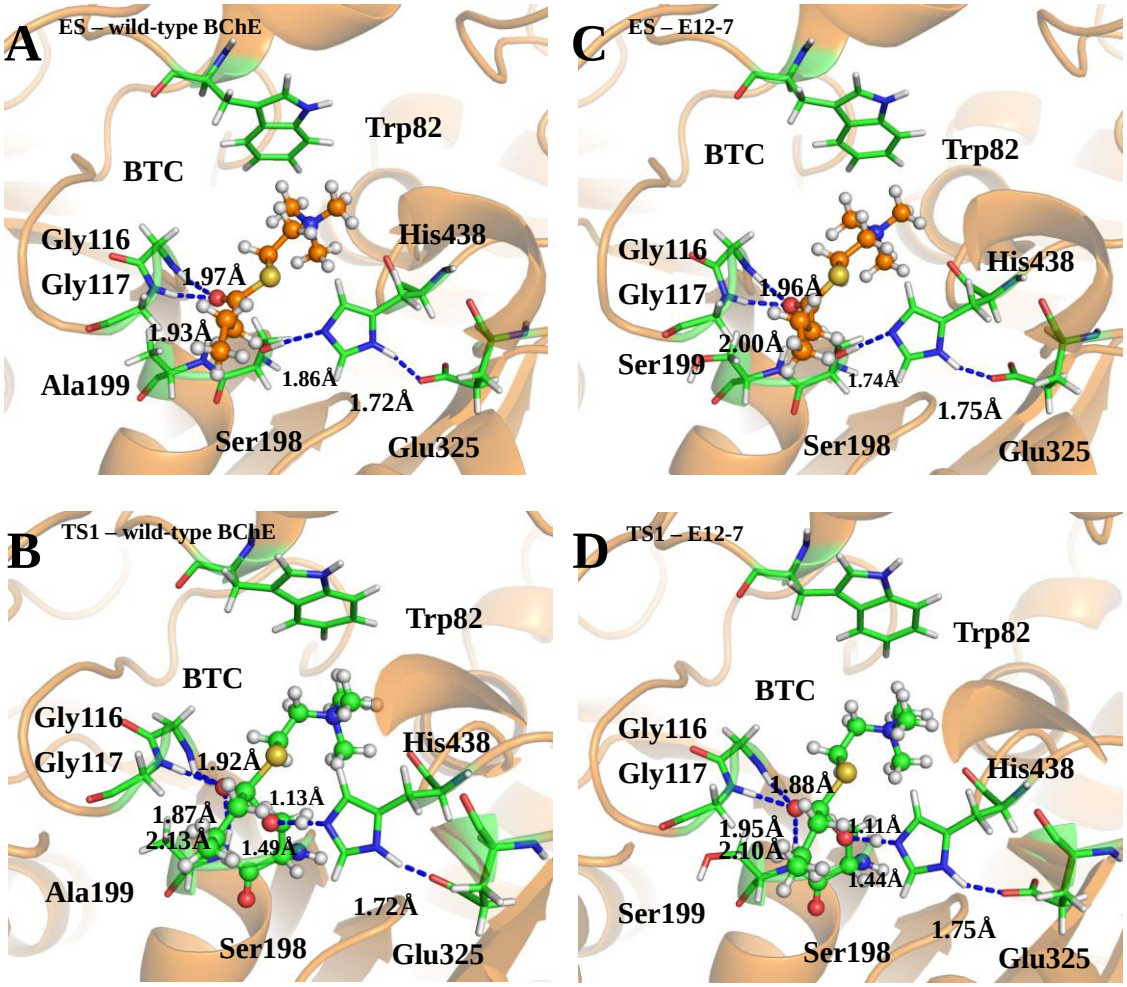


Figure 2.5 The energy-minimized ES and TS1 structures for BTC interacting with wild-type human BChE and E12-7.

2.5 Kinetic parameters

Based on the computational insights, we carried out *in vitro* experimental tests, including the protein expression and enzyme activity assays, on the A199S/A328W/Y332G (E14-1), A199S/F227A/A328W/Y332G (E14-2), A199S/S287G/A328W/Y332G (E14-3), A199S/F227A/S287G/A328W/E441D (E12-4), and A199S/F227A/S287G/A328W/Y332G (E12-7) mutants. To minimize the possible systematic experimental errors of the kinetic data, we also expressed the wild-type enzyme and performed the kinetic analysis along with the mutants under the same experimental conditions, and compared the catalytic efficiencies of the mutants to the

corresponding catalytic efficiencies of the wild-type enzyme against various substrates. Depicted in Figures 2.6 to 2.10 are the measured kinetic data. Summarized in Table 1 are the determined kinetic parameters.

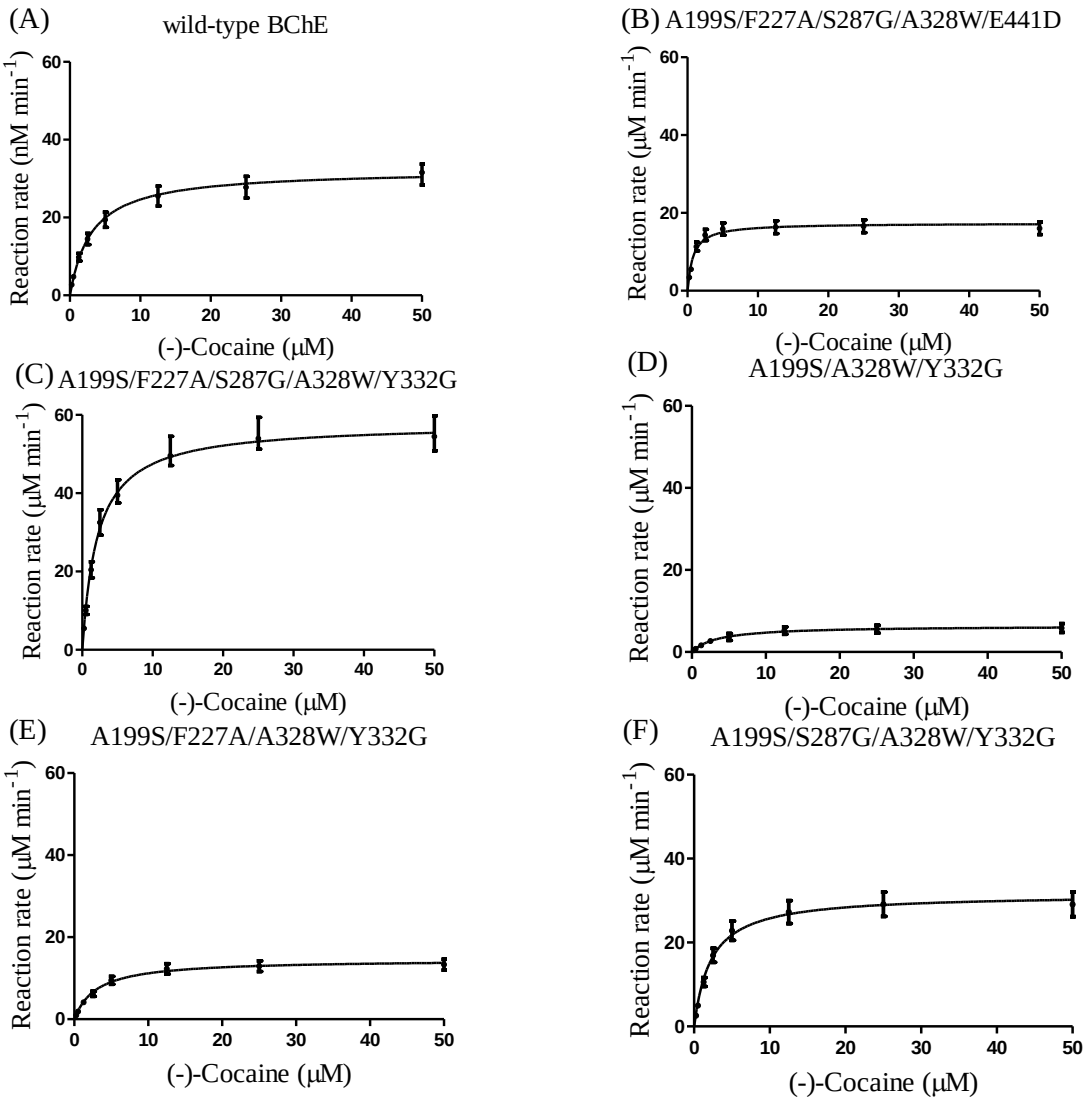


Figure 2.6 Kinetic data for (-)-cocaine hydrolysis catalyzed by wild-type human BChE and various BChE mutants.

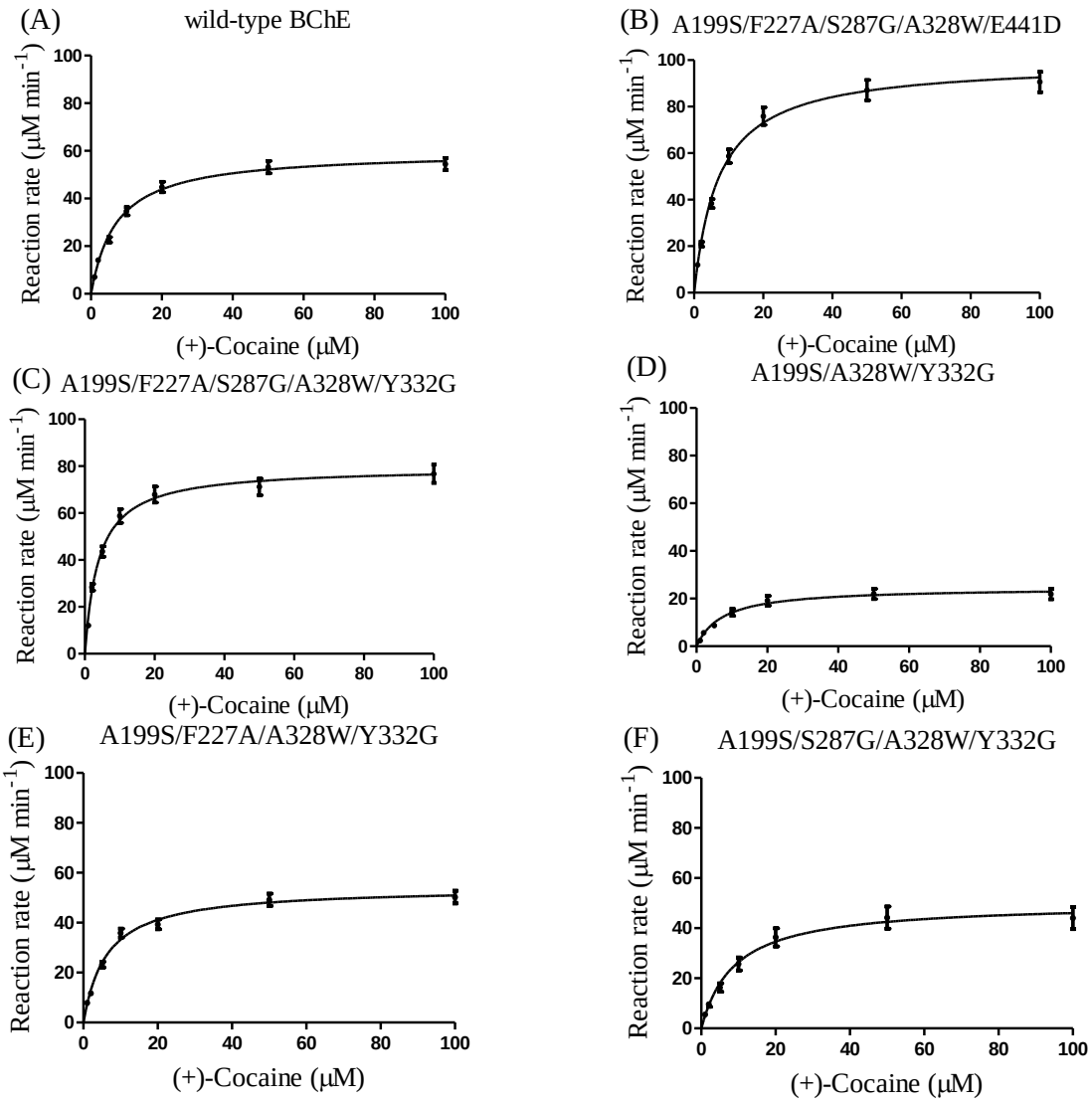


Figure 2.7 Kinetic data for (+)-cocaine hydrolysis catalyzed by wild-type human BChE and various BChE mutants.

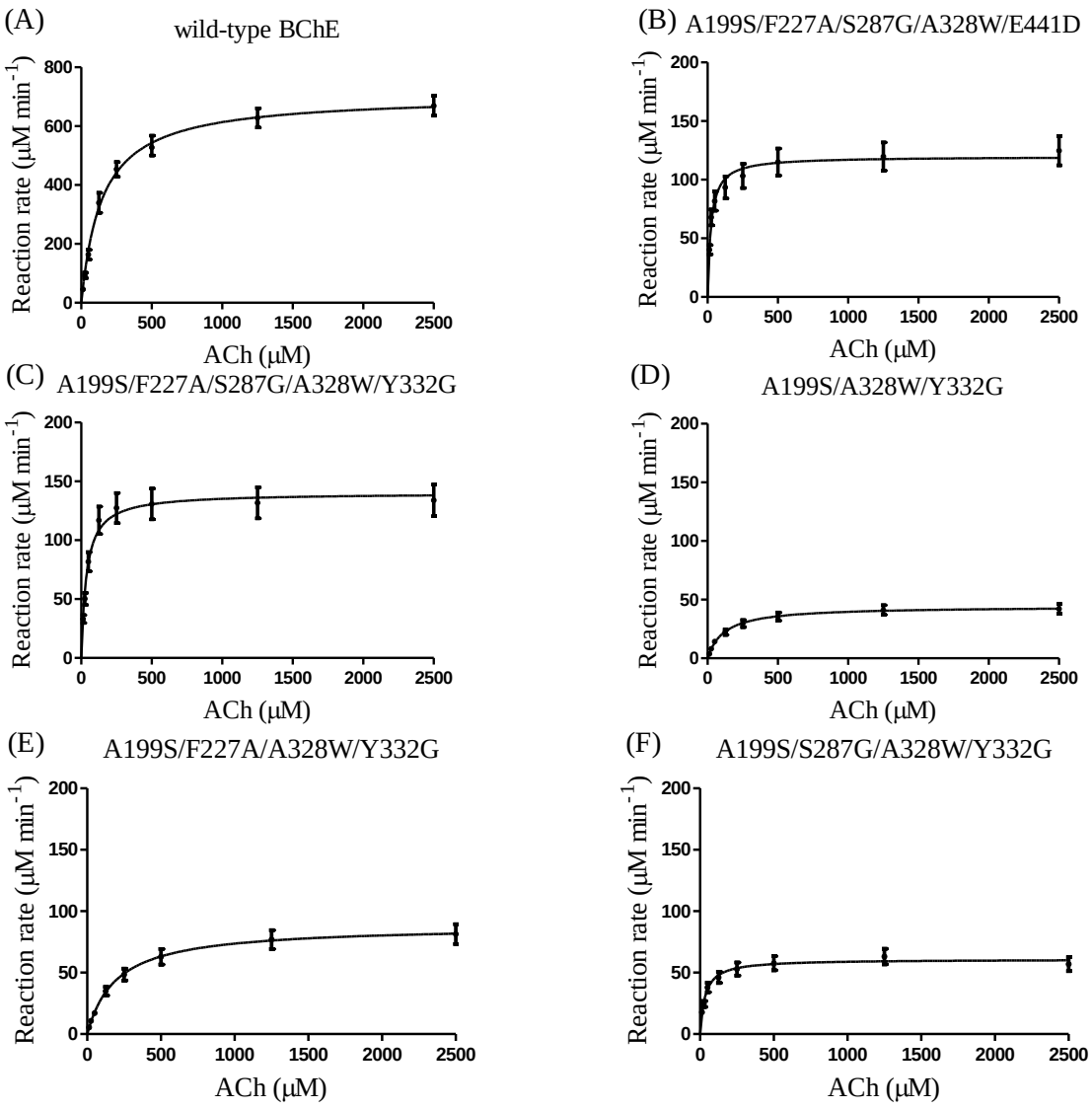


Figure 2.8 Kinetic data for ACh hydrolysis catalyzed by wild-type human BChE and various BChE mutants.

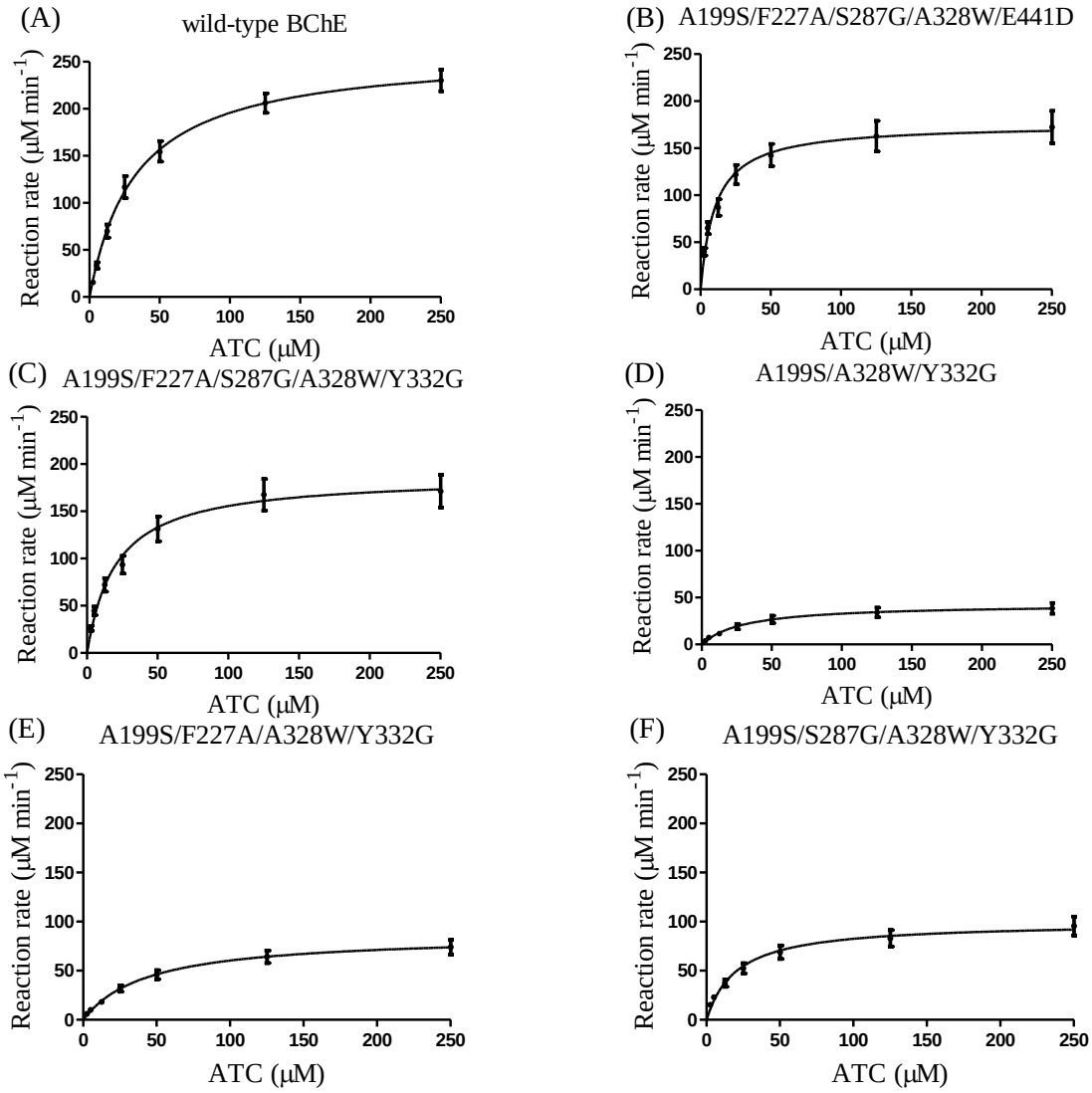


Figure 2.9 Kinetic data for ATC hydrolysis catalyzed by wild-type human BChE and various BChE mutants.

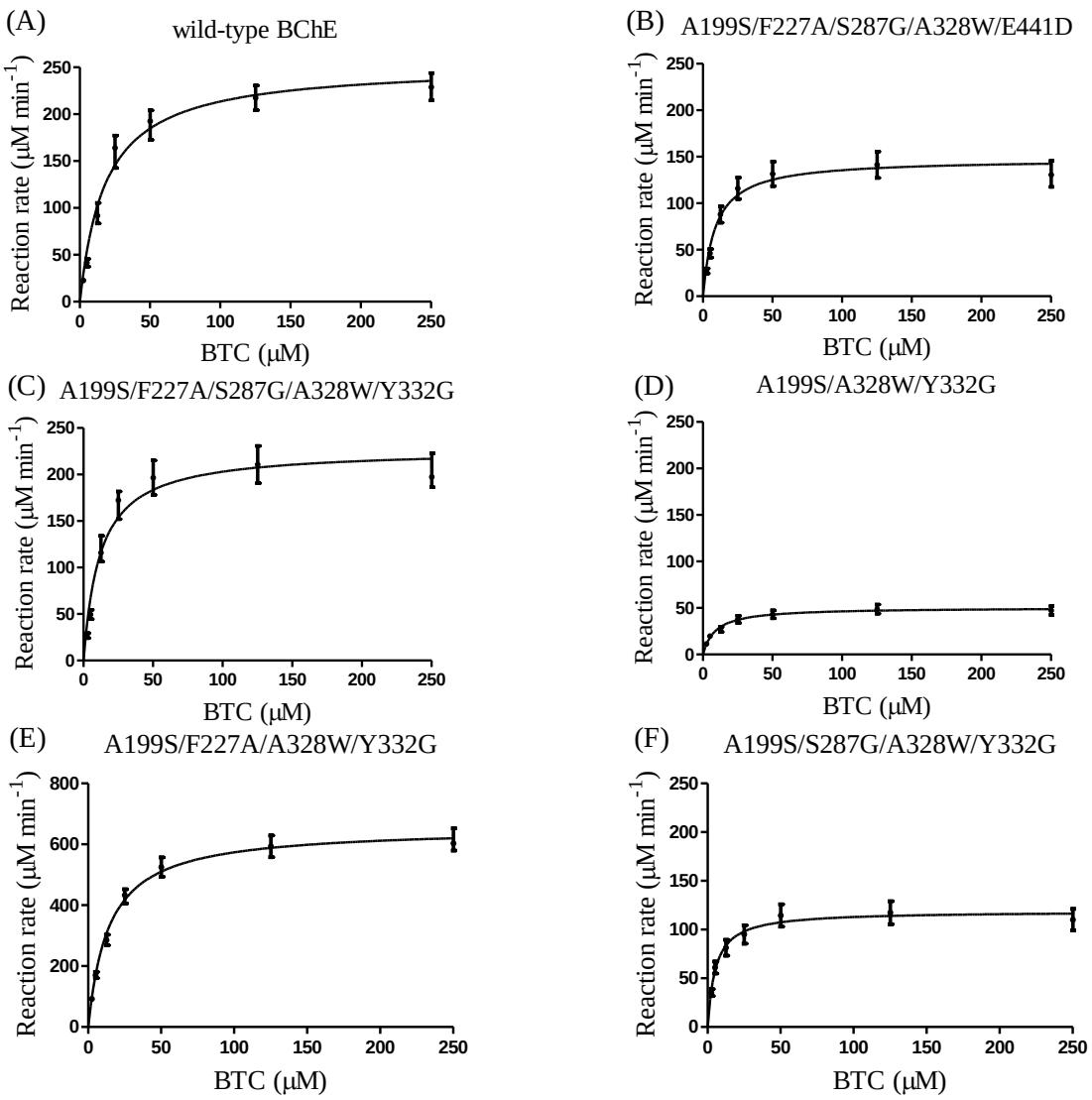


Figure 2.10 Kinetic data for BTC hydrolysis catalyzed by wild-type human BChE and various BChE mutants.

Table 2.1 Kinetic parameters determined for (-)-cocaine, (+)-cocaine, ACh, ATC, and BTC hydrolyses catalyzed by wild-type BChE and its mutants.

Substrate	Enzyme [a]	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M ($M^{-1}min^{-1}$)	RCE[b]
(-)-cocaine	wild-type BChE[c]	4.5	4.1	9.1×10^5	1
	A199S/A328W/Y332G	5.1	560	1.1×10^8	121
	A199S/F227A/A328W/Y332G	4.4	1560	3.6×10^8	396
	A199S/S287G/A328W/Y332G	3.1	3,060	9.9×10^8	1,080
	A199S/F227A/S287G/A328W/E441D	1.1	1,730	1.6×10^9	1,730
	A199S/F227A/S287G/A328W/Y332G	3.1	5,700	1.8×10^9	2,020
(+)-cocaine	wild-type BChE	4.7	6,420	1.4×10^9	1
	A199S/A328W/Y332G	5.0	2,820	5.6×10^8	0.40
	A199S/F227A/A328W/Y332G	4.4	6,060	1.4×10^9	1.01
	A199S/S287G/A328W/Y332G	6.3	5,620	8.9×10^8	0.65
	A199S/F227A/S287G/A328W/E441D	4.7	10,800	2.3×10^9	1.68
	A199S/F227A/S287G/A328W/Y332G	4.6	8,990	2.0×10^9	1.43
ACh	wild-type BChE[d]	148	61,200	4.1×10^8	1
	A199S/A328W/Y332G	156	4,190	2.7×10^7	0.066
	A199S/F227A/A328W/Y332G	189	7,430	3.9×10^7	0.095
	A199S/S287G/A328W/Y332G	36	5,320	1.5×10^8	0.37
	A199S/F227A/S287G/A328W/E441D	27	10,400	3.9×10^8	0.95
	A199S/F227A/S287G/A328W/Y332G	37	11,900	3.2×10^8	0.78

Table 2.1 (continued)

ATC	wild-type BChE[e]	33	20,200	6.1×10^8	1
	A199S/A328W/Y332G	31	3,410	1.1×10^8	0.18
	A199S/F227A/A328W/Y332G	41	6,870	1.6×10^8	0.26
	A199S/S287G/A328W/Y332G	21	7,880	3.7×10^8	0.60
	A199S/F227A/S287G/A328W/E441D	12	14,000	1.2×10^9	1.99
	A199S/F227A/S287G/A328W/Y332G	20	14,800	7.2×10^8	1.19
BTC	wild-type BChE[f]	17	29,500	1.7×10^9	1
	A199S/A328W/Y332G	8.9	6,100	6.8×10^8	0.39
	A199S/F227A/A328W/Y332G	11	74,700	6.8×10^9	3.91
	A199S/S287G/A328W/Y332G	5.3	14,400	2.7×10^9	1.57
	A199S/F227A/S287G/A328W/E441D	8.9	17,800	2.0×10^9	1.15
	A199S/F227A/S287G/A328W/Y332G	13	28,000	2.2×10^9	1.24

[a]Unless indicated otherwise, all kinetic parameters listed in this table were determined in the present study.
[b]RCE refers to the relative catalytic efficiency (k_{cat}/K_M), *i.e*. the ratio of the k_{cat}/K_M value of the mutant to that of wild-type BChE against the same substrate.
[c]Data for wild-type BChE from reference.(*79*)
[d]The k_{cat} value for wild-type BChE was reported in reference.(*100*)
[e]The k_{cat} value for wild-type BChE from reference.(*101*)
[f]The k_{cat} value for wild-type BChE was reported in reference.(*101*)

Based on the kinetic parameters summarized in Table 2.1, all of the five BChE mutants examined in this study have a considerably improved catalytic efficiency (k_{cat}/K_M) against (-)-cocaine, with the improvement ranging from 121 to 2020-fold. The same mutations do not dramatically improve the catalytic efficiencies of human BChE against the other substrates. Within the five mutants examined, the values of the relative catalytic efficiency (RCE), *i.e.* the ratio of the catalytic efficiency of the mutant to that of the wild-type BChE, range from 0.4 to 1.68 for (+)-cocaine, 0.066 to 0.95 for ACh, 0.18 to 1.99 for ATC, and 0.39 to 3.91 for BTC. The largest RCE value is associated with the

A199S/F227A/S287G/A328W/E441D mutant for both (+)-cocaine (1.68-fold) and ATC (1.99-fold), and the A199S/F227A/A328W/Y332G mutant for BTC (3.91-fold).

For ACh (which is the only known endogenous substrate of BChE in the body), all of the BChE mutants actually have a slightly lower catalytic efficiency compared to the wild-type BChE. So, all of these mutants only have a considerably improved catalytic efficiency against (-)-cocaine without any improvement on the catalytic efficiency of BChE against ACh. As a result, the catalytic efficiencies of these BChE mutants against (-)-cocaine are all higher than the corresponding catalytic efficiencies against ACh, as seen in Table 2.1.

The observed substrate selectivity of these mutants may be used to address a potential question concerning whether the enzyme therapy using a high-activity mutant of human BChE would significantly affect the cholinergic transmission and, thus, produce adverse effects. In fact, previous studies evaluating wide-type human BChE as a prophylaxis against chemical warfare nerve agents found no autonomic or motor impairment in rats, guinea pigs, or primates, even with the high doses raising the plasma enzyme levels for 50 to 100-fold.(*102*-*107*) This is not surprising due to several factors. First, the molar concentrations of acetylcholinesterase (AChE) and BChE in blood are roughly similar (*108*) and BChE has a lower catalytic efficiency against ACh compared to AChE. Second, cholinergic synapses in the brain are insulated from plasma enzymes by the blood-brain barrier and, thus, the exogenous enzymes in plasma would not reach the brain. In addition, peripheral cholinergic synapses are densely packed with AChE. It has been known that mouse neuromuscular junction has 5×10^{19} catalytic AChE subunits per cc, *i.e.* ~0.1 mM (*109*), whereas mouse plasma BChE levels are below 0.1 μM (*108*). For these reasons, even high levels of plasma BChE activity are unlikely to affect motor transmission. The current observation that none of these high-activity mutants of human BChE has an improved catalytic efficiency against ACh compared to the wild-type BChE gives us additional confidence in development of an enzyme therapy by using one of these BChE mutants, particularly the A199S/F227A/S287G/A328W/Y332G mutant with the highest catalytic efficiency against (-)-cocaine.

2.6 Conclusion

Both the computational modeling and experimental kinetic analysis have consistently revealed that all of the five BChE mutants examined in this study only have a considerably improved catalytic efficiency against (-)-cocaine, without dramatically improving the catalytic efficiency against any of the other substrates examined compared to the wild-type BChE. In particular, all of these BChE mutants have an even slightly lower catalytic efficiency against ACh compared to the wild-type BChE. The observation of the substrate selectivity gives us additional confidence in development of an enzyme therapy by using one of these BChE mutants, particularly the A199S/F227A/S287G/A328W/Y332G mutant (E12-7) with the highest catalytic efficiency against (-)-cocaine.

Chapter Three: Kinetic Characterization of Human Butyrylcholinesterase Mutants for Hydrolysis of Cocaethylene

Summary

It is known that majority of cocaine users also consume alcohol. Alcohol can react with cocaine to produce a significantly more cytotoxic compound, cocaethylene. Hence, a truly valuable cocaine-metabolizing enzyme for cocaine abuse/overdose treatment should be efficient for not only cocaine itself, but also cocaethylene. The catalytic parameters (k_{cat} and K_M) of human BChE and two mutants (E14-3 and E12-7) for cocaethylene have been characterized in this chapter, for the first time, in comparison with those for cocaine. Based on the obtained kinetic data, wild-type human BChE has a lower catalytic activity for cocaethylene (k_{cat} = 3.3 min^{-1}, K_M = 7.5 μM, and k_{cat}/K_M = 4.40 × 10^5 M^{-1} min^{-1}) compared to its catalytic activity for (-)-cocaine. E14-3 and E12-7 have a considerably improved catalytic activity against cocaethylene compared to the wild-type BChE. E12-7 is identified as the most efficient enzyme for hydrolyzing cocaethylene in addition to its high activity for (-)-cocaine. E12-7 has an 861-fold improved catalytic efficiency for cocaethylene (k_{cat} = 3600 min^{-1}, K_M = 9.5 μM, and k_{cat}/K_M = 3.79 × 10^8 M^{-1} min^{-1}). It has been demonstrated that E12-7 as an exogenous enzyme can indeed rapidly metabolize cocaethylene in rats. Further kinetic modeling has suggested that E12-7 with an identical concentration as that of the endogenous BChE in human plasma can effectively eliminate (-)-cocaine, cocaethylene, and norcocaine in simplified kinetic models of cocaine abuse and overdose associated with the concurrent use of cocaine and alcohol. The studies described in this chapter have been published.(*110*)

3.1 Overview of possible treatment of combined cocaine-alcohol abuse using high-activity mutants

In humans, cocaine is metabolized through hydrolysis catalyzed by plasma enzyme BChE that catalyzes hydrolysis at the benzoyl ester group (Figure 3.1), hydrolysis by two liver carboxylesterases (hCE-1 and hCE-2) that catalyze hydrolysis at the methyl ester and the benzoyl ester, respectively, and oxidation by liver microsomal cytochrome P450 (CYP) 3A4 to produce norcocaine which has similar physiological effects as of

cocaine.(*60, 111*) BChE-catalyzed hydrolysis of cocaine at the benzoyl ester is the primary cocaine-metabolizing pathway which is most suitable for amplification. Unfortunately, wild-type BChE has a low catalytic efficiency against naturally occurring (-)-cocaine (k_{cat} = 4.1 min^{-1} and K_M = 4.5 μM).(*74, 79, 96, 112, 113*)

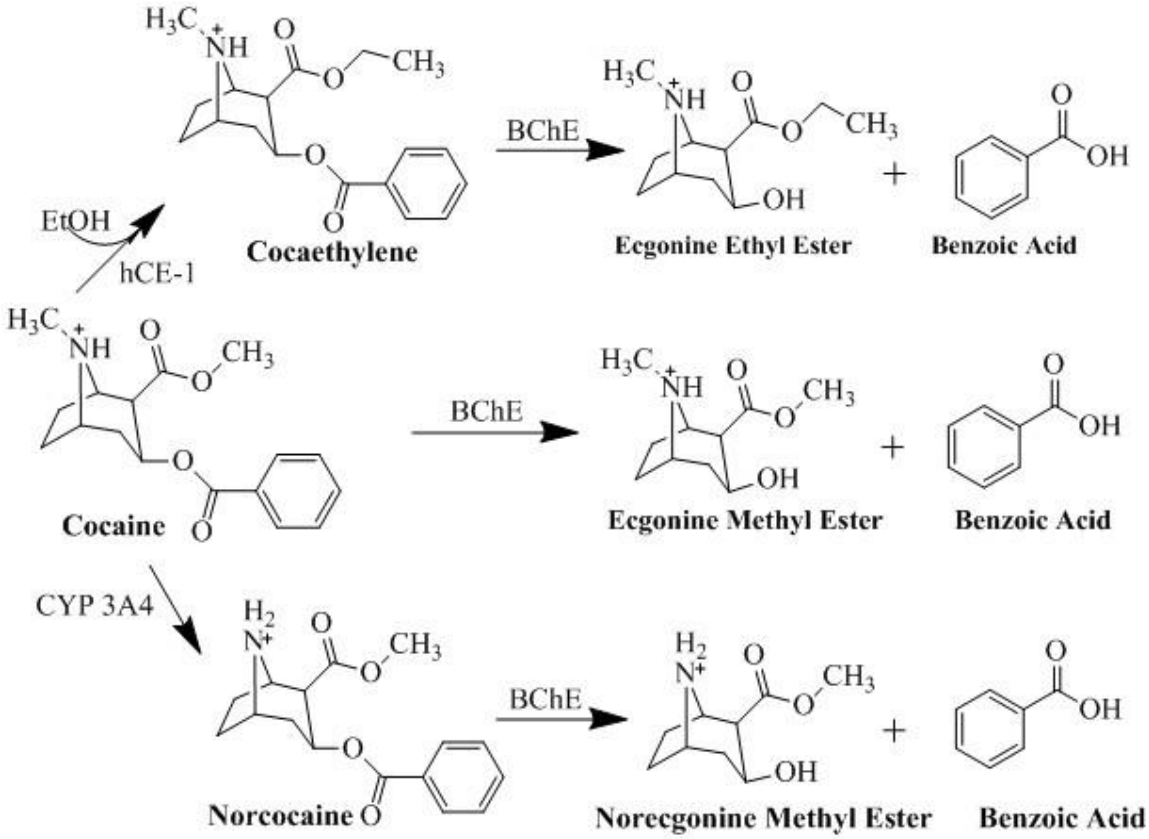


Figure 3.1 Cocaine metabolites produced in humans through hydrolysis by BChE, oxidation by cytochrome P450 (CYP) 3A4, and reaction of cocaine with alcohol (catalyzed by liver hCE-1).

As an additional challenge to cocaine abuse treatment, statistical data report that the majority of cocaine users (*e.g.* 92% as of August 2013) (*114*) also consume alcohol (which always refers to ethanol in this report). Alcohol can react with cocaine under hCE-1 catalysis to produce a significantly more cytotoxic compound, cocaethylene, through transesterification. With alcohol co-administration, ~24% (intravenous), ~34% (oral), or ~18% (smoked) of cocaine is converted to cocaethylene through transesterification.(*115*)

Hence, a truly valuable mutant of human BChE for anti-cocaine enzyme therapy development should be efficient for not only cocaine, but also norcocaine and cocaethylene.

Our computationally designed mutations of human BChE have led to at least 1000-fold improved catalytic efficiency against (-)-cocaine (*76, 84-89*) and norcocaine (*116*). The first one of our designed high-activity mutants of human BChE, *i.e*. the A199S/S287G/A328W/Y332G mutant (*84*), has been recognized as a *true* cocaine hydrolase (CocH) suitable for testing in humans.(*90, 91*) The A199S/S287G/A328W/Y332G mutant is currently in double-blind, placebo-controlled clinical trials in humans by Teva Pharmaceutical Industries Ltd for cocaine abuse treatment.(*17*) Our more recently designed new mutants (*76, 89*) of human BChE are even more effective against (-)-cocaine. However, it has been unknown whether any of these mutants can also catalyze the hydrolysis of cocaethylene. To our best knowledge, we have not seen a report on the kinetic parameters for cocaethylene hydrolysis catalyzed by wild-type human BChE or any of these BChE mutants. What has been known in literature is that cocaethylene produces more euphoria and possesses a longer half-life than that of cocaine.(*115, 117-120*)

One might reasonably expect that the BChE mutants with a considerably improved catalytic efficiency against cocaine should also have a considerably improved catalytic efficiency against cocaethylene. However, the recently reported kinetic analysis of the BChE mutants against acetylcholine (ACh), the only known natural substrate of BChE in the body, revealed that the mutations did not improve the catalytic efficiency of BChE against ACh.(*83, 88*) In fact, the catalytic efficiency of the examined BChE mutants against ACh is slightly lower than that of the wild-type BChE. So, it is unknown whether any of the BChE mutants reported so far has a significantly improved catalytic efficiency against cocaethylene compared to the wild-type BChE.

In the present study, we have characterized the catalytic activity of wild-type human BChE and our discovered A199S/S287G/A328W/Y332G mutant (denoted as E14-3 for convenience) and A199S/F227A/S287G/A328W/Y332G mutant (denoted as E12-7 for convenience) of human BChE against cocaethylene, in comparison with the corresponding catalytic activities against (-)-cocaine and norcocaine. The obtained kinetic

data have demonstrated that the BChE mutants examined in this study have not only a considerably improved catalytic efficiency against (-)-cocaine and norcocaine, but also a considerably improved catalytic efficiency against cocaethylene *in vitro* and *in vivo* compared to the wild-type BChE. Further kinetic modeling has demonstrated that these BChE mutants can effectively hydrolyze (-)-cocaine, cocaethylene and norcocaine at the same time in simplified kinetic models of combined cocaine-alcohol abuse.

3.2 Materials and methods

3.2.1 Molecular modeling

Cocaethylene binding with human BChE and mutants was modeled by using our previously simulated structures of the same enzymes.(*43, 76, 84-87, 89*) Our previous molecular dynamics (MD) simulations(*88*) on the structures of enzyme-cocaine/norcocaine complexes (*116*) started from the X-ray crystal structure deposited in the Protein Data Bank (pdb code: 1P0P). For each enzyme (human BChE or mutant), cocaethylene was docked into the possible active site of the enzyme by using the AutoDock 4.2 program (*121*), as we previously did for the enzyme binding with (-)-cocaine and norcocaine.(*116*) During the docking process, the Solis and Wets local search method (*122*) was used for the conformational search and the Lamarkian genetic algorithm (LGA) (*121*) was employed to deal with the enzyme-ligand interactions. The grid size was set to be 120 × 120 × 120. The finally obtained enzyme-cocaethylene binding structures were the ones with the lowest binding free energies.

3.2.2 Enzyme preparation and *in vitro* activity assays

Both wild-type and mutants of human BChE were expressed and their enzyme activities against cocaethylene and (-)-cocaine were assayed at the same time under the same experimental conditions so that the activity against cocaethylene can be compared with that against (-)-cocaine for each enzyme. For the purpose of *in vitro* activity assays, the proteins (wild-type human BChE and mutants) were expressed in human embryonic kidney (HEK) 293F cells. Cells at the density of ~1 × 10^6 cells/ml were transfected by 293fectin reagent-DNA complexes at the ratio of 2 μl : 1 μg per ml of the cells. Cells were cultured for five more days. The culture medium was harvested, and the protein was

purified by using a two-step purification procedure (ion exchange chromatography followed by affinity chromatography), as described previously in detail.(*116*) The purified protein was dialyzed against phosphate-buffered saline and stored at 4 ℃ or -80 ℃.

The catalytic activities of the enzymes against cocaethylene and (-)-cocaine were determined by performing a UV-Vis spectrophotometric assay. Using the UV-Vis spectrophotometric assay, the catalytic activities of the enzymes against cocaethylene and (-)-cocaine were determined at the same time under the same experimental conditions. The enzymatic reaction was initiated by adding 180 μl of a substrate (cocaethylene or (-)-cocaine) solution to 20 μl of an enzyme solution. The final initial cocaethylene/(-)-cocaine concentrations were as follows: 100, 50, 20, 10, 5, 2, and 1 μM. The reaction temperature was 25 ℃, and the buffer used was 0.1 M potassium phosphate (pH 7.4). The initial rates of the enzymatic hydrolysis of cocaethylene/(-)-cocaine in various initial substrate concentrations were estimated by following the change in the intrinsic absorbance peak of cocaethylene/(-)-cocaine at 230 nm (see below for the UV-Vis absorption spectra) with time using a GENios Pro Microplate Reader (TECAN, Research Triangle Park, NC) with the XFluor software. The initial reaction rates were estimated 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 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 ℃. Each rat was used only once. 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.

3.2.4 Characterization of cocaethylene clearance accelerated by E12-7

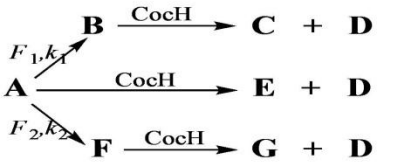
Cocaethylene (formulated in the salt form of cocaethylene fumarate) was provided by the National Institute on Drug Abuse (NIDA) Drug Supply Program (Bethesda, MD), and the E12-7 material used for *in vivo* studies in rats were prepared in our recently reported study (*123*) developing and using stable CHO-S cells. Our *in vitro* assays revealed that E12-7 expressed in the CHO-S cells had the same catalytic activities of that expressed in HEK 293F cells. General anesthetic isoflurane was utilized with nose cone during the administration of cocaethylene and E12-7 (or saline). Rats were injected with saline or 0.15 mg/kg of E12-7 through the tail vein 1 min before i.v. injection of 3 mg/kg cocaethylene (~7 μmol/kg). Four rats were used for each set of experiments (n=4), About 50 to 75 μl of blood from saphenous veins was collected into capillary tubes and immediately diluted in 100 μl of 250 μM paraoxon at 2, 5, 15, 30, 60, 90, 120, 150, and 180 min after the i.v. injection of cocaethylene. Paraoxon is an irreversible BChE inhibitor that can stop the enzymatic hydrolysis of cocaethylene between sampling and analysis. The diluted blood samples were stored at -70 ℃ and assayed using a High-Performance Liquid Chromatographic (HPLC) method.

Benzoic acid is the product of cocaethylene hydrolysis catalyzed by the enzyme (wild-type BChE or E12-7). The standard benzoic acid for the HPLC analysis was purchased through Sigma Aldrich (Sigma Aldrich, St. Louis, MO). To assay the cocaethylene and benzoic acid concentrations in the blood samples, the frozen whole blood samples were thawed on ice for 3 hours. Then 150 μl of mobile phase (26% acetonitrile and 74% water containing 0.1% TFA) was mixed with each sample, and 50 μl of 10% $HClO_4$ was added to break the blood cell membrane. The mixture was vortexed for 1 min and then centrifuged at 25,000 g for 15 min, and the supernatant was transferred to an autosampler vial of which 200 μl was injected into the chromatographic system. Chromatography was performed using a Waters 1525 binary HPLC pump (Waters Corporation, Milford, MA), a Waters 2487 dual λ absorbance detector, a Waters 2475 multi λ fluorescence detector, and a Waters 717 plus autosampler. The flow rate was 1 ml/min. The eluent was monitored at 230 nm for absorbance of benzoic acid and at 315 nm for fluorescence of cocaethylene while exciting at 230 nm. The cocaethylene peaks appeared at 11.6 min, and the benzoic acid peaks occurred at 12.7 min. The

concentrations of cocaethylene and benzoic acid were determined by comparing the corresponding HPLC peak areas with those of authentic standards.

3.2.5 Kinetic modeling

Kinetic modeling of (-)-cocaine in humans was performed by use of a MatLab program (developed in house) (*116*, *124*, *125*) in a way similar to that of our recently developed pharmacokinetic modeling of (-)-cocaine in the presence of a cocaine-metabolizing enzyme.(*82, 116*) The previously used kinetic models did not involve cocaethylene. By using a one-compartment model, the present kinetic modeling also accounted for the transformation of (-)-cocaine to cocaethylene and the subsequent cocaethylene hydrolysis in the presence of a cocaine-metabolizing enzyme. Given in Figure 3.2 are the reaction scheme and kinetic equations used in the present study. In the kinetic model depicted in Figure 3.2, the function of alcohol in the transesterification is similar to a co-factor of hCE-1, and the kinetic modeling is based on the assumption that the alcohol concentration is high enough to reach the saturation in which the reaction rate no longer can increase with further increasing the alcohol concentration.



A: Cocaine
B: Cocaethylene
C: Ecgonine Ethyl Ester
D: Benzoic Acid
E: Ecgonine Methyl Ester
F: Norcocaine
G: Norecgonine Methyl Ester

$$\frac{dA(t)}{dt} = -F_1 A(t)/(k_1 + A(t)) - F_2 A(t)/(k_2 + A(t)) - V_{max} A(t)/(K_M + A(t))$$

$$\frac{dB(t)}{dt} = F_1 A(t)/(k_1 + A(t)) - V'_{max} B(t)/(K'_M + B(t))$$

$$\frac{dC(t)}{dt} = V'_{max} B(t)/(K'_M + B(t))$$

$$\frac{dD(t)}{dt} = V_{max} A(t)/(K_M + A(t)) + V'_{max} B(t)/(K'_M + B(t)) + V''_{max} F(t)/(K''_M + F(t))$$

$$\frac{dE(t)}{dt} = V_{max} A(t)/(K_M + A(t))$$

$$\frac{dF(t)}{dt} = F_2 A(t)/(k_2 + A(t)) - V''_{max} F(t)/(K''_M + F(t))$$

$$\frac{dG(t)}{dt} = V''_{max} F(t)/(K''_M + F(t))$$

Figure 3.2 Reaction scheme and kinetic equations used in the kinetic modeling. *X(t)* is the concentration of **X** at time *t* (**X** = **A** to **G**). $V_{max} = k_{cat}[E]$ (for cocaine hydrolysis), $V'_{max} = k'_{cat}[E]$ (for cocaethylene hydrolysis), and $V''_{max} = k''_{cat}[E]$ (for norcocaine hydrolysis) in

which [E] is the concentration of the enzyme (CocH) hydrolyzing all of the three substrates (cocaine, cocaethylene and norcocaine). F_1 and k_1 represent the kinetic parameters for the transesterification reaction of cocaine with alcohol (catalyzed by hCE-1) to produce cocaethylene when the alcohol concentration is high enough to reach the saturation; in this reaction, the function of alcohol is similar to that of a co-factor. F_2 and k_2 refer to the kinetic parameters for cocaine oxidation (catalyzed by cytochrome P450 3A4) to norcocaine. V'_{max}, $V''_{max,}$ K'_M, and K''_M values used in the modeling were based on the reported overall enzyme activities (*126*) and the enzyme distributions in the body.(*127*)

3.3 Insights from molecular docking

Molecular docking enabled us to understand how cocaethylene may bind with human BChE and the mutants in comparison with (-)-cocaine binding with the same enzymes. As seen in Figure 3.1, the only structural difference between cocaethylene and (-)-cocaine is that the methyl group on the methyl ester of (-)-cocaine is replaced by an ethyl group in cocaethylene. According to the enzyme-substrate binding structures obtained from molecular docking, the binding mode for each enzyme (wild-type human BChE or its mutant) with cocaethylene is essentially the same as that with (-)-cocaine, particularly for the crucial interactions between the carbonyl oxygen of the substrate and the oxyanion hole (consisting of residues #116, #117, and #199) of the enzyme. The minor structural difference between cocaethylene and (-)-cocaine does not significantly change the binding mode with the BChE or mutant. In particular, there is always only one hydrogen bond between the carbonyl oxygen of the substrate and the oxyanion hole (G117 backbone) of wild-type BChE no matter whether the substrate is cocaethylene or (-)-cocaine, and there are always two hydrogen bonds between the carbonyl oxygen of the substrate and the oxyanion hole (G117 backbone and S199 side chain) of the mutant no matter whether the substrate is cocaethylene or (-)-cocaine. Depicted in Figure 3.3 are the obtained enzyme-substrate binding structures for cocaethylene and (-)-cocaine with wild-type human BChE and a representative mutant (E12-7). The binding structures with E14-3 (not shown) are similar to those with E12-7 in terms of the overall hydrogen bonding with the oxyanion hole.

The docking structures depicted in Figure 3.3 indicate that, regardless of whether the substrate is cocaethylene or (-)-cocaine, the hydroxyl group of S199 side chain in the mutant forms an additional, strong hydrogen bond with the substrate compared to that in the wild-type BChE. Based on this common feature, the same amino-acid mutations that can significantly improve the catalytic efficiency of human BChE against (-)-cocaine may be expected to significantly improve the catalytic efficiency of the enzyme against cocaethylene. Hence, the BChE mutants concerned in the present study are expected to have a significantly improved catalytic efficiency against cocaethylene, although it has been known that these BChE mutants do not have an improved catalytic efficiency against ACh.(*88*)

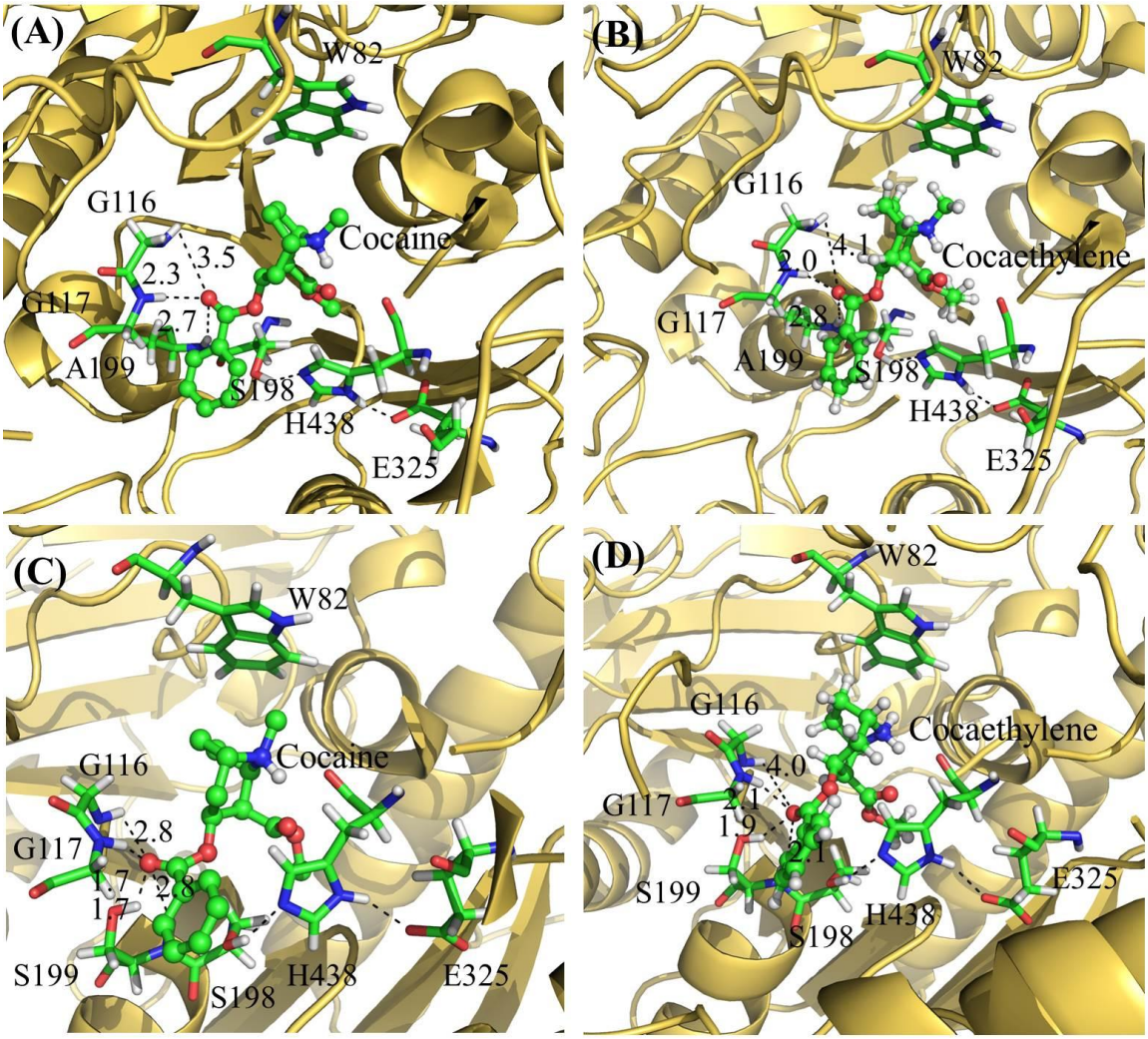


Figure 3.3 Docked structures of the wild-type BChE and E12-7 binding with cocaethylene and (-)-cocaine: (A) Wild-type human BChE binding with (-)-cocaine; (B) Wild-type human BChE binding with cocaethylene; (C) E12-7 binding with (-)-cocaine; (D) E12-7 binding with cocaethylene. Indicated in the figure are the key distances (in Å) of the carbonyl oxygen of the substrate with the hydrogen atoms of the oxyanion hole.

3.4 Kinetic parameters

In light of the computational insights, we carried out *in vitro* experimental tests, including the protein expression and enzyme activity assays, on wild-type human BChE, E14-3, and E12-7. The *in vitro* assays were based on our observation (Figure 3.4) that cocaethylene also had an UV-Vis absorption peak at 230 nm as (-)-cocaine, and that the absorption at 230 nm is linearly proportional to the concentration of cocaethylene or (-)-cocaine. The *in vitro* assays enabled us to determine the catalytic activity of the enzymes against cocaethylene in comparison with the corresponding activity against (-)-cocaine. To minimize the possible systematic experimental errors of the kinetic data, for each enzyme the catalytic activities against both cocaethylene and (-)-cocaine were assayed at the same time under the same experimental conditions so as to reliably determine the catalytic activity of the enzyme against cocaethylene relative to the known activity against (-)-cocaine. Depicted in Figure 3.5 are the measured kinetic data. Summarized in Table 1 are the kinetic parameters of the enzymes against cocaethylene in comparison with those against (-)-cocaine and norcocaine.

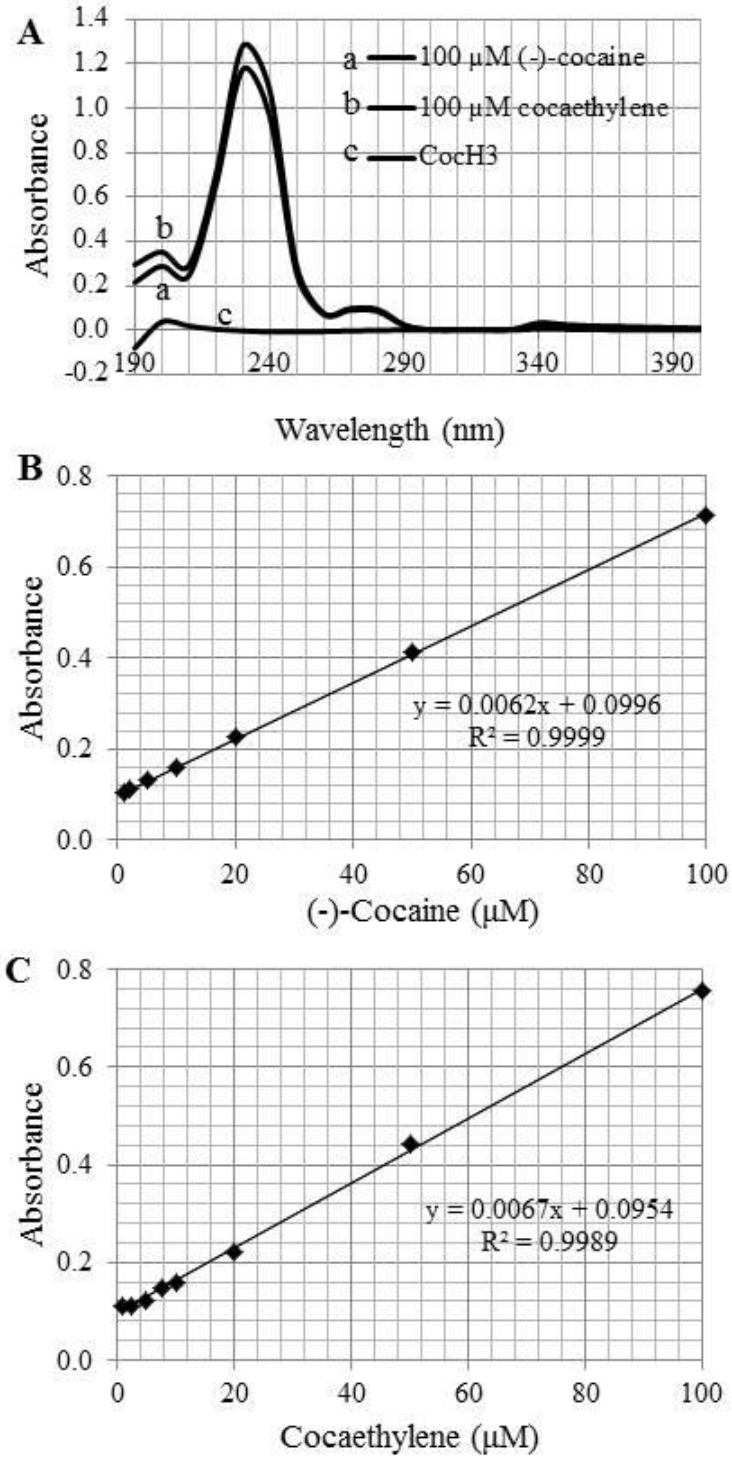
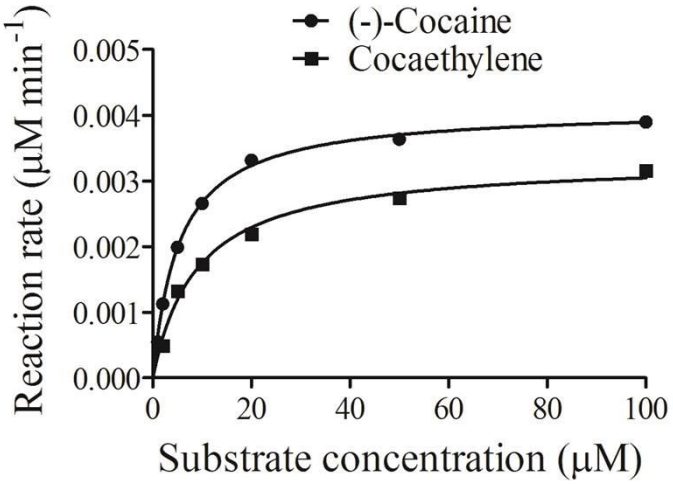
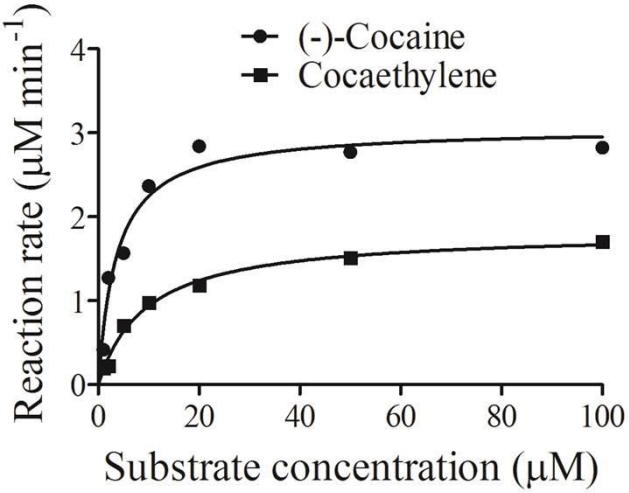


Figure 3.4 UV–visible absorptions of the enzyme and substrates. (A) UV-Vis absorption of (-)-cocaine, cocaethylene, and E12-7. (B) Plot of the absorption at 230 nm *versus* the concentration of (-)-cocaine. (C) Plot of the absorption at 230 nm *versus* the concentration of cocaethylene.

(A) wild-type BChE



(B) E14-3



(C) E12-7

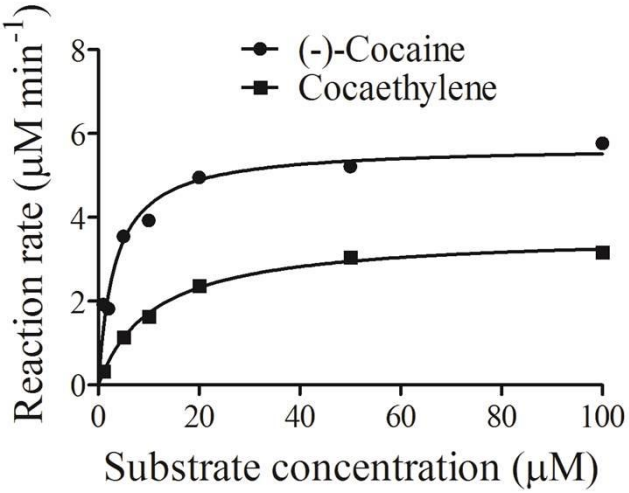


Figure 3.5 Kinetic data obtained in vitro for enzymatic hydrolysis of (-)-cocaine and cocaethylene: (A) wild-type human BChE; (B) E14-3; (C) E12-7. To minimize the possible systematic experimental errors of the kinetic data, each enzyme's catalytic activities against both cocaethylene and (-)-cocaine were assayed at the same time under the same experimental conditions so as to reliably determine the kinetic parameters of the enzyme against cocaethylene relative to those against (-)-cocaine. The reaction rate (represented in μM min^{-1} per nM enzyme) was determined by measuring the rate of the change of the absorbance at 230 nm.

A survey of the kinetic parameters summarized in Table 1 reveals that both E14-3 and E12-7 examined in this study have a considerably improved catalytic efficiency (k_{cat}/K_M) against cocaethylene. Wild-type BChE has a slightly lower catalytic activity against cocaethylene (k_{cat} = 3.3 min^{-1}, K_M = 7.5 μM, and k_{cat}/K_M = 4.40 × 10^5 M^{-1} min^{-1}) compared to its catalytic activity against (-)-cocaine (k_{cat} = 4.1 min^{-1}, K_M = 4.5 μM, and k_{cat}/K_M = 9.11 × 10^5 M^{-1} min^{-1}). According to the kinetic parameters summarized in Table 1, E14-3 and E12-7 indeed have a significantly improved catalytic efficiency against cocaethylene compared to the wild-type BChE: 517-fold for E14-3 and 861-fold for E12-7. In comparison with the catalytic activities of the same mutant for different substrates, for both E14-3 and E12-7, the catalytic efficiency of the enzyme for cocaethylene is lower than that for (-)-cocaine, but higher than that for norcocaine. Within the enzymes examined in this study, the most efficient BChE mutant (E12-7) against cocaethylene is the same as the most efficient mutant against norcocaine and (-)-cocaine. E12-7 has an 861-fold improved catalytic efficiency against cocaethylene, 1080-fold improved catalytic efficiency against norcocaine, and a 2020-fold improved catalytic efficiency against (-)-cocaine. So, E12-7 is identified as the most promising enzyme (BChE mutant) for metabolizing all of the three toxic substrates: cocaethylene, (-)-cocaine, and norcocaine.

Table 3.1 Kinetic parameters determined *in vitro* for (-)-cocaine, norcocaine and cocaethylene hydrolyses catalyzed by wild-type BChE and its mutants.

Substrate	Enzyme[a]	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M ($M^{-1}min^{-1}$)	RCE[d]
(-)-Cocaine[b]	WT BChE	4.5	4.1	9.11×10^5	1
	E14-3	3.1	3,060	9.87×10^8	1,080
	E12-7	3.1	5,700	1.84×10^9	2,020
Norcocaine[b]	WT BChE	15	2.8	1.87×10^5	1
	E14-3	12	766	6.38×10^7	343
	E12-7	13	2,610	2.01×10^8	1,080
Cocaethylene[c]	WT BChE	7.5	3.3	4.40×10^5	1
	E14-3	8.0	1,820	2.28×10^8	517
	E12-7	9.5	3,600	3.79×10^8	861

[a]The enzyme under the study was wild-type human BChE (WT BChE), A199S/S287G/A328W/Y332G mutant (E14-3), or A199S/F227A/S287G/A328W/Y332G mutant (E12-7).
[b]Data for wild-type BChE against (-)-cocaine came from reference (*79*), data for E14-3 against (-)-cocaine came from reference (*88*), data for E12-7 against (-)-cocaine came from reference (*76*), and data for all enzymes against norcocaine came from reference (*116*).
[c]All of the kinetic data for cocaethylene were determined in the present study for the first time.
[d]RCE refers to the relative catalytic efficiency (k_{cat}/K_M), *i.e.* the ratio of the k_{cat}/K_M value of the mutant to that of wild-type BChE against the same substrate.

In addition, we also wanted to know whether alcohol has a significant effect on the catalytic activity of the enzymes examined in this study. For this purpose, the catalytic efficiency of E14-3 (as an example) for cocaine hydrolysis was determined in the presence of alcohol (800 mg/dL) in comparison with the corresponding activity in the absence of alcohol. The data depicted in Figure 3.6 revealed that alcohol (even at the

concentration as high as 800 mg/dL or 0.174 M) does not significantly affect the enzyme activity.

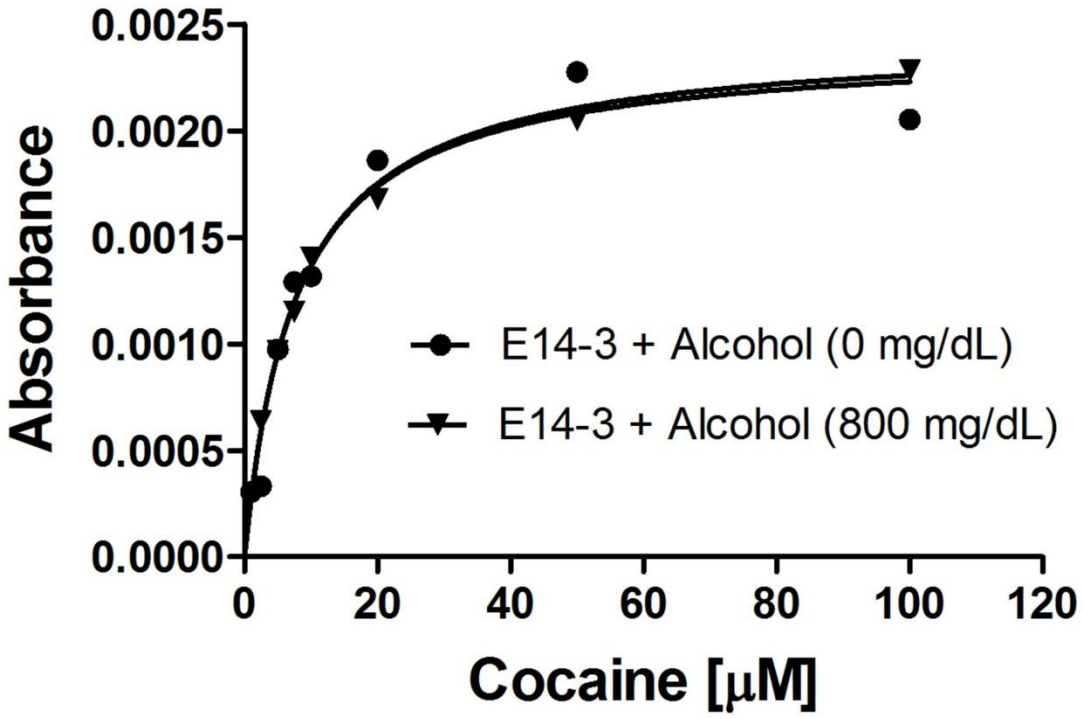


Figure 3.6 Kinetic data obtained *in vitro* for E14-3-catalyzed hydrolysis of (-)-cocaine in the absence and presence of alcohol, showing that alcohol with the concentration as high as 800 mg/dl or 0.174 M does not significantly modify the catalytic efficiency of the enzyme. The concentrations of the enzyme used for the two experiments were exactly the same. The changes in the UV absorption at 230 nm represent the changes in the cocaine concentrations.

3.5 Cocaethylene clearance accelerated by E12-7

Our recently reported *in vivo* studies (*116, 123*) have demonstrated that E12-7 can efficiently metabolize (-)-cocaine and norcocaine in rats. In light of the encouraging *in vitro* activity data discussed above, we would like to know whether E12-7 can also efficiently metabolize cocaethylene in rats. We characterized the pharmacokinetic profiles of cocaethylene clearance with and without the presence of E12-7 in rats. Four rats (n=4) were injected with saline, followed by i.v. injection of 3 mg/kg cocaethylene.

Another set of four rats (n=4) were injected with 0.15 mg/kg E12-7, followed by i.v. injection of 3 mg/kg cocaethylene. The E12-7 dose was 0.15 mg/kg which 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, see discussion below) in plasma at ~2 min after the i.v. injection of E12-7 according to our previous study.(*123*) For each rat, the blood was sampled at 2, 5, 15, 30, 60, 90, 120, 150, and 180 min after the cocaethylene injection. The *in vivo* data are depicted in Figure 3.7.

E12-7 can hydrolyze cocaethylene to produce benzoic acid and ecgonine ethyl ester, and greatly accelerate the clearance of cocaethylene from the body. The control curves in Figure 3.7 reflect the overall effects of all possible cocaethylene elimination pathways.(*99*) As seen in Figure 3.7, in the control rats, the average concentration of cocaethylene at the first time point (2 min) was ~2.7 μM, while the average concentration of benzoic acid (metabolite) was ~0.3 μM. In the presence of E12-7, the average concentration of cocaethylene at ~2 min in the blood sample was below the detectable level (~0.1 μM, see Figure 3.7A), while the average concentration of benzoic acid at the first time point (2 min) was ~7.4 μM (Figure 3.7B). Most of the cocaethylene was hydrolyzed by E12-7 between the i.v. cocaethylene injection and the first blood sampling at 2 min after the injection. The E12-7-caused dramatic changes in both the cocaethylene and benzoic acid concentrations clearly indicated that cocaethylene was metabolized rapidly to benzoic acid in the presence of E12-7. Notably, as shown in Figure 3.7B, the benzoic acid concentration in plasma decreased with time. This is because most of the cocaethylene had already been hydrolyzed by E12-7 before the first time point (2 min) so that further generation of benzoic acid after 2 min was negligible compared to the benzoic acid elimination from plasma.

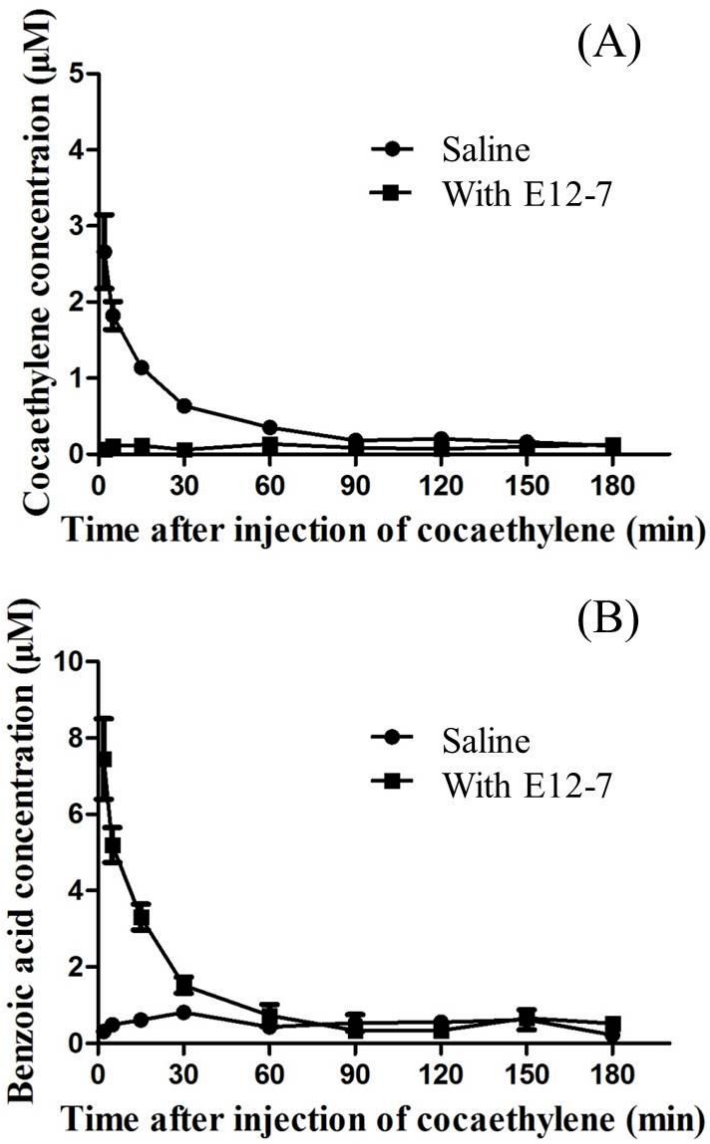


Figure 3.7 Cocaethylene clearance accelerated by E12-7. Time-dependent concentrations of cocaethylene (A) and benzoic acid (B) in blood. Benzoic acid is the product of E12-7-catalyzed hydrolysis of cocaethylene. Saline or 0.15 mg/kg E12-7 was injected i.v. in rats (n=4) 1 min before the i.v. injection of 3 mg/kg cocaethylene.

It should be mentioned that the total plasma concentration of cocaethylene and benzoic acid (~7.5 μM) in the presence of E12-7 (when the benzoic acid concentration was higher) was higher than that (3.0 μM) in the absence of E12-7 (when the cocaethylene concentration was higher). This observation might be associated with the

potentially different distribution volumes of cocaethylene and benzoic acid in the body. Cocaethylene is an amine drug which can readily cross cell membranes under physiological condition, while benzoic acid primarily exists in the benzoate ion state under the physiological conditions. So, benzoic acid is expected to have a relatively smaller distribution volume compared to cocaethylene.

3.6 Effects of E12-7 on the pharmacokinetics of (-)-cocaine and cocaethylene

With E12-7 identified as the most active enzyme (BChE mutant) for cocaethylene in addition to its previously known high catalytic activities for (-)-cocaine and norcocaine, we further carried out kinetic modeling of cocaine metabolism using the kinetic equations shown in Figure 3.2 in the presence of alcohol and three enzymes: CocH (which refers to either wild-type human BChE or E12-7) in human plasma; hCE-1; and CYP 3A4. Concerning CocH, a typical adult has a blood volume of ~5 L.(*82*) Previously reported concentrations of endogenous BChE protein in human plasma ranged from 4 to 7 mg/L (*128-130*), giving an average value of ~6 mg/L or ~0.07 μM in terms of the total BChE protein concentration (denoted as [E]), assuming that a tetramer of human BChE has four active sites.(*131, 132*) According to the kinetic data summarized in Table 3.1, we should have V_{max} = 0.29 μM min^{-1} and K_M = 4.5 μM for the wild-type BChE against (-)-cocaine, and V'_{max} = 0.23 μM min^{-1} and K'_M = 7.5 μM for the wild-type BChE against cocaethylene, and V''_{max} = 0.20 μM min^{-1} and K''_M = 15 μM for the wild-type BChE against norcocaine, when [E] = 0.07 μM. These kinetic parameters were used in our modeling with the wild-type BChE. Similarly, for E12-7, according to the kinetic data summarized in Table 3.1, we should have V_{max} = 400 μM min^{-1} and K_M = 3.1 μM against (-)-cocaine, V'_{max} = 250 μM min^{-1} and K'_M = 9.5 μM against cocaethylene, and V''_{max} = 180 μM min^{-1} and K''_M = 13 μM against norcocaine, when [E] = 0.07 μM.

For (-)-cocaine transesterification to cocaethylene in the presence of a sufficiently high concentration of alcohol, it has been known that hCE-1 and its (-)-cocaine transesterification-specific activity exist in not only liver, but also other tissues. (-)-Cocaine transesterification-specific activity in isolated kidney microsomes was even greater than that measured in the liver microsomes.(*133*) It has also been known that (-)-cocaine can diffuse in the body very rapidly to reach the equilibrium.(*82*) It is reasonable

to assume that (-)-cocaine, cocaethylene, and norcocaine distributions in the blood and other tissues can rapidly reach the equilibrium during the metabolic reactions. Thus, it was roughly estimated that F_1 = 12.5 μM min^{-1} and k_1 = 0.56 mM for (-)-cocaine transesterification to cocaethylene, according to the available experimental data including the enzyme activity (*134*) and the enzyme distribution in the body (*127*) and an assumption that the average hCE-1 density in the whole human body is ~50% of the hCE-1 density in the human liver. Similarly, it was estimated that F_2 = 14.4 μM min^{-1} and k_2 = 2.7 mM for the enzymatic oxidation of (-)-cocaine to norcocaine.(*116*) 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.

The first set of initial concentrations used in the kinetic modeling include: $A(0)$ (the initial concentration of (-)-cocaine) = 1 to 100 μM while $B(0)$ (the initial concentration of cocaethylene) = 0 and $F(0)$ (the initial concentration of norcocaine) = 0. Depicted in Figure 3.8 are the time-dependent concentrations of (-)-cocaine, cocaethylene and norcocaine when $A(0)$ = 10 μM in the presence of alcohol, hCE-1, CYP 3A4 and wild-type human BChE (Figure 3.8A to C) or alcohol, hCE-1, CYP 3A4 and E12-7 (Figure 3.8D to F). As seen in Figure 3.8A to C, in the presence of alcohol, hCE-1, CYP 3A4 and wild-type human BChE (without administration of any exogenous enzyme), (-)-cocaine has an area under the curve (AUC) of 178 μM min and a half-life ($t_{1/2}$) of 14 min, cocaethylene has an AUC of 152 μM min and a half-life of 67 min, and norcocaine has an AUC of 37 μM min and a half-life of 99 min. The modeling data suggest that cocaethylene can exist in the body for a much longer time compared to (-)-cocaine itself because the endogenous wild-type BChE has a relatively lower catalytic activity against cocaethylene.

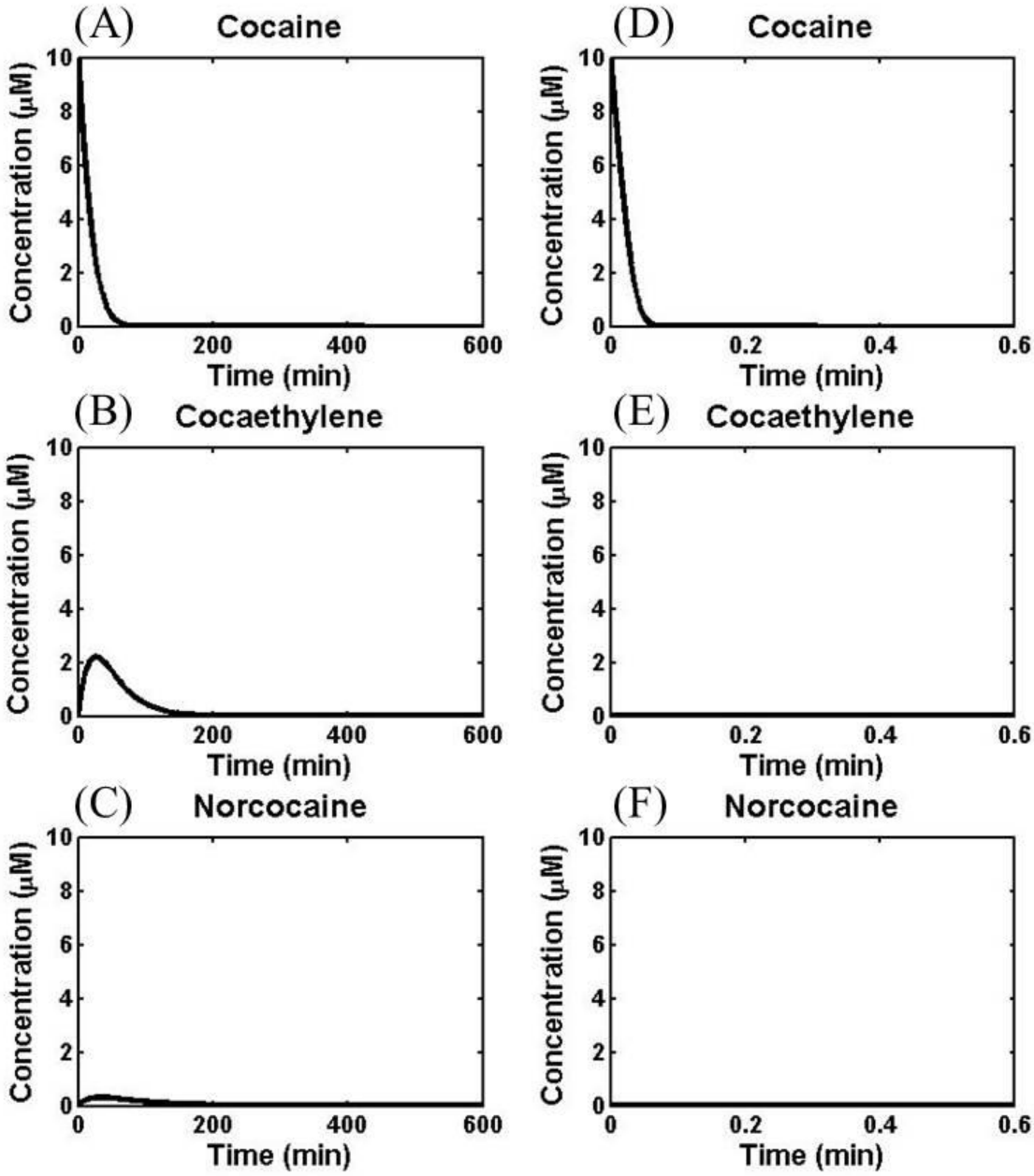


Figure 3.8 Kinetics of (-)-cocaine and its metabolites in the presence of alcohol and various enzymes. The modeled concentrations (*in silico* data) of (-)-cocaine (A), cocaethylene (B), and norcocaine (C) in human blood when the initial (-)-cocaine concentration is 10 μM in the presence of alcohol, hCE-1, CYP 3A4 and wild-type human BChE, and concentrations of (-)-cocaine (D), cocaethylene (E), and norcocaine (F) in human blood when the initial (-)-cocaine concentration is 10 μM in the presence of alcohol, hCE-1, CYP 3A4 and E12-7.

Based on the kinetic modeling, cumulatively, about 39% of (-)-cocaine has been metabolized to cocaethylene and then ecgonine ethyl ester and benzoic acid when $A(0)$ = 10 μM. Further, the modeling data summarized in Table 3.2 indicate that percentage contribution of (-)-cocaine transesterification to cocaethylene, as well as the AUC and $t_{1/2}$ of (-)-cocaine, cocaethylene and norcocaine, should increase with increasing the initial (-)-cocaine concentration. The data of the kinetic modeling are in reasonable agreement with the experimental observations that ~18% to ~34% of cocaine is converted to cocaethylene through transesterification when the peak concentration of cocaine in human plasma is 1 to 2 μM. According to the modeling data in Table 2, when $A(0)$ = 1 or 2 μM, about 27% or 29% of (-)-cocaine has been converted to cocaethylene and then ecgonine ethyl ester and benzoic acid.

Table 3.2 Kinetic parameters obtained from *in silico* modeling for (-)-cocaine and norcocaine hydrolyses catalyzed by wild-type human BChE and its mutants. *A*(0) is the initial concentration of (-)-cocaine. Coca% refers to the percentage contribution of (-)-cocaine metabolism through hCE-1-catalyzed transesterification to cocaethylene. Coc, Coca, and Norc represent (-)-cocaine, cocaethylene, and norcocaine, respectively.

A(0) (μM)	hCE-1, CYP 3A4, and wild-type BChE							hCE-1, CYP 3A4, and E12-7						
	AUC(μM·min)			$t_{1/2}$(min)			Coca%	AUC(μM·min)			$t_{1/2}$(min)			Coca%
	Coc	Coca	Norc	Coc	Coca	Norc		Coc	Coca	Norc	Coc	Coca	Norc	
1	12	8.9	2.5	8.5	54	91	27%	0.009	0.000	0.000	0.007	0.051	0.078	0.02%
2	26	19	5.3	9.3	56	92	29%	0.021	0.000	0.000	0.008	0.053	0.079	0.03%
3	41	31	8.4	10	57	93	30%	0.035	0.000	0.000	0.009	0.054	0.081	0.03%
4	58	44	12	11	59	94	32%	0.051	0.000	0.000	0.010	0.056	0.083	0.03%
5	75	59	15	11	60	95	33%	0.070	0.000	0.000	0.011	0.057	0.084	0.03%
6	94	74	19	12	62	96	35%	0.092	0.000	0.000	0.013	0.058	0.086	0.03%
7	114	92	23	13	63	97	36%	0.116	0.000	0.000	0.014	0.060	0.088	0.04%
8	134	110	28	13	64	98	37%	0.142	0.000	0.000	0.015	0.062	0.089	0.04%
9	155	130	32	14	66	99	38%	0.171	0.000	0.000	0.016	0.064	0.091	0.04%
10	178	152	37	14	67	99	39%	0.203	0.000	0.000	0.018	0.066	0.093	0.05%
20	432	451	91	17	83	107	47%	0.656	0.001	0.000	0.030	0.083	0.111	0.07%
30	728	925	156	19	100	114	53%	1.359	0.001	0.000	0.042	0.100	0.129	0.10%
40	1051	1599	229	21	117	119	56%	2.312	0.002	0.000	0.055	0.117	0.147	0.12%
50	1395	2495	310	22	134	124	59%	3.514	0.003	0.001	0.068	0.134	0.165	0.15%
60	1755	3627	398	23	151	129	62%	4.965	0.004	0.001	0.079	0.150	0.183	0.17%
70	2129	5007	493	24	169	133	64%	6.665	0.005	0.001	0.093	0.166	0.200	0.20%
80	2515	6642	594	25	187	137	65%	8.614	0.007	0.002	0.104	0.182	0.217	0.22%
90	2912	8540	702	26	206	141	66%	10.81	0.008	0.002	0.117	0.198	0.234	0.24%
100	3319	10705	817	26	224	145	68%	13.26	0.010	0.002	0.130	0.213	0.250	0.27%

In the presence of alcohol, hCE-1, CYP 3A4 and E12-7 (exogenous enzyme with [E] = 0.07 μM), when $A(0)$ = 10 μM, (-)-cocaine only has an AUC of 0.203 μM·min and a half-life of 0.018 min, and both cocaethylene and norcocaine have the AUC values smaller than 0.001 μM·min, as shown in Figure 3.8D to F. Both the AUC and $t_{1/2}$ values are all negligible when E12-7 is administered as an exogenous enzyme (or provided *via* gene therapy (*135*)) with the E12-7 concentration being the same as that of the endogenous BChE ([E] = 0.07 μM). As seen in Table 3.2, for all of the three toxic compounds, *i.e.* (-)-cocaine, cocaethylene and norcocaine, the AUC and $t_{1/2}$ increase with increasing the initial (-)-cocaine concentration, but not dramatically. In particular, even if $A(0)$ = 100 μM, the half-life of (-)-cocaine is still only 0.130 min, and the half-life of cocaethylene is still only 0.213 min in the presence of E12-7. Clearly, all of the three toxic compounds can be eliminated effectively and rapidly at the same time when E12-7 is administered as an exogenous enzyme with the E12-7 concentration being the same as that of the endogenous BChE ([E] = 0.07 μM) in the simplified kinetic model.

The initial concentrations used in the above kinetic modeling may represent the possible cocaine abuse treatment conditions in which E12-7 is administered prior to the cocaine administration. For possible cocaine overdose treatment using an exogenous cocaine-metabolizing enzyme, the cocaine abusers have already taken cocaine and alcohol, and converted some cocaine to cocaethylene and norcocaine before the enzyme administration. In order to know whether E12-7 is also efficacious in hydrolysis of cocaethylene, in addition to (-)-cocaine and norcocaine, for the cocaine overdose treatment, we performed an additional, simplified kinetic modeling by assuming that 34% (-)-cocaine has been converted to cocaethylene, 5% (-)-cocaine has been converted to norcocaine, and 61% (-)-cocaine remains as (-)-cocaine when t = 0. Depicted in Figure 3.9 are data obtained from the simplified kinetic modeling when $A(0)$ = 61 μM, $B(0)$ = 34 μM, and $F(0)$ = 5 μM. As seen in Figure 3.8A to C, in the absence of E12-7, (-)-cocaine and cocaethylene may last in the body for a long time (>200 min). As seen in Figure 3.8D to F, in the presence of 0.07 μM E12-7, all of the three toxic compounds may be eliminated completely in ~0.2 min. These data qualitatively suggest that E12-7 should be effective for cocaine overdose treatment even in the case of combined use of cocaine and alcohol.

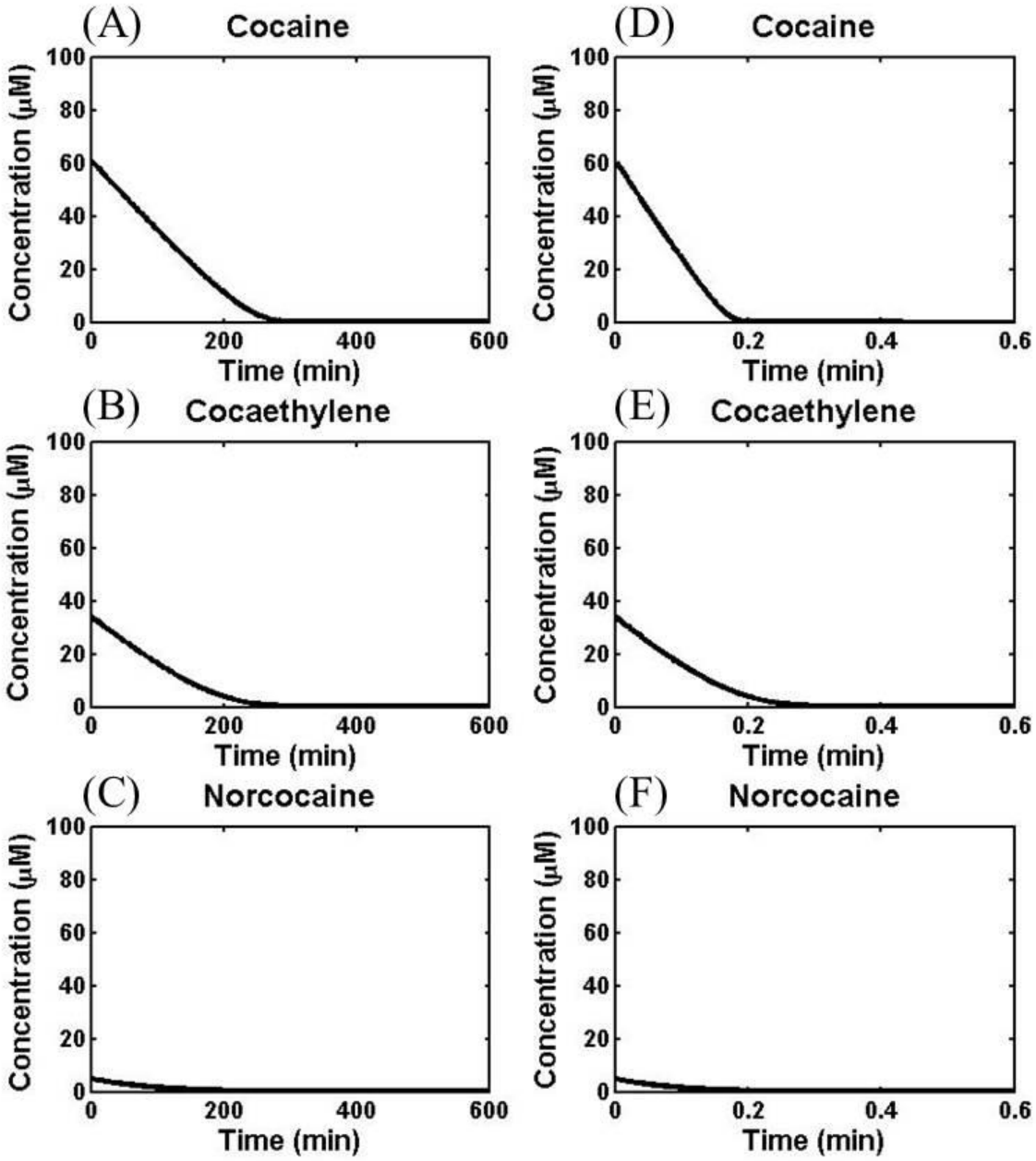


Figure 3.9 The modeled concentrations (*in silico* data) of (-)-cocaine, cocaethylene, and norcocaine in human blood when the initial concentrations of (-)-cocaine, cocaethylene, and norcocaine are 61, 34, and 5 μM, respectively. (A) to (C) refer to the time-dependent concentrations in the presence of 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.

3.7 Conclusion

The catalytic activity of human BChE and two BChE mutants (E14-3 and E12-7) for cocaethylene has been characterized in comparison with the corresponding catalytic activity for cocaine. The kinetic data reveal that wild-type human BChE has a relatively lower catalytic activity against cocaethylene (k_{cat} = 3.3 min^{-1}, K_M = 7.5 μM, and k_{cat}/K_M = 4.40 × 10^5 M^{-1} min^{-1}) compared to its catalytic activity against (-)-cocaine (k_{cat} = 4.1 min^{-1}, K_M = 4.5 μM, and k_{cat}/K_M = 9.11 × 10^5 M^{-1} min^{-1}). It has been shown that E14-3 and E12-7 have not only a considerably improved catalytic efficiency against cocaine and norcocaine, but also a considerably improved catalytic efficiency against cocaethylene, compared to the wild-type BChE. The most efficient enzyme (E12-7, *i.e.* the A199S/F227A/S287G/A328W/Y332G mutant of human BChE) against cocaethylene is the same as the most efficient one against norcocaine in addition to the known high catalytic activity against (-)-cocaine. E12-7 has an 861-fold improved catalytic efficiency against cocaethylene (k_{cat} = 3600 min^{-1}, K_M = 9.5 μM, and k_{cat}/K_M = 3.79 × 10^8 M^{-1} min^{-1}). Thus, E12-7 is identified as the most promising enzyme for hydrolyzing for all three toxic compounds, *i.e.* (-)-cocaine, cocaethylene and norcocaine. It has been demonstrated that E12-7 as an exogenous enzyme can indeed rapidly metabolize cocaethylene, in addition to cocaine and norcocaine, in rats. Further kinetic modeling has suggested that E12-7 with a concentration similar to that of the endogenous BChE in human plasma can effectively eliminate all of the three toxic compounds in simplified kinetic models of cocaine abuse and overdose associated with the concurrent use of cocaine and alcohol.

Chapter Four: Evaluation of the Catalytic Activity of E12-7 against Cocaine in the Presence of Commonly Used Small-Molecule Drugs

Summary

BChE was once called "nonspecific cholinesterase" due to its wide range of substrates and inhibitors. The effect of BChE and its mutants on cocaine metabolism could possibly be reversed by some BChE inhibitors. Therefore, it is necessary to know the inhibitory activity of various small-molecule drugs against BChE and its mutants. Four classes of drugs intimately involved in cocaine abuse and its treatment are included in this study. For the investigation described in this chapter, possible drug-drug interactions between the promising BChE mutant E12-7 and some commonly used therapeutic agents were examined *in vitro* for the first time in order to know whether the examined therapeutic agents would significantly affect the catalytic activity of E12-7 against cocaine when they are co-administered with the enzyme. Most of the agents examined did not show significant inhibition on E12-7. However, some tricyclic antidepressants (TCA) were found as inhibitors of E12-7 with K_i in the range of 0.24-1.22 μM. Besides, TCA appeared to enhance cocaine-induced toxicity and, therefore, should be avoided or used with caution when BChE or its mutant is applied for cocaine abuse treatment.

4.1 The importance of drug-enzyme interaction study

Adverse drug reaction is one of the major causes of morbidity and mortality.(*136*) Drug-enzyme interaction study could benefit clinical outcomes and safety of medication treatments, especially for the high-risk and challenging population.

In comparison with the high selectivity of AChE, BChE was once called "nonspecific cholinesterase" due to its wide range of substrates and inhibitors, including acetylcholine, butyrylcholine, succinylcholine, cocaine, aspirin, organophosphates and cholinesterase inhibitors for Alzheimer's disease treatment.(*73, 137, 138*) The effect of BChE and its mutants on cocaine metabolism could possibly be reversed by some potent BChE inhibitors. Therefore, it is interesting to know the inhibitory activity of various types of small-molecule drugs on the enzymes. Four classes of drugs intimately involved

in cocaine abuse or its treatment are chosen in this study, including the drugs possibly co-abused with cocaine, medications for the treatment of those co-abused drugs, the current medications used for cocaine overdose treatment in emergency department, or the possible medications used to alleviate the symptoms of cocaine withdrawal. To be specific, there are several stimulants might co-abused with cocaine, such as alcohol, nicotine, caffeine, heroin (its metabolite morphine), and methamphetamine. It is necessary to know whether the co-abused drugs and the medications prescribed for those co-abused drugs would affect the catalytic activity of the enzyme against cocaine. Besides, although there are no medications available specific for cocaine, medications such as fast-onset benzodiazepines and phentolamine are given to patients to control cocaine overdose-induced symptoms in the emergency department.(*46*) When come to cocaine withdrawal, depression is one of the major symptoms. The medications effective in depression treatment might also alleviate the symptoms of cocaine withdrawal. In light of the promising data obtained from the previous chapters, E12-7 may be developed as a valuable therapeutic enzyme for cocaine abuse treatment. When necessary, the combination of enzyme therapy and currently existing treatments might give a better clinical outcome and, therefore, it is necessary to know whether those existing medications would compromise the enzymatic activity.

In this study, possible drug-drug interactions between E12-7 and some commonly used therapeutic agents were examined *in vitro* for the first time in order to know whether the examined therapeutic agents would significantly affect the catalytic activity of E12-7 against cocaine when they are co-administered. Although the *in vitro* study could not represent what really happens in human body, it can give us some insights into the potential drug interactions which may occur in people.

4.2 Materials and methods

4.2.1 Enzyme preparation and other materials

E12-7 enzyme materials used for *in vitro* studies was prepared in our recently reported study (*123*) developing and using stable CHO-S cells. The protein was purified by using a two-step purification procedure (ion exchange chromatography followed by

affinity chromatography), as described previously in detail.(*116*) The purified protein was dialyzed against phosphate-buffered saline and stored at -80 ℃.

[^{3}H](-)-cocaine (20-50 Ci/mmol) was purchased from PerkinElmer Life and Sciences (Waltham, MA). Other drugs were obtained from Sigma-Aldrich (St. Louis, MO), National Institute on Drug Abuse (NIDA) Drug Supply Program (Bethesda, MD) and Tocris Bioscience (Minneapolis, MN). Dimethy sulfoxide, and ethanol were used to dissolve the drugs only when water failed and only at concentrations that shown not to interfere with enzyme activity.

4.2.2 *In vitro* activity assays

For determining the catalytic activity of the E12-7 against (-)-cocaine in the absence or presence of commonly co-administered drugs, we used a sensitive radiometric assay based on toluene extraction of [^{3}H](-)-cocaine labeled on its benzene ring (*99*). In brief, E12-7 (10 ng/mL) was incubated with the tested drug in 0.1 M phosphate buffer (pH 7.4) for 15 min at 25^{o}C, and then varying concentrations of (-)-cocaine (mixed with trace amount of [^{3}H](-)-cocaine) was added to initiate the enzymatic reaction. 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. [^{3}H]benzoic acid (a product of (-)-cocaine hydrolysis) was extracted by 1 ml of toluene and measured by scintillation counting. Finally, the measured (-)-cocaine concentration-dependent radiometric data were analyzed by using the standard Michaelis-Menten kinetics so that the catalytic parameters were determined.

IC_{50} values of some compounds for E12-7 were determined by using a similar assay. 5 μM (-)-cocaine was used as the substrate. Compounds were tested in the range of 0.04 – 20 μM for amitriptyline and doxepin, 0.1–32 μM for nortriptyline, 1–400 μM for ketamine, 2–800 μM for naloxone, and 10–4000 μM for methamphetamine. IC_{50} was calculated using GraphPad Prism software, and K_i for a competitive inhibitor was converted from obtained IC_{50} by the Cheng-Prusoff equation.(*139*)

$$K_i = \frac{\mathrm{IC_{50}}}{1 + [\mathrm{S}]/K_\mathrm{M}}$$

4.3 Drug-E12-7 interaction

Four classes of drugs that most intimately involved in cocaine abuse and its treatment were chosen in the study: I) the drugs usually co-abused with cocaine; II) medications approved for those co-abused drugs; III) the medications used for cocaine overdose treatment in emergency room; IV) the possible medications used to alleviate the symptoms of cocaine withdrawal. These drug concentrations represent 1/10, 1/3 and 1× the pharmacologically relevant blood concentrations in human. The 1× drug level represents the peak blood or plasma concentration of the drug ($C_{max\ or}\ C_{ss}$) when taking the maximum recommended dose.

4.3.1 Class I: Drugs commonly co-abused with cocaine

Six possibly co-abused drugs, including alcohol, nicotine, morphine, methamphetamine, caffeine and ketamine were chosen as the representative of this class. The pharmacological blood concentrations of drugs were from the study of *Brim et al. (140)* and the wikipedia (caffeine). Nicotine, morphine and caffeine did not show any inhibition at the examined concentrations, while methamphetamine and ketamine demonstrated slight inhibition on E12-7 only at the maximum concentration of the drugs (Table 4.1).

The effect of alcohol was discussed in Chapter Three, and E14-3 was used as an example. The catalytic efficiency of E14-3 for cocaine hydrolysis was determined in the presence of alcohol in comparison with the corresponding activity in the absence of alcohol. The data depicted in Figure 3.6 revealed that alcohol (even at the concentration as high as 800 mg/dL or 0.174 M) does not significantly affect the enzyme activity.

Table 4.1 Class I drug concentrations and tested interactions with E12-7. k_{cat} and K_M were determined for three separate experiments. ns: no significant interaction found.

Drugs	**Pharmacological blood or plasma concentrations**	**k_{cat} (min^{-1})**	**K_M (μM)**	**k_{cat}/K_M (μM^{-1} min^{-1})**	**Significant interaction**
Nicotine	0 μM	5700	3.10	1840	ns
	0.12 μM	5660	3.01	1880	
	0.36 μM	5620	2.99	1880	
	1.2 μM	5700	2.97	1920	
Morphine	0 μM	5700	3.10	1840	ns
	0.18 μM	5570	3.12	1790	
	0.54 μM	5530	2.98	1860	
	1.8 μM	5750	3.09	1860	
Methamphetamine	0 μM	5700	3.10	1840	
	6.7 μM	5800	3.37	1720	
	20.1 μM	5610	3.37	1660	
	67 μM	5550	4.31	1290	
Caffeine	0	5700	3.10	1840	ns
	5.15 μM	5720	3.13	1830	
	15.4 μM	5700	3.11	1830	
	51.5 μM	5800	3.21	1810	
Ketamine	0	5700	3.10	1840	
	0.35 μM	5780	3.12	1850	
	1.05 μM	5730	3.21	1780	
	3.5 μM	5720	4.16	1380	

4.3.2 Class II: Medications approved for the commonly co-abused drugs with cocaine

There are several medications available on market for treatment of alcohol, nicotine, and opioid abuse. For example, disulfiram is one of the drugs for treatment of chronic alcoholism by inhibition of acetaldehyde dehydrogenase. Varenicline, as a nicotinic receptor partial agonist, is a prescription medication used to treat nicotine addiction.

Naltrexone, as an opioid receptor antagonist, is primarily used for the management of alcohol dependence and opioid dependence, such as heroin or morphine. Naloxone is a pure opioid antagonist and used to counter the effects of opioid overdose. The pharmacological blood concentrations of drugs (1 ×) were from the studies.(*140-142*)

According to the data shown in Table 4.2, the kinetic parameters of E12-7 for cocaine hydrolysis were not altered by those compounds, except for naloxone at the highest concentration of 10 μM causing slight increase in K_M value and decrease in catalytic efficiency of E12-7, compared with the parameters of E12-7 in the absence of drugs.

Table 4.2 Class II drug concentrations and tested interactions with E12-7. k_{cat} and K_M were determined for three separate experiments. ns: no significant interaction found.

Drugs	**Pharmacological blood or plasma concentrations**	**k_{cat} (min^{-1})**	**K_M (μM)**	**k_{cat}/K_M (μM^{-1} min^{-1})**	**Significant interaction**
Naltrexone	0	5700	3.10	1840	ns
	25 nM	5970	3.11	1920	
	75 nM	5900	2.99	1970	
	250 nM	5950	3.14	1900	
Naloxone	0	5700	3.10	1840	
	1 μM	5550	3.02	1840	
	3 μM	5480	3.18	1720	
	10 μM	5250	4.27	1230	
Varenicline	0	5700	3.10	1840	ns
	1.89 nM	5540	3.09	1790	
	5.69 nM	5500	3.02	1820	
	18.9 nM	5590	3.09	1810	
Disulfiram	0	5700	3.10	1840	ns
	0.14 μM	5660	2.98	1900	
	0.42 μM	5610	3.00	1870	
	1.4 μM	5700	3.04	1880	

4.3.3 Class III: Medications used for cocaine overdose treatment in emergency department

Although there is no medication available specific for cocaine so far, a standard protocol in emergency department is employed mainly to treat patients by relieving cocaine-induced symptoms, especially chest pain and possible heart attack. Fast-onset benzodiazepines (diazepam, and midazolam) and phentolamine are the first line medications for cocaine overdose treatment. The 1× drug concentrations are from the package insert for REGITINE® (phentolamine mesilate) and studies (*140, 143, 144*).

In light of the promising data obtained from the previous chapters, E12-7 may be developed as a valuable therapeutic enzyme for cocaine abuse treatment. The combination of enzyme therapy and current treatments might give a better clinical outcome, therefore, it is necessary to know whether those existing medications would compromise the enzymatic activity. Fortunately, the tested drugs in Table 4.3, including diazepam, oxazepam (a metabolite of diazepam), midazolam, and phentolamine, did not show any significant inhibitory activity on E12-7 for cocaine hydrolysis.

Table 4.3 Class III drug concentrations and tested interactions with E12-7. k_{cat} and K_M were determined for three separate experiments. ns: no significant interaction found.

Drugs	**Pharmacological blood or plasma concentrations**	**k_{cat} (min^{-1})**	**K_M (μM)**	**k_{cat}/K_M (μM^{-1} min^{-1})**	**Significant interaction**
Diazepam	0	5700	3.10	1840	ns
	0.81 μM	5990	3.18	1880	
	2.43 μM	6060	3.27	1850	
	8.1 μM	5840	3.52	1660	
Oxazepam	0 μM	5700	3.10	1840	ns
	0.21 μM	5720	3.16	1810	
	0.63 μM	5690	3.12	1820	
	2.09 μM	5750	3.13	1840	
Midazolam	0	5700	3.10	1840	ns
	0.64 μM	5660	3.03	1870	
	1.90 μM	5670	3.14	1810	
	6.4 μM	5520	3.17	1740	
Phentolamine	0	5700	3.10	1840	ns
	39.1 nM	5850	3.03	1930	
	117 nM	5880	3.09	1900	
	391 nM	5800	3.21	1810	

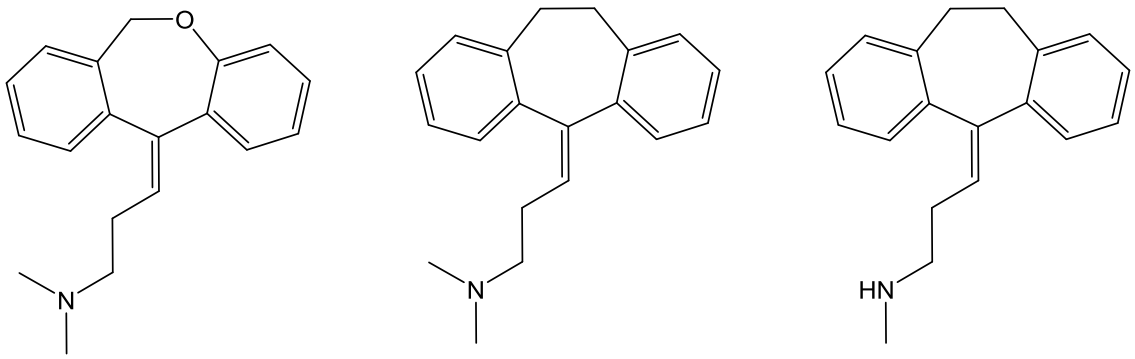
4.3.4 Class IV: Medications that could be used to alleviate the symptoms of cocaine withdrawal

Depression is one of the major symptoms of cocaine withdrawal. Hence, the medications effective in depression treatment might also alleviate the symptoms of cocaine withdrawal. Tricyclic antidepressants (TCA, such as doxepin, amitriptyline and nortriptyline) and monoamine oxidase inhibitors (phenelzine) are the important classes of antidepressants. The 1× drug concentrations are based on the package insert for NARDIL (phenelzine sulfate) and Nortriptyline hydrochloride, as well as the known studies (*145, 146*).

Among the drugs on the list, amitriptyline and doxepin significantly affect the catalytic activity of E12-7 against cocaine when they are co-administered, which is consistent with the report about amitriptyline.(*73, 147, 148*) TCA share the similar chemical structure with each other (shown in Figure 4.1), and also have the similarity as the structure of BChE inhibitors reported by Zheng et al.(*149*) Therefore, it is not surprising to note that doxepin and nortriptyline also inhibit the catalytic activity of E12-7 for cocaine hydrolysis.

Table 4.4 Class IV drug concentrations and tested interactions with E12-7. k_{cat} and K_M were determined for three separate experiments. ns: no significant interaction found.

Drugs	**Pharmacological blood or plasma concentrations**	**k_{cat} (min^{-1})**	**K_M (μM)**	**k_{cat}/K_M (μM^{-1} min^{-1})**	**Significant interaction**
Phenelzine	0	5700	3.10	1840	ns
	8.45 nM	5700	3.20	1780	
	25.4 nM	5870	3.26	1800	
	84.5 nM	5740	3.26	1760	
Doxepin	0	5700	3.10	1840	
	71.6 nM	5960	3.94	1510	
	215 nM	5790	4.99	1160	
	716 nM	5340	8.37	640	
Amitriptyline	0	5700	3.10	1840	
	90.1 nM	5740	3.64	1580	
	270 nM	5840	5.83	1000	
	901 nM	6000	12.20	490	
Nortriptyline	0	5700	3.10	1840	
	57 nM	5780	3.28	1760	
	171 nM	5930	3.78	1570	
	570 nM	5900	4.84	1220	



Doxepin Amitriptyline Nortriptyline

Figure 4.1 Chemical structures of doxepin, amitriptyline, and nortriptyline.

4.4 Inhibitory activities of compounds for E12-7

4.4.1 Doxepin, amitriptyline and nortriptyline for E12-7

According to the above kinetic parameters of E12-7, K_M increased and k_{cat} decreased or increased slightly (less than 6.3% at the highest drug concentration) in the presence of doxepin, amitriptyline, or nortriptyline. However, the change in k_{cat} might be due to the experimental errors, suggesting that these drugs are likely competitive inhibitors.

The examined TCA including doxepin, amitriptyline, and nortriptyline exhibited strong inhibition on E12-7 with K_i in the range of 0.24-1.22 μM, as shown in Table 4.5. The overall binding of E12-7 with those three compounds may be similar to its binding with BTC or cocaine. In general, as a tertiary or secondary amine, doxepin, amitriptyline, and nortriptyline exist mainly in protonated form under physiological pH that resembles choline. The positively charged quaternary ammonium group is bound to a choline binding site and/or the tricyclic part interacts with the hydrophobic pocket of the enzyme.(*148, 150*) The order of inhibitory potency is amitriptyline > doxepin > nortriptyline for E12-7, which is probably due to the difference in their chemical structures.

Table 4.5 IC_{50} and inhibitory constant K_i of compounds for E12-7.

Enzyme	Compound	IC_{50} (μM)	K_i (μM)	Plasma C_{max} (μM)
E12-7	Doxepin	0.73	0.28	0.72
	Amitriptyline	0.62	0.24	0.90
	Nortriptyline	3.19	1.22	0.57
	Ketamine	15.9	6.08	3.5
	Naloxone	24.8	9.50	10
	Methamphetamine	258	98.6	67

K_i is defined as the concentration of inhibitor that is required to decrease the maximum rate of the reaction by a half. If the K_i value is much larger than the maximum plasma drug concentration from typical dosing, then the drug is not likely to inhibit the activity of the enzyme. From the data in Table 4.5, the K_i values for doxepin and amitriptyline for E12-7 are ~3-fold lower than the corresponding maximum drug concentration in plasma. Thus, it is very likely that doxepin and amitriptyline slow down the cocaine hydrolysis catalyzed by E12-7 and affect *in vivo* efficacy of E12-7 in cocaine abuse treatment. Fortunately, amitriptyline is a reversible inhibitor of BChE (*147*), thus it is expected that the enzyme activity will recover once the drug is cleared from plasma. However, studies have shown that TCA appears to enhance cocaine-induced toxicity (*38*, *151*) and, therefore, TCA should be used with caution when BChE or its mutant is applied for cocaine abuse treatment.

4.4.2 Methamphetamine, ketamine, and naloxone for E12-7

The data in Table 4.1 and 4.2 have revealed that methamphetamine, ketamine, and naloxone at their maximum drug concentrations increased the K_M values and decreased the catalytic efficiency of E12-7 by 30-40%. Their K_i values were further determined to be 98.59 μM, 6.08 μM, and 9.5 μM, respectively (Table 4.5), which are a little higher than or roughly equal to their corresponding maximum drug concentrations in plasma. Therefore, methamphetamine, ketamine, and naloxone are likely to slightly affect the catalytic activity of exogenous E12-7 in metabolism of cocaine.

4.5 Conclusion

Four classes of drugs intimately involved in cocaine abuse and its treatment were examined in this study. Most of the agents did not significantly inhibit E12-7. However, some tricyclic antidepressants (TCA) examined in this study did inhibit E12-7 with K_i in the range of 0.24-1.22 μM. Besides, TCA appears to enhance cocaine-induced toxicity, therefore it should be avoided or used with caution when BChE or its mutant is applied for cocaine abuse treatment. Fortunately, the current medications for cocaine overdose treatment in the ED do not comprise the catalytic activity of E12-7 for cocaine hydrolysis. Therefore the combination of enzyme therapy and current treatment for cocaine overdose might be feasible and give a better clinical outcome. Additional *in vivo* studies in rodents are necessary in order to predict the likelihood of drug-enzyme interactions in humans.

Chapter Five: Characterization of a Highly Efficient New Mutant of Human Butyrylcholinesterase Specific for Cocaine Detoxification

Summary

It is known that the aforementioned E12-7 (which is the A199S/F227A/S287G/A328W/Y332G mutant of human BChE) has ~2000-fold improved catalytic efficiency against (-)-cocaine compared to the wild-type BChE. It is highly desirable to further improve the catalytic activity of the enzyme against (-)-cocaine in order to decrease the required dose and also the cost for effective treatment of cocaine abuse. Through a novel and systematic computational design approach using E12-7 as a standard reference, a new BChE mutant (A199S/F227A/P285Q/S287G/A328W/Y332G), denoted as E20-7, with further improved *in vitro* activity and *in vivo* potency compared to E12-7 was designed and discovered. The *in vitro* catalytic activity of E20-7 against cocaine and *in vivo* potency in metabolizing cocaine were well characterized in the study described in this chapter. The newly discovered mutant E20-7 (k_{cat}/K_M = 4.1 × 10^9 M^{-1} min^{-1}) shows 2.2-fold further improvement in catalytic efficiency against (-)-cocaine compared to E12-7 (k_{cat}/K_M = 1.8 × 10^9 M^{-1} min^{-1}), or 4,450-fold improved catalytic efficiency against (-)-cocaine compared to the wild-type BChE (k_{cat}/K_M = 9.1 × 10^5 M^{-1} min^{-1}). Extensive *in vivo* studies have demonstrated that E20-7 as an exogenous enzyme can indeed rapidly metabolize cocaine in mice and rats, can protect mice from the acute toxicity induced by a lethal dose of cocaine, and efficiently prevent cocaine from entering CNS and producing the physiological effects. E20-7 is identified as the most efficient cocaine hydrolase known so far, and may be developed as a valuable therapeutic agent for cocaine abuse treatment.

5.1 Overview of the strategy to design and discover high-activity mutants of human BChE

As well known, computational design of high-activity mutants of an enzyme is extremely challenging, particularly when the chemical reaction process is rate-determining for the enzymatic reaction.(*92, 94, 95*) To computationally design a mutant enzyme with an improved catalytic activity for a given substrate, one needs to design

possible amino-acid mutations that can accelerate the rate-determining step of the catalytic reaction process (*96, 150, 152*) while other steps of the reaction are not slowed down by the mutations. The fundamental reaction pathway for BChE-catalyzed hydrolysis of (-)-cocaine was uncovered by extensive molecular dynamics (MD) simulations (*96, 150*) and reaction-coordinate calculations (*150, 152*) using quantum mechanics (QM) and hybrid quantum mechanics/molecular mechanics (QM/MM).(*153-157*) The computational studies revealed that the rate-determining step of (-)-cocaine hydrolysis catalyzed by the A328W/Y332A and A328W/Y332G mutants of BChE is the first step of the chemical reaction process.(*84, 92, 96, 150*) Therefore, starting from the A328W/Y332A or A328W/Y332G mutant, rational design of BChE mutants against (-)-cocaine has been focused on decreasing the energy barrier for the first reaction step without significantly affecting the other steps. Our research group has developed unique computational strategies and protocols based on the virtual screening of rate-determining transition states of the enzymatic reaction to design enzyme mutants with improved catalytic activity.(*76, 84-89*) The computational design was followed by *in vitro* experiments, including site-directed mutagenesis, protein expression, and enzyme activity assays. The integrated computational-experimental studies have led to discovery of a series of BChE mutants with a significantly improved catalytic efficiency against (-)-cocaine (*76, 84-89*). The first one of our designed and discovered high-activity mutants of human BChE, *i.e*. the A199S/S287G/A328W/Y332G mutant (*84*), was validated by an independent group of scientists (*90, 91*). This human BChE mutant fused with albumin is currently in double-blind, placebo-controlled clinical trial Phase II in humans by Teva Pharmaceutical Industries Ltd for cocaine abuse treatment.(*17*) Our recently designed and discovered new mutants (*76, 89*) of human BChE are even more active against (-)-cocaine *in vitro* and *in vivo*. The designed BChE mutant E12-7 shows ~2,000-fold improved catalytic efficiency against cocaine, compared to the wide-type BChE. The *in vivo* data of previously designed BChE mutants indicates that the catalytic efficiency correlates with their efficacy in practical protection effects from cocaine-induced acute toxicity, which may guide the future direction of the rational design of high efficient BChE mutants in order to further improve the efficacy in the cocaine abuse treatment.(*88*) Since the costs for protein-based drugs are usually much higher than small-molecule

based drugs, it is highly desirable to further improve the catalytic efficiency of the enzyme against cocaine in order to decrease the required dose of the enzyme, and the costs of the enzyme therapy as well. The general concept of the computational design strategy (*76, 84-89*) was employed (using E12-7 as a standard reference) to design and discover new BChE mutants with further improved catalytic efficiency against (-)-cocaine.

5.2 *In vitro* characterization of E20-7 for their activities against (-)-cocaine and acetylcholine

5.2.1 Materials and methods for *in vitro* studies

5.2.1.1 Materials used for *in vitro* studies

Cloned *pfu* DNA polymerase and *Dpn I* endonuclease were obtained from Stratagene (La Jolla, CA). [^{3}H](-)-cocaine (50 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA). All oligonucleotides were synthesized by the Eurofins MWG Operon (Huntsville, Alabama). The QIAprep Spin Plasmid Miniprep Kit and Qiagen plasmid purification kit and QIAquick PCR purification kit were obtained from Qiagen (Santa Clarita, CA). Chinese Hamster Ovary (CHO) cells were obtained from Life Technologies (Grand Island, NY). 3, 3′, 5, 5′-Tetramethylbenzidine (TMB) was obtained from Sigma (Saint Louis, Missouri). Anti-BChE (mouse monoclonal antibody, Product # HAH002-01) was purchased from AntibodyShop (Gentofte, Denmark) and goat anti-mouse IgG HRP conjugate was from Zymed (San Francisco, CA).

5.2.1.2 Site-directed mutagenesis

Site-directed mutagenesis of human BChE cDNA was performed to get desired mutations by using the QuickChange method (*158*). E20-7 cDNA was generated by performing polymerase chain reaction (PCR) with Pfu polymerase, using E12-7 cDNA in a pRc/CMV expression plasmid as the template and primers designed for P285Q mutation. The primers used for P285Q mutation were as follows (the bases corresponding to introduced mutation are shown in bold):

F-primer GTT GTC CCC TAT GGG ACT **CAG** TTG TCA GTA AAC TTT GGT CCG ACC

R-primer GGT CGG ACC AAA GTT TAC TGA CAA **CTG** AGT CCC ATA GGG GAC AAC

The PCR product was treated with *Dpn I* endonuclease to digest the parental DNA template. Modified plasmid DNA was transformed into *Escherichia coli*, amplified, and purified. The DNA sequences of the mutants were confirmed by DNA sequencing.

5.2.1.3 Protein expression

The enzymes, including wild-type BChE, E12-7, and E20-7, were expressed in Chinese Hamster Ovary (CHO) cells. Cells at the density of ~1 × 10^6 cells/ml were transfected by TransIT-PRO® Transfection Kit and cultured for five more days. The medium was harvested for the BChE activity assays. The wild-type BChE, E12-7 and E20-7 were expressed and their enzyme activity against (-)-cocaine were assayed at the same time under the same experimental conditions. The wild-type BChE and E12-7 were used as standard references.

5.2.1.4 Protein purification

Purification of the enzyme in the medium was achieved by a two-step purification using ion-exchange chromatography followed by affinity chromatography, as described previously in detail.(*116*) The purified protein was dialyzed against phosphate-buffered saline and stored at 4 ℃ or -80 ℃.

5.2.1.5 Enzyme activity assays

To measure (-)-cocaine and benzoic acid, the product of (-)-cocaine hydrolysis catalyzed by BChE, we used sensitive radiometric assays based on toluene extraction of [^{3}H](-)-cocaine labeled on its benzene ring (*99*). In brief, to initiate the enzymatic reaction, 100 nCi of [^{3}H](-)-cocaine was mixed with 100 μl of culture medium. The enzymatic reactions proceeded at room temperature (25^{o}C) with varying concentrations of (-)-cocaine. 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. [^{3}H]benzoic acid was extracted by 1 ml of toluene and measured by scintillation counting. Finally, the measured (-)-cocaine concentration-dependent radiometric data were analyzed by using the standard Michaelis-Menten kinetics so that the catalytic

parameters were determined along with the enzyme concentration determined by a well-established standard enzyme-linked immunosorbent assay (ELISA) protocol (*84*) and also the Bradford assay with the two step-purified enzyme. The BChE activity assays with [^{3}H]ACh are similar to the assays with [^{3}H](-)-cocaine. The primary difference is that the enzymatic reaction was stopped by addition of 200 µl of 0.2 M HCl containing 2 M NaCl and that the product was [^{3}H]acetic acid for the ACh hydrolysis.

5.2.2 *In vitro* activity of E20-7 against (-)-cocaine and ACh

Based on the computational design, we carried out *in vitro* experimental tests, including site-directed mutagenesis, protein expression, and *in vitro* enzyme activity assays, on wild-type BChE, E12-7 and E20-7 mutants of human BChE. To minimize the possible systematic experimental errors of the *in vitro* kinetic data, we expressed the enzymes and performed kinetic studies with wild-type BChE, E12-7 and E20-7 under the same conditions and compared the catalytic efficiencies of E20-7 against (-)-cocaine and ACh to the corresponding catalytic efficiencies of wild-type BChE and E12-7. Michaelis-Menten kinetics of the enzymatic hydrolysis of (-)-cocaine or ACh was determined by performing the sensitive radiometric assays using [^{3}H](-)-cocaine (labeled on its benzene ring) or [^{3}H]ACh (labeled on its acetyl group) with varying concentrations of substrate. Depicted in Figures 5.1 are the measured kinetic data of E20-7 for (-)-cocaine and ACh. Summarized in Table 5.1 are the obtained kinetic parameters of E20-7 for hydrolysis of (-)-cocaine and ACh in comparison with the known kinetic parameters of wild-type BChE and E12-7 against (-)-cocaine and ACh.

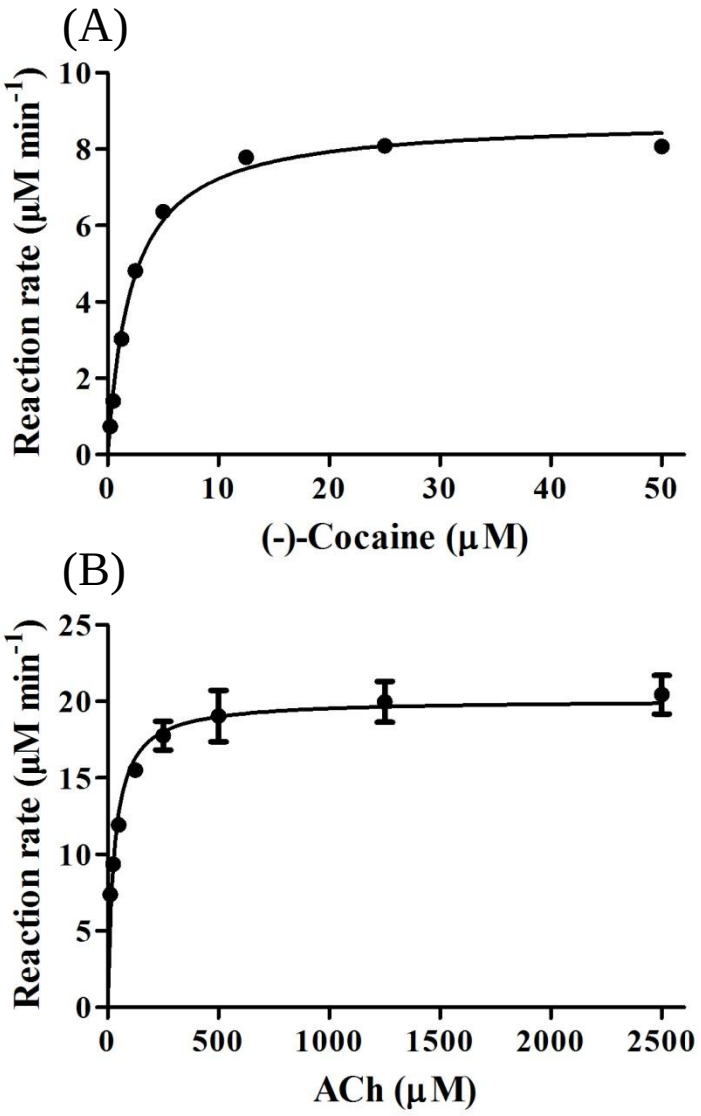


Figure 5.1 Kinetic data for (-)-cocaine (A) and ACh (B) hydrolysis catalyzed by E20-7. The reaction rate was represented in μM min^{-1} per nM enzyme.

As shown in Table 5.1, the newly discovered mutant E20-7 (k_{cat} = 8790 min^{-1}, K_M = 2.2 μM, and k_{cat}/K_M = 4.1 × 10^9 M^{-1} min^{-1}) shows a 2.2-fold improvement in catalytic efficiency against (-)-cocaine compared to E12-7 (k_{cat} = 5700 min^{-1}, K_M = 3.1 μM, and k_{cat}/K_M = 1.8 × 10^9 M^{-1} min^{-1}), or a 4,450-fold improved catalytic efficiency against (-)-cocaine compared to the wild-type BChE (k_{cat} = 4.1 min^{-1}, K_M = 4.5 μM, and k_{cat}/K_M = 9.1 × 10^5 M^{-1} min^{-1}). AChE has been recognized as the most efficient hydrolytic enzyme,

with its catalytic activity for ACh (k_{cat} = 702,000 min^{-1}, K_M = 90 μM, and k_{cat}/K_M = 7.8 × 10^9 M^{-1} min^{-1}) approaching the limit allowed by the diffusion of the substrate.(*159, 160*) The obtained catalytic efficiency of E20-7 for (-)-cocaine hydrolysis is comparable to that of AChE for ACh hydrolysis.

Compared to E12-7 (A199S/F227A/S287G/A328W/Y332G), the additional P285Q mutation in E20-7 (A199S/F227A/P285Q/S287G/A328W/Y332G) significantly decreases the K_M values and slightly increases the k_{cat} of E20-7 against (-)-cocaine. The new mutation P285Q should not produce any new hydrogen bonds in the transition state. However, it may indirectly affect the existing hydrogen bonding between the carbonyl oxygen of (-)-cocaine and the oxyanion hole (consisting of residuals G116, G117, and S199) in the transition states, thus further stabilizing the transition states and improving the catalytic activity for (-)-cocaine.

Table 5.1 Kinetic parameters determined *in vitro* for enzymatic hydrolysis of (-)-cocaine and ACh.

Substrate	**Enzyme**[a]	**K_M (μM)**	**k_{cat} (min^{-1})**	**k_{cat}/K_M ($M^{-1}min^{-1}$)**	**RCE**[b]
(-)-cocaine	WT BChE [c]	4.5	4.1	9.1×10^5	1
	E12-7	3.1	5700	1.8×10^9	2020
	E20-7	2.2	8790	4.1×10^9	4450
ACh	WT BChE [d]	148	61200	4.1×10^8	1
	E12-7	37	11900	3.2×10^8	0.78
	E20-7	29	20100	6.9×10^8	1.68
	AChE[e]	90	702,000	7.8×10^9	19

[a]Unless indicated otherwise, all kinetic parameters listed in this table were determined in the present study.
[b]RCE refers to the relative catalytic efficiency (k_{cat}/K_M), *i.e.* the ratio of the k_{cat}/K_M value of the mutant to that of wild-type BChE against the same substrate.
[c]Data for wild-type BChE from reference.(*79*)
[d]The k_{cat} value for wild-type BChE was reported in reference.(*100*)
[e] Data for AChE from reference.(*160*)

Regarding the catalytic activity against ACh (shown in Figures 5.1), we obtained k_{cat} = 20,100 min^{-1} and K_M = 29 μM for E20-7. Compared to the wild-type BChE (k_{cat}/K_M = 4.1 × 10^8 M^{-1} min^{-1}), both E12-7 and E20-7 listed in Table 5.1 have significantly lower k_{cat} and K_M values against ACh. Overall, the catalytic efficiency (k_{cat}/K_M = 6.9 × 10^8 M^{-1} min^{-1}) of E20-7 against ACh is slightly higher than that of the wild-type BChE (~1.68-fold).

Furthermore, it is interesting to notice that, the catalytic efficiency of wild-type BChE against ACh (k_{cat}/K_M = 4.1 × 10^8 M^{-1} min^{-1}) is ~450-fold higher than that of the same enzyme against (-)-cocaine (k_{cat}/K_M = 9.1 × 10^5 M^{-1} min^{-1}). However, for both E12-7 and E20-7, the catalytic efficiency against (-)-cocaine (k_{cat}/K_M = 1.8 × 10^9, 4.1 × 10^9 M^{-1} min^{-1}) is ~5-fold higher than that of the same enzyme against ACh (k_{cat}/K_M = 3.2 × 10^8, 6.9 × 10^8 M^{-1} min^{-1}). As a result, E20-7 has not only a significantly improved catalytic efficiency against (-)-cocaine, but also the desirable selectivity for (-)-cocaine over ACh. It is known that ACh is the only natural substrate for both AChE and BChE in human body.(*161*) However, it is unlikely that E20-7 administrated as an exogenous enzyme would affect the motor transmission. First of all, the exogenous enzyme in plasma would not enter the brain. Besides, E20-7 has a ~10-fold lower catalytic efficiency against ACh than AChE, and peripheral cholinergic synapses are densely packed with AChE.

5.3 *In vivo* characterization of E20-7 for its potency in metabolizing cocaine

5.3.1 Materials and subjects for *in vivo* studies

Cocaine hydrochloride was provided by the National Institute on Drug Abuse (NIDA) Drug Supply Program (Bethesda, MD) and prepared in saline for *in vivo* studies. The benzoic acid used for the standard in the HPLC analysis was purchased through Sigma Aldrich (St. Louis, MO). The E20-7 material used for *in vivo* studies were prepared in our recently reported study (*123*) using stable CHO-S cells, purified using ion exchange and affinity chromatography and diluted to the required concentration in phosphate buffered saline (PBS) for injection.

Sprague-Darley (male or female) rats (200-250 g), male CD-1 mice, and Swiss Webster male mice (27-30 g) were ordered from Harlan (Indianapolis, IN). The rats were housed initially in 2 to 4 rats per cage. The mice were housed in groups of 2 to 5 mice per

page. All the animals were allowed ad libitum access to food and water and were maintained on a 12-hour light and dark cycle with lights on at 8 AM in a room kept at a temperature of 21 to 22 ℃. Each animal was only used once. Experiments were performed in the same colony room in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. The experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Kentucky.

5.3.2 Characterization of cocaine clearance accelerated by E20-7

5.3.2.1 Animal procedure

Sprague-Darley rats (male, 200-250 g) were used in this study. General anesthetic isoflurane was utilized with nose cone during the i.v. administration of enzyme E20-7 (or PBS) and cocaine. To characterize cocaine (i.v. administrated) clearance accelerated by E20-7, four rats were injected with PBS through tail vein 1 min before i.v. injection of 5 mg/kg cocaine, and other four rats were injected with the enzyme E20-7 followed by i.v. injection of the same dose of cocaine. About 50 to 75 μl of blood from saphenous veins was collected into capillary tubes and immediately mixed in 100 μl of 250 μM paraoxon and 10 U heparin at 2, 5, 10, 15, 30, and 60 min after the i.v. injection of cocaine. To characterize cocaine (i.p. administrated) clearance accelerated by E20-7, five rats were injected with PBS through tail vein 1 min before i.p. injection of 25 mg/kg cocaine, and other five rats were injected with the enzyme E20-7 followed by i.p. injection of the same dose of cocaine. The blood was sampled at 5, 10, 15, 20, 30, 40, 60, 90, 120, and 150 min after the i.p. administration of cocaine and treated with paraoxon. Paraoxon is an irreversible BChE inhibitor that can stop the enzymatic hydrolysis of cocaine between sampling and analysis. The diluted blood samples were stored at -70 ℃ and assayed by using a High-Performance Liquid Chromatographic (HPLC) method.

Benzoic acid is the product of cocaine hydrolysis catalyzed by the enzyme E20-7. To assay the cocaine and benzoic acid concentrations in the blood samples, the frozen whole blood samples were thawed on ice for 3 hours. Then 150 μl of mobile phase (26% acetonitrile and 74% water containing 0.1% TFA) was mixed with each sample, and 50 μl of 10% $HClO_4$ was added to break the blood cell membrane. The mixture was

vortexed for 1 min and then centrifuged at 25,000 g for 15 min, and the supernatant was transferred to an autosampler vial of which 200 μl was injected into the chromatographic system. Chromatography was performed using a Waters 1525 binary HPLC pump (Waters Corporation, Milford, MA), a Waters 2487 dual λ absorbance detector, a Waters 2475 multi λ fluorescence detector, and a Waters 717 plus autosampler. The flow rate was 1 ml/min. The eluent was monitored at 230 nm for absorbance of benzoic acid and at 315 nm for fluorescence of cocaine while exciting at 230 nm. The cocaine peaks appeared at 10.5 min, and the benzoic acid peaks occurred at 14.5 min. The concentrations of cocaine and benzoic acid were determined by comparing the corresponding HPLC peak areas with those of authentic standards.

5.3.2.2 Cocaine clearance accelerated by E20-7

In order to examine the *in vivo* potency of E20-7 for metabolizing cocaine, we characterized the pharmacokinetic profiles of cocaine clearance in the absence or presence of E20-7 in rats by using a chromatographic assay. The rats (n=4) were injected with PBS, 0.01 or 0.02 mg/kg E20-7, followed by i.v. injection of 5 mg/kg cocaine. E20-7 can hydrolyze cocaine to produce benzoic acid and ecgonine methyl ester, and greatly accelerate the clearance of cocaine from the body. It has been known that the endogenous BChE in rats is very inefficient in metabolizing cocaine and, for this reason, cocaine was mainly metabolized by liver carboxylesterase in the blood to produce benzoylecgonine and methanol in rats.(*99*) E20-7 accelerated cocaine clearance dose-dependently as depicted in Figure 5.2. The control curves in Figure 5.2 reflect the overall effects of the all cocaine elimination pathways. In the control rats, the average concentration of cocaine at the first time point (2 min) was 7.4 μM, while the average concentration of benzoic acid (metabolite) was 0.5 μM. With a very low dose (0.01 mg/kg) of E20-7 pretreatment, the initial cocaine peak (2.9 μM) was slightly lowered than the control (7.4 μM). When the rats were given 0.02 mg/kg E20-7 prior to the cocaine challenge, the initial cocaine concentration at 2 min was decreased to a very low level (0.6 μM). The corresponding initial benzoic acid peak was elevated, indicating that the accelerated clearance is due to the activity of administrated E20-7 in the plasma. The high potency of E20-7 for metabolizing cocaine in rats is consistent with its high *in vitro* catalytic activity against

cocaine. This E20-7 dose (0.02 mg/kg) used is lowered than the previously used dose of E12-7 (0.1 mg/kg) (*123*) to produce the same effect.

It should be mentioned that the total plasma concentration of cocaine and benzoic acid (~11.6 μM) in the presence of E20-7 (when the benzoic acid concentration was higher) was higher than that (~7.6 μM) in the absence of E20-7 (when the cocaine concentration was higher). This observation might be associated with the potentially different distribution volumes of cocaine and benzoic acid in the body. As well known, cocaine is an amine drug which can readily cross cell membranes under physiological condition, while benzoic acid primarily exists in the benzoate ion under physiological condition. So, benzoic acid is expected to have a relatively smaller distribution volume compared to cocaine.

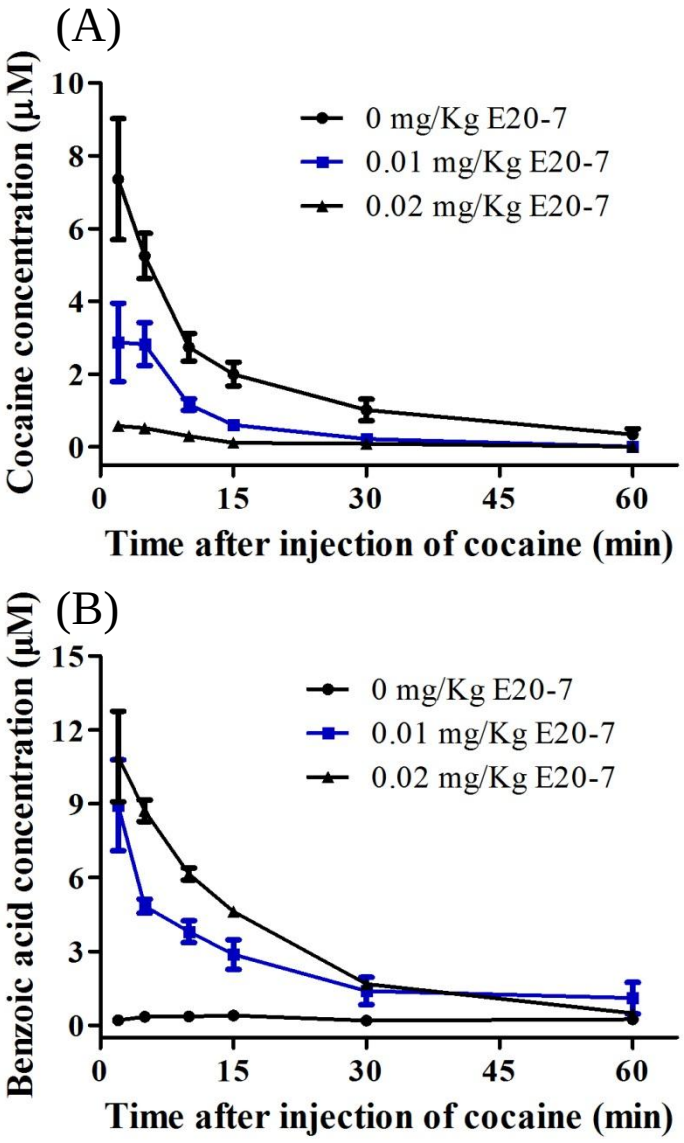


Figure 5.2 Cocaine (i.v. administrated) clearance accelerated by E20-7. Time-dependent concentrations of cocaine (A) and benzoic acid (B) in cocaine clearance accelerated by E20-7 expressed in stable CHO-S cells. E20-7 or PBS was i.v. injected 1 min before 5 mg/kg cocaine i.v. administration (n=4). The blood was sampled from 2 to 60 min after the injection of cocaine. The concentrations of cocaine in the blood samples were quantitated in duplicate by using an HPLC.

The effect of E20-7 on acceleration of cocaine clearance was also evaluated in a similar experiment, with cocaine being administrated through intraperitoneal injection.

The rats (n=5) were injected with PBS or E20-7 through tail vein, followed by i.p. injection of 25 mg/kg cocaine. Similar effects were observed as depicted in Figure 5.3. In the control rats, cocaine concentration peaked (2.2 μM) at 15 min after i.p. cocaine administration. With a very low dose (0.04 mg/Kg) of E20-7, the peak concentration of cocaine was as low as 0.15 μM; when the dose of E20-7 was increased to 0.10 mg/Kg, the peak concentration of cocaine was below the detectable level. The corresponding benzoic acid concentration was elevated due to the treatment of E20-7. Two doses of E20-7 gave the similar levels of benzoic acid, only differs in the peak time.

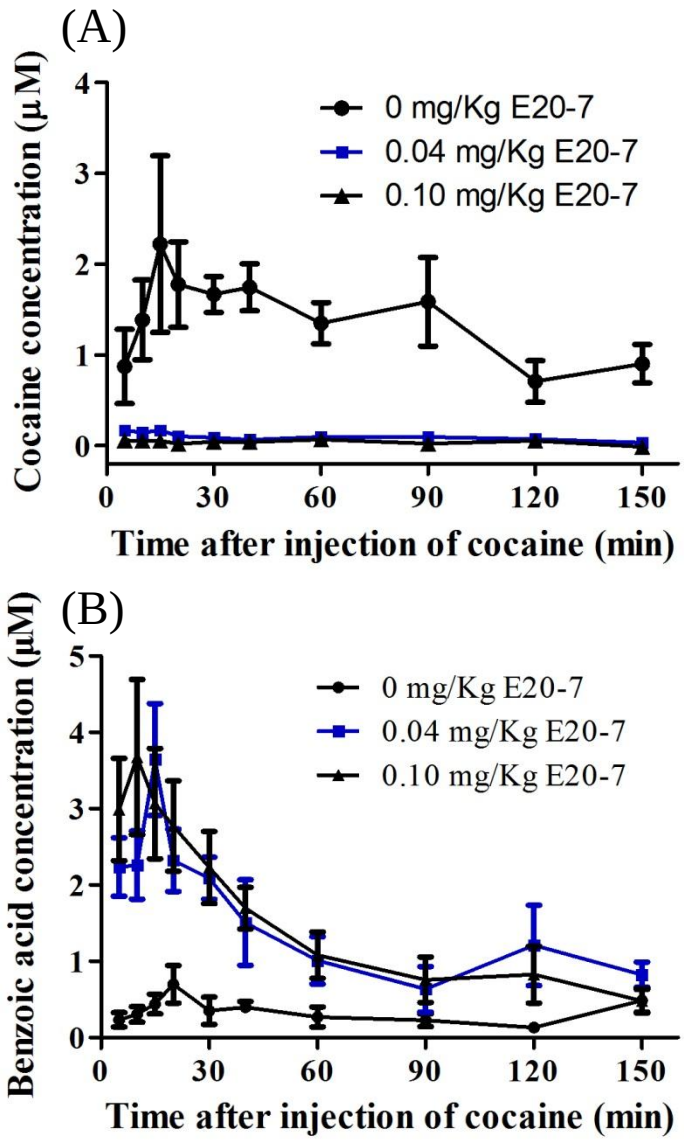


Figure 5.3 Cocaine (i.p. administrated) clearance accelerated by E20-7. Time-dependent concentrations of cocaine (A) and benzoic acid (B) in cocaine clearance accelerated by E20-7 expressed in stable CHO-S cells. E20-7 or PBS was i.v. injected 1 min before 25 mg/kg cocaine i.p. administration (n=5). The blood was sampled from 5 to 150 min after the injection of cocaine. The concentrations of cocaine in the blood samples were quantitated in duplicate by using an HPLC.

To summarize, the E20-7-caused dramatic changes in both the cocaine and benzoic acid concentrations clearly indicated that cocaine was rapidly metabolized to benzoic acid in the presence of E20-7. The pretreatment of E20-7 greatly accelerate the clearance of cocaine from the rats, no matter how cocaine is administrated. The high potency of E20-7 in metabolism of cocaine correlates with its high catalytic activity against cocaine. The minimum dose of E20-7 required to quickly clear cocaine from body is expected to be lowered than that of E12-7 to produce the same effect.

5.3.3 Protection effects of E20-7 against acute toxicity of a lethal dose of cocaine

5.3.3.1 Animal procedure

Cocaine hydrochloride and purified enzyme E20-7 were prepared to the required concentration and administered at a volume of 0.2 mL/mouse. Cocaine-induced acute toxicity was characterized by the occurrence of convulsion and lethality. Cocaine-induced convulsion was defined as loss of righting posture for at least 5 sec with the simultaneous presence of clonic limb movements (*162*). Lethality was defined as cessation of observed movement and respiration.

CD-1 mice (male, 27-30 g) were used in this study. PBS or E20-7 at different doses (n=6) was injected through tail vein 1 min before intraperitoneal administration of cocaine 180 mg/kg (LD_{100}). After administration of cocaine, mice were immediately placed individually in cages for observation. The presence or absence of convulsions and lethality were recorded for 60 min after cocaine administration.

5.3.3.2 Protection effects of E20-7

The purified E20-7 was used to study their *in vivo* activity in protection of mice from acute toxicity of a lethal dose of cocaine (180 mg/kg, LD_{100}). As shown in Figure 5.4, for the control experiments without administration of the enzyme, intraperitoneal (i.p.) administration of 180 mg/kg cocaine produced lethality in all tested mice (n=6). Pretreatment with E20-7 (*i.e.* 1 min prior to cocaine administration) dose-dependently protected mice against cocaine-induced convulsion and lethality. In particular, E20-7 at a small dose of 0.2 mg/kg produced full protection in mice after receiving a lethal dose (180 mg/kg) of cocaine. So, the minimum dose of the enzyme required to produce full protection of the mice from the acute toxicity of 180 mg/kg cocaine was determined to be 0.2 mg/kg for E20-7. The minimum dose of E12-7 required to produce full protection of the mice from 180 mg/kg cocaine induced acute toxicity is a dose between 0.33 mg/kg and 1 mg/kg (*76*), therefore E20-7 is more potent than E12-7 in their practical protection effects, which is consistent with their *in vitro* catalytic efficiencies against cocaine. The *in vivo* data depicted in Figure 5.4 suggest that E20-7 is indeed promising for cocaine detoxification in therapeutic treatment of cocaine abuse.

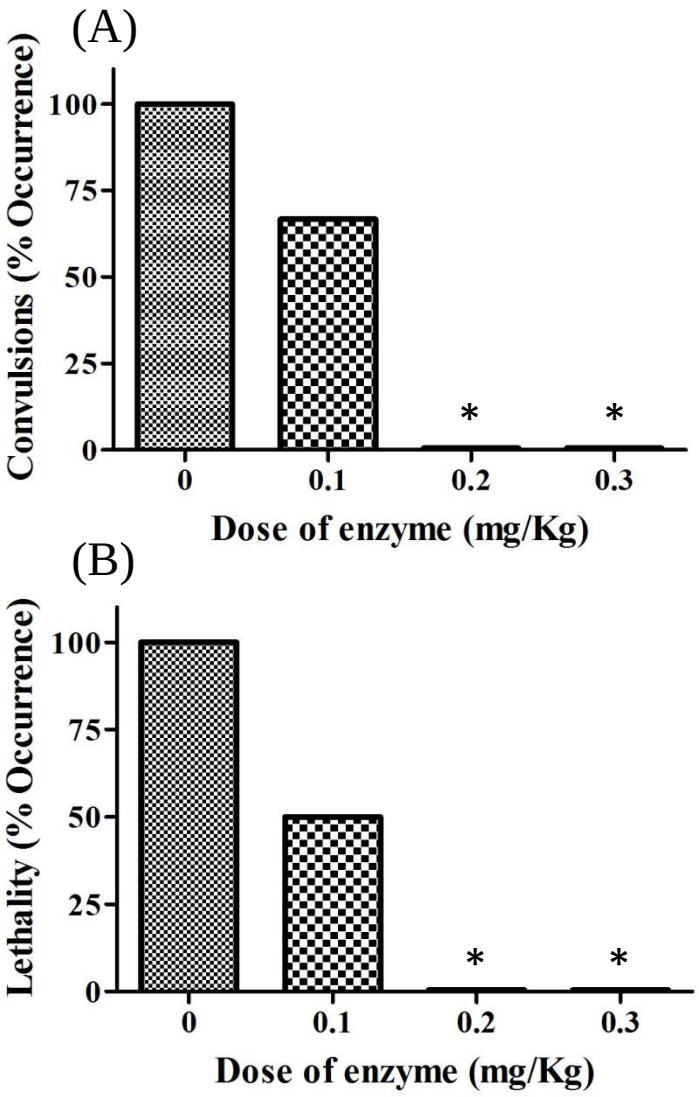


Figure 5.4 Potency of protection effects of E20-7 against acute toxicity of a lethal dose of cocaine. The PBS or E20-7 was administered i.v. 1 min before i.p. administration of 180 mg/kg cocaine (n=6). The asterisks represent significant differences from the conditions of mice pretreated with PBS ($p<0.05$).

5.3.4 Effects of E20-7 on the hyperactivity induced by cocaine

5.3.4.1 Animal procedure

The effect of E20-7 on cocaine-induced hyperactivity was evaluated by using a video-tracking system at the University of Kentucky's Rodent Behavior Core (RBC). The locomotor activity test was performed in high density, non-porous plastic chambers measuring 50 cm (L) × 50 cm (W) × 38 cm (H) in a light- and sound-attenuating behavioral test enclosure (San Diego Instruments, San Diego, CA). Cumulative distance traveled and speed was recorded by EthoVision XT video tracking system (Noldus Information Technology, Wageningen, Netherlands) to represent the locomotor activity. The test session was 60 min long and data was collected in 5-min bins. Male Swiss Webster mice were introduced to the test chambers for habituation (90 min long per session) on two consecutive days before the test day. On the test day, mice were allowed to acclimate to the test chambers for 60 minutes, and the total distance traveled during this period of time was used to determine the basal activity. Then E20-7 or PBS was administered through intravenous (i.v.) injection, followed by intraperitoneal (i.p.) injection of 25 mg/kg cocaine or saline (n=8). After the cocaine/saline administration, mice were immediately returned to the test chamber for activity monitoring in the following session of 60 minutes.

5.3.4.2 Effects of E20-7 on the hyperactivity induced by cocaine

The potency of E20-7 in elimination of the physiological effects of cocaine was evaluated in Male Swiss Webster mice. Based on the results shown in Figure 5.6, compared with the baseline in the control group (treated with i.v. PBS and i.p. saline), without pretreatment of E20-7, 25 mg/kg cocaine (i.p.) induced rather strong hyperactivity in mice. The group injected with 0.04 mg/kg E20-7 still displayed slight hyperactivity, but the extent and duration of hyperactivity were decreased considerably. Furthermore, 0.10 mg/kg E20-7 completely eliminated the hyperactivity induced by 25 mg/kg cocaine, as seen in Figure 5.6. The minimum dose of E20-7 required to completely suppress the hyperactivity induced by 25 mg/kg cocaine was 0.10 mg/kg.

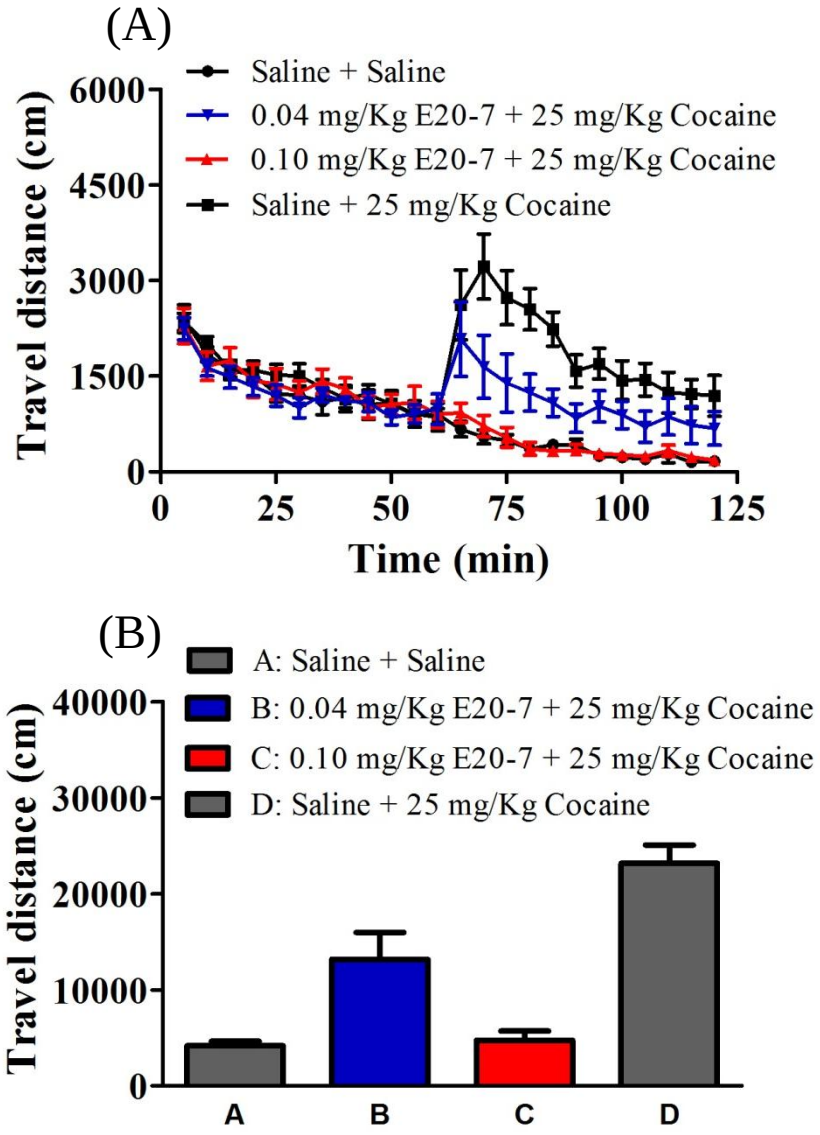


Figure 5.5 Effects of the exogenous enzyme E20-7 on cocaine-induced hyperactivity. PBS or E20-7 was injected i.v. into mice through tail veins 1 min before i.p. injection of saline or cocaine. Four groups were scheduled, and each group had eight mice (n=8). Group A were treated with i.v. PBS and i.p. saline; Group B were treated with i.v. 0.04 mg/kg E20-7 and i.p. 25 mg/kg cocaine; Group C were treated with i.v. 0.10 mg/kg E20-7 and i.p. 25 mg/kg cocaine. Group D were treated with i.v. PBS and i.p. 25 mg/kg cocaine.

Volkow *et al.* reported that, for humans, "*at least 47% of dopamine transporter has to be blocked for subjects to perceive cocaine's effects*"(*163*). Cocaine diffuse across the blood brain barrier is very fast so that there is an equilibrium distribution between plasma and brain. The threshold concentrations of cocaine in human brain and plasma required to produce physiological effects were estimated to be 0.22±0.07 μM and 0.24±0.08 μM, respectively, in light of a recently reported cocaine pharmacokinetic modeling.(*82*) When the cocaine concentration in brain and plasma do not reach the "threshold" values because of the administration of an efficient cocaine-metabolizing enzyme, one may consider that the enzyme has *effectively* prevented cocaine from entering the brain and producing the detectable physiological effects. The hyperactivity induced by cocaine correlate with the corresponding plasma cocaine concentration. As shown in Figure 5.3, with the pretreatment of 0.04 mg/kg E20-7, the blood cocaine concentrations in rats for the first 15 min after cocaine administration were under ~0.2 μM, close to the threshold value for humans. With the pretreatment of 0.10 mg/kg E20-7, the blood cocaine concentrations in the investigated period were in a very low level (under ~0.05 μM), which is much lower than the threshold value to produce measurable physiological effects. So, 0.10 mg/kg E20-7 completely eliminated the hyperactivity induced by 25 mg/kg cocaine. Base on the above analysis, E20-7 has shown great potential in rapidly accelerating cocaine metabolism in plasma and completely eliminating cocaine-induced hyperactivity, indicating that E20-7 can effectively prevent cocaine from entering brain and producing the physiological effects.

5.4 Conclusion

This study reveals that the newly discovered E20-7 is more active against cocaine than E12-7 both *in vitro* and *in vivo*. The catalytic activity of E20-7 for cocaine hydrolysis is considerably improved compared to the wild-type BChE, and comparable to that of the most efficient hydrolytic enzyme AChE for ACh hydrolysis. Extensive *in vivo* studies in rodents have demonstrated that administration of E20-7 can indeed rapidly metabolize cocaine, can protect the mice from the acute toxicity induced by lethal dose of cocaine, and efficiently prevent cocaine from entering brain and producing the

physiological effects. In particular, the catalytic efficiency of the enzymes correlates with their practical efficacy in cocaine detoxification. Therefore, the higher the catalytic activity, the lower the dose required for effective detoxification, and the lower the cost for treatment of cocaine abuse. E20-7 is identified as the most efficient cocaine hydrolase at this point of time, and may be developed as a valuable therapeutic agent for cocaine abuse treatment.

Chapter Six: Concluding Remarks and Future Plan

Human BChE has been recognized as a promising candidate of the enzyme therapy to detoxify cocaine. However, the low activity of wide-type human BChE against cocaine limits its potential therapeutic use in cocaine abuse treatment. Dr. Zhan's lab previously designed and discovered a series of high-activity mutants of human BChE. In particularly, E12-7 has a 2000-fold improvement in catalytic efficiency against cocaine, compared to wide-type BChE.

This dissertation was mainly focused to address the possible concerns in further development of those promising BChE mutants for cocaine abuse treatment, such as whether the administration of this exogenous enzyme will affect the cholinergic system, whether it can efficiently hydrolyze the cocaine's toxic metabolites, and whether the commonly used therapeutic agents will significantly affect the catalytic activity of designed BChE mutants against cocaine when they are co-administered. Both computational modeling and experimental kinetic analysis on the catalytic activity of the previously designed BChE mutants (includes E12-7) against the various substrates, including acetylcholine (the only natural substrate in the body) and cocaine metabolite cocaethylene, were carried out to demonstrate the substrate selectivity of BChE mutants for cocaine and cocaethylene over acetylcholine. Meanwhile, possible drug-drug interactions between the promising enzyme E12-7 and some commonly used agents were investigated *in vitro*, providing insights for the further development of E12-7 into an effective treatment for cocaine abuse. In addition, further computational design using E12-7 as a standard reference was followed by extensive wet experimental tests, including both *in vitro* and *in vivo* assays, leading to a more promising BChE mutant E20-7. The identified mutants of human BChE are expected to be valuable candidates for development of a more efficient enzyme therapy for cocaine abuse.

6.1 Summary of the major conclusions obtained from this investigation

1) Both the computational modeling and experimental kinetic analysis have consistently revealed that all the examined BChE mutants in this study have a

considerably improved catalytic efficiency against (-)-cocaine, without dramatically improving the catalytic efficiency against any of the other examined substrates (including acetylcholine, acetylthiocholine, butyrylthiocholine, and (+)-cocaine), compared to the wild-type BChE. The previously designed BChE mutants showed desired selectivity for (-)-cocaine over neurotransmitter acetylcholine.

2) Based on the obtained kinetic data, E14-3 and E12-7 have a considerably improved catalytic activity against cocaethylene compared to the wild-type BChE. E12-7 is identified as the most promising enzyme for hydrolyzing for all three toxic compounds, *i.e.* (-)-cocaine, cocaethylene, and norcocaine.

3) According to the *in vitro* drug-enzyme interaction data, most of the tested drugs did not display significant inhibition on E12-7. However, some tricyclic antidepressants (TCA) at high concentrations did inhibit the enzyme and, thus, should be avoided or used with caution. Importantly, the current drugs for cocaine overdose treatment in the ED do not affect the catalytic activity of E12-7 for cocaine hydrolysis.

4) The newly discovered mutant E20-7 is more active for cocaine than E12-7 both *in vitro* and *in vivo*. The catalytic activity of E20-7 for cocaine hydrolysis is comparable to that of the most efficient hydrolytic enzyme AChE for ACh hydrolysis. Extensive *in vivo* studies in rodents have demonstrated the practical efficacy of E20-7 in cocaine detoxification and the correlation between the practical efficacy and the *in vitro* catalytic efficiency. E20-7 may be developed as a valuable therapeutic agent for cocaine abuse treatment.

6.2 Future plan of this investigation

1) *In vitro* characterization of E20-7 against other potential substrates or for the interactions with commonly used small-molecule drugs, as what we have done for E12-7 and other BChE mutants;

2) Additional *in vivo* study in rodents is necessary in order to predict the likelihood of drug-enzyme interactions in human;

3) Further development of human BChE mutants for cocaine addiction treatment should be focused on improving the biological half-life while maintaining the high catalytic activity against (-)-cocaine.

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Education

Jan. 2010 - Present **University of Kentucky**
Ph.D. Candidate in Pharmaceutical Sciences,
Advisor: Chang-Guo Zhan, Ph.D., Professor

Sep. 2005 - Jul. 2008 **University of Chinese Academy of Sciences**
Master in Analytical Chemistry,
Advisor: Xiangjun Li, Ph.D., Professor

Sep. 2001- Jul. 2005 **Anhui Normal University**
Bachelor in Chemistry

Research Experience

Oct. 2009- Present **University of Kentucky**

1) Design and characterization of human butyrylcholinesterase mutants for treatment of cocaine overdose and addiction;
2) Rational design and discovery of human microsomal prostaglandin E synthase-1 (mPGES-1) inhibitors as next-generation anti-inflammatory drugs;
3) Rational drug design of phosphodiesterase-2 (PDE2) inhibitors as novel memory enhancers and anxiolytic drugs.

May. 2006 - Jul. 2008 **University of Chinese Academy of Sciences**

1) Selective detection of dopamine and influence of magnetic field on electrochemical reactions of dopamine;
2) Qualitative and quantitative analysis of flavonoids and isoflavones by Capillary Electrophoresis;

Honors and Awards

Nov. 2013 AAPS Graduate Student Award in Drug Discovery and Development Interface

Patents

1. Chang-Guo Zhan, Fang Zheng, Wenchao Yang, Liu Xue, **Shurong Hou**. "High Activity Mutants of Butyrylcholinesterase for Cocaine Hydrolysis". *U.S. Patent 8592193*, filed Feb. 17, 2012, and issued Nov. 26, 2013.

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1. **Shurong Hou**, Max Zhan, Xirong Zheng, Chang-Guo Zhan, Fang Zheng. "Kinetic Characterization of Human Butyrylcholinesterase Mutants for Hydrolysis of Cocaethylene". *Biochemical Journal*, 2014, 460: 447-457.
2. Fang Zheng, Liu Xue, **Shurong Hou**, Junjun Liu, Max Zhan, Wenchao Yang, Chang-Guo Zhan. "A Highly Efficient Cocaine-detoxifying Enzyme Obtained by Computational Design". *Nature Communications*, 2014, 5: 3457.
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4. Lei Fang, **Shurong Hou**, Liu Xue, Fang Zheng, Chang-Guo Zhan. "Amino-acid Mutations to Extend the Biological Half-life of a Therapeutically Valuable Mutant of Human Butyrylcholinesterase". *Chemico-Biological Interactions*, 2014 214: 18-25.
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8. Liu Xue*, **Shurong Hou*** (*co-first authors), Wenchao Yang, Lei Fang, Fang Zheng, and Chang-Guo Zhan. "Catalytic Activities of a Cocaine Hydrolase Engineered from Human Butyrylcholinesterase against (+)- and (-)-Cocaine". *Chemico-Biological Interactions*, **2013**, 203 (1): 57-62.

9. Liyi Geng, Yang Gao, Xiabin Chen, **Shurong Hou**, Chang-Guo Zhan, Zoran Radic, Robin J. Parks, Stephen J. Russell, Linh Pham, Stephen Brimijoin. "Gene Transfer of Mutant Mouse Cholinesterase Provides High Lifetime Expression and Reduced Cocaine Responses with No Evident Toxicity". *PLoS One*, 2013, 8(6): e67446
10. Liu Xue, Mei-Chuan Ko, Min Tong, Wenchao Yang, **Shurong Hou**, Lei Fang, Junjun Liu, Fang Zheng, James H. Woods, Hsin-Hsiung Tai and Chang-Guo Zhan. "Design, Preparation, and Characterization of High-Activity Mutants of Human Butyrylcholinesterase Specific for Detoxification of Cocaine". *Molecular Pharmacology*, 2011, 79(2): 290-297.
11. Fang Zheng, Wenchao Yang, Liu Xue, **Shurong Hou**, Junjun Liu, and Chang-Guo Zhan. "Design of High-Activity Mutants of Human Butyrylcholinesterase against (−)-Cocaine: Structural and Energetic Factors Affecting the Catalytic Efficiency". *Biochemistry*, 2010, 49(42): 9113-9119.
12. **Shurong Hou**, Na Zheng, Haiyan Feng, Xiangjun Li and Zhuobin Yuan. "Determination of Dopamine in the Presence of Ascorbic Acid using Poly(3,5-dihydroxy benzoic acid) Film Modified Electrode". *Analytical Biochemistry*, 2008, 381(2): 179-184.
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16. Haiyan Feng, Xiangjun Li, **Shurong Hou**, Na Zheng, Zhongbo Hu and Zhuobin Yuan. "On-line Concentration of Trace Genistein by Acid Barrage Stacking in Capillary Electrophoresis with UV Detection". *Chinese Chemical Letters*, 2008, 19(8): 973-976.
17. Ruijun Jing, Xiaoying Jiang, **Shurong Hou**, Xiangjun Li, Zhuobin Yuan. "Determination of Quercetin, Luteolin, Kaempferol and Isoquercitrin in Stamen Nelumbinis by Capillary Zone Electrophoresis-Ultraviolet Detection". *Chinese Journal of Analytical Chemistry*, 2007, 35(8): 1187-1190.

Meeting Presentations and Contributions

Podium presentation

1. **Shurong Hou**, Xinyun Zhao, Ying Wu, Oreoluwa O. Adedoyin, Hoon Cho, Hsin-Hsiung Tai, Charles Loftin, and Chang-Guo Zhan. "Rational Design and Discovery of Novel, Potent and Selective mPGES-1 Inhibitors for Development of Next-Generation of Anti-inflammatory Drugs". *AAPS*, San Antonio, Texas, Nov. **2013**.

Poster presentations and abstracts

1. **Shurong Hou**, Liu Xue, Wenchao Yang, Lei Fang, Zheng Fang and Chang-Guo Zhan. Rational Design of Human Butyrylcholinesterase Mutants Specific for Cocaine Detoxification. *AAPS*, Chicago, Illinois, Oct. **2012**.
2. **Shurong Hou**, Wenchao Yang, Liu Xue, Chang-Guo Zhan. Characterization of Catalytic Activities of High-Activity Mutants of Human Butyrylcholinesterase against (-)-Cocaine and (+)-Cocaine. *AAPS*, Washington, D.C., Oct. **2011**.
3. **Shurong Hou**, Wenchao Yang, Liu Xue, Chang-Guo Zhan. Characterization of Mutants of Human Butyrylcholinesterase against (-)-Cocaine and (+)-Cocaine. *AAPS*, New Orleans, Louisiana, Nov. **2010**.
4. Liu Xue, Wenchao Yang, **Shurong Hou**, Fang Zheng, Lei Fang, Xi Chen, Junjun Liu, and Chang-Guo Zhan. Rational Engineering of Human Butyrylcholinesterase for Selectively Improving the Catalytic Efficiency against Cocaine. AAPS, New Orleans, Louisiana, Nov. 2010.
5. Fang Zheng, Wenchao Yang, Liu Xue, **Shurong Hou**, Chang-Guo Zhan. Structural and Energetic Factors Affecting Catalytic Activity of Human Butyrylcholinesterase Mutants against (-)-Cocaine. AAPS, New Orleans, Louisiana, Nov. 2010.