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Metabolic Enzymes of Cocaine Metabolite Benzoylecgonine

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Abstract

Cocaine is one of the most addictive drugs without a U.S. Food and Drug Administration (FDA)-approved medication. Enzyme therapy using an efficient cocaine-metabolizing enzyme is recognized as the most promising approach to cocaine overdose treatment. The actual enzyme, known as RBP-8000, under current clinical development for cocaine overdose treatment is our previously designed T172R/G173Q mutant of bacterial cocaine esterase (CocE). The T172R/G173Q mutant is effective in hydrolyzing cocaine, but inactive against benzoylecgonine (a major, biologically active metabolite of cocaine) at all. Unlike cocaine itself, benzoylecgonine has an unusually stable zwitterion structure resistant to further hydrolysis in the body and environment. In fact, benzoylecgonine can last in the body for a very long time (a few days) and, thus, is responsible for the long-term toxicity of cocaine and a commonly used marker for drug addiction diagnosis in the pre-employment drug tests. Because CocE and its mutants are all active against cocaine and inactive against benzoylecgonine, one might simply assume that other enzymes that are active against cocaine are also inactive against benzoylecgonine. Here, through combined computational modeling and experimental studies, we demonstrate for the first time that human butyrylcholinesterase (BChE) is actually active against benzoylecgonine, and that a rationally designed BChE mutant can not only more efficiently accelerate cocaine hydrolysis, but also significantly hydrolyze benzoylecgonine *in vitro* and *in vivo*. This sets the stage for advanced studies to design more efficient mutant enzymes valuable for development of an ideal cocaine overdose enzyme therapy and for benzoylecgonine detoxification in environment.

Graphical Abstract

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Notes

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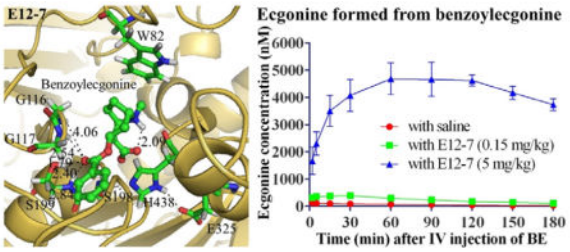

Keywords

Benzoylecgonine; metabolic enzyme; drug abuse; environment; hydrolysis

Development of a truly effective medication for treatment of drug abuse is a grand challenge.[1, 2] As well known, a truly effective addiction medication must be able to effectively block physiological effects of the drug and prevent relapse during abstinence without affecting normal functions of the brain. The currently available pharmacological approaches to drug addiction treatment either affect normal functions of some brain proteins (receptors or transporters) or are unable to prevent relapse. It is highly desired to develop a better therapeutic strategy which effectively prevent relapse without affecting normal functions of the brain proteins. Cocaine abuse is a compelling example of how traditional pharmacodynamic approach (using an agonist or antagonist) is difficult to work.[3] Despite decades of efforts, none of pharmacodynamic agents tested so far has been proven effective for treatment of cocaine addiction or overdose. The inherent difficulties of antagonizing physiological effects of cocaine in the central nervous system have led to exploring protein-based pharmacokinetic approaches using biologics like vaccines, monoclonal antibodies, and enzymes.[3, 4] Of the pharmacokinetic approaches, pharmacokinetic approach using an efficient metabolic enzyme is recognized as a truly promising strategy for treatment of cocaine overdose and addiction.[3, 5–7] In particular, each enzyme molecule can degrade multiple drug molecules, depending on the catalytic parameters of the enzyme such as the turnover number (catalytic rate constant k_{cat}), which is remarkably different from the well-known stoichiometric binding of an antibody with drug. Nevertheless, efficient and thermally stable cocaine-metabolizing enzymes have been designed and developed recently as potential candidates of therapies for cocaine overdose and addiction.[8–13] These computationally designed enzymes, that are mutants of human butyrylcholinesterase (BChE) or bacterial cocaine esterase (CocE), can rapidly convert cocaine to physiologically inactive ecgonine methyl ester (EME) and benzoic acid. In particular, a thermally stable CocE mutant (T172R/G173Q),[12] known as RBP-8000,[14] is currently in clinical development for cocaine overdose treatment; the human clinical trial Phase IIa has been completed, showing that the CocE mutant is safe and efficacious for accelerating the cocaine conversion to EME and benzoic acid in humans.[14]

A potential concern for practical clinical use of the enzyme therapy for cocaine overdose is that cocaine is also converted to benzoylecgonine (BE) by endogenous liver carboxylesterase-1 (hCE-1) in the body (see Figure 1). BE is actually a more potent vasoconstrictor compared to drugs like cocaine, norcocaine, and norepinephrine,[15] and

cerebral artery segments are significantly more sensitive to BE than to cocaine and the other cocaine metabolites.[16] More importantly, compared to cocaine itself, BE can exist in the body for a considerably longer period of time. For this reason, in human drug tests, BE is commonly used as a cocaine marker for diagnosis of cocaine addiction. For cocaine overdose treatment in an emergence department (ED), a patient intakes cocaine before going to the ED and, thus, a lot of cocaine molecules have already been converted to BE in the body prior to administration of an exogenous enzyme.[15] Hence, an ideal enzyme therapy for cocaine overdose should detoxify not only cocaine itself (which is mainly responsible for the acute toxicity and lethality), but also its long-lasting metabolite BE (which is mainly responsible for the long-term toxicity of cocaine). However, unlike cocaine, BE is a zwitterion extremely difficult to hydrolyze in not only human body, but also environment.[17] It has been extremely challenging to efficiently hydrolyze BE for both cocaine overdose treatment and BE detoxification in environment. In fact, CocE and its T172R/G173Q mutant (RBP-8000)[12] are highly efficient against cocaine and norcocaine, but inactive against BE.[18] To the best of our knowledge, no metabolic enzyme of BE has ever been reported in literature. Here we report the first observation of metabolic enzymes of BE that are human butyrylcholinesterase (BChE) and two rationally designed mutants of human BChE. The BChE mutants have improved catalytic efficiency against not only cocaine, but also BE compared to the wild-type BChE. These findings provide a promising starting point for future development of an ideal enzyme therapy for cocaine overdose and for BE detoxification in environment.

Results and Discussion

Identification of enzymes hydrolyzing BE

Our search for a metabolic enzyme of BE started from molecular docking (using a protocol described previously[19]) which enabled us to predict whether BE may bind with various serine esterases, including acetylcholinesterase, BChE and various mutants available in our lab, in a way suitable for the enzymatic hydrolysis of cocaine benzoyl ester group. Potential BE-hydrolyzing enzymes predicted by molecular docking were assayed *in vitro* for their catalytic activity against BE (see Methods section). Because it has been known that CocE and the T172R/G173Q mutant are active against cocaine and inactive against BE, one might simply assume that BChE and its mutants that are active against cocaine are also inactive against BE. Surprisingly, unlike CocE and its mutants, both molecular docking and *in vitro* activity assays consistently revealed that BChE and our rationally designed mutant enzymes, *i.e.* the A199S/S287G/A328W/Y332G mutant (E14-3)[8] and A199S/F227A/S287G/A328W/Y332G mutant (E12-7)[9] of human BChE, are all active against BE. E12-7 is also known as cocaine hydrolase-3 (CocH3), and E14-3 is also known as CocH1, in literature.[19, 20]

According to the enzyme-substrate binding structures obtained from molecular docking, BChE and the two mutants all can bind with BE in a similar way as their binding with (−)-cocaine, suitable for the desired enzymatic hydrolysis of (−)-cocaine benzoyl ester group. Depicted in Figure 2A and B are the obtained structures of wild-type BChE binding with (−)-cocaine and BE, showing that the backbone NH groups of G117 and A199 residues (within the oxyanion hole) are all close to the common carbonyl oxygen of the substrates

such that they may form two favorable hydrogen bonds with the carbonyl oxygen of the substrate in the anticipated transition state[8] during the enzymatic reaction process for both (−)-cocaine and BE. Further, according to the E12-7-BE binding structure depicted in Figure 2C, the carbonyl oxygen of BE is also close to the hydroxyl group of S199 side chain; similar structure (not shown) was also obtained for the E14-3-BE binding. Thus, the carbonyl oxygen of BE may form three hydrogen bonds with G117 and S199 residues of the enzyme (E12-7 or E14-3) within the oxyanion hole in the anticipated transition state during the enzymatic reaction process, suggesting that E12-7 (and E14-3) may have significantly improved catalytic activity against BE compared to wild-type BChE. Based on the modeling insights, we decided to carry out *in vitro* enzyme activity assays on human BChE, E14-3, and E12-7 against BE. Depicted in Figure 2D to F are data obtained from *in vitro* kinetic analysis on human BChE, E14-3, and E12-7 against BE. According to the kinetic data in Figure 2D to F, wild-type human BChE has a catalytic rate constant (k_{cat}) of 3.6 min^{-1} against BE comparable to its k_{cat} against (−)-cocaine (k_{cat} = 4.1 min^{-1}). The main difference is that it has a relatively larger Michaelis-Menten constant value (K_M = 83 μM) against BE compared to its K_M against (−)-cocaine (K_M = 4.5 μM). Notably, despite of the relatively lower catalytic efficiency (k_{cat}/K_M) against BE compared to its own efficiency against (−)-cocaine, the catalytic efficiency of human BChE against BE is actually higher than that of the known most active anti-cocaine catalytic antibody 15A10 (k_{cat} = 2.3 min^{-1} and K_M = 220 μM) against (−)-cocaine.[21, 22]

Interestingly, compared to wild-type human BChE, both enzymes E14-3 and E12-7 that have considerably improved catalytic activities against (−)-cocaine and norcocaine also have significantly improved catalytic activity against BE. As seen in Figure 2, k_{cat} = 23 min^{-1} for E14-3, k_{cat} = 65 min^{-1} for E12-7, and k_{cat} = 3.6 min^{-1} for the wild-type BChE against BE. So, E14-3 and E12-7 have improved the k_{cat} of the wild-type BChE against BE by ~6-fold and ~18-fold, respectively. Meanwhile, E14-3 and E12-7 have slightly larger K_M values (133 μM for E14-3 and 207 μM for E12-7). The improvement in k_{cat} against BE is significant, although not as much as that (>1000-fold improvement) against (−)-cocaine.[19]

Effects of E12-7 on the pharmacokinetics of (−)-cocaine and BE

Following the identification of endogenous metabolic enzyme of BE (*i.e.* BChE) and its improved mutants (E14-3 and E12-7), we wanted to know whether E12-7 may significantly accelerate BE metabolism in the body. For this purpose, we first carried out kinetic modeling of cocaine and its metabolites (including norcocaine and BE). The kinetic model was based on our previous kinetic modeling[19] without accounting for BE formation and metabolism. Using the catalytic parameters for wild-type BChE- and E12-7-catalyzed hydrolysis of BE determined in the present study, we were able to expand the previous kinetic model[19] so that we may model the possible effects of E12-7 on the kinetic profile of BE when the plasma concentration of E12-7 is the same as that of endogenous BChE in human plasma ([E] = 0.07 μM)[19] or higher. Briefly, kinetic modeling of (−)-cocaine metabolism was carried out by using the kinetic equations shown in Figure 3 in the presence of three enzymes: BChE or CocH (which refers to either wild-type human BChE or E12-7) in human plasma; liver carboxylesterase; and CYP 3A4. Concerning CocH, a typical adult has a blood volume of ~5 L.[23] Previously reported concentrations of endogenous BChE protein in human plasma

ranged from 4 to 7 mg/L,[24–26] 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.[27, 28] According to the known kinetic data reported previously[19] and obtained in the present study, we should have V_{max} = 0.29 μM min^{-1} and K_M = 4.5 μM for the wild-type BChE against (−)-cocaine, V''_{max} = 0.20 μM min^{-1} and K''_M = 15 μM for the wild-type BChE against norcocaine, and V'''_{max} = 0.25 μM min^{-1} and K'''_M = 83 μM for the wild-type BChE against BE 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 reported previously[19] and obtained in the present study, we should have V_{max} = 400 μM min^{-1} and K_M = 3.1 μM against (−)-cocaine, V''_{max} = 180 μM min^{-1} and K''_M = 13 μM against norcocaine, and V'''_{max} = 4.55 μM min^{-1} and K'''_M = 207 μM against BE when [E] = 0.07 μM. It was reported that non-specific carboxylesterase in humans and rodents is responsible for catalyzing the hydrolysis of the methyl ester group of (−)-cocaine to BE, and the reaction follows the simple Michaelis-Menten kinetics with K_M = 116 μM.[29] The human carboxylesterase mainly exists in liver, as well as other tissues. It is known that (−)-cocaine can diffuse in the body very rapidly to reach the equilibrium.[23] Thus, it is reasonable to assume that (−)-cocaine, BE, and norcocaine distributions in the blood and other tissues can rapidly reach the equilibrium during the metabolic reactions. It was roughly estimated that F_3 = 4.5 μM min^{-1} and k_3 = 116 μM for (−)-cocaine hydrolysis to BE, according to the available experimental data including the enzyme activity[30] and the enzyme distribution in the body.[31] In our previous study, it has been estimated that F_2 = 14.4 μM min^{-1} and k_2 = 2.7 mM for the enzymatic oxidation of (−)-cocaine to norcocaine.[19] These roughly estimated kinetic parameters were used in our kinetic modeling with various initial concentrations; our additional modeling tests revealed that kinetic modeling using different values of the catalytic parameters would lead to the same qualitative conclusions mentioned below.

For possible treatment of cocaine overdose using an exogenous cocaine-metabolizing enzyme, the cocaine abusers have already taken (−)-cocaine, and converted some (−)-cocaine to BE and norcocaine before the enzyme administration. In order to know whether E12-7 is also efficacious in hydrolysis of BE, in addition to (−)-cocaine and norcocaine, for the cocaine overdose treatment, we performed an additional, simplified kinetic modeling by assuming that the initial concentration (when t = 0) is 10 μM for (−)-cocaine, 45 μM for BZ, and 5 μM for norcocaine. It was reported that 45% (−)-cocaine was converted to BE, 40% (−)-cocaine was converted to EME, and 5% (−)-cocaine was converted to norcocaine in humans.[32, 33] So, in practical cocaine overdose treatment, a patient could have (−)-cocaine, norcocaine, and BE remained in the body when he/she is available for the treatment using an enzyme. The specific concentrations of (−)-cocaine, norcocaine, and BE remained in the body will be dependent on specific dose of cocaine and how soon a patient is available for the treatment after the cocaine administration. The simplified kinetic modeling described here is merely for illustration. In fact, our further kinetic modeling (data not shown) using different initial concentrations of (−)-cocaine, norcocaine, and BE led to the qualitatively the same conclusion concerning the effectiveness of the enzyme in accelerating metabolism of (−)-cocaine, norcocaine, and BE.

Depicted in Figure 4 are data obtained from the simplified kinetic modeling when $A(0)$ (the initial concentration of (−)-cocaine) = 10 μM, $B(0)$ (the initial concentration of BE) = 45

μM, and $F(0)$ (the initial concentration of norcocaine) = 5 μM. As seen in Figure 4A to C, in the absence of E12-7, (−)-cocaine has a half-life ($t_{1/2}$) of 28 min ($t_{1/2}$ = 28 min), and $t_{1/2}$ = 65 min for norcocaine and $t_{1/2}$ = 318 min for BE. As seen in Figure 4D to F, in the presence of 0.07 μM E12-7, both (−)-cocaine and norcocaine are eliminated completely in less than 1 min. However, there was still a significant amount of BE left (AUC of BE = 2270 μM·min), but the half-life of BE is reduced significantly from 318 min to 36 min. These data qualitatively suggest that E12-7 can be effective for cocaine overdose treatment as it can completely break down (−)-cocaine and norcocaine, prevent (−)-cocaine from converting to BE, and significantly decrease the half-life and AUC of BE. So, E12-7 can lower the BE concentration in plasma through accelerating not only (−)-cocaine benzoyl ester hydrolysis, but also BE hydrolysis.

To validate the effectiveness of E12-7 in accelerating BE hydrolysis *in vivo*, four groups of rats (four rats, *i.e.* n=4, for each group) were used in this study. The obtained calibration curves for detecting BE and its hydrolysis product ecgonine are depicted in Figure 5, and the obtained *in vivo* data are shown in Figure 6. The first three groups of rats were injected intravenously (IV) with 0.15 mg/kg E12-7 or 5 mg/kg E12-7 or saline, followed by IV injection of 2 mg/kg BE one minute after the enzyme/saline injection. The dose of 0.15 mg/kg E12-7 led to an E12-7 concentration of ~3 mg/L (which is about a half of the average concentration of the endogenous BChE in human[19]) in plasma at ~2 min after IV injection of E12-7 according to our previous study.[34] The dose of 5 mg/kg resulted in a plasma E12-7 concentration between ~0.4 μM at 5 min and ~0.1 μM at 180 min (Figure 6D). The final group of rats were injected with 2 mg/kg ecgonine (metabolite of BE), instead of 2 mg/kg BE, in order to know the elimination profile of ecgonine. For each rat, blood samples were collected at 2, 5, 10, 30, 60, 90, 120, 150, and 180 min after the BE or ecgonine injection. The collected blood samples were analyzed by using a sensitive LC-MS/MS method to determine the blood concentrations of BE and ecgonine. In addition, blood samples collected from the rats injected with 5 mg/kg E12-7 were also analyzed for the E12-7 concentrations.

Statistical analysis using the two-way analysis of variance (ANOVA) method implemented in the SigmaPlot software (Systat Software, San Jose, CA) revealed that $p < 0.001$, *i.e.* significant differences exist in the blood concentrations of BE and ecgonine between different experimental groups (treated with saline or 0.15 mg/kg E12-7 or 5 mg/kg E12-7). Further, according to the two-way ANOVA with *post hoc* Dunnett's testing, the differences in the mean BE concentrations between the high-dose group (treated with 5 mg/kg E12-7) and the control group (treated with saline) were significant ($p < 0.001$), although the differences between the low-dose group (0.15 mg/kg E12-7) and the control group were insignificant ($p = 0.117$). So, according to the *in vivo* data depicted in Figure 6, E12-7 dose-dependently accelerated BE hydrolysis to ecgonine. The enzyme-accelerated BE clearance (Figure 6A) is consistent with the increased production of BE metabolite ecgonine (Figure 6B). In comparison with the ecgonine elimination profile shown in Figure 6C, the data depicted in Figure 6B revealed that ecgonine was formed continuously until BE was consumed.

Further discussion

This is the first identification of metabolic enzymes of BE, demonstrating that plasma enzyme BChE is the endogenous metabolic enzyme of BE and the BChE mutant E12-7 with significantly improved catalytic efficiency against BE compared to wild-type BChE may be used as an exogenous enzyme to effectively accelerate BE hydrolysis in the body for cocaine overdose treatment. Compared to RBP-8000 (the T172R/G173Q mutant of CocE)[12, 14] under current clinical development for cocaine overdose treatment, E12-7 is not only more efficient against cocaine,[9] but also effective in hydrolyzing BE. Hence, E12-7 should be a more promising therapeutic candidate for cocaine overdose treatment in comparison with the T172R/G173Q mutant of CocE, although one would like to further improve the catalytic efficiency against BE for cocaine overdose treatment.

In addition, our most recently reported study[35] demonstrated a novel protein form of E12-7 (or CocH3), denoted as CocH3-Fc or catalytic antibody analog, which is an Fc-fused CocH3 dimer (CocH3-Fc) constructed by using CocH3 to replace the Fab region of human immunoglobulin G1. A single dose of CocH3-Fc significantly accelerated cocaine metabolism in rats after 20 days and, hence, block cocaine-induced physiological and toxic effects for a long period of time.[20] Thus, taking all of these together, E12-7 (or CocH3) in its Fc-fusion protein form may be used as a truly promising therapeutic candidate for treatment of both cocaine addiction and overdose.

Further, the findings in this study provide an exciting starting point for future efforts to rationally design and discover new BChE mutants with further improved catalytic efficiency against BE, in addition to cocaine itself and other toxic metabolites.[19, 36] A highly efficient enzyme which can efficiently convert BE to biologically inactive metabolites is valuable for not only cocaine overdose treatment, but also BE detoxification in environment because BE widely exists in environment worldwide[17] including drinking water due to cocaine abuse. For example, an efficient BE-hydrolyzing enzyme could be used along with osmosis membranes of water filters to convert BE to inactive metabolites such that the filtered water becomes BE-free. A transgenic aquatic plant with the gene of an efficient BE-hydrolyzing enzyme could also be developed to express the enzyme in the plant so as to hydrolyze BE in water contacting the plant.

Materials and Methods

Molecular modeling

BE binding with human BChE and its mutants was modeled by using our previously modeled structures of the same enzymes.[10, 19, 37–42] Our previously reported molecular dynamics (MD) simulations[10] on the structures of enzyme-cocaine complexes[19] started from the X-ray crystal structure available in the Protein Data Bank (pdb code: 1P0P). For each enzyme (human BChE or mutant), BE was docked into the active site of the enzyme by using the AutoDock 4.2 program,[43] as we previously did for the enzyme binding with (−)-cocaine, norcocaine, and cocaethylene.[19] During the docking process, the Solis and Wets local search method[44] was used for the conformational search and the Lamarkian genetic algorithm (LGA)[43] was employed to deal with the enzyme-ligand interactions. The grid size

was set to be 120 × 120 × 120. The finally obtained enzyme-BE binding structures were the ones with the lowest binding free energies.

In vitro enzyme activity assays

All enzymes (wild-type human BChE, E14-3, and E12-7) were prepared as described previously,[19] and the catalytic activities of the purified enzymes against BE (and (−)-cocaine for comparison) were determined by performing a UV-Vis spectrophotometric assay. Using the UV-Vis spectrophotometric assay, we determined the catalytic activity of the enzymes against BE and (−)-cocaine at the same time under the same experimental conditions except the concentration of the enzymes and substrates, to make sure that the known enzyme activity against (−)-cocaine can be reproduced. The enzymatic reaction was initiated by adding 180 μl of a substrate (BE or (−)-cocaine) solution to 20 μl of an enzyme solution. The final reaction systems of BE have the initial BE concentrations of 500, 300, 200, 100, 50, 20, and 10 μM, whereas the final reaction systems of (−)-cocaine have initial (−)-cocaine concentrations of 50, 30, 20, 10, 5, 2, and 1 μM. The reaction temperature was 25 °C, and the buffer used was 0.1 M potassium phosphate (pH 7.4). The initial reaction rates of the enzymatic hydrolysis of BE/(−)-cocaine in various initial substrate concentrations were estimated according to the change in the intrinsic absorbance peak of BE/(−)-cocaine at 230 nm with time using a GENios Pro Microplate Reader (TECAN, Research Triangle Park) with the XFluor software. The initial reaction rates were calculated from the linear portions of the progress curves. All assays were carried out in triplicate. The Michaelis-Menten kinetic analysis was performed by using Prism 5 (GraphPad Software Inc., San Diego, CA) to determine the V_{max} and K_M values.

Kinetic modeling

Kinetic modeling of (−)-cocaine in humans was performed by use of a MatLab program (developed in house)[19, 45, 46] in a way similar to that of our recently developed pharmacokinetic modeling of (−)-cocaine in the presence of a cocaine-metabolizing enzyme.[19, 23] The previously used kinetic model[19] did not involve BE. By using a one-compartment model, the present kinetic modeling also accounted for the transformation of (−)-cocaine to BE and the subsequent BE hydrolysis in the presence of wide-type BChE or a highly efficient cocaine-metabolizing enzyme E12-7. The specific concentration of the enzyme (~0.07 μM which is the average concentration of the endogenous BChE in humans) and the initial concentrations of (−)-cocaine (10 μM), norcocaine (5 μM), and BE (45 μM) used in the kinetic modeling are discussed in the Results and Discussion section.

Subjects for *in vivo* studies

Male Sprague-Darley rats (200–250 g) were ordered from the Harlan and were housed initially in 2 to 4 rats per cage. All rats were allowed *ad libitum* access to food and water and were maintained on a 12-hour light and dark cycle with lights on at 8 AM in a room kept at a temperature of 21 to 22 °C. Experiments were performed in a same colony room in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Kentucky.

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

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

Characterization of BE clearance accelerated by E12-7 in rats by use of LC-MS/MS method

Benzoylecgonine (BE)-D3, which was used as internal standards (IS), was purchased from the Cerilliant. Heparin, HPLC-grade methanol and acetonitrile were obtained from the Thermo Fisher Scientific. Ammonium formate, ammonium hydroxide, and formic acid were ordered from the Sigma-Aldrich. Mixed cation exchange model solid phase extraction cartridges (Oasis MCX 1 cc Vac Cartridge, 10 mg) were obtained from the Waters.

Preparation of blood samples for mass spectrometry—The frozen blood samples were thawed on dry bath at 25 °C for 15 min. Then, equal volume of internal standard solution (100 nM) as the whole blood was mixed with each blood sample. The solution was vortex-mixed for 1 min and then centrifuged for 15 min at 13,000 rpm, and the supernatant was transferred to another clean centrifugal tube. After being added 350 μl formic acid (4%), samples were vortexed for 1 min and centrifuged at 13,000 for 15 min; and then the supernatants were submitted to solid-phase extraction. Oasis MCX 1 cc Vac Cartridges were preconditioned with 1 ml methanol followed by 1 ml water. Loaded cartridges were washed twice with 1 ml methanol, and the contents were eluted twice with 500 μl methanol/water solution (95:5, v/v, with 7.5% ammonium hydroxide). Eluates were evaporated to dryness at 35 °C using a vacuum concentrator, reconstituted in 74 μl mobile phase A/B (95:5, v/v), and centrifuged at 13,000 rpm for 15 min. Supernatants were transferred to vials and stored refrigerated until analysis by LC-Q-TOF.

Preparation of stock and calibration standards—A series of combined BE and ecgonine solutions were prepared, with concentrations 50, 100, 500, 1000, 2000, 5000, 10000 nM for each analyte. Calibration standards were prepared by adding 74 μl different concentrations of combined standard solutions into 174 μl blood mixture (74 μl whole blood + 100 μl paraoxon solution) from untreated Sprague-Dawley rats. The same method as described above was used to extract BE, ecgonine, and BE-D3.

Liquid chromatographic and mass spectrometric conditions—A Shimadzu HPLC system, consisting of a DGU-20A/3R degasser, LC-20AD binary pumps, CBM-20A

controller, and SIL-20A/HT auto sampler, was used in this study (Shimadzu). The mass spectrometer utilized for this work was an AB SCIEX tripleTOF™ 5600 (AB SCIEX, Redwood City, CA).

The chromatographic analysis was carried out on an Atlantis T3 (100Å, 3 μm, 2.1 mm X 150 mm I.D) column (Waters). Mobile phase A consisted of 2 mM ammonium formate, pH 3.0 and mobile phase B consisted of mobile phase A: acetonitrile (10:90, v/v). The flow rate was set at 0.2 ml/min. A 5 μl injection of each sample was loaded on to the column. The gradient program was as follow: 5% B at 0 min, hold 5% B for 3 min, then B increased at 20% at 4 min, 40% B at 14 min, 90% B at 16 min, hold 90% B for 4 min, 5% B at 21 min, and re-equilibrate at 5% B for 3 min.[47] The total run time was 24 min, and the column temperature was maintained at room temperature (~21 °C). The auto sampler temperature was maintained at 15°C, and the auto sampler injection needle was washed with 200 μL mobile phase A: mobile phase B (95:5, v/v) after each sample injection.

The mass spectrometer was run in positive ion and high sensitivity mode under the following conditions and settings: positive ions were generated in the source using liquid nitrogen. Ion source gas 1 (GS1) and 2 (GS2) were set at 25 and 35 respectively, and curtain gas (CUR) was set at 40. Source gas temperature was set at 500 °C. Ion spray voltage floating (ISVF) was 3000 V. Compound-specific ionization parameters, Ion transition, declustering potential (DP), collision energies (CE), collision energy spread (CES), ion release delay (IRD), and ion release width (IRW), were optimized and summarized in table 1. Analyst® TF 1.7 software (AB SCIEX) was used for instrument control, data acquisition. MultiQuant™ 3.0 software (AB SCIEX) was used for quantitative analysis.

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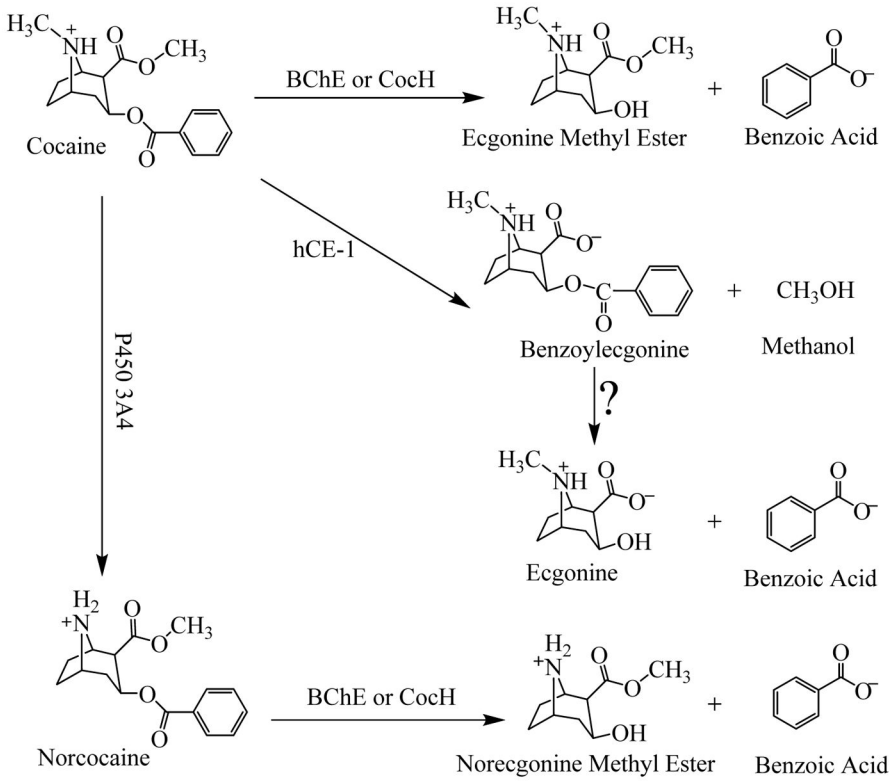

Figure 1.

Metabolic pathways of cocaine in humans and animals with metabolic enzymes including BChE, hCE-1, and oxidation by cytochrome P450 (CYP) 3A4.

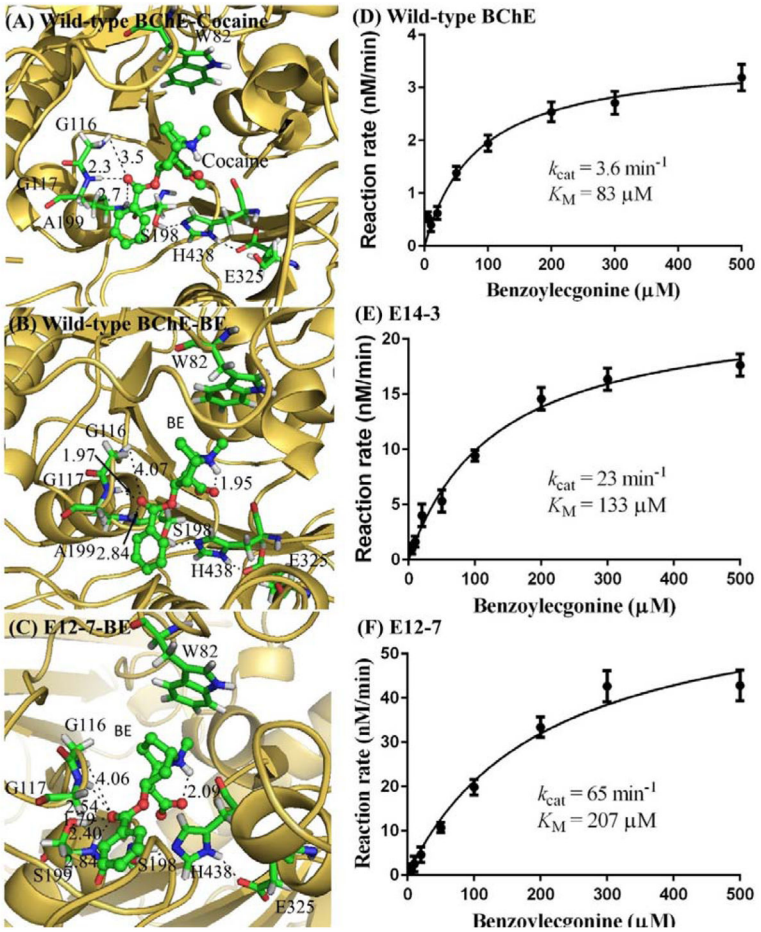

Figure 2.

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

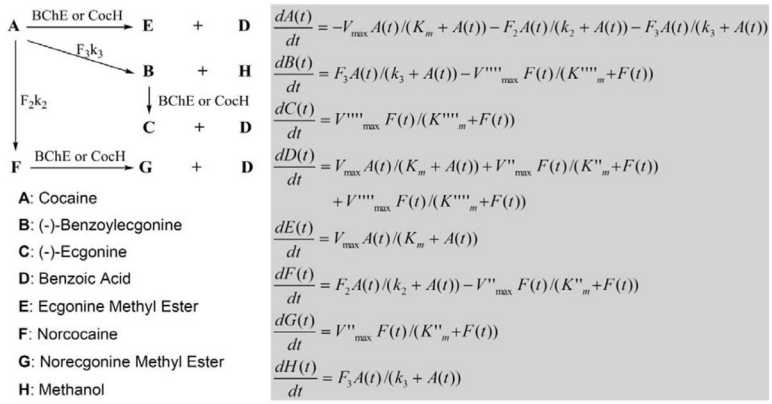

Figure 3.
Reaction scheme and kinetic equations used in the kinetic modeling

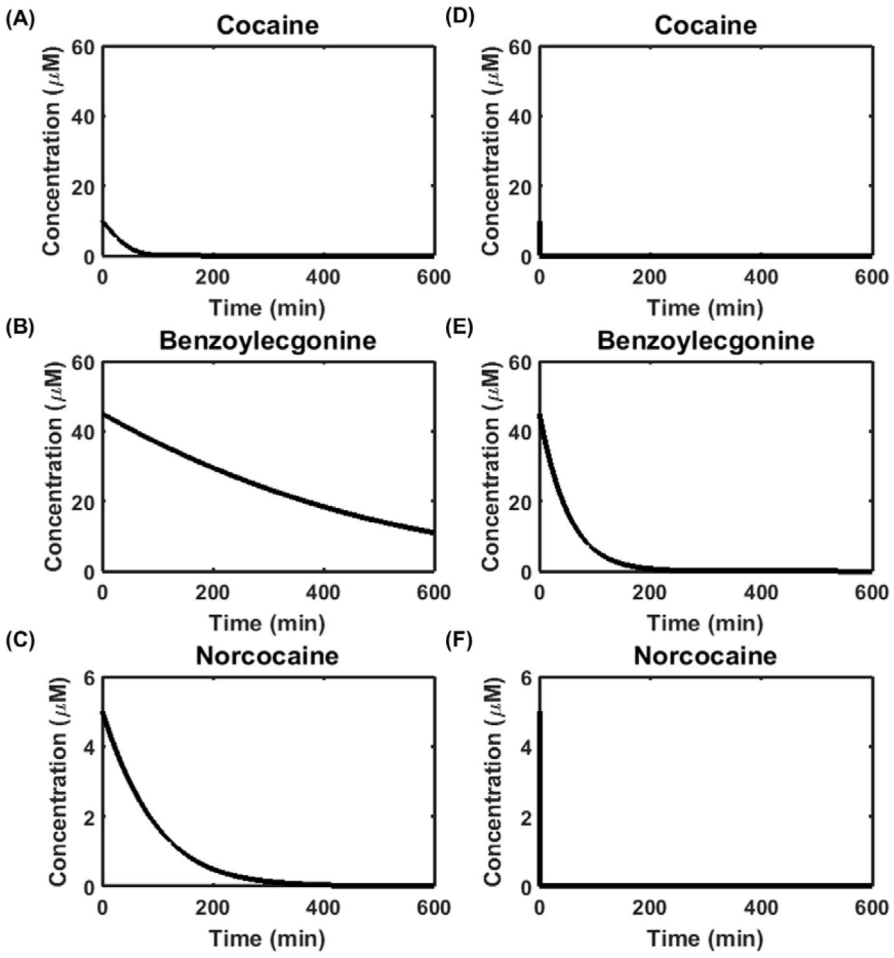

Figure 4.

The modeled concentrations of (−)-cocaine, BE, and norcocaine in human blood when the initial concentrations of (−)-cocaine, BE, and norcocaine are 10, 45, and 5 μM, respectively. (A) to (C) refer to the time-dependent concentrations in the presence of wild-type human BChE (without E12-7), whereas (D) to (F) refer to the time-dependent concentrations in the presence of 0.07 μM E12-7.

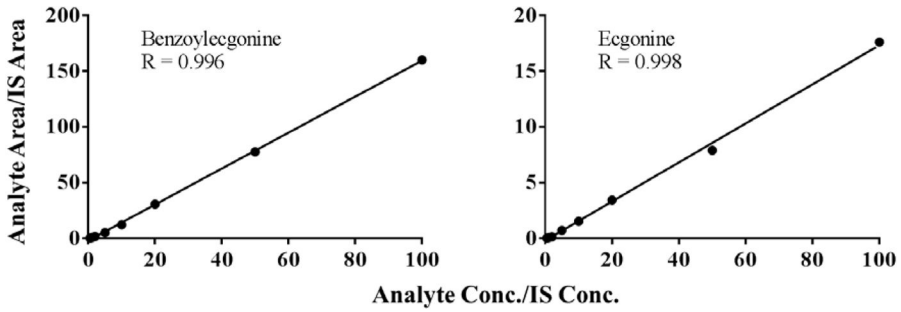

Figure 5.

LC-MS/MS calibration curves for BE and ecgonine. Calibration curves were established by calculating the ratios of the peak area for analyte to that for the internal standard (BE-D3 or ecgonine-D3) and plotting the ratio as a function of the ratio of the analyte concentration to the internal standard. The recovery rate of the internal standard was 81.4% for BE-D3 or 84.2% for ecgonine-D3; all were determined in six replicates at 100 nM (0.1 μM). X-axis (Analyte Conc. / IS Conc.) refers to the ratio of the analyte concentration to the corresponding internal standard concentration (IS Conc. = 0.1 μM for each internal standard compound). Y-axis (Analyte Area / IS Area) represents the ratio of the measured area for the analyte to that for the corresponding internal standard. For each compound (BE or ecgonine), data were fitted to a linear curve using the least-squares analysis with 1/x weighting.

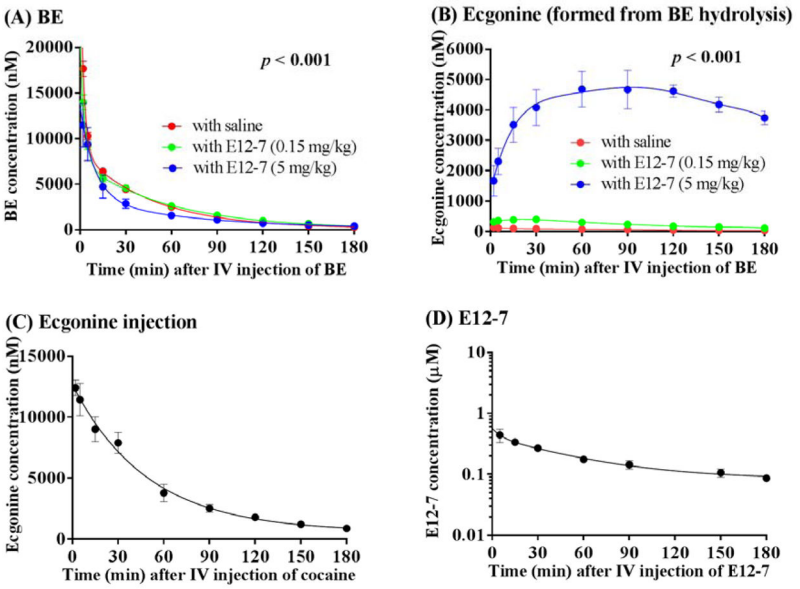

Figure 6.

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