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High Activity Mutants of Butyrylcholinesterase for Cocaine Hydrolysis

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Zhan, Chang-Guo; Zheng, Fang; and Yang, Wenchao, "High Activity Mutants of Butyrylcholinesterase for Cocaine Hydrolysis" (2011). Pharmaceutical Sciences Faculty Patents. 1. https://uknowledge.uky.edu/ps_patents/1

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US007892537B1

(12) United States Patent

Zhan et al.

(10) **Patent No.:** (45) **Date of Patent:**

US 7,892,537 B1 Feb. 22, 2011

(54) HIGH ACTIVITY MUTANTS OF BUTYRYLCHOLINESTERASE FOR COCAINE HYDROLYSIS

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(*) Notice: Subject to any disclaimer, the term of this

patent is extended or adjusted under 35

U.S.C. 154(b) by 0 days.

(21) Appl. No.: 12/767,128

(22) Filed: Apr. 26, 2010

Related U.S. Application Data

- (60) Division of application No. 12/685,341, filed on Jan. 11, 2010, now Pat. No. 7,740,840, which is a continuation-in-part of application No. 12/192,394, filed on Aug. 15, 2008, now Pat. No. 7,731,957, which is a division of application No. 11/243,111, filed on Oct. 4, 2005, now Pat. No. 7,438,904.
- (51) Int. Cl. A61K 38/46 (2006.01) C12N 9/16 (2006.01)
- (52) **U.S. Cl.** **424/94.6**; 435/196; 435/197

See application file for complete search history.

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(57) ABSTRACT

Butyrylcholinesterase (BChE) polypeptide variants of the presently-disclosed subject matter have enhanced catalytic efficiency for (–)-cocaine, as compared to wild-type BChE. Pharmaceutical compositions of the presently-disclosed subject matter include a BChE polypeptide variant having an enhanced catalytic efficiency for (–)-cocaine. A method of the presently-disclosed subject matter for treating a cocaine-induced condition includes administering to an individual an effective amount of a BChE polypeptide variant, as disclosed herein, to lower blood cocaine concentration.

5 Claims, No Drawings

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HIGH ACTIVITY MUTANTS OF BUTYRYLCHOLINESTERASE FOR COCAINE HYDROLYSIS

RELATED APPLICATIONS

This application is a division of and claims benefit to U.S. patent application Ser. No. 12/685,341, now allowed, filed Jan. 11, 2010 now U.S. Pat. No. 7,740,840, which is a continuation-in-part of U.S. patent application Ser. No. 12/192, 394 filed Aug. 15, 2008 now U.S. Pat. No. 7,731,957, which is a divisional of U.S. patent application Ser. No. 11/243,111, filed Oct. 4, 2005 and now issued as U.S. Pat. No. 7,438,904. The entire disclosures contained in U.S. patent application Ser. No. 12/192,394, U.S. patent application Ser. No. 11/243, 111, and U.S. Pat. No. 7,438,904 are incorporated herein by this reference.

GOVERNMENT INTEREST

Subject matter described herein was made with government support under Grant Number R01DA013930 awarded by the National Institute on Drug Abuse (NIDA) of the National Institutes of Health (NIH). The government has certain rights in the described subject matter.

TECHNICAL FIELD

The presently-disclosed subject matter relates to butyryl-cholinesterase variant polypeptides, and in particular, butyrylcholinesterase mutants having amino acid substitutions.

INTRODUCTION

Cocaine abuse is a major medical and public health problem that continues to defy treatment. The disastrous medical and social consequences of cocaine addiction, such as violent crime, loss in individual productivity, illness, and death, have made the development of an effective pharmacological treatment a high priority. However, cocaine mediates its reinforcing and toxic effects by blocking neurotransmitter reuptake and the classical pharmacodynamic approach has failed to yield small-molecule receptor antagonists due to the difficulties inherent in blocking a blocker. An alternative to receptor-based approaches is to interfere with the delivery of cocaine to its receptors and accelerate its metabolism in the body.

The dominant pathway for cocaine metabolism in primates is butyrylcholinesterase (BChE)-catalyzed hydrolysis at the benzoyl ester group (Scheme 1).

 $\label{eq:Scheme 1.} Schematic repesentation of BChE-catalyzed hydrolysis at the benzoyl ester group.$

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-continued

$$H_3C$$
 H_3C
 H_3C

Only 5% of the cocaine is deactivated through oxidation by the liver microsomal cytochrome P450 system. Cocaine hydrolysis at benzoyl ester group yields ecgonine methyl ester, whereas the oxidation produces norcocaine. The metabolite ecgonine methyl ester is a biologically inactive metabolite, whereas the metabolite norcocaine is hepatotoxic and a local anesthetic. BChE is synthesized in the liver and widely distributed in the body, including plasma, brain, and lung. Extensive experimental studies in animals and humans demonstrate that enhancement of BChE activity by administration of exogenous enzyme substantially decreases cocaine half-life.

Enhancement of cocaine metabolism by administration of BChE has been recognized to be a promising pharmacokinetic approach for treatment of cocaine abuse and dependence. However, the catalytic activity of this plasma enzyme 55 is three orders-of-magnitude lower against the naturally occurring (-)-cocaine than that against the biologically inactive (+)-cocaine enantiomer. (+)-cocaine can be cleared from plasma in seconds and prior to partitioning into the central nervous system (CNS), whereas (-)-cocaine has a plasma half-life of approximately 45-90 minutes (for a relatively low dose of cocaine), long enough for manifestation of the CNS effects which peak in minutes. Under the overdose condition, BChE is saturated with (-)-cocaine and, thus, the plasma 65 half-life of (-)-cocaine will be longer. Hence, BChE mutants with high activity against (-)-cocaine are highly desired for use in humans. Although some BChE mutants with increased

catalytic activity over wild-type BChE have previously been generated, there exists a need for mutant BChE with even higher catalytic activity.

SUMMARY

The presently-disclosed subject matter meets some or all of the above-identified needs, as will become evident to those of ordinary skill in the art after a study of information provided in this document.

This Summary describes several embodiments of the presently-disclosed subject matter, and in many cases lists variations and permutations of these embodiments. This Summary is merely exemplary of the numerous and varied embodiments. Mention of one or more representative features of a given embodiment is likewise exemplary. Such an embodiment can typically exist with or without the feature(s) mentioned; likewise, those features can be applied to other embodiments of the presently-disclosed subject matter, whether listed in this Summary or not. To avoid excessive repetition, this Summary does not list or suggest all possible combinations of such features.

The presently-disclosed subject matter includes butyrylcholinesterase (BChE) polypeptide variants. In some embodiments the amino acid sequence of the BChE polypeptide variant includes an amino acid sequence selected from the group consisting of: SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, and 32, as set forth herein.

The presently-disclosed subject matter further includes a pharmaceutical composition that includes a butyrylcholinesterase polypeptide variant and a suitable pharmaceutical carrier

The presently-disclosed subject matter further includes a method of treating a cocaine-induced condition, which 35 includes administering to an individual an effective amount of BChE polypeptide variant or a pharmaceutical composition comprising a BChE polypeptide variant, as described herein, to lower blood cocaine concentration. In some embodiments, the BChE polypeptide variant exhibits a one-hundred-fold or more increase in cocaine hydrolysis catalytic efficiency compared to wild-type butyrylcholinesterase.

BRIEF DESCRIPTION OF THE SEQUENCE LISTING

SEQ ID NO: 1 is a nucleotide sequence encoding a butyrylcholinesterase (BChE) polypeptide variant of SEQ ID NO: 2;

SEQ ID NO: 2 is an amino acid sequence encoding a BChE polypeptide variant having the following amino acid substitutions, as compared to wild type BChE: A199S, F227A, P285A, S287G, A328W, and Y332G;

SEQ ID NO: 3 is a nucleotide sequence encoding a butyrylcholinesterase (BChE) polypeptide variant of SEQ ID NO: 4;

SEQ ID NO: 4 is an amino acid sequence encoding a BChE polypeptide variant having the following amino acid substitutions, as compared to wild type BChE: A199S, F227A, P285S, S287G, A328W, and Y332G;

SEQ ID NO: 5 is a nucleotide sequence encoding a butyrylcholinesterase (BChE) polypeptide variant of SEQ ID NO: 6;

SEQ ID NO: 6 is an amino acid sequence encoding a BChE polypeptide variant having the following amino acid substitutions, as compared to wild type BChE: A199S, F227A, P285Q, S287G, A328W, and Y332G;

SEQ ID NO: 7 is a nucleotide sequence encoding a butyrylcholinesterase (BChE) polypeptide variant of SEQ ID NO: 8;

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SEQ ID NO: 8 is an amino acid sequence encoding a BChE polypeptide variant having the following amino acid substitutions, as compared to wild type BChE: A199S, F227P, S287G, A328W, and Y332G.

SEQ ID NO: 9 is a nucleotide sequence encoding a butyrylcholinesterase (BChE) polypeptide variant of SEQ ID NO: 10

SEQ ID NO: 10 is an amino acid sequence encoding a BChE polypeptide variant having the following amino acid substitutions, as compared to wild type BChE: A199S, F227A, P285G, S287G, A328W, and Y332G;

SEQ ID NO: 11 is a nucleotide sequence encoding a butyrylcholinesterase (BChE) polypeptide variant of SEQ ID NO: 12:

SEQ ID NO: 12 is an amino acid sequence encoding a BChE polypeptide variant having the following amino acid substitutions, as compared to wild type BChE: A199S, F227A, L286M, S287G, A328W, and Y332G;

SEQ ID NO: 13 is a nucleotide sequence encoding a butyrylcholinesterase (BChE) polypeptide variant of SEQ ID NO: 14:

SEQ ID NO: 14 is an amino acid sequence encoding a BChE polypeptide variant having the following amino acid substitutions, as compared to wild type BChE: A199S, P285Q, S287G, A328W, and Y332G;

SEQ ID NO: 15 is a nucleotide sequence encoding a butyrylcholinesterase (BChE) polypeptide variant of SEQ ID NO: 16:

SEQ ID NO: 16 is an amino acid sequence encoding a BChE polypeptide variant having the following amino acid substitutions, as compared to wild type BChE: A199S, P285I, S287G, A328W, and Y332G;

SEQ ID NO: 17 is a nucleotide sequence encoding a butyrylcholinesterase (BChE) polypeptide variant of SEQ ID NO: 18;

SEQ ID NO: 18 is an amino acid sequence encoding a BChE polypeptide variant having the following amino acid substitutions, as compared to wild type BChE: A199S, F227G, S287G, A328W, and Y332G;

SEQ ID NO: 19 is a nucleotide sequence encoding a butyrylcholinesterase (BChE) polypeptide variant of SEQ ID NO: 20;

SEQ ID NO: 20 is an amino acid sequence encoding a BChE polypeptide variant having the following amino acid substitutions, as compared to wild type BChE: A199S, P285S, S287G, A328W, and Y332G;

SEQ ID NO: 21 is a nucleotide sequence encoding a butyrylcholinesterase (BChE) polypeptide variant of SEQ ID NO: 22;

SEQ ID NO: 22 is an amino acid sequence encoding a BChE polypeptide variant having the following amino acid substitutions, as compared to wild type BChE: A199S, F227V, S287G, A328W, and Y332G;

SEQ ID NO: 23 is a nucleotide sequence encoding a 55 butyrylcholinesterase (BChE) polypeptide variant of SEQ ID NO: 24:

SEQ ID NO: 24 is an amino acid sequence encoding a BChE polypeptide variant having the following amino acid substitutions, as compared to wild type BChE: A199S, P285G, S287G, A328W, and Y332G;

SEQ ID NO: 25 is a nucleotide sequence encoding a butyrylcholinesterase (BChE) polypeptide variant of SEQ ID NO: 26;

SEQ ID NO: 26 is an amino acid sequence encoding a BChE polypeptide variant having the following amino acid substitutions, as compared to wild type BChE: A199S, F2271, S287G, A328W, and Y332G;

SEQ ID NO: 27 is a nucleotide sequence encoding a butyrylcholinesterase (BChE) polypeptide variant of SEQ ID NO: 28:

SEQ ID NO: 28 is an amino acid sequence encoding a BChE polypeptide variant having the following amino acid substitutions, as compared to wild type BChE: A199S, F227L, S287G, A328W, and Y332G;

SEQ ID NO: 29 is a nucleotide sequence encoding a butyrylcholinesterase (BChE) polypeptide variant of SEQ ID NO: 30:

SEQ ID NO: 30 is an amino acid sequence encoding a BChE polypeptide variant having the following amino acid substitutions, as compared to wild type BChE: A199S, L286M, S287G, A328W, and Y332G;

SEQ ID NO: 31 is a nucleotide sequence encoding a butyrylcholinesterase (BChE) polypeptide variant of SEQ ID NO: 32; and SEQ ID NO: 32 is an amino acid sequence encoding a BChE polypeptide variant having the following

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polypeptide variants disclosed herein each have enhanced catalytic efficiency for (–)-cocaine, as compared to wild-type BChE. The presently-disclosed subject matter further includes a pharmaceutical composition including a butyryl-cholinesterase polypeptide variant, as described herein, and a suitable pharmaceutical carrier. The presently-disclosed subject matter further includes a method of treating a cocaine-induced condition comprising administering to an individual an effective amount of a butyrylcholinesterase polypeptide variant, as disclosed herein, to lower blood cocaine concentration.

In some embodiments, the BChE polypeptide variant is selected from a BChE polypeptide variants set forth in Table 1. Table 1 also includes the SEQ ID NOs associated with the identified BChE polypeptide variants, as well as a summary of the approximate fold increase in catalytic efficiency against (–)-cocaine for the identified BChE polypeptide variants, as compared to wild type BChE.

TABLE 1

			BChE Po	lypeptide '	Variants a	nd Associa	ated SEQ I	D NOs		
Variant .			Amino	Acid Subs	stitution			Catalytic Efficiency (k_{cat}/K_M) against (-)-cocaine (Approximate Fold	Nucleic Acid SEQ ID	Amino Acid SEQ ID
Number	199	227	285	286	287	328	332	Increase)a	NO:	NO:
1	A199S	F227A	P285A	-	S287G	A328W	Y332G	4080	1	2
2	A199S	F227A	P285S	-	S287G	A328W	Y332G	3700	3	4
3	A199S	F227A	P285Q	-	S287G	A328W	Y332G	3590	5	6
4	A199S	F227P		-	S287G	A328W	Y332G	1860	7	8
5	A199S	F227A	P285G	-	S287G	A328W	Y332G	2420	9	10
6	A199S	F227A	-	L286M	S287G	A328W	Y332G	2120	11	12
7	A199S	-	P285Q	-	S287G	A328W	Y332G	2220	13	14
8	A199S	-	P285I	-	S287G	A328W	Y332G	830	15	16
9	A199S	F227G	-	-	S287G	A328W	Y332G	2010	17	18
10	A199S	-	P285S	-	S287G	A328W	Y332G	1240	19	20
11	A199S	F227V	-	-	S287G	A328W	Y332G	950	21	22
12	A199S	-	P285G	-	S287G	A328W	Y332G	1250	23	24
13	A199S	F227I	-	-	S287G	A328W	Y332G	1240	25	26
14	A199S	F227L	-	-	S287G	A328W	Y332G	1100	27	28
15	A199S	-	-	L286M	S287G	A328W	Y332G	740	29	30
16	A199S	F227A	P285K	-	S287G	A328W	Y332G	1540	31	32

^aThe approximate ratio of the k_{car}/K_M value for the BChE mutant to that for the wild-type BChE against (–)-cocaine.

amino acid substitutions, as compared to wild type BChE: A199S, F227A, P285K, S287G, A328W, and Y332G.

DESCRIPTION OF EXEMPLARY EMBODIMENTS

The details of one or more embodiments of the presently-disclosed subject matter are set forth in this document. Modifications to embodiments described in this document, and other embodiments, will be evident to those of ordinary skill in the art after a study of the information provided in this document. The information provided in this document, and particularly the specific details of the described exemplary embodiments, is provided primarily for clearness of understanding and no unnecessary limitations are to be understood therefrom. In case of conflict, the specification of this document, including definitions, will control.

The presently-disclosed subject matter includes butyrylcholinesterase (BChE) polypeptide variants. The BChE

The terms "polypeptide", "protein", and "peptide", which
are used interchangeably herein, refer to a polymer of the
protein amino acids, or amino acid analogs, regardless of its
size or function. Although "protein" is often used in reference
to relatively large polypeptides, and "peptide" is often used in
reference to small polypeptides, usage of these terms in the art
overlaps and varies. The term "polypeptide" as used herein
refers to peptides, polypeptides, and proteins, unless otherwise noted. The terms "protein", "polypeptide", and "peptide" are used interchangeably herein when referring to a gene
product. Thus, exemplary polypeptides include gene products, naturally occurring proteins, homologs, orthologs, paralogs, fragments and other equivalents, variants, and analogs
of the foregoing.

The term "variant" refers to an amino acid sequence that is different from the reference polypeptide by one or more amino acids, e.g., one or more amino acid substitutions. For

example a butyrylcholinesterase (BChE) polypeptide variant differs from wild-type BChE by one or more amino acid substitutions, i.e., mutations.

The terms "polypeptide fragment" or "fragment", when used in reference to a reference polypeptide, refers to a 5 polypeptide in which amino acid residues are deleted as compared to the reference polypeptide itself, but where the remaining amino acid sequence is usually identical to the corresponding positions in the reference polypeptide. Such deletions can occur at the amino-terminus, carboxy-terminus of the reference polypeptide, or alternatively both. A fragment can also be a "functional fragment," in which case the fragment retains some or all of the activity of the reference polypeptide as described herein. For example, a functional fragment of a particular BChE polypeptide variant retains 15 some or all of the cocaine hydrolysis activity, i.e., the catalytic efficiency for (-)-cocaine, of the particular BChE polypeptide variant. In this regard, the term "BChE polypeptide variant" is inclusive of functional fragments of the BChE polypeptide variant. Such fragments are typically are at least 20 about 300, 325, 350, 375, 400, 425, 450, 475, 500, 525, or 550 amino acids long. One or more residues from about 1 to 67 and/or one or more residues from about 443 to 574 can be removed without substantially affecting the catalytic activity of the BChE polypeptide variant. As such, the term "BChE 25 polypeptide variant" is inclusive of functional fragments wherein one or more residues from 1 to 67 and/or one or more residues from 443 to 574 is truncated relative to the fulllength BChE polypeptide variant.

The BChE polypeptide variant (e.g., SEQ ID NOS: 2, 4, 6, 30 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, and 32) can be formulated in a pharmaceutical composition along with a suitable pharmaceutical carrier known to one skilled in the art.

The present BChE variant polypeptides can be used in 35 treating a cocaine-induced condition by administering to an individual, an effective amount of a BChE variant polypeptides, (e.g., SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, and 32), to lower blood cocaine concentration. The BChE polypeptide variant can be administered in the 40 form of a pharmaceutical composition in which the BChE polypeptide variant is included with a suitable pharmaceutical carrier. Treatment of a cocaine-induced condition using one of the aforementioned BChE polypeptide variants can be in a manner that will be understood by those skilled in the art. 45

The preferred dose for administration of a BChE polypeptide variant or pharmaceutical composition in accordance with the presently-described subject matter is that amount which will be effective in lowering (-)-cocaine concentration in a patient's bloodstream, and one would readily recognize 50 that this amount will vary greatly depending on the nature of cocaine consumed, e.g., injected or inhaled, and the condition of a patient. An "effective amount" of butyrylcholinesterase polypeptide variant or pharmaceutical composition to be used in accordance with the presently-disclosed subject matter is 55 intended to mean a nontoxic but sufficient amount of the agent, such that the desired prophylactic or therapeutic effect is produced. Thus, the exact amount of the enzyme or a particular agent that is required will vary from subject to subject, depending on the species, age, and general condition 60 of the subject, the severity of the condition being treated, the particular carrier or adjuvant being used and its mode of administration, and the like. Similarly, the dosing regimen should also be adjusted to suit the individual to whom the composition is administered and will once again vary with 65 age, weight, metabolism, etc. of the individual. Accordingly, the "effective amount" of any particular butyrylcholinest8

erase polypeptide variant, or pharmaceutical composition thereof, will vary based on the particular circumstances, and an appropriate effective amount may be determined in each case of application by one of ordinary skill in the art using only routine experimentation.

The presently-disclosed subject matter is further illustrated by the following specific but non-limiting examples. The following examples may include compilations of data that are representative of data gathered at various times during the course of development and experimentation related to the presently-disclosed subject matter.

EXAMPLES

Embodiments of the BChE polypeptide variants of the presently-disclosed subject matter were made and studied using the following experimental procedure.

Site-directed mutagenesis of human BChE cDNA was performed by the QuikChange method of Braman, J.; Papworth, C.; Greener, A. *Methods Mol. Biol.* 1996, 57, 5731, incorporated herein by this reference. Mutations were generated from wild-type human BChE in a pRc/CMV expression plasmid in accordance with Xie, W.; Altamirano, C. V.; Bartels, C. F.; Speirs, R. J.; Cashman, J. R.; Lockridge, O. *Mol. Pharmacol.* 1999, 55, 83, each of which is incorporated herein by this reference. The expression plasmid pRc/CMV was kindly provided by Dr. O. Lockridge, University of Nebraska Medical Center (Omaha, Nebr.).

Using plasmid DNA as template and primers with specific base-pair alterations, mutations were made by polymerase chain reaction with Pfu DNA polymerase, for replication fidelity. The PCR product was treated with Dpn I endonuclease to digest the parental DNA template. Cloned pfu DNA polymerase and Dpn I endonuclease were obtained from Stratagene (La Jolla, Calif.). Modified plasmid DNA was transformed into *Escherichia coli*, amplified, and purified. The DNA sequences of the mutants were confirmed by DNA sequencing. All oligonucleotides were synthesized by the Integrated DNA Technologies, Inc. The QIAprep Spin Plasmid Miniprep Kit and Qiagen plasmid purification kit and QIAquick PCR purification kit were obtained from Qiagen (Santa Clarita, Calif.).

BChE mutants 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 2000 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 a BChE activity assay.

Human embryonic kidney 293T/17 cells were from ATCC (Manassas, Va.). Dulbecco's modified Eagle's medium (DMEM) was purchased from Fisher Scientific (Fairlawn, N.J.). Oligonucleotide primers were synthesized by the Integrated DNA Technologies and Analysis Facility of the University of Kentucky. 3,3',5,5'-Tetramethylbenzidine (TMB) was obtained from Sigma (Saint Louis, Mo.). Anti-butyrylcholinesterase (mouse monoclonal antibody, Product # HAH002-01) was purchased from AntibodyShop (Gentofte, Denmark) and Goat anti-mouse IgG HRP conjugate from Zymed (San Francisco, Calif.).

To measure cocaine and benzoic acid, the product of cocaine hydrolysis by BChE, sensitive radiometric assays based on toluene extraction of [³H]-(-)-cocaine labeled on its benzene ring were used in accordance with Zheng, F.; Yang, W.; Ko, M.-C.; Liu, J.; Cho, H.; Gao, D.; Tong, M.; Tai, H.-H.;

Woods, J. H.; Zhan, C.-G. "Most Efficient Cocaine Hydrolase Designed by Virtual Screening of Transition States", *J. Am. Chem. Soc.* 2008, 130, 12148-12155, which is incorporated herein by this reference. ³H-(–)-cocaine (50 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, Mass.). 5

In brief, to initiate reactions, 100 nCi of [3H]-(-)-cocaine was mixed with 100 µl of culture medium. Reactions proceeded at room temperature (25° C.) with varying concentrations of (–)-cocaine. Reactions were stopped by adding 300 μl of 0.02 M HCl, which neutralized the liberated benzoic acid 10 while ensuring a positive charge on the residual cocaine. [3H]benzoic acid was extracted by 1 ml of toluene and measured by scintillation counting. Finally, the measured (-)cocaine concentration-dependent radiometric data were analyzed by using the standard Michaelis-Menten kinetics so that 15 the catalytic efficiency $(k_{\it cat}/K_{\it M})$ was determined, along with the use of an enzyme-linked immunosorbent assay (ELISA) described in by Zheng, F.; Yang, W.; Ko, M.-C.; Liu, J.; Cho, H.; Gao, D.; Tong, M.; Tai, H.-H.; Woods, J. H.; Zhan, C.-G. "Most Efficient Cocaine Hydrolase Designed by Virtual 20 Screening of Transition States", J. Am. Chem. Soc. 2008, 130, 12148-12155.

The catalytic efficiency (k_{ca}/K_M) of the BChE polypeptide variants of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, and 32 are set forth in Table 2.

TABLE 2

Cat	alytic Efficiency	(k KM) of BChE Polyr	eptide Variants
Variant Number	Amino Acid SEQ ID NO:	Catalytic Efficiency against (-)-cocaine (M ⁻¹ min ⁻¹)	Catalytic Efficiency against (-)-cocaine (Approximate Fold Increase) ^a
1	2	3.72×10^{9}	4080
2	4	3.37×10^{9}	3700
3	6	3.27×10^{9}	3590
4	8	1.69×10^{9}	1860
5	10	2.20×10^{9}	2420
6	12	1.93×10^{9}	2120
7	14	2.02×10^{9}	2220
8	16	7.56×10^{8}	830
9	18	1.83×10^{9}	2010
10	20	1.13×10^{9}	1240
11	22	8.65×10^{8}	950
12	24	1.14×10^{9}	1250
13	26	1.13×10^{9}	1240
14	28	1.00×10^{9}	1100
15	30	6.74×10^{8}	740
16	32	1.40×10^{9}	1540

 $^a{\rm The~approximate~ratio}$ of the ${\rm k}_{cal}/{\rm K}_M$ value for the BChE mutant to that for the wild-type BChE against (–)-cocaine.

The catalytic efficiencies (k_{cad}/K_M) of the BChE polypeptide variants were found to be between about 6.74×10^8 and $3.72\times10^9 {\rm M}^{-1}~{\rm min}^{-1}$, which is about 740 to about 4080 times the k_{cad}/K_M value ($9.11\times10^5 {\rm M}^{-1}~{\rm min}^{-1}$) of the wild-type BChE.

Enzyme-linked immunosorbent assays (ELISA) were preformed as follows. The ELISA buffers used were the same as those described in the literature such as Brock, A.; Mortensen, V.; Loft, A. G. R.; Nergaard-Pedersen, B. *J. Clin. Chem. Clin. Biochem.* 1990, 28, 221-224; and Khattab, A. D.; Walker, C. H.; Johnston, G.; Siddiqui, M. K. Saphier, P. W. *Environmental Toxicology and Chemistry* 1994, 13, 1661-1667, both of which are incorporated herein by this reference. The coating buffer was 0.1 M sodium carbonate/bicarbonate buffer, pH 9.5. The diluent buffer (EIA buffer) was potassium phosphate monobasic/potassium phosphate monohydrate buffer, pH 7.5, containing 0.9% sodium chloride and 0.1% bovine serum

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albumin. The washing buffer (PBS-T) was 0.01 M potassium phosphate monobasic/potassium phosphate monohydrate buffer, pH 7.5, containing 0.05% (v/v) Tween-20. All the assays were performed in triplicate. Each well of an ELISA microtiter plate was filled with 100 µl of the mixture buffer consisting of 20 µl culture medium and 80 µl coating buffer. The plate was covered and incubated overnight at 4° C. to allow the antigen to bind to the plate. The solutions were then removed and the wells were washed four times with PBS-T. The washed wells were filled with 200 ul diluent buffer and kept shaking for 1.5 h at room temperature (25° C.). After washing with PBS-T for four times, the wells were filled with 100 μl antibody (1:8000) and were incubated for 1.5 h, followed by washing for four times. Then, the wells were filled with 100 µl goat anti-mouse IgG HRP conjugate complex diluted to a final 1:3000 dilution, and were incubated at room temperature for 1.5 h, followed by washing for four times. The enzyme reactions were started by addition of 100 µl substrate (TMB) solution. The reactions were stopped after 15 min by the addition of 100 μl of 2 M sulfuric acid, and the absorbance was read at 460 nm using a Bio-Rad ELISA plate reader.

While the terms used herein are believed to be well understood by one of ordinary skill in the art, the definitions set forth herein are provided to facilitate explanation of the presently-disclosed subject matter.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the presently-disclosed subject matter belongs. Although any methods, devices, and materials similar or equivalent to those described herein can be used in the practice or testing of the presently-disclosed subject matter, representative methods, devices, and materials are now described.

Following long-standing patent law convention, the terms "a", "an", and "the" refer to "one or more" when used in this application, including the claims. Thus, for example, reference to "a cell" includes a plurality of such cells, and so forth.

Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about". Accordingly, unless indicated to the contrary, the numerical parameters set forth in this specification and claims are approximations that can vary depending upon the desired properties sought to be obtained by the presently-disclosed subject matter.

As used herein, the term "about," when referring to a value or to an amount of mass, weight, time, volume, concentration or percentage is meant to encompass variations of in some embodiments±50%, in some embodiments±40%, in some embodiments±20%, in some embodiments±20%, in some embodiments±5%, in some embodiments±5%, in some embodiments±0.5%, and in some embodiments±0.1% from the specified amount, as such variations are appropriate to perform the disclosed method.

Throughout this document, various references are mentioned. All such references are incorporated herein by references

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<220> FEATURE:

<223> OTHER INFORMATION: mutant of human BChE

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<212> TYPE: PRT <213> ORGANISM: Artificial Sequence

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<223> OTHER INFORMATION: mutant of human BChE

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Ile Tyr Gly Gly Gly Phe Gln Thr Gly Thr Ser Ser Leu His Val Tyr 115 120 125
Asp Gly Lys Phe Leu Ala Arg Val Glu Arg Val Ile Val Val Ser Met 130 135 140
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Pro	Ala	Leu	Glu	Phe 405	Thr	Lys	Lys	Phe	Ser 410	Glu	Trp	Gly	Asn	Asn 415	Ala	
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n Gl
n Leu Ala Leu Gl
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Arg Ser Ile Val Lys Arg Trp Ala Asn Phe Ala Lys Tyr Gly Asn Pro 465 470 475 480	
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Glu Gln Lys Tyr Leu Thr Leu Asn Thr Glu Ser Thr Arg Ile Met Thr 500 505 510	
Lys Leu Arg Ala Gln Gln Cys Arg Phe Trp Thr Ser Phe Phe Pro Lys 515 520 525	
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Lys	Leu	Arg 515	Ala	Gln	Gln	СЛа	Arg 520	Phe	Trp	Thr	Ser	Phe 525	Phe	Pro	Lys
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What is claimed is:

- 1. A butyrylcholinesterase polypeptide variant comprising the amino acid sequence of SEQ ID NO: 4.
 - 2. A pharmaceutical composition comprising: a butyrylcholinesterase polypeptide variant comprising the samino acid sequence of SEQ ID NO: 4; and
 - a suitable pharmaceutical carrier.
- 3. A method of treating a cocaine-induced condition comprising administering to an individual an effective amount of the butyrylcholinesterase polypeptide variant of claim 1 to lower blood cocaine concentration.
- **4**. The method of claim **3**, wherein said butyrylcholinesterase polypeptide variant exhibits a one-hundred-fold or more increase in cocaine hydrolysis catalytic efficiency compared to butyrylcholinesterase.
- **5**. A method of treating a cocaine-induced condition comprising administering to an individual an effective amount of the pharmaceutical composition of claim **2** to lower blood cocaine concentration.

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