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Substrate Selectivity of High-Activity Mutants of Human Butyrylcholinesterase

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Abstract

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

Introduction

Cocaine is a widely abused drug¹ without an FDA-approved medication. The disastrous medical and social consequences of cocaine abuse have made a high priority the development of a feasible anti-cocaine medication^{2–3}. An ideal anti-cocaine medication would be to accelerate cocaine metabolism producing biologically inactive metabolites *via* a route similar to the primary cocaine-metabolizing pathway, *i.e.* cocaine hydrolysis catalyzed by butyrylcholinesterase (BChE) in plasma^{4–9}. Unfortunately, wild-type BChE has a low catalytic activity against naturally occurring (–)-cocaine ($k_{\text{cat}} = 4.1 \text{ min}^{-1}$ and $K_{\text{M}} = 4.5$

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μM)^{10–14}. It is interesting for anti-cocaine medication development to design a human BChE mutant as a cocaine hydrolase (CocH) with significantly improved catalytic activity against (–)-cocaine.

As well known, computational design of high-activity mutants of an enzyme is extremely challenging, particularly when the chemical reaction process is rate-determining for the enzymatic reaction^{15–17}. To computationally design a mutant enzyme with an improved catalytic activity for a given substrate, one needs to design possible amino-acid mutations that can accelerate the rate-determining step of the catalytic reaction process^{11, 18–19} while other steps of the reaction are not slowed down by the mutations. The fundamental reaction pathway for BChE-catalyzed hydrolysis of (–)-cocaine was uncovered by extensive molecular dynamics (MD) simulations^{11, 18} and reaction-coordinate calculations^{18–19} using quantum mechanics (QM) and hybrid quantum mechanics/molecular mechanics (QM/MM).^{20–24} The computational studies revealed^{11, 15, 18, 25} that the rate-determining step of (–)-cocaine hydrolysis catalyzed by the A328W/Y332A and A328W/Y332G mutants of BChE is the first step of the chemical reaction process. Therefore, starting from the A328W/Y332A or A328W/Y332G mutant, rational design of BChE mutants against (–)-cocaine has been focused on decreasing the energy barrier for the first reaction step without significantly affecting the other steps. We have developed unique computational strategies and protocols based on the virtual screening of rate-determining transition states of the enzymatic reaction to design enzyme mutants with improved catalytic activity^{25–31}. The computational design was followed by *in vitro* experiments, including site-directed mutagenesis, protein expression, and enzyme activity assays. The integrated computational-experimental studies have led to discovery of a series of BChE mutants with a significantly improved catalytic efficiency against (–)-cocaine^{25–31}. The first one of our designed and discovered high-activity mutants of human BChE, *i.e.* the A199S/S287G/A328W/Y332G mutant²⁵, was validated by an independent group of scientists^{32–33} 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 treatment³⁴. Our recently designed and discovered new mutants^{28, 30} of human BChE are even more active against (–)-cocaine *in vitro* and *in vivo*, without knowing whether these mutations also considerably increase the catalytic efficiencies against other substrates.

The present study was focused on the substrate selectivity of our discovered high-activity mutants of human BChE. We carried out both computational modeling and experimental kinetic analysis on the catalytic activities of the promising BChE mutants against other known substrates, including acetylcholine (ACh), acetylthiocholine (ATC), butyrylthiocholine (BTC), and (+)-cocaine, in comparison with the corresponding catalytic activity against (–)-cocaine. In particular, ACh is the only known natural substrate of BChE in the body. The catalytic activities of wild-type BChE against ACh, ATC, BTC, and (+)-cocaine are all much higher than that against (–)-cocaine. So, we wanted to know whether the same amino-acid mutations designed to considerably increase the catalytic activity of BChE against (–)-cocaine also considerably increase the catalytic activities of BChE against other substrates. The obtained kinetic data have demonstrated that the computationally

designed mutations only considerably improve the catalytic efficiency of human BChE against (–)-cocaine, without significantly improving the catalytic activities against other substrates.

Methods

Molecular modeling

Various substrates interacting with human BChE and its mutants were modeled for their enzyme-substrate binding complexes (denoted as ES's) and transition states for the initial reaction step (denoted as TS1's) by using the same modeling strategy and approach that we used to study (–)-cocaine interacting with the enzymes^{25–31}. The general strategy of performing an energy minimization or molecular dynamics (MD) simulation on a transition-state structure of an enzymatic reaction using a classical force field (molecular mechanics) has been described and justified in our recent reports^{25, 28, 30–31, 35}. During the energy minimization or MD simulation on the TS1 structure, the lengths of transition bonds (*i.e.* the covalent bonds that are being broken or formed gradually during the initial reaction step) were restrained while all other geometric parameters were allowed to move. The transition-bond lengths used in our modeling of the TS1 structures for each pair of enzyme and substrate were based on our previously reported molecular modeling and QM/MM reaction-coordinate calculations on BChE-catalyzed hydrolysis of (–)-cocaine or ACh or ATC^{28, 31, 36}. Specifically, the transition-bond lengths used in our modeling of the TS1 structures with (–)-cocaine or (+)-cocaine were the same as those in the QM/MM-optimized TS1 geometry for BChE-catalyzed hydrolysis of (–)-cocaine²⁸. The transition-bond lengths used in our modeling of the TS1 structures with ACh were the same as those in the QM/MM-optimized TS1 geometry for BChE-catalyzed hydrolysis of ACh³⁶. The transition-bond lengths used in our modeling of the TS1 structures with ATC or BTC were the same as those in the QM/MM-optimized TS1 geometry for BChE-catalyzed hydrolysis of ATC³⁷. The amino-acid mutations and the minor structural difference in the substrate were not expected to significantly change the transition-bond lengths based on our previous experience in the transition-state simulations^{16–17, 25, 28, 30, 35}. As discussed in our previous computational studies related to the transition-state modeling^{16–17, 25, 28, 30, 35}, the computational procedures for modeling a TS1 structure were the same as those for modeling the corresponding ES structure, except for keeping the transition bond lengths restrained during the energy minimization or MD simulation on the TS1 structure. Technically, each transition-bond length in a TS1 structure was restrained through defining a new type of covalent bond whose force constant was a half of the normal covalent bond between the two atoms. It should be pointed out that the sole purpose of performing such type of computational modeling on a transition state was to examine the hydrogen bonding interaction between the carbonyl group of substrate and the oxyanion hole of the enzyme. We were only interested in the modeled structures, as the total energies calculated in this way would be meaningless. The modeled structures were used to estimate the hydrogen bonding energies (HBE) by using a HBE equation²⁵ used in our earlier studies.

The initial structures of BChE and the mutants used in the molecular modeling were prepared on the basis of our previous MD simulation^{11, 25, 31} on the enzyme-substrate

complex for wild-type BChE binding with (–)-cocaine. Our previous MD simulations on the enzyme-substrate complexes started from the X-ray crystal structure³⁸ deposited in the Protein Data Bank (pdb code: 1POP). The general procedure for carrying out the MD simulations on the enzyme-substrate interactions in water was essentially the same as that used in our previously reported computational studies on other complexes^{17, 25, 30–31}. Each starting structure was neutralized by adding a counter ion (chloride ion) and was solvated in an orthorhombic box of TIP3P water molecules with a minimum solute-wall distance of 10 Å (which means that the shortest distance between an atom of the enzyme-substrate complex and the boundary of the box is longer than 10 Å). The obtained box sizes of the solvated systems were about 99 Å × 92 Å × 87 Å. All of the energy minimizations and MD simulations (using Newton's equations of motion) were performed by using the Sander module of Amber11 package³⁹. The solvated systems were carefully equilibrated and fully energy-minimized. First, the solvent molecules were energy-minimized for 5000 steps (including 2500 steps using the steepest descent method and 2500 steps using the conjugate gradient method) with the ligand and enzyme restrained. Second, the solvent, ligand, and side chains of the enzyme were energy-minimized for 1000 steps (including 500 steps using the steepest descent method and 500 steps using the conjugate gradient method) with the backbone of enzyme restrained. Finally the whole system was energy-minimized for 5000 steps (including 2500 steps using the steepest descent method and 2500 steps using the conjugate gradient method). These systems were gradually heated from T = 10 K to T = 298.15 K in 30 ps before running the MD simulation at T = 298.15 K for 1 ns or longer, making sure that we obtained a stable MD trajectory for each of the simulated structures. The time step used for the MD simulations was 2 fs. Periodic boundary conditions in the NPT ensemble at T = 298.15 K with Berendsen temperature coupling and P = 1 atm with isotropic molecule-based scaling were applied. The SHAKE algorithm was used to fix all covalent bonds containing hydrogen atoms. The non-bonded pair list was updated every 10 steps. The particle mesh Ewald (PME) method was used to treat long-range electrostatic interactions. A residue-based cutoff of 10 Å was utilized to the non-covalent interactions. The final snapshot of the stable MD trajectory was fully energy-minimized using the steepest descent method for 5000 steps and then using the conjugate gradient method until the convergence criterion for the energy gradient of 0.1 kcal·mol^{–1}·Å^{–1} was achieved.

Experimental methods

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

For determining the catalytic activity of the enzymes against (–)-cocaine, we used a sensitive radiometric assay based on toluene extraction of [³H](–)-cocaine labeled on its benzene ring⁴⁰. In brief, to initiate the enzymatic reaction, 100 nCi of [³H](–)-cocaine along

with (–)-cocaine was mixed with 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 (a product of (–)-cocaine hydrolysis) was extracted by 1 ml of toluene and measured by scintillation counting. Finally, the measured (–)-cocaine concentration-dependent radiometric data were analyzed by using the standard Michaelis-Menten kinetics so that the catalytic parameters were determined along with the use of a well-established standard enzyme-linked immunosorbent assay (ELISA) protocol²⁷. The enzyme activity assay with [³H]ACh was similar to the assay with [³H](–)-cocaine. The primary difference is that the enzymatic reaction was stopped by addition of 200 µl of 0.2 M HCl containing 2 M NaCl and that the product was [³H]acetic acid for the ACh hydrolysis. All measurements were performed at room temperature.

The catalytic activities of the enzymes against ATC, BTC, and (+)-cocaine were determined by UV-Vis spectrophotometric assays using a GENios Pro Microplate Reader (TECAN, Research Triangle Park, NC) with the XFluor software. (+)-Cocaine were dissolved in water to make a 0.1 M stock containing 34 mg/ml. Aliquots were frozen at –20°C, thawed once, and discarded. The reaction rate of (+)-cocaine hydrolysis was measured at 25°C by recording the time-dependent absorption at 230 nm. ATC and BTC stock solutions of 0.2 M were prepared in water and frozen at –20°C. The reaction rates were measured at 25°C by recording the time-dependent absorption at 450 nm in the presence of 1 mM dithiobisnitrobenzoic acid, in 0.1 M potassium phosphate, pH 7.2.

Results and Discussion

Insights from molecular modeling

Molecular modeling enabled us to understand how human BChE and its mutants interact with ACh, ATC, BTC, (+)-cocaine, and (–)-cocaine in the ES and TS1 structures. According to the modeling, for (–)-cocaine interacting with wild-type BChE, there is only one hydrogen bond (H-bond) between the carbonyl oxygen of (–)-cocaine and the oxyanion hole (G116, G117, and A199) in the ES structure, and there are two H-bonds in the TS1 structure, as seen in Figure 1A and 1B; more detailed computational data are provided as Supplementary Information. With the A199S/F227A/S287G/A328W/Y332G mutant (E12-7), there are two H-bonds between the carbonyl oxygen of (–)-cocaine and the oxyanion hole (G116, G117, and S199) in the ES structure and three H-bonds in the TS1 structure, as seen in Figure 1C and 1D. The extra H-bond in both the ES and TS1 structures is with the hydroxyl group of S199 after the A199S mutation. The modeled ES and TS1 structures suggest that this mutant should have a significantly higher catalytic activity against (–)-cocaine compared to the wild-type BChE.

For (–)-cocaine interacting with other mutants examined (*i.e.* the A199S/A328W/Y332G, A199S/F227A/A328W/Y332G, A199S/S287G/A328W/Y332G, and A199S/F227A/S287G/A328W/E441D mutants), the modeled ES and TS1 structures are all qualitatively similar to the corresponding ES and TS1 structures with the A199S/F227A/S287G/A328W/Y332G mutant in terms of the number of H-bonds. Thus, we only depict the ES and TS1 structures with the A199S/F227A/S287G/A328W/Y332G mutant in Figure 1 as a typical example of

the five mutants examined. The modeled ES and TS1 structures qualitatively suggest that each of these mutants should have a considerably higher catalytic activity against (–)-cocaine compared to the wild-type BChE.

For other substrates (including ACh, ATC, BTC, and (+)-cocaine) interacting with the wild-type BChE or anyone of the aforementioned mutants, there are always two H-bonds between the carbonyl oxygen of the substrate and the oxyanion hole (G116, G117, and A/S199) in the ES structure and three H-bonds in the TS1 structure. Depicted in Figures 2 to 5 are the modeled ES and TS1 structures with wild-type BChE and a representative mutant (A199S/F227A/S287G/A328W/Y332G). More detailed computational data are provided as Supplementary Information. The hydroxyl group of S199 in the mutant does not form an extra H-bond in the ES or TS1 structure for the mutant interacting with any substrate other than (–)-cocaine, suggesting that these BChE mutants should not have dramatically improved catalytic activities against ACh, ATC, BTC, and (+)-cocaine compared to the wild-type BChE.

Generally speaking, structural information obtained from MD simulations on a protein-ligand complex may be used to analyze the interaction energies.^{41–42} Based on the MD-simulated H···O distances between the carbonyl oxygen of substrate and the oxyanion hole, we estimated the total HBE (tHBE) values in the ES and TS1 structures for the wild-type BChE- and E12-7-catalyzed hydrolysis of the five substrates (see Supplementary Information for the data). The calculated tHBE values indicate that, for a given enzymatic hydrolysis, the tHBE value in the TS1 structure is always lower than that in the corresponding ES structure; the lower tHBE value (minus value) means the stronger overall hydrogen bonding. So, the TS1 structure is always stabilized by hydrogen bonding between the carbonyl oxygen of substrate and the oxyanion hole. With the structure of the reaction system changing from ES to TS1, the calculated tHBE value decreases about only 1 kcal/mol for wild-type BChE-catalyzed hydrolysis of (–)-cocaine (or we have Δ tHBE $\approx$ –1 kcal/mol), and about 13–15 kcal/mol for E12-7-catalyzed hydrolysis of (–)-cocaine (or we have Δ tHBE $\approx$ –13–15 kcal/mol). In other words, the Δ tHBE value decreases for about 12–14 kcal/mol from the wild-type BChE to E12-7 for (–)-cocaine. So, the amino-acid mutations in E12-7 have dramatically enhanced the overall hydrogen bonding between the carbonyl oxygen of substrate and the oxyanion hole in the TS1 structure for the (–)-cocaine hydrolysis. In comparison, the changes in the Δ tHBE values calculated for (+)-cocaine, ACh, ATC, and BTC are all much smaller than the change of the Δ tHBE value calculated for (–)-cocaine.

Based on the modeling results, the computationally examined five sets of mutations (A199S/A328W/Y332G, A199S/F227A/A328W/Y332G, A199S/S287G/A328W/Y332G, A199S/F227A/S287G/A328W/E441D, and A199S/F227A/S287G/A328W/Y332G) are all expected to considerably improve the catalytic efficiency of human BChE against (–)-cocaine, without considerably improving the catalytic efficiency of human BChE against ACh, ATC, BTC, or (+)-cocaine.

Kinetic parameters

Based on the computational insights, we carried out *in vitro* experimental tests, including the protein expression and enzyme activity assays, on the A199S/A328W/Y332G, A199S/F227A/A328W/Y332G, A199S/S287G/A328W/Y332G, A199S/F227A/S287G/A328W/E441D, and A199S/F227A/S287G/A328W/Y332G mutants of human BChE. To minimize the possible systematic experimental errors of the kinetic data, we also expressed the wild-type enzyme and performed the kinetic analysis along with the mutants under the same experimental conditions, and compared the catalytic efficiencies of the mutants to the corresponding catalytic efficiencies of the wild-type enzyme against various substrates. Depicted in Figures 6 to 10 are the measured kinetic data. Summarized in Table 1 are the determined kinetic parameters.

Based on the kinetic parameters summarized in Table 1, all of the five BChE mutants examined in this study have a considerably improved catalytic efficiency (k_{cat}/K_M) against (–)-cocaine, with the improvement ranging from 121 to 2020-fold. The same mutations do not dramatically improve the catalytic efficiencies of human BChE against the other substrates. Within the five mutants examined, the values of the relative catalytic efficiency (RCE), *i.e.* the ratio of the catalytic efficiency of the mutant to that of the wild-type BChE, range from 0.4 to 1.68 for (+)-cocaine, 0.066 to 0.95 for ACh, 0.18 to 1.99 for ATC, and 0.39 to 3.91 for BTC. The largest RCE value is associated with the A199S/F227A/S287G/A328W/E441D mutant for both (+)-cocaine (1.68-fold) and ATC (1.99-fold), and the A199S/F227A/A328W/Y332G mutant for BTC (3.91-fold).

For ACh (which is the only known endogenous substrate of BChE in the body), all of the BChE mutants actually have a slightly lower catalytic efficiency compared to the wild-type BChE. So, all of these mutants only have a considerably improved catalytic efficiency against (–)-cocaine without any improvement on the catalytic efficiency of BChE against ACh. As a result, the catalytic efficiencies of these BChE mutants against (–)-cocaine are all higher than the corresponding catalytic efficiencies against ACh, as seen in Table 1.

The observed substrate selectivity of these mutants may be used to address a potential question concerning whether the enzyme therapy using a high-activity mutant of human BChE would significantly affect the cholinergic transmission and, thus, produce adverse effects. In fact, previous studies evaluating wide-type human BChE as a prophylaxis against chemical warfare nerve agents found no autonomic or motor impairment in rats, guinea pigs, or primates, even with the high doses raising the plasma enzyme levels for 50 to 100-fold^{43–48}. This is not surprising due to several factors. First, the molar concentrations of acetylcholinesterase (AChE) and BChE in blood are roughly similar⁴⁹ and BChE has a lower catalytic efficiency against ACh compared to AChE. Second, cholinergic synapses in the brain are insulated from plasma enzymes by the blood-brain barrier and, thus, the exogenous enzymes in plasma would not reach the brain. In addition, peripheral cholinergic synapses are densely packed with AChE. It has been known that mouse neuromuscular junction has 5×10^{19} catalytic AChE subunits per cc, *i.e.* ~0.1 mM,⁵⁰ whereas mouse plasma BChE levels are below 0.1 μM .⁴⁹ For these reasons, even high levels of plasma BChE activity are unlikely to affect motor transmission. The current observation that none

of these high-activity mutants of human BChE has an improved catalytic efficiency against ACh compared to the wild-type BChE gives us additional confidence in development of an enzyme therapy by using one of these BChE mutants, particularly the A199S/F227A/S287G/A328W/Y332G mutant with the highest catalytic efficiency against (–)-cocaine.

Conclusion

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
The energy-minimized ES and TS1 structures for (–)-cocaine interacting with wild-type human BChE and its A199S/F227A/S287G/A328W/Y332G mutant (E12-7)

Figure 2.
The energy-minimized ES and TS1 structures for (+)-cocaine interacting with wild-type human BChE and its A199S/F227A/S287G/A328W/Y332G mutant (E12-7)

Figure 3.
The energy-minimized ES and TS1 structures for ACh interacting with wild-type human BChE and its A199S/F227A/S287G/A328W/Y332G mutant (E12-7)

Figure 4.
The energy-minimized ES and TS1 structures for ATC interacting with wild-type human BChE and its A199S/F227A/S287G/A328W/Y332G mutant (E12-7)

Figure 5.
The energy-minimized ES and TS1 structures for BTC interacting with wild-type human BChE and its A199S/F227A/S287G/A328W/Y332G mutant (E12-7)

Figure 6.
Kinetic data for (-)-cocaine hydrolysis catalyzed by wild-type human BChE and various BChE mutants

Figure 7.
Kinetic data for (+)-cocaine hydrolysis catalyzed by wild-type human BChE and various BChE mutants

Figure 8.

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

Figure 9.
Kinetic data for ATC hydrolysis catalyzed by wild-type human BChE and various BChE mutants

Figure 10.
Kinetic data for BTC hydrolysis catalyzed by wild-type human BChE and various BChE mutants

Table 1

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

Substrate	Enzyme ^a	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M ($M^{-1}min^{-1}$)	RCE ^b
(–)-cocaine	wild-type BChE ^c	4.5	4.1	9.1×10^5	1
	A199S/A328W/Y332G	5.1	560	1.1×10^8	121
	A199S/F227A/A328W/Y332G	4.4	1560	3.6×10^8	396
	A199S/S287G/A328W/Y332G	3.1	3,060	9.9×10^8	1,080
	A199S/F227A/S287G/A328W/E441D	1.1	1,730	1.6×10^9	1,730
	A199S/F227A/S287G/A328W/Y332G	3.1	5,700	1.8×10^9	2,020
	wild-type BChE	4.7	6,420	1.4×10^9	1
(+)cocaine	A199S/A328W/Y332G	5.0	2,820	5.6×10^8	0.40
	A199S/F227A/A328W/Y332G	4.4	6,060	1.4×10^9	1.01
	A199S/S287G/A328W/Y332G	6.3	5,620	8.9×10^8	0.65
	A199S/F227A/S287G/A328W/E441D	4.7	10,800	2.3×10^9	1.68
	A199S/F227A/S287G/A328W/Y332G	4.6	8,990	2.0×10^9	1.43
	wild-type BChE ^d	148	61,200	4.1×10^8	1
	A199S/A328W/Y332G	156	4,190	2.7×10^7	0.066
ACh	A199S/F227A/A328W/Y332G	189	7,430	3.9×10^7	0.095
	A199S/S287G/A328W/Y332G	36	5,320	1.5×10^8	0.37
	A199S/F227A/S287G/A328W/E441D	27	10,400	3.9×10^8	0.95
	A199S/F227A/S287G/A328W/Y332G	37	11,900	3.2×10^8	0.78
	wild-type BChE ^e	33	20,200	6.1×10^8	1
	A199S/A328W/Y332G	31	3,410	1.1×10^8	0.18
	A199S/F227A/A328W/Y332G	41	6,870	1.6×10^8	0.26
ATC	A199S/S287G/A328W/Y332G	21	7,880	3.7×10^8	0.60
	A199S/F227A/S287G/A328W/E441D	12	14,000	1.2×10^9	1.99
	A199S/F227A/S287G/A328W/Y332G	20	14,800	7.2×10^8	1.19

Substrate	Enzyme ^a	K _M (μM)	k _{cat} (min ⁻¹)	k _{cat} /K _M (M ⁻¹ min ⁻¹)	RCE ^b
BTC	wild-type BChE ^f	17	29,500	1.7 × 10 ⁹	1
	A199S/A328W/Y332G	8.9	6,100	6.8 × 10 ⁸	0.39
	A199S/F227A/A328W/Y332G	11	74,700	6.8 × 10 ⁹	3.91
	A199S/S287G/A328W/Y332G	5.3	14,400	2.7 × 10 ⁹	1.57
	A199S/F227A/S287G/A328W/E441D	8.9	17,800	2.0 × 10 ⁹	1.15
	A199S/F227A/S287G/A328W/Y332G	13	28,000	2.2 × 10 ⁹	1.24

^aUnless indicated otherwise, all kinetic parameters listed in this table were determined in the present study.

^bRCE 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.

^cData for wild-type BChE from reference¹⁰.

^dThe k_{cat} value for wild-type BChE was reported in reference³².

^eThe k_{cat} value for wild-type BChE from reference⁵¹.

^fThe k_{cat} value for wild-type BChE was reported in reference⁵².