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Catalytic activities of a cocaine hydrolase engineered from human butyrylcholinesterase against (+)- and (–)-cocaine

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Abstract

It can be argued that an ideal anti-cocaine medication would be one that accelerates cocaine metabolism producing biologically inactive metabolites *via* a route similar to the primary cocaine-metabolizing pathway, *i.e.* hydrolysis catalyzed by butyrylcholinesterase (BChE) in plasma. However, wild-type BChE has a low catalytic efficiency against naturally occurring (–)cocaine. Interestingly, wild-type BChE has a much higher catalytic activity against unnatural (+)cocaine. According to available positron emission tomography (PET) imaging analysis using [¹¹C](–)cocaine and [¹¹C](+)cocaine tracers in human subjects, only [¹¹C](–)cocaine was observed in the brain, whereas no significant [¹¹C](+)cocaine signal was observed in the brain. The available PET data imply that an effective therapeutic enzyme for treatment of cocaine abuse could be an exogenous cocaine-metabolizing enzyme with a catalytic activity against (–)cocaine comparable to that of wild-type BChE against (+)cocaine. Our recently designed A199S/F227A/S287G/A328W/Y332G mutant of human BChE has a considerably improved catalytic efficiency against (–)cocaine and has been proven active *in vivo*. In the present study, we have characterized the catalytic activities of wild-type BChE and the A199S/F227A/S287G/A328W/Y332G mutant against both (+)- and (–)-cocaine at the same time under the same experimental conditions. Based on the obtained kinetic data, the A199S/F227A/S287G/A328W/Y332G mutant has a similarly high catalytic efficiency ($k_{\text{cat}}/K_{\text{M}}$) against (+)- and (–)-cocaine, and indeed has a catalytic efficiency ($k_{\text{cat}}/K_{\text{M}} = 1.84 \times 10^9 \text{ M}^{-1} \text{ min}^{-1}$) against (–)cocaine comparable to that ($k_{\text{cat}}/K_{\text{M}} = 1.37 \times 10^9 \text{ M}^{-1} \text{ min}^{-1}$) of wild-type BChE against (+)cocaine. Thus, the mutant may be used to effectively prevent (–)cocaine from entering brain and producing physiological effects in the enzyme-based treatment of cocaine abuse.

1 Introduction

Cocaine is known as the most reinforcing drug of abuse [1,2,3]. There is still no FDA-approved medication specific for cocaine addiction or overdose treatment. The disastrous medical and social consequences of cocaine addiction have made a high priority the development of an anti-cocaine medication [4,5,6]. A promising anti-cocaine medication strategy is to accelerate cocaine metabolism producing biologically inactive metabolites *via* a route similar to the primary cocaine-metabolizing pathway, *i.e.* hydrolysis catalyzed by butyrylcholinesterase (BChE) in plasma [4,7,8,9,10,11]. However, wild-type BChE has a

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low catalytic efficiency ($k_{\text{cat}} = 4.1 \text{ min}^{-1}$ and $K_M = 4.5 \text{ }\mu\text{M}$) against naturally occurring (–)-cocaine [12,13,14,15,16]. It is highly desirable to develop a mutant of human BChE which can be regarded as a cocaine hydrolase (CocH) with a significantly improved catalytic activity against (–)-cocaine.

It should be mentioned that cocaine has two enantiomers: naturally occurring (–)-cocaine which is biologically active, and unnatural (+)-cocaine which is biologically inactive. Interestingly, wild-type BChE has a much higher catalytic activity against (+)-cocaine compared to its catalytic activity against (–)-cocaine. According to the positron emission tomography (PET) imaging analysis using tracer doses of [^{11}C](–)-cocaine and [^{11}C](+)-cocaine in human subjects [17,18], (+)-cocaine can be cleared from plasma in seconds and prior to partitioning into the central nervous system (CNS) due to the presence of endogenous BChE in plasma. Hence, no significant [^{11}C](+)-cocaine signal was observed in the brain. In contrast, (–)-cocaine has a plasma half-life of ~45–90 min, long enough for manifestation of the CNS effects which peak in minutes and for PET detection [14,17,18]. The available PET data imply that an effective therapeutic enzyme for treatment of cocaine abuse could be a BChE mutant (or an equivalent cocaine-metabolizing enzyme) with a catalytic activity against (–)-cocaine comparable to that of wild-type BChE against (+)-cocaine. Hence, it has been our goal in this research direction to design and discover such a desirable high-activity mutant of human BChE for anti-cocaine medication development [19,20].

It has been extremely challenging to design an appropriate enzyme with a high catalytic efficiency for a given drug of abuse, particularly when the chemical process is rate determining. Nevertheless, we have developed novel computational strategies and protocols [21,22,23,24,25,26,27,28] for rational design of high-activity enzymes against a given substrate through virtual screening of transition states, leading to discovery of a set of BChE mutants, known as cocaine hydrolases (CocHs), with at least 1,000-fold improved catalytic efficiency against (–)-cocaine compared to wild-type BChE [29,30,31,32]. The first one of our reported (and patented) high-activity mutants of BChE, *i.e.* the A199S/S287G/A328W/Y332G mutant [24,31], was validated *in vitro* and *in vivo* by other research groups, *e.g.* Brimijoin *et al.* [33] who concluded that this BChE mutant is “*a true CocH with a catalytic efficiency that is 1,000-fold greater than wild-type BChE*” and that this mutant (fused with human serum albumin to extend the plasma half-life) selectively blocked the cocaine-induced toxicity and reinstatement of drug seeking in rats that had previously self-administered cocaine [33]. The albumin-fused A199S/S287G/A328W/Y332G mutant of BChE has been denoted differently as Albu-CocH, TV-1380, or AlbuBChE by different research groups. The albumin-fused A199S/S287G/A328W/Y332G mutant of BChE has been tested extensively in rodents and primates for its safety, cocaine overdose rescue, PK/PD properties, and efficacy in cocaine addiction treatment [33,34,35,36,37,38]. The albumin-fused A199S/S287G/A328W/Y332G mutant of BChE is currently in double-blind, placebo-controlled human clinical trials by Teva Pharmaceutical Industries Ltd for cocaine addiction treatment [38]. The clinical data available so far have consistently demonstrated that the albumin-fused A199S/S287G/A328W/Y332G mutant of BChE is safe and efficacious for humans in cocaine addiction treatment [38]. Without immunogenicity and any unacceptable side effects, the enzyme administration using the albumin-fused A199S/S287G/A328W/Y332G mutant significantly decreased the cocaine preference, the desire to take cocaine again, and the overall drug liking [38]. So, both the preclinical and clinical data have consistently shown that our rationally designed and discovered CocHs are promising candidates for anti-cocaine medication development [39].

Further, our more recently designed and discovered new mutants of human BChE, particularly the A199S/F227A/S287G/A328W/Y332G mutant known as the “most efficient

cocaine hydrolase (CocH)" in literature [29,40], have an even higher catalytic efficiency ($k_{\text{cat}} = 5,700 \text{ min}^{-1}$ and $K_M = 3.1 \text{ }\mu\text{M}$) compared to the above-mentioned A199S/S287G/A328W/Y332G mutant in the clinical trials. *In vivo* studies [29,32] have confirmed that the A199S/F227A/S287G/A328W/Y332G mutant is indeed more potent in protection of mice from acute toxicity of a lethal dose of cocaine (180 mg/kg, i.p.). The minimum i.v. dose of enzyme required for fully protecting mice from acute toxicity of a lethal dose of cocaine (180 mg/kg, i.p.) is 0.03 mg/mouse for the A199S/S287G/A328W/Y332G mutant [32] and 0.01 mg/mouse for the A199S/F227A/S287G/A328W/Y332G mutant [29]. It would be interesting to explore further preclinical and clinical studies on the A199S/F227A/S287G/A328W/Y332G mutant.

In order to guide future further preclinical and clinical studies on the A199S/F227A/S287G/A328W/Y332G mutant as well as future rational design of new mutants of human BChE, we would like to first address a couple of key questions. Does this mutant have a catalytic activity against (–)-cocaine comparable to that of wild-type BChE against (+)-cocaine? Do the A199S/F227A/S287G/A328W/Y332G mutations also significantly change the catalytic activity of human BChE against (+)-cocaine? To address these questions, in the present study, we performed further modeling studies on the enzyme-substrate interactions and carried out the kinetic analysis on both wild-type human BChE and the A199S/F227A/S287G/A328W/Y332G mutant against both (+)-cocaine and (–)-cocaine at the same time under the same experimental conditions so that the kinetic parameters can be compared appropriately. The computational and experimental data lead to clear answers to the above questions and provide valuable insights.

2 Materials and Methods

The (+)-cocaine binding with BChE and mutants was modeled by using the same modeling approach which we used to study (–)-cocaine interacting with the enzymes [24,25,31,32]. Briefly, the initial structures of BChE and the mutant used in the molecular modeling were prepared based on our previous molecular dynamics (MD) simulation [32] on the enzyme-substrate complex for wild-type BChE binding with (–)-cocaine. Our previous MD simulations [32] on the enzyme-substrate complexes started from the X-ray crystal structure [41] deposited in the Protein Data Bank (pdb code: 1POP). The general procedure for carrying out the MD simulations on the enzyme-substrate complexes in water is essentially the same as that used in our previously reported computational studies on other complexes [24,25,31,32]. Each aforementioned starting structure was neutralized by adding chloride counterions and was solvated in an orthorhombic box of TIP3P water molecules with a minimum solute-wall distance of 10 Å. All of the MD simulations were performed by using the Sander module of Amber11 package [42]. The solvated systems were carefully equilibrated and fully energy-minimized. These systems were gradually heated from $T = 10 \text{ K}$ to $T = 298.15 \text{ K}$ in 30 ps before running the MD simulation at $T = 298.15 \text{ K}$ 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 $T = 298.15 \text{ K}$ with Berendsen temperature coupling and $P = 1 \text{ atm}$ 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 energy-minimized.

The protein materials of both wild-type human BChE and the A199S/F227A/S287G/A328W/Y332G mutant were prepared simultaneously in the same way as we described previously [29,32]. Briefly, the proteins were expressed in human embryonic kidney cell

line 293T/17. Cells were grown to 80–90% confluence in 6-well dishes and then transfected by Lipofectamine 2,000 complexes of 4 μg plasmid DNA per each well. Cells were incubated at 37° C in a CO₂ incubator for 24 hours and cells were moved to 60-mm culture vessel and cultured for four more days. The culture medium [10% fetal bovine serum in Dulbecco's modified Eagle's medium (DMEM)] was harvested for the enzyme activity assays using both the sensitive radiometric assay with [³H]-(-)-cocaine labeled on its benzene ring (for the enzyme activity against (-)-cocaine) and UV-Vis spectrophotometric assay (for the enzyme activity against both (+)- and (-)-cocaine).

The radiometric assay was based on toluene extraction of product [³H]-benzoic acid. The toluene extraction of [³H]-benzoic acid is a well-established solvent-partitioning procedure with water and toluene phases. At a very low pH, [³H]-benzoic acid is protonated and the protonated [³H]-benzoic acid exists in toluene phase, while [³H]-(-)-cocaine exists in water phase. To measure benzoic acid, the product of (-)-cocaine hydrolysis catalyzed by BChE, a sensitive radiometric assay was used to get the K_M and V_{max} values based on toluene extraction of [³H]-(-)cocaine labeled on its benzene ring. To initiate the enzymatic reaction, 100 nCi of [³H]-(-)cocaine was mixed with 100 μl of culture medium. The enzymatic reactions proceeded at room temperature (25°C) with varying concentrations of (-)-cocaine. The reactions were stopped by adding 200 μl of 0.1 M HCl, which neutralized the liberated benzoic acid while ensuring a positive charge on the residual (-)-cocaine. [³H]-benzoic acid was extracted by 1 ml of toluene and measured by scintillation counting.

Using the UV-Vis spectrophotometric assay, the catalytic activities of the enzymes against (+)- and (-)-cocaine were determined at the same time under the same experimental conditions. The temperature was 25°C, and the buffer used was 0.1 M potassium phosphate (pH 7.2). The initial rates of the enzymatic hydrolysis of (+)/(-)-cocaine in various initial concentrations were estimated by following the change in the intrinsic absorbance of (+)/(-)-cocaine at 230 nm with time using a GENios Pro Microplate Reader (TECAN, Research Triangle Park, NC) in our own lab with the XFluor software. The initial rates were estimated from the linear portions of the progress curves and spanned no longer than 15 min.

All assays were performed in triplicate. The kinetic data were analyzed by using the standard Michaelis-Menten kinetics so that the catalytic parameters (k_{cat} and K_M) were determined along with the use of an enzyme-linked immunosorbent assay (ELISA) described previously [29].

3 Results and Discussion

3.1 Insights from modeling studies

The same modeling approach which we used to study (-)-cocaine interacting with BChE and mutants [24,25,31,32] was employed to study how (+)-cocaine binds with the proteins. Depicted in Figure 1 are the energy-minimized structures of the enzyme-substrate complexes. The binding structures depicted in Figure 1 indicate that the crucial interactions between the carbonyl oxygen of (+)-cocaine and the oxyanion hole (residues #116, #117, and #199) in the A199S/F227A/S287G/A328W/Y332G mutant (Figure 1D) are very similar to the corresponding interactions in the wild-type (Figure 1C) in terms of the hydrogen bonding (two hydrogen bonds with the backbone NH groups of residues #117 and #199 in each structure). The side chain of S199 in the A199S/F227A/S287G/A328W/Y332G mutant has no hydrogen bond with the carbonyl oxygen of (+)-cocaine. The role of residue #199 in the enzyme-(+)-cocaine binding is remarkably different from the role of residue #199 in the corresponding enzyme-(-)-cocaine binding (Figure 1A and B). The backbone of residue #199 cannot form a hydrogen bond with (-)cocaine, whereas the hydroxyl group of S199 side chain in the mutant forms a strong hydrogen bond with (-)-cocaine. Overall, there are

two hydrogen bonds between the carbonyl oxygen of substrate and the oxyanion hole of the enzyme in each of the three complexes: wild-type BChE binding with (+)-cocaine and the A199S/F227A/S287G/A328W/Y332G mutant binding with (–)cocaine and (+)-cocaine. In comparison, there is only one hydrogen bond between the carbonyl oxygen of (–)-cocaine and the oxyanion hole of wild-type BChE. The molecular modeling results suggest that the A199S/F227A/S287G/A328W/Y332G mutant has a catalytic activity against (–)cocaine comparable to that of wild-type BChE against (+)-cocaine, and that the A199S/F227A/S287G/A328W/Y332G mutations should not considerably change the catalytic activity of wild-type BChE against (+)-cocaine.

3.2 Kinetic parameters

To minimize the possible systematic experimental errors of the *in vitro* kinetic data, we expressed the enzymes and performed kinetic studies for both wild-type BChE and the A199S/F227A/S287G/A328W/Y332G mutant at the same time under the same conditions in order to compare their activities appropriately. In this way, the well-established kinetic parameters for wild-type human BChE against (–)cocaine can be used as a standard reference. Whereas the radiometric assay was used for kinetic analysis on the enzymes against (–)cocaine, the UV-Vis spectrophotometric assay was used for kinetic analysis on the enzymes against both (+)- and (–)cocaine. In fact, there was no significant difference in the determined catalytic activities between the radiometric and UV-Vis spectrophotometric assays, indicating that the two types of kinetic assays are reliable.

Depicted in Figure 2 are the kinetic data. Summarized in Table 1 are the determined kinetic parameters. As seen in Table 1, the A199S/F227A/S287G/A328W/Y332G mutations only slightly change the catalytic efficiency ($k_{\text{cat}}/K_{\text{M}}$) of human BChE against (+)-cocaine from $1.37 \times 10^9 \text{ M}^{-1} \text{ min}^{-1}$ to $1.95 \times 10^9 \text{ M}^{-1} \text{ min}^{-1}$, although the same mutations improve the catalytic efficiency of the same enzyme against (–)cocaine by ~2020-fold. As a result, the A199S/F227A/S287G/A328W/Y332G mutant has a similarly high catalytic efficiency ($k_{\text{cat}}/K_{\text{M}}$) against (+)- and (–)cocaine.

It should be noted that an early study reported by Sun *et al.* [43] determined the catalytic activities of wild-type BChE against both (+)- and (–)cocaine at the same time. They determined that $k_{\text{cat}} = 6,423 \pm 24 \text{ min}^{-1}$ and $K_{\text{M}} = 8.5 \pm 0.5 \text{ }\mu\text{M}$ for wild-type BChE against (+)-cocaine, whereas $k_{\text{cat}} = 3.9 \pm 0.8 \text{ min}^{-1}$ and $K_{\text{M}} = 9.0 \pm 0.3 \text{ }\mu\text{M}$ for wild-type BChE against (–)cocaine. In more recent studies on BChE with (–)cocaine, the catalytic activity of wild-type BChE against (–)cocaine was characterized more accurately, with $k_{\text{cat}} = 4.1 \text{ min}^{-1}$ and $K_{\text{M}} = 4.5 \text{ }\mu\text{M}$ [12]. Apparently, the earlier study [43] systematically overestimated the K_{M} values for wild-type BChE against both (+)- and (–)cocaine. Our current kinetic analysis on wild-type BChE against (+)- and (–)cocaine indicated that $K_{\text{M}} = 4.7 \text{ }\mu\text{M}$ for wild-type BChE against (+)-cocaine while $K_{\text{M}} = 4.5$ for wild-type BChE against (–)cocaine, and there was no significant difference between our determined k_{cat} values and the previously reported k_{cat} values.

The kinetic parameters summarized in Table 1 reveal that the A199S/F227A/S287G/A328W/Y332G mutant of human BChE indeed has a catalytic efficiency ($k_{\text{cat}}/K_{\text{M}} = 1.84 \times 10^9 \text{ M}^{-1} \text{ min}^{-1}$) against (–)cocaine comparable to that ($k_{\text{cat}}/K_{\text{M}} = 1.37 \times 10^9 \text{ M}^{-1} \text{ min}^{-1}$) of wild-type BChE against (+)-cocaine. Due to the similar catalytic efficiency of the A199S/F227A/S287G/A328W/Y332G mutant against (–)cocaine and wild-type BChE against (+)-cocaine, and in light of the aforementioned PET data with (+)-cocaine in literature [14,17,18], one can reasonably hypothesize that the A199S/F227A/S287G/A328W/Y332G mutant may be used to effectively prevent (–)cocaine from entering brain and producing physiological effects. In particular, if one could repeat the PET imaging analysis on (–)cocaine pharmacokinetics using the A199S/F227A/S287G/A328W/Y332G mutant as an

exogenous enzyme with a plasma concentration being the same as the endogenous BChE, the time course of the brain concentration of (–)-cocaine would be comparable to that of the brain concentration of (+)-cocaine without the exogenous enzyme (with the endogenous BChE only). It would be extremely interesting to carry out such type of PET imaging analysis with the A199S/F227A/S287G/A328W/Y332G mutant in near future to test this hypothesis and determine the detailed effects of the A199S/F227A/S287G/A328W/Y332G mutant on (–)cocaine pharmacokinetics in human subjects.

4 Conclusion

Based on the obtained kinetic data, the A199S/F227A/S287G/A328W/Y332G mutant has a similarly high catalytic efficiency (k_{cat}/K_M) against (+)- and (–)-cocaine, and indeed has a catalytic efficiency ($k_{\text{cat}}/K_M = 1.84 \times 10^9 \text{ M}^{-1} \text{ min}^{-1}$) against (–)-cocaine comparable to that ($k_{\text{cat}}/K_M = 1.37 \times 10^9 \text{ M}^{-1} \text{ min}^{-1}$) of wild-type BChE against (+)-cocaine. Due to the similar catalytic efficiency of the A199S/F227A/S287G/A328W/Y332G mutant against (–)-cocaine and wild-type BChE against (+)-cocaine, and in light of the available PET data with (+)-cocaine in literature, one can reasonably expect that the A199S/F227A/S287G/A328W/Y332G mutant may be used to effectively prevent (–)-cocaine from entering brain and producing physiological effects in the enzyme-based treatment of cocaine abuse.

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Research Highlights

We determine catalytic activities of a cocaine hydrolase for (+)- and (–)-cocaine

The cocaine hydrolase has a similar catalytic efficiency for (+)- and (–)-cocaine

The hydrolase activity for (–)-cocaine is similar to that of BChE for (+)-cocaine.

The cocaine hydrolase is expected to be effective in treatment of cocaine abuse.

Figure 1.

Modeled enzyme-substrate binding structures: (A) wild-type human BChE with (–)-cocaine; (B) the A199S/F227A/S287G/A328W/Y332G mutant (denoted as CocH) with (–)-cocaine; (C) wild-type BChE with (+)-cocaine; (D) the A199S/F227A/S287G/A328W/Y332G mutant (denoted as CocH) with (+)-cocaine.

Figure 2.

Kinetic data for the hydrolysis of (-)-cocaine or (+)-cocaine catalyzed by wild-type human BChE or the A199S/F227A/S287G/A328W/Y332G mutant: (A) wild-type BChE against (-)-cocaine; (B) the A199S/F227A/S287G/A328W/Y332G mutant against (-)-cocaine; (C) wild-type BChE against (+)-cocaine; (D) the A199S/F227A/S287G/A328W/Y332G mutant against (+)-cocaine.

Table 1

Kinetic parameters determined for the enzymatic hydrolysis of (+)- and (-)-cocaine catalyzed by wild-type BChE and the A199S/F227A/S287G/A328W/Y332G mutant.

Substrate	Enzyme ^a	K _M (μM)	k _{cat} (min ⁻¹)	k _{cat} /K _M (M ⁻¹ min ⁻¹)	Relative k _{cat} /K _M
(+)cocaine	wild-type BChE	4.7	6,420	1.37 × 10 ⁹	1
	A199S/F227A/S287G/A328W/Y332G	4.6	8,990	1.95 × 10 ⁹	1.43
(-)cocaine	wild-type BChE	4.5	4.1	9.1 × 10 ⁵	1
	A199S/F227A/S287G/A328W/Y332G	3.1	5,700	1.84 × 10 ⁹	2,020

^aThe k_{cat} value for wild-type BChE came from ref.[12] or [33].