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Kinetic Characterization of High-Activity Mutants of Human Butyrylcholinesterase for Cocaine Metabolite Norcocaine

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Abstract

It has been known that cocaine produces the toxic and physiological effects through not only cocaine itself but also norcocaine formed from cocaine oxidation catalyzed by microsomal cytochrome P450 3A4 in the human liver. The catalytic parameters (k_{cat} and K_{M}) of human butyrylcholinesterase (BChE) and its three mutants (*i.e.* the A199S/S287G/A328W/Y332G, A199S/F227A/S287G/A328W/E441D, and A199S/F227A/S287G/A328W/Y332G mutants) for norcocaine have been characterized in the present study, for the first time, in comparison with those for cocaine. Based on the obtained kinetic data, wild-type human BChE has a significantly lower catalytic activity for norcocaine ($k_{\text{cat}} = 2.8 \text{ min}^{-1}$, $K_{\text{M}} = 15 \text{ }\mu\text{M}$, and $k_{\text{cat}}/K_{\text{M}} = 1.87 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$) compared to its catalytic activity for (–)-cocaine. The BChE mutants examined in this study have considerably improved catalytic activities against both cocaine and norcocaine compared to the wild-type enzyme. Within the enzymes examined in this study, the A199S/F227A/S287G/A328W/Y332G mutant (CocH3) is identified as the most efficient enzyme for hydrolyzing both cocaine and norcocaine. CocH3 has a 1080-fold improved catalytic efficiency for norcocaine ($k_{\text{cat}} = 2610 \text{ min}^{-1}$, $K_{\text{M}} = 13 \text{ }\mu\text{M}$, and $k_{\text{cat}}/K_{\text{M}} = 2.01 \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$) and a 2020-fold improved catalytic efficiency for cocaine. It has been demonstrated that CocH3 as an exogenous enzyme can rapidly metabolize norcocaine, in addition to cocaine, in rats. Further kinetic modeling has suggested that CocH3 with an identical concentration as that of the endogenous BChE in human plasma can effectively eliminate both cocaine and norcocaine in a simplified kinetic model of cocaine abuse.

Keywords

Butyrylcholinesterase; protein drug; drug detoxification; hydrolysis; kinetics

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AUTHOR CONTRIBUTION

Max Zhan performed the molecular docking, *in vitro* studies and kinetic modeling, and drafted the paper. Shurong Hou carried out the *in vivo* tests and trained Max Zhan to perform the *in vitro* assays. Fang Zheng and Chang-Guo Zhan designed the project and finalized the paper.

Introduction

Cocaine is a widely abused and hepatotoxic drug.[1] Disastrous medical and social consequences of cocaine abuse have made it a high priority to develop a feasible anti-cocaine medication.[2–3] Generally speaking, pharmacological treatment for drug abuse can be either pharmacodynamic or pharmacokinetic. The traditional pharmacodynamic approaches to cocaine addiction treatment include possible medications to target a specific subtype of transporters/receptors, which affect various neurotransmitter systems, such as dopaminergic, serotonergic, noradrenergic, cholinergic, glutamatergic, GABAergic, and opioidergic pathways, and modulate neurological processes.[4] However, despite of decades of effort, pharmacodynamic treatment of cocaine abuse has been proven very elusive. There is still no FDA (U.S. Food and Drug Administration)-approved medication available. The inherent difficulties in antagonizing cocaine in central nervous system (CNS) have led to the development of alternative approaches to alter the pharmacokinetics of cocaine in a favorable manner by tightly binding or rapidly metabolizing cocaine.

An ideal anti-cocaine medication would accelerate cocaine metabolism producing biologically inactive metabolites.[4] The primary pathway for metabolism of cocaine in primates is hydrolysis at the benzoyl ester or methyl ester group (Scheme 1).[5–10] Benzoyl ester hydrolysis generates ecgonine methyl ester (EME), whereas the methyl ester hydrolysis yields benzoylecgonine (BE). The major cocaine-metabolizing enzymes in humans are butyrylcholinesterase (BChE) which catalyzes benzoyl ester hydrolysis and two liver carboxylesterases (denoted by hCE-1 and hCE-2) that catalyze hydrolysis at the methyl ester and the benzoyl ester, respectively. Among the three, BChE is the principal cocaine hydrolase (CocH) in human serum. Hydrolysis accounts for most of cocaine metabolism in humans. The remaining cocaine is metabolized through oxidation by the liver microsomal cytochrome P450 (3A4) system, producing norcocaine which has similar physiological effects to that of cocaine.[10–11] The hepatotoxic activity of cocaine is associated with further oxidative reactions of norcocaine.[12]

Metabolite EME is known to be the least pharmacologically active of the cocaine metabolites and may even cause vasodilation.[7] Thus, hydrolysis of cocaine at the benzoyl ester by an enzyme is the pathway most suitable for amplification. Unfortunately, wild-type BChE has a low catalytic activity against naturally occurring (–)-cocaine ($k_{\text{cat}} = 4.1 \text{ min}^{-1}$ and $K_{\text{M}} = 4.5 \text{ }\mu\text{M}$).[13–17] Furthermore, norcocaine (which always refers to (–)-norcocaine in the present report, unless stated otherwise) produced by the P450-catalyzed oxidation of (–)-cocaine appears to be as pharmacologically active as (–)-cocaine itself. In addition, norcocaine is hepatotoxic, and causes vasoconstriction, lowering the seizure threshold.[11] It is interesting for anti-cocaine medication development to develop a mutant of human BChE as a CocH with significantly improved catalytic activities against both (–)-cocaine and norcocaine.

To design a mutant of human BChE with an improved catalytic activity against (–)-cocaine, our group recently 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 an improved catalytic activity.[18–24] 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 considerably improved catalytic efficiency against (–)-cocaine.[18–24] The first one of our designed high-activity mutants of human BChE, *i.e.* the A199S/S287G/A328W/Y332G mutant,[18] was validated by an independent group of scientists[25–26] who concluded that this mutant is “*a true Coch with a catalytic efficiency that is 1,000-fold greater than wild-type BChE*”. This BChE mutant is currently in double-blind, placebo-controlled clinical trials in humans by Teva Pharmaceutical Industries Ltd for cocaine abuse treatment.[4] Our more recently designed new mutants[21, 23] 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 norcocaine. To our best knowledge, we have not seen a report on the kinetic parameters for (–)-norcocaine hydrolysis catalyzed by wild-type human BChE or any of these BChE mutants, although (–)-norcocaine was characterized as an inhibitor for human BChE-catalyzed hydrolysis of cocaine.[15]

As mentioned in the above background, (–)-cocaine produces the physiological effects through not only (–)-cocaine itself, but also norcocaine formed from the P450-catalyzed oxidation of (–)-cocaine. A truly valuable mutant of human BChE for anti-cocaine enzyme therapy development should be efficient for not only (–)-cocaine, but also norcocaine. It is crucial for (–)-cocaine detoxification to efficiently hydrolyze norcocaine to biologically inactive metabolites. 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 norcocaine. However, our 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.[24] The catalytic efficiency of the examined BChE mutants against ACh is even slightly lower than that of the wild-type BChE. Hence, it is unclear whether these BChE mutants have a significantly improved catalytic efficiency against norcocaine compared to the wild-type BChE.

Here we report a kinetic analysis on the catalytic activity of wild-type human BChE and our discovered high-activity mutants of human BChE (*i.e.* the A199S/S287G/A328W/Y332G, A199S/F227A/S287G/A328W/E441D, and A199S/F227A/S287G/A328W/Y332G mutants) against norcocaine, in comparison with the corresponding catalytic activity against (–)-cocaine. The obtained kinetic data have demonstrated that the BChE mutants examined in this study have not only a considerably improved catalytic efficiency against (–)-cocaine but also a considerably improved catalytic efficiency against norcocaine *in vitro* and *in vivo* compared to the wild-type BChE. Further kinetic modeling has demonstrated that these BChE mutants can effectively hydrolyze both (–)-cocaine and norcocaine in a simplified kinetic model of cocaine abuse.

Methods

Molecular modeling

Norcocaine binding with human BChE and mutants was modeled by using our previously modeled structures of the same enzymes.[18–24] Our previous molecular dynamics (MD) simulations[24] on the structures of enzyme-substrate complexes started from the X-ray crystal structure[27] deposited in the Protein Data Bank (pdb code: 1P0P). For each enzyme (human BChE or mutant), norcocaine was docked into the possible active site of the enzyme by using the AutoDock 4.2 program.[28] For comparison, the similar docking was also performed on (–)-cocaine binding with the same enzymes. During the docking process, a conformational search was performed using the Solis and Wets local search method,[29] and the Lamarkian genetic algorithm (LGA)[28] was applied to deal with the enzyme-ligand interactions. Among a series of docking parameters, the grid size was set to be $120 \times 120 \times 120$. The finally obtained enzyme-substrate binding structures were the ones with the lowest binding free energies.

Enzyme preparation and *in vitro* activity assays

Both wild-type and mutants of human BChE were expressed and their enzyme activities against norcocaine and (–)-cocaine were assayed at the same time under the same experimental conditions so that the activity against norcocaine 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 $\sim 1 \times 10^6$ cells/ml were transfected by 293 fectin reagent-DNA complexes at the ratio of 2 μ l : 1 μ g 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). Specifically, the medium was diluted with the same volume of 20 mM Tris-HCl, pH 7.4. Equilibrated QFF anion exchanger was added to diluted medium in 1% of its volume and incubated at 4°C with occasional stirring for 1 h. The suspension was then packed in a column and the medium was allowed to flow through rapidly with the aid of suction of (50–100 ml/min). The QFF resin was repacked again in a washing buffer after the entire medium was excluded. After washing the column with 20 mM Tris-HCl, pH 7.0, overnight at 4°C, the enzyme was eluted by 20 mM Tris-HCl, pH 7.0, plus 0.3 M NaCl. The eluate was desalted to 20 mM Tris-HCl, pH 7.0, by Millipore centrifugal filter device. The desalted eluate was applied to a hydroxyapatite column (Clarkson Chem. Co., Williamsport, PA) (2.5×22 cm), which was packed with fibrous cellulose powder CF11 at a ratio of 1:1. The column was washed by 20 mM Tris-HCl, pH 7.0, and then the enzyme was eluted by 10 mM sodium phosphate buffer, pH 7.0, containing 0.3 M NaCl. The purified protein was dialyzed against phosphate-buffered saline and stored at 4°C or –80°C.

The catalytic activities of the enzymes against norcocaine and (–)-cocaine were determined by a UV-Vis spectrophotometric assay. Using the UV-Vis spectrophotometric assay, the catalytic activities of the enzymes against norcocaine and (–)-cocaine were determined at the same time under the same experimental conditions. The enzymatic reaction was initiated by adding 180 μ l of a substrate (norcocaine or (–)-cocaine) solution to 20 μ l of an enzyme

solution. The final initial norcocaine/(–)-cocaine concentrations were as follows: 100, 50, 20, 10, 5, 2, and 1 μ M. The reaction temperature was 25°C, and the buffer used was 0.1 M potassium phosphate (pH 7.4). The initial rates of the enzymatic hydrolysis of norcocaine/(–)-cocaine in various initial substrate concentrations were estimated by following the change in the intrinsic absorbance peak of norcocaine/(–)-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 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.

Subjects for *in vivo* studies

Male Sprague-Darley rats (200–250 g) were ordered from Harlan (Harlan, Indianapolis, IN) and were housed initially in 2 to 4 rats per cage. All rats were allowed ad libitum access to food and water and were maintained on a 12-hour light and dark cycle with lights on at 8 AM in a room kept at a temperature of 21 to 22°C. 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.

Characterization of norcocaine clearance accelerated by Coch3

Norcocaine was provided by the National Institute on Drug Abuse (NIDA) Drug Supply Program (Bethesda, MD), and the Coch3 material used for *in vivo* studies in rats were prepared in our recently reported study[30] developing and using stable CHO-S cells. Our *in vitro* assays revealed that Coch3 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 norcocaine and Coch3 (or saline). One set of rats were injected with saline through tail vein 1 min before i.v. injection of a given dose of norcocaine (see below for the norcocaine doses tested), and another set of rats (n=4) were injected with 0.15 mg/kg of Coch3 followed by i.v. injection of the same dose of norcocaine. About 50 to 75 μ l of blood from saphenous veins was collected into capillary tubes and immediately diluted in 100 μ l of 250 μ M paraoxon at 2, 5, 10, 15, 30, 60, 90, 120, 150, and 180 min after the i.v. injection of norcocaine. Paraoxon is an irreversible BChE inhibitor that can stop the enzymatic hydrolysis of norcocaine between sampling and analysis. The diluted blood samples were stored at –70°C and assayed by using a High-Performance Liquid Chromatographic (HPLC) method.

Benzoic acid is the product of norcocaine hydrolysis catalyzed by the enzyme (wild-type BChE or Coch3). The standard benzoic acid for the HPLC analysis was purchased through Sigma Aldrich (Sigma Aldrich, St. Louis, MO). To assay the norcocaine and benzoic acid concentrations in the blood samples, the frozen whole blood samples were thawed on ice for 3 hours. Then 150 μ l of mobile phase (23% acetonitrile and 77% water containing 0.1% TFA) was mixed with each sample, and 50 μ l of 10% HClO₄ 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 μ l 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 norcocaine when exciting at 230 nm. The norcocaine peaks appeared at 11.9 min, and the benzoic acid peaks occurred at 15.9 min. The concentrations of norcocaine and benzoic acid were determined by comparing the corresponding HPLC peak areas with those of authentic standards.

Kinetic modeling

Kinetic modeling of (–)-cocaine in humans was performed by use of a MatLab program (developed in house)[31–32] in a way similar to that of our recently developed pharmacokinetic modeling of (–)-cocaine in the presence of a cocaine-metabolizing enzyme. [33] The previously used kinetic model[33] was focused on the hydrolysis of (–)-cocaine. By using a one-compartment model, the present kinetic modeling accounted for not only the (–)-cocaine hydrolysis, but also the (–)-cocaine oxidation to norcocaine and the subsequent norcocaine hydrolysis in the presence of a cocaine-metabolizing enzyme. Given in Table 1 are the reaction scheme and kinetic equations used in the present study.

Results and Discussion

Insights from molecular modeling

Molecular docking enabled us to understand how norcocaine may bind with human BChE and the mutants in comparison with (–)-cocaine binding with the same enzymes. As seen in Figure 1, the only structural difference between norcocaine and (–)-cocaine is that the methyl group on the nitrogen atom of (–)-cocaine is replaced by a hydrogen atom in norcocaine. 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 norcocaine is the same as that with (–)-cocaine, particularly for the crucial interactions between the carbonyl oxygen of the substrate and the oxyanion hole (residues #116, #117, and #199) of the enzyme. The minor structural difference between norcocaine 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 norcocaine or (–)-cocaine, and there are always two hydrogen bonds between the carbonyl oxygen (G117 backbone and S199 side chain) of the substrate and the oxyanion hole of the mutant no matter whether the substrate is norcocaine or (–)-cocaine. Depicted in Figure 2 are the obtained enzyme-substrate binding structures for norcocaine and (–)-cocaine with wild-type human BChE and a representative mutant (the A199S/F227A/S287G/A328W/Y332G mutant, denoted as Coch3). The binding structures with the A199S/S287G/A328W/Y332G and A199S/F227A/S287G/A328W/E441D mutants (not shown) are similar to those with the A199S/F227A/S287G/A328W/Y332G mutant in terms of the overall hydrogen bonding with the oxyanion hole.

The modeling results depicted in Figure 2 indicate that, no matter whether the substrate is norcocaine 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 norcocaine. Hence, all of the BChE mutants concerned in the present study are expected to have a significantly improved catalytic efficiency against norcocaine, although it has been known that these BChE mutants do not have an improved catalytic efficiency against ACh.[24]

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 and the A199S/S287G/A328W/Y332G, A199S/F227A/S287G/A328W/E441D, and A199S/F227A/S287G/A328W/Y332G mutants. The *in vitro* assays were based on our observation (Figure 3) that norcocaine 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 norcocaine or (–)-cocaine. The *in vitro* assays enabled us to determine the catalytic activity of the enzymes against norcocaine 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 norcocaine 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 norcocaine relative to the known activity against (–)-cocaine. Depicted in Figure 4 are the measured kinetic data, and summarized in Table 2 are the determined kinetic parameters.

A survey of the kinetic parameters summarized in Table 2 reveals that all of the three BChE mutants (CocH1 to 3) examined in this study have a considerably improved catalytic efficiency ($k_{\text{cat}}/K_{\text{M}}$) against both (–)-cocaine and norcocaine. Against (–)-cocaine, these mutants have a 1080 to 2020-fold improved catalytic efficiency compared to the wild-type enzyme. Notably, wild-type BChE has a significantly lower catalytic activity against norcocaine ($k_{\text{cat}} = 2.8 \text{ min}^{-1}$, $K_{\text{M}} = 15 \text{ }\mu\text{M}$, and $k_{\text{cat}}/K_{\text{M}} = 1.87 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$) compared to its catalytic activity against (–)-cocaine ($k_{\text{cat}} = 4.1 \text{ min}^{-1}$, $K_{\text{M}} = 4.5 \text{ }\mu\text{M}$, and $k_{\text{cat}}/K_{\text{M}} = 9.11 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$). Compared to its kinetic parameters (k_{cat} and K_{M}) for (–)-cocaine hydrolysis, the wild-type BChE has a slightly smaller k_{cat} value and a significantly larger K_{M} value for norcocaine hydrolysis. The significantly larger K_{M} value is associated with the lack of favorable hydrophobic interaction with the methyl group on the nitrogen atom of (–)-cocaine (see Figure 2). As a result, the catalytic efficiency ($k_{\text{cat}}/K_{\text{M}}$) of wild-type BChE against norcocaine is ~five-fold lower than that against (–)-cocaine.

According to the kinetic parameters summarized in Table 2, all of the three BChE mutants (CocH1 to 3) indeed have a significantly improved catalytic efficiency against norcocaine compared to the wild-type BChE, with the improvement ranging from 343 to 1080-fold: 343-fold for CocH1, 798-fold for CocH2, and 1080-fold for CocH3. In comparison between the kinetic activities of the enzymes against the two different substrates, the K_{M} value

against norcocaine also qualitatively correlates with that against (–)-cocaine. This correlation is consistent with the modeled binding structures discussed above. As noted in the modeled enzyme-substrate binding structures, the wild-type BChE and the mutants bind with norcocaine in the same mode as that with (–)-cocaine, which means that the change of the binding affinity from (–)-cocaine to norcocaine is mainly due to lack of the favorable hydrophobic interaction with the methyl group on the nitrogen atom of (–)-cocaine for a given enzyme (wild-type BChE or mutant). The lack of hydrophobic interaction with the methyl group systematically decreases the binding affinity with norcocaine and, thus, systematically increases the corresponding K_M value. The k_{cat} values against norcocaine also have some correlation with those against (–)-cocaine, but with an exception concerning Coch2 *versus* Coch1. Based on the kinetic data in Table 2, $k_{cat}(\text{Coch2}) > k_{cat}(\text{Coch1})$ for norcocaine, whereas $k_{cat}(\text{Coch2}) < k_{cat}(\text{Coch1})$ for (–)-cocaine. Overall, the improvement in the catalytic efficiency (k_{cat}/K_M) against norcocaine correlates well with that against (–)-cocaine.

Within the BChE mutants examined in this study, the most efficient BChE mutant (Coch3) against norcocaine is the same as the most efficient mutant against (–)-cocaine. Coch3 has a 1080-fold improved catalytic efficiency against norcocaine and a 2020-fold improved catalytic efficiency against (–)-cocaine. So, Coch3 is identified as the most promising enzyme (BChE mutant) for metabolizing both (–)-cocaine and norcocaine.

Norcocaine clearance accelerated by Coch3

Our recently reported *in vivo* studies[30] have demonstrated that Coch3 can efficiently metabolize (–)-cocaine in rats. In light of the encouraging *in vitro* activity data discussed above, we would like to know whether Coch3 can also efficiently metabolize norcocaine in rats. Hence, we characterized the pharmacokinetic profiles of norcocaine clearance with and without the presence of Coch3 in rats. The rats were injected with saline (for the control animals) or Coch3, followed by i.v. injection of norcocaine. The Coch3 dose was 0.15 mg/kg which led to a Coch3 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 Coch3 according to our previous study.[30] Concerning the dose of norcocaine, we first tried to use 3 mg/kg in the control animals. It turned out that 3 mg/kg norcocaine was highly toxic and even lethal; within the three rats injected with 3 mg/kg norcocaine, the third died in 2 min and saphenous veins of the other two rats became unclear to locate, which made it difficult to collect blood samples from saphenous veins. We decided not to test additional rats under this dose condition (saline and 3 mg/kg norcocaine). The observation suggested that 3 mg/kg norcocaine was significantly more toxic than 5 mg/kg (–)-cocaine, as we were able to inject 5 mg/kg (–)-cocaine to rats and take blood samples without any problem.[30] Hence, we had to lower the norcocaine dose to 2 mg/kg. Four rats (n=4) were injected with saline, followed by i.v. injection of 2 mg/kg norcocaine. Other four rats (n=4) were injected with 0.15 mg/kg Coch3, followed by i.v. injection of 2 mg/kg norcocaine. For each rat, the blood was sampled at 2, 5, 10, 15, 30, 60, 90, 120, 150, and 180 min after the norcocaine injection. The *in vivo* data are depicted in Figure 5.

CocH3 can hydrolyze norcocaine to produce benzoic acid and norecgonine methyl ester, and greatly accelerate the clearance of norcocaine from the body. The control curves in Figure 5 reflect the overall effects of all possible norcocaine elimination pathways.[34] As seen in Figure 5, in the control rats, the average concentration of norcocaine at the first time point (2 min) was 2.9 μM , while the average concentration of benzoic acid (metabolite) was 0.5 μM . In the presence of CocH3, the average concentration of norcocaine at ~2 min in the blood sample was below the detectable level (~0.1 μM , see Figure 5A), while the average concentration of benzoic acid at the first time point (2 min) was 5.7 μM (Figure 5B). Most of the norcocaine was hydrolyzed by CocH3 between the i.v. norcocaine injection and the first blood sampling at 2 min after the injection. The CocH3-caused dramatic changes in both the norcocaine and benzoic acid concentrations clearly indicated that norcocaine was metabolized rapidly to benzoic acid in the presence of CocH3. Notably, as shown in Figure 5B, the benzoic acid concentration in plasma decreased with time. This is because most of the norcocaine had already been hydrolyzed by CocH3 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.

It should be mentioned that the total plasma concentration of norcocaine and benzoic acid (5.8 μM) in the presence of CocH3 (when the benzoic acid concentration was higher) was higher than that (3.4 μM) in the absence of CocH3 (when the norcocaine concentration was higher). This observation might be associated with the potentially different distribution volumes of norcocaine and benzoic acid in the body. Norcocaine 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 norcocaine.

Effects of CocH3 on the pharmacokinetics of (–)-cocaine and norcocaine

With CocH3 identified as the most efficient enzyme (BChE mutant) for both (–)-cocaine and norcocaine, we further carried out kinetic modeling of cocaine metabolism using the kinetic equations summarized in Table 1 in the presence of two enzymes: one is CocH (which refers to either wild-type human BChE or CocH3) in human plasma, and the other is cytochrome P450 (CYP) 3A4 in human liver. Concerning CocH, a typical adult has a blood volume of ~5 L.[33] Concerning the CocH concentration (denoted as [E]), previously reported concentrations of endogenous BChE protein in human plasma ranged from 4 to 7 mg/L.[35–37] For example, the BChE concentration in human plasma should be ~5 mg/L according to Bartels *et al.*[36] and ~7 mg/L according to Polhuijs *et al.*,[35] giving an average value of ~6 mg/L or ~0.07 μM in terms of the total BChE protein concentration. Further, both native acetylcholinesterase (AChE) and BChE exist in tetramer. Previously reported computational simulations of AChE and BChE tetramer structures[38] suggested that a tetramer of AChE/BChE should have only two truly active sites and that the other two active sites of the tetramer should be dysfunctional due to their restricted accessibility to the substrate. If this is the case, ~6 mg/L BChE tetramer should have an active-site concentration of ~0.035 μM . [33] On the other hand, available experimental data concerning the number of active sites in a BChE tetramer (~340 KDa) are controversy in literature. Earlier experimental data reported by Muensch *et al.*[39] demonstrated that a tetramer of

human BChE should have two active sites, similar to the AChE tetramer which should have two active sites according to the experimental data reported by Gordon *et al.*[40] In contrast, subsequent experimental data reported by other researchers[41–42] indicated that a tetramer of human BChE should have four active sites. With four active sites in a BChE tetramer, the active-site concentration of endogenous BChE in human plasma should be $\sim 0.07 \mu\text{M}$. Hence, $[E] = 0.07 \mu\text{M}$ was used in the kinetic modeling discussed below; we also performed the kinetic modeling with $[E] = 0.035 \mu\text{M}$ and obtained qualitatively similar results.

According to the kinetic data summarized in Table 2, we should have $V_{\max} = 0.28 \mu\text{M min}^{-1}$ and $K_M = 4.5 \mu\text{M}$ for the wild-type BChE against (–)-cocaine, and $V'_{\max} = 0.20 \mu\text{M min}^{-1}$ and $K'_M = 15 \mu\text{M}$ for the wild-type BChE against norcocaine when $[E] = 0.07 \mu\text{M}$. These kinetic parameters were used in our modeling with the wild-type BChE. Similarly, for Coch3, according to the kinetic data summarized in Table 2, we should have $V_{\max} = 400 \mu\text{M min}^{-1}$ and $K_M = 3.1 \mu\text{M}$ against (–)-cocaine, and $V'_{\max} = 180 \mu\text{M min}^{-1}$ and $K'_M = 13 \mu\text{M}$ against norcocaine when $[E] = 0.07 \mu\text{M}$.

For (–)-cocaine oxidation catalyzed by CYP 3A4 in human liver, the CYP 3A4 enzyme in a typical human body may oxidize (–)-cocaine to norcocaine at an overall rate of $\sim 72 \mu\text{mol min}^{-1}$ in the liver according to the available experimental data including the enzyme activity[43] and the enzyme distribution in the body.[44] Further, it has been known that (–)-cocaine can diffuse in the body very rapidly to reach the equilibrium.[33] It is reasonable to assume that (–)-cocaine and norcocaine distributions in the blood and liver can rapidly reach the equilibrium during the metabolic reactions. With the rapid equilibrium assumption, in comparison with the hydrolysis reactions in $\sim 5 \text{ L}$ blood, CYP 3A4 should have an effective $V''_{\max}$ value of $\sim 72/5 = \sim 14.4 \mu\text{M min}^{-1}$ for the (–)-cocaine oxidation. In addition, the available experimental kinetic data also revealed that $K''_M = \sim 2.7 \text{ mM}$ for the enzymatic oxidation of (–)-cocaine (the average value for humans).[43] These kinetic parameters ($V''_{\max} = 14.4 \mu\text{M min}^{-1}$ and $K''_M = 2.7 \text{ mM}$) were used in our kinetic modeling with various initial concentrations: $A(0)$ (the initial concentration of (–)-cocaine) = 1 to 100 μM while $B(0)$ (the initial concentration of norcocaine) = 0.

Depicted in Figure 6 are the time-dependent concentrations of (–)-cocaine and norcocaine when the initial (–)-cocaine concentration is 5 μM (a representative one of the cocaine addiction conditions) in the presence of both CYP 3A4 and wild-type human BChE (Figure 6A and 6B) or both CYP 3A4 and Coch3 (Figure 6C and 6D). As seen in Figure 6A and 6B, in the presence of both CYP 3A4 and wild-type human BChE (without administration of any exogenous enzyme), (–)-cocaine has an area under the curve (AUC) of 107 $\mu\text{M}\cdot\text{min}$ and a half-life ($t_{1/2}$) of 17 min, and norcocaine has an AUC of 44 $\mu\text{M}\cdot\text{min}$ and a half-life of 108 min. The modeling data suggest that norcocaine can exist in the body for a relatively longer time compared to (–)-cocaine itself because the endogenous wild-type BChE has a significantly lower catalytic activity against norcocaine. Based on the kinetic modeling, cumulatively, about 11.5% of (–)-cocaine has been metabolized to norcocaine and then norecgonine methyl ester and benzoic acid. The modeled overall contribution of (–)-cocaine oxidation to norcocaine is qualitatively consistent with the previous studies by Nayak *et al.* [45] using [^3H]cocaine in rats, as they demonstrated that norcocaine constituted 10 to 20%

of the drugs (cocaine and norcocaine) in the brain of the chronically treated rats at the tested times (0.25, 0.5, 1, 2, and 4 hr) after the i.v. injection of cocaine. Further, the modeling data summarized in Table 3 indicate that percentage contribution of (–)-cocaine oxidation to norcocaine, as well as the AUC and $t_{1/2}$ of both (–)-cocaine and norcocaine, should increase with increasing the initial (–)-cocaine concentration.

In the presence of both CYP 3A4 and CocH3 (exogenous enzyme with $[E] = 0.07 \mu\text{M}$), (–)-cocaine only has an AUC of $0.070 \mu\text{M}\cdot\text{min}$ and a half-life of 0.012 min, and norcocaine has an AUC of $0.000027 \mu\text{M}\cdot\text{min}$ and a half-life of 0.084 min, as shown in Figure 6C and 6D. Both the AUC and $t_{1/2}$ values are negligible when CocH3 is administered as an exogenous enzyme (or provided *via* gene therapy[46]) with the CocH3 concentration being the same as that of the endogenous BChE ($[E] = 0.07 \mu\text{M}$). As seen in Table 3, the AUC and $t_{1/2}$ of both (–)-cocaine and norcocaine increase with increasing the initial (–)-cocaine concentration, but not dramatically. In particular, even if $A(0) = 100 \mu\text{M}$, the half-life of (–)-cocaine is only 0.13 min, and the half-life of norcocaine is only 0.25 min in the presence of CocH3. Clearly, both (–)-cocaine and norcocaine can be eliminated effectively and rapidly at the same time when CocH3 is administered as an exogenous enzyme with the CocH3 concentration being the same as that of the endogenous BChE ($[E] = 0.07 \mu\text{M}$) in the simplified kinetic model.

Conclusion

The catalytic activity of human BChE and its three mutants (CocH1 to 3) for norcocaine has been characterized in comparison with the corresponding catalytic activity for cocaine. The kinetic data reveal that wild-type human BChE has a significantly lower catalytic activity against norcocaine ($k_{\text{cat}} = 2.8 \text{ min}^{-1}$, $K_{\text{M}} = 15 \mu\text{M}$, and $k_{\text{cat}}/K_{\text{M}} = 1.87 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$) compared to its catalytic activity against (–)-cocaine ($k_{\text{cat}} = 4.1 \text{ min}^{-1}$, $K_{\text{M}} = 4.5 \mu\text{M}$, and $k_{\text{cat}}/K_{\text{M}} = 9.11 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$). It has been shown that the BChE mutants examined in this study have not only a considerably improved catalytic efficiency against cocaine but also a considerably improved catalytic efficiency against norcocaine compared to the wild-type BChE. The most efficient BChE mutant (CocH3, *i.e.* the A199S/F227A/S287G/A328W/Y332G mutant) against norcocaine is the same as the most efficient one against cocaine. CocH3 has a 1080-fold improved catalytic efficiency against norcocaine ($k_{\text{cat}} = 2610 \text{ min}^{-1}$, $K_{\text{M}} = 13 \mu\text{M}$, and $k_{\text{cat}}/K_{\text{M}} = 2.01 \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$) and a 2020-fold improved catalytic efficiency against cocaine ($k_{\text{cat}} = 5700 \text{ min}^{-1}$, $K_{\text{M}} = 3.1 \mu\text{M}$, and $k_{\text{cat}}/K_{\text{M}} = 1.84 \times 10^9 \text{ M}^{-1} \text{ min}^{-1}$). Thus, CocH3 is identified as the most efficient enzyme for hydrolyzing both cocaine and norcocaine. It has been demonstrated that CocH3 as an exogenous enzyme can indeed rapidly metabolize norcocaine, in addition to cocaine, in rats. Further kinetic modeling has suggested that CocH3 with a concentration similar to that of the endogenous BChE in human plasma can effectively eliminate both cocaine and norcocaine in a simplified kinetic model of cocaine abuse.

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Figure 1. Cocaine metabolites produced in humans through hydrolysis by BChE, which is the primary endogenous cocaine hydrolase (CocH) in humans, and oxidation by cytochrome P450 (CYP) 3A4.

Figure 2.

Docked structures of the wild-type BChE and CocH3 binding with norcocaine and (–)cocaine: (A) Wild-type human binding with (–)cocaine; (B) Wild-type human binding with norcocaine; (C) CocH3 binding with (–)cocaine; (D) CocH3 binding with norcocaine. 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.

Figure 3.

(A) UV-Vis absorption of (-)-cocaine, norcocaine, and CocH3. (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 norcocaine.

Figure 4.

Kinetic data obtained for enzymatic hydrolysis of (-)-cocaine and norcocaine: (A) wild-type human BChE; (B) CocH1; (C) CocH2; (D) CocH3. To minimize the possible systematic experimental errors of the kinetic data, for each enzyme the catalytic activities against both norcocaine 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 norcocaine relative to those against (-)-cocaine. The reaction rate (represented in μM min⁻¹ per nM enzyme) was determined by measuring the change of the absorbance at 230 nm per minute.

Figure 5. Norcocaine clearance accelerated by CocH3: Time-dependent concentrations of norcocaine (A) and benzoic acid (B) in blood. Benzoic acid is the product of CocH3-catalyzed hydrolysis of norcocaine. Saline or 0.15 mg/kg CocH3 was injected i.v. in rats (n=4) 1 min before the i.v. injection of 2 mg/kg norcocaine.

Figure 6.

The modeled concentrations of (–)-cocaine (A) and norcocaine (B) in blood when the initial (–)-cocaine concentration is 5 μM in the presence of both CYP 3A4 and wild-type human BChE, and concentrations of (–)-cocaine (C) and norcocaine (D) in blood when the initial (–)-cocaine concentration is 5 μM in the presence of both CYP 3A4 and Coch3.

Table 1
Reaction scheme and kinetic equations used in the kinetic modeling.

Reaction scheme ^a	Kinetic equations ^{a,b}
<div><div><div><div><div>A</div><div>P450</div></div><div><div>CocH</div><div>$(V_{\max}, K_M)$</div></div><div><div>D + E</div></div></div><div><div><div>B</div><div>$(V'_{\max}, K'_M)$</div></div><div><div>CocH</div><div>$(V'_{\max}, K'_M)$</div></div><div><div>C + D</div></div></div></div><div><div><div>A</div><div>$(V''_{\max}, K''_M)$</div></div><div><div>B</div></div></div></div> <div><div>A: Cocaine</div><div>B: Norcocaine</div><div>C: Norecgonine Methyl Ester</div><div>D: Benzoic Acid</div><div>E: Ecgonine Methyl Ester</div></div>	<div>$\frac{dA(t)}{dt} = -V_{\max}A(t)/(K_M + A(t)) - V''_{\max}A(t)/(K''_M + A(t))$$\frac{dB(t)}{dt} = V''_{\max}A(t)/(K''_M + A(t)) - V'_{\max}B(t)/(K'_M + B(t))$$\frac{dC(t)}{dt} = V'_{\max}B(t)/(K'_M + B(t))$$\frac{dD(t)}{dt} = V_{\max}A(t)/(K_M + A(t)) + V'_{\max}B(t)/(K'_M + B(t))$$\frac{dE(t)}{dt} = V_{\max}A(t)/(K_M + A(t))$</div>

^a $V_{\max} = k_{\text{cat}}[E]$ and $V'_{\max} = k'_{\text{cat}}[E]$ in which $[E]$ is the concentration of the enzyme (CocH) catalyzing hydrolysis of both cocaine and norcocaine. $V''_{\max} = k''_{\text{cat}}[E'']$ in which $[E']$ is the concentration of the cytochrome P450 (CYP) 3A4 catalyzing oxidation of cocaine into norcocaine.

^b $A(t)$ is the concentration of **A** at time t . $B(t)$ is the concentration of **B** at time t . $C(t)$ is the concentration of **C** at time t . $D(t)$ is the concentration of **D** at time t . $E(t)$ is the concentration of **E** at time t .

Table 2
Kinetic parameters determined for (–)-cocaine and norcocaine hydrolyses catalyzed by wild-type human BChE and its mutants.

Enzyme ^d	(–)-Cocaine ^b				Norcocaine ^c			
	K_M (μM)	k_{cat} (min ^{–1})	k_{cat}/K_M (M ^{–1} min ^{–1})	RCE ^d	K_M (μM)	k_{cat} (min ^{–1})	k_{cat}/K_M (M ^{–1} min ^{–1})	RCE ^d
WT BChE	4.5	4.1	9.11×10^5	1	15	2.8	1.87×10^5	1
CocH1	3.1	3,060	9.87×10^8	1,080	12	766	6.38×10^7	343
CocH2	1.1	1,730	1.57×10^9	1,730	7.6	1,130	1.49×10^8	798
CocH3	3.1	5,700	1.84×10^9	2,020	13	2,610	2.01×10^8	1,080

^aThe enzyme under the study was wild-type human BChE (WT BChE), A199S/S287G/A328W/Y332G mutant (CocH1), A199S/F227A/S287G/A328W/E441D mutant (CocH2), or A199S/F227A/S287G/A328W/Y332G mutant (CocH3).

^bData for wild-type BChE against (–)-cocaine came from reference [13], data for CocH1 an CocH2 against (–)-cocaine came from reference [24], and data for CocH3 against (–)-cocaine came from reference [21].

^cAll of the kinetic data for norcocaine were determined in the present study for the first time.

^dRCE 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.

Table 3

Kinetic parameters determined for (–)-cocaine and norcocaine hydrolyses catalyzed by wild-type human BChE and its mutants.

<i>A</i> (0) (μM) ^a	CYP 3A4 and wild-type BChE					CYP 3A4 and CocH3				
	AUC(μM·min)		<i>t</i> _{1/2} (min)		Norc% ^d	AUC(μM·min)		<i>t</i> _{1/2} (min)		Norc% ^d
	Coc ^b	Norc ^c	Coc ^b	Norc ^c		Coc ^b	Norc ^c	Coc ^b	Norc ^c	
1	16	6.5	11.2	100	8.5%	0.009	0.000003	0.0066	0.078	0.00%
2	35	14	12.7	102	9.3%	0.021	0.000008	0.0077	0.080	0.01%
3	56	23	13.9	104	10.0%	0.035	0.000013	0.0090	0.081	0.01%
4	81	33	15.4	105	10.7%	0.051	0.000019	0.0101	0.083	0.01%
5	107	44	16.5	108	11.5%	0.070	0.000027	0.0115	0.084	0.01%
6	137	57	17.8	109	12.2%	0.092	0.000035	0.0129	0.086	0.01%
7	169	70	19.3	112	12.8%	0.116	0.000044	0.0139	0.088	0.01%
8	203	85	20.4	114	13.5%	0.142	0.000054	0.0153	0.089	0.01%
9	239	101	22.0	116	14.2%	0.171	0.000065	0.0162	0.091	0.01%
10	278	118	23.2	118	14.8%	0.203	0.000077	0.0175	0.093	0.01%
20	775	348	33.7	139	20.6%	0.657	0.000248	0.0300	0.111	0.02%
30	1440	693	41.7	159	25.4%	1.361	0.000513	0.0424	0.129	0.02%
40	2237	1163	49.5	179	29.6%	2.315	0.000870	0.0550	0.147	0.03%
50	3142	1772	55.5	199	33.1%	3.520	0.001320	0.0667	0.165	0.04%
60	4136	2534	60.7	219	36.3%	4.975	0.001862	0.0801	0.183	0.04%
70	5207	3465	65.0	239	39.1%	6.680	0.002495	0.0928	0.200	0.05%
80	6343	4580	68.9	259	41.6%	8.635	0.003218	0.1044	0.218	0.06%
90	7536	5893	72.6	280	43.8%	10.841	0.004030	0.1165	0.234	0.06%
100	8780	7417	75.8	300	45.8%	13.296	0.004932	0.1290	0.251	0.07%

^aThe initial concentration of (–)-cocaine.

^bCoc represents (–)-cocaine.

^cNorc represents norcocaine.

^dNorc% refers to the percentage contribution of (–)-cocaine metabolism through oxidation to norcocaine.