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High Activity Mutants of Butyrylcholinesterase for Cocaine Hydrolysis

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(12) United States Patent

Zhan et al.

(54) HIGH ACTIVITY MUTANTS OF BUTYRYLCHOLINESTERASE FOR COCAINE HYDROLYSIS

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- (60) Division of application No. 13/005,213, filed on Jan. 12, 2011, now Pat. No. 8,206,703, which is a division of application No. 12/767,128, filed on Apr. 26, 2010, now Pat. No. 7,892,537, which is a 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. *C07H 21/04* (2006.01)
- (52) U.S. Cl. USPC 536/23.2; 435/196

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(56) **References Cited**

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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.

2 Claims, No Drawings

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. 13/005,213, now allowed, filed Jan. 12, 2011 now U.S. Pat. No. 8,206,703, which is a divi-¹⁰ sional of U.S. patent application Ser. No. 12/767,128, now allowed, filed Apr. 26, 2010, now issued as U.S. Pat. No. 7,892,537, which is a divisional of U.S. patent application $_{15}$ Ser. No. 12/685,341, filed Jan. 11, 2010, now issued as U.S. Pat. No. 7,740,840, which is a continuation-in-part of copending U.S. patent application Ser. No. 12/192,394 filed Aug. 15, 2008, now issued as U.S. Pat. No. 7,731,957, which ²⁰ is a divisional of U.S. patent application Ser. No. 11/243,111, filed Oct. 4, 2005, now issued as U.S. Pat. No. 7,438,904. The entire disclosures contained in U.S. patent application Ser. 25 No. 12/767,128, U.S. Pat. No. 7,740,840, U.S. Pat. No. 7,731, 957, 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 butyrylcholinesterase 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 ⁵⁰ 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 receptorbased 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 65 is butyrylcholinesterase (BChE)-catalyzed hydrolysis at the benzoyl ester group (Scheme 1).

Scheme 1. Schematic representation of BChE-catalyzed hydrolysis at the benzoyl ester group.



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 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 5 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 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 15 generated, there exists a need for mutant BChE with even higher catalytic activity.

SUMMARY

²⁰ 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 pres- 25 ently-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 ³⁵ 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 ⁴⁰ 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 car-⁴⁵ rier.

The presently-disclosed subject matter further includes a method of treating a cocaine-induced condition, which includes administering to an individual an effective amount of BChE polypeptide variant or a pharmaceutical composition ⁵⁰ 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. ⁵⁵

BRIEF DESCRIPTION OF THE SEQUENCE LISTING

SEQ ID NO: 1 is a nucleotide sequence encoding a butyryl- 60 cholinesterase (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;

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 55 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 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 5 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 15 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, 20 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 25 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 30 NO: 32; and

SEQ ID NO: 32 is an amino acid sequence encoding a BChE polypeptide variant having the following 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 presentlydisclosed 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 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 butyrylcholinesterase polypeptide variant, as described herein, and a suitable pharmaceutical carrier. The presently-disclosed subject matter further includes a method of treating a cocaineinduced 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 P	olypeptide	e Variants	and Assoc	iated SEQ	ID NOs		
Variant			Amin	o Acid Sul	ostitution			Catalytic Efficiency (k _{ca} /K _M) against (-)-cocaine (Approximate	Nucleic Acid SEQ ID	Amino Acid SEQ ID
Number	199	227	285	286	287	328	332	Fold 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_{cat}/K_M value for the BChE mutant to that for the wild-type BChE against (-)-cocaine.

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 5 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 10 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 15 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 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 25 by the following specific but non-limiting examples. 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 30 polypeptide as described herein. For example, a functional fragment of a particular BChE polypeptide variant retains 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 vari- 35 ant" is inclusive of functional fragments of the BChE polypeptide variant. Such fragments are typically are at least 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 40 removed without substantially affecting the catalytic activity of the BChE polypeptide variant. As such, the term "BChE 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 full- 45 length BChE polypeptide variant.

The BChE polypeptide variant (e.g., SEQ ID NOS: 2, 4, 6, 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 50 art.

The present BChE variant polypeptides can be used in 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, 55 24, 26, 28, 30, and 32), to lower blood cocaine concentration. The BChE polypeptide variant can be administered in the 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 60 one of the aforementioned BChE polypeptide variants can be in a manner that will be understood by those skilled in the art.

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

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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 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 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 age, weight, metabolism, etc. of the individual. Accordingly, the "effective amount" of any particular butyrylcholinesterase polypeptide variant, or pharmaceutical composition 20 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 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

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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 15 cocaine hydrolysis by BChE, sensitive radiometric assays based on toluene extraction of $[^{3}H]$ -(-)-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.). 25

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 30 of 0.02 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. Finally, the measured (-)cocaine concentration-dependent radiometric data were analyzed by using the standard Michaelis-Menten kinetics so that the catalytic efficiency (k_{cat}/K_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. 40 "Most Efficient Cocaine Hydrolase Designed by Virtual Screening of Transition States", J. Am. Chem. Soc. 2008, 130, 12148-12155.

The catalytic efficiency (k_{cat}/K_{M}) of the BChE polypeptide variants of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 45 24, 26, 28, 30, and 32 are set forth in Table 2.

TABLE 2

Cat	talytic Efficiency	(k_{cat}/K_M) of BChE Pol	ypeptide Variants	_ 5
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	6
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	6
14	28	1.00×10^9	1100	

10TABLE 2-continued

Ca	talytic Efficiency	(k_{car}/K_M) of BChE Pol	lypeptide Variants	
Variant Number	Amino Acid SEQ ID NO:	Catalytic Efficiency against (-)-cocaine (M ⁻¹ min ⁻¹)	Catalytic Efficiency against (–)-cocaine (Approximate Fold Increase) ^a	
15 16	30 32	6.74×10^8 1.40×10^9	740 1540	

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

The catalytic efficiencies (k_{cat}/K_{M}) of the BChE polypeptide variants were found to be between about 6.74×10^8 and $3.72 \times 10^9 M^{-1} min^{-1}$, which is about 740 to about 4080 times the k_{cat}/K_M value (9.11×10⁵M⁻¹ 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 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 µl 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 ul substrate (TMB) solution. The reactions were stopped after 15 min by the addition of 100 µl of 2 M sulfuric acid, and the ⁵⁰ 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 pres-55 ently-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 presentlydisclosed subject matter belongs. Although any methods, 60 devices, and materials similar or equivalent to those described herein can be used in the practice or testing of the presentlydisclosed 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

SEQUENCE LISTING

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or percentage is meant to encompass variations of in some embodiments $\pm 50\%$, in some embodiments $\pm 40\%$, in some embodiments $\pm 30\%$, in some embodiments $\pm 20\%$, in some embodiments $\pm 10\%$, in some embodiments $\pm 5\%$, in some embodiments $\pm 1\%$, in some embodiments $\pm 0.5\%$, and in some embodiments $\pm 0.1\%$ 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 reference.

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17

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 Phe Gly Gly Gly Thr Lys Asp IIe

 Asp IIe
 Thr Lys Asp IIe

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concinaca

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Glu	Ala	Pro	Gly	Asn	Met	Gly	Leu	Phe	Asp	Gln	Gln	Leu	Ala	Leu	Gln			

Trp Val Gln Lys Asn Ile Ala Phe Gly Gly Asn Pro Lys Ser Val Thr Leu Pro Gly Glu Ser Ser Gly Ala Ala Ser Val Ser Leu His Leu Leu Ser Pro Gly Ser His Ser Leu Pro Pro Gly Ser His Ser Gly Ser Ala Asn Ala Pro Trp Ala Val Thr Ser Leu Glu Ala Arg 225 Ser Ala Asn Ala Pro Trp Ala Val Thr Ser Leu Ala Arg 225 Ser Ala Asn Ala Pro Cys Leu Arg Ser Arg Glu Ala Arg 235 Glu Ala Pro Cys Arg Cys Arg Cys Glu Ala Pro Cys C
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Ile Leu Phe His Tyr Thr Asp Trp Val Asp Asp Gln Arg Pro Glu Asn 370 375 380
Tyr Arg Glu Ala Leu Gly Asp Val Val Gly Asp Tyr Asn Phe Ile Cys 385 390 395 400
Pro Ala Leu Glu Phe Thr Lys Lys Phe Ser Glu Trp Gly Asn Asn Ala 405 410 415
Phe Phe Tyr Tyr Phe Glu His Arg Ser Ser Lys Leu Pro Trp Pro Glu 420 425 430
Trp Met Gly Val Met His Gly Tyr Glu Ile Glu Phe Val Phe Gly Leu 435 440 445
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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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<220> FEATURE:

<223> OTHER INFORMATION: mutant of human BChE

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Tro	Met	Glv	420 Val	Met	His	Glv	Tvr	425 Glu	Ile	Glu	Phe	Val	430 Phe	Glv	Leu
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Gly Leu Lys Ile Phe Phe Pro Gly Val Ser Glu Phe 355 360	Gly Lys Glu Ser 365
Ile Leu Phe His Tyr Thr Asp Trp Val Asp Asp Gln 370 375 380	Arg Pro Glu Asn
Tyr Arg Glu Ala Leu Gly Asp Val Val Gly Asp Tyr 385 390 395	Asn Phe Ile Cys 400
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Phe Phe Tyr Tyr Phe Glu His Arg Ser Ser Lys Leu	Pro Trp Pro Glu 430
Trp Met Gly Val Met His Gly Tyr Glu Ile Glu Phe	Val Phe Gly Leu
Pro Leu Glu Arg Arg Asp Asn Tyr Thr Lys Ala Glu	Glu Ile Leu Ser
450 455 460 Arg Ser Ile Val Lys Arg Trp Ala Asn Phe Ala Lys	Tyr Gly Asn Pro
465 470 475	480
Asn Glu Thr Gln Asn Asn Ser Thr Ser Trp Pro Val 485 490	Phe Lys Ser Thr 495
Glu Gln Lys Tyr Leu Thr Leu Asn Thr Glu Ser Thr 500 505	Arg Ile Met Thr 510
Lys Leu Arg Ala Gln Gln Cys Arg Phe Trp Thr Ser 515 520	Phe Phe Pro Lys 525
Val Leu Glu Met Thr Gly Asn Ile Asp Glu Ala Glu 530 535 540	Trp Glu Trp Lys
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tatgcaaatt cttgctgtca gaacatagat caaagttttc cag	getteca tggateagag 240
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gcagetteag ttageetgea tttgetttet eetggaagee atteattgtt eaceagagee	660
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Leu Thr Lys Trp Ser Asp Ile Trp Asn Ala Thr Lys Tyr Ala Asn Ser 50 55 60	
Cys Cys Gln Asn Ile Asp Gln Ser Phe Pro Gly Phe His Gly Ser Glu 65 70 75 80	
Met Trp Asn Pro Asn Thr Asp Leu Ser Glu Asp Cys Leu Tyr Leu Asn 85 90 95	
Val Trp Ile Pro Ala Pro Lys Pro Lys Asn Ala Thr Val Leu Ile Trp 100 105 110	

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Glu	Ala	Pro	Gly	Asn 165	Met	Gly	Leu	Phe	Asp 170	Gln	Gln	Leu	Ala	Leu 175	Gln		
Trp	Val	Gln	Lys 180	Asn	Ile	Ala	Ala	Phe 185	Gly	Gly	Asn	Pro	Lys 190	Ser	Val		
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Leu	Ser	Pro	Gly	Ser	His	Ser 215	Leu	Phe	Thr	Arg	Ala 220	Ile	Leu	Gln	Ser		
Gly	Ser	Val	Asn	Ala	Pro	Trp	Ala	Val	Thr	Ser	Leu	Tyr	Glu	Ala	Arg		
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Ile	Leu 370	Phe	His	Tyr	Thr	Asp 375	Trp	Val	Asp	Asp	Gln 380	Arg	Pro	Glu	Asn		
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Pro	Leu 450	Glu	Arg	Arg	Asp	Asn 455	Tyr	Thr	Lys	Ala	Glu 460	Glu	Ile	Leu	Ser		
Arg 465	Ser	Ile	Val	Гла	Arg 470	Trp	Ala	Asn	Phe	Ala 475	Lys	Tyr	Gly	Asn	Pro 480		
Asn	Glu	Thr	Gln	Asn	Asn	Ser	Thr	Ser	Trp	Pro	Val	Phe	Lys	Ser	Thr		
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Lys	Leu	Ara	500 Ala	Gln	Gln	Cys	Ara	505 Phe	Tro	Thr	Ser	Phe	510 Phe	Pro	Lys		
	_	515					520	_				525		_	-		
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Thr	Leu	Phe 195	Gly	Glu	Ser	Ser	Gly 200	Ala	Ala	Ser	Val	Ser 205	Leu	His	Leu	
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Trp Met Gly Val Met His Gly Tyr Glu Ile Glu Phe Val Phe Gly 435 440 445	y Leu
Pro Leu Glu Arg Arg Asp Asn Tyr Thr Lys Ala Glu Glu Ile Lev 450 455 460	ı Ser
Arg Ser Ile Val Lys Arg Trp Ala Asn Phe Ala Lys Tyr Gly As 465 470 475	1 Pro 480
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Glu Gln Lys Tyr Leu Thr Leu Asn Thr Glu Ser Thr Arg Ile Met	= Thr
Lys Leu Arg Ala Gln Gln Cys Arg Phe Trp Thr Ser Phe Phe Pro	о Гла
Val Leu Glu Met Thr Gly Asn Ile Asp Glu Ala Glu Trp Glu Trp	о ГЛа
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73

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Thr	Leu	Phe 195	Gly	Glu	Ser	Ser	Gly 200	Ala	Ala	Ser	Val	Ser 205	Leu	His	Leu
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Asn	Arg	Thr	Leu	Asn 245	Leu	Ala	Lys	Leu	Thr 250	Gly	Суз	Ser	Arg	Glu 255	Asn
Glu	Thr	Glu	Ile 260	Ile	Гла	Сүз	Leu	Arg 265	Asn	Lys	Asp	Pro	Gln 270	Glu	Ile
Leu	Leu	Asn 275	Glu	Ala	Phe	Val	Val 280	Pro	Tyr	Gly	Thr	Lys 285	Leu	Gly	Val
Asn	Phe 290	Gly	Pro	Thr	Val	Asp 295	Gly	Asp	Phe	Leu	Thr 300	Asp	Met	Pro	Asp
Ile 305	Leu	Leu	Glu	Leu	Gly 310	Gln	Phe	Lys	Lys	Thr 315	Gln	Ile	Leu	Val	Gly 320
Val	Asn	Lys	Asp	Glu 325	Gly	Thr	Trp	Phe	Leu 330	Val	Gly	Gly	Ala	Pro 335	Gly
Phe	Ser	Lys	Asp 340	Asn	Asn	Ser	Ile	Ile 345	Thr	Arg	Lys	Glu	Phe 350	Gln	Glu
Gly	Leu	Lys 355	Ile	Phe	Phe	Pro	Gly 360	Val	Ser	Glu	Phe	Gly 365	Lys	Glu	Ser
Ile	Leu 370	Phe	His	Tyr	Thr	Asp 375	Trp	Val	Aab	Asp	Gln 380	Arg	Pro	Glu	Asn
Tyr 385	Arg	Glu	Ala	Leu	Gly 390	Asp	Val	Val	Gly	Asp 395	Tyr	Asn	Phe	Ile	Cys 400
Pro	Ala	Leu	Glu	Phe 405	Thr	Гла	Гла	Phe	Ser 410	Glu	Trp	Gly	Asn	Asn 415	Ala
Phe	Phe	Tyr	Tyr 420	Phe	Glu	His	Arg	Ser 425	Ser	Lys	Leu	Pro	Trp 430	Pro	Glu
Trp	Met	Gly 435	Val	Met	His	Gly	Tyr 440	Glu	Ile	Glu	Phe	Val 445	Phe	Gly	Leu
Pro	Leu 450	Glu	Arg	Arg	Asp	Asn 455	Tyr	Thr	Lys	Ala	Glu 460	Glu	Ile	Leu	Ser
Arg 465	Ser	Ile	Val	Lys	Arg 470	Trp	Ala	Asn	Phe	Ala 475	Lys	Tyr	Gly	Asn	Pro 480
Asn	Glu	Thr	Gln	Asn 485	Asn	Ser	Thr	Ser	Trp 490	Pro	Val	Phe	Lys	Ser 495	Thr
Glu	Gln	Lys	Tyr 500	Leu	Thr	Leu	Asn	Thr 505	Glu	Ser	Thr	Arg	Ile 510	Met	Thr
Lys	Leu	Arg 515	Ala	Gln	Gln	Сүз	Arg 520	Phe	Trp	Thr	Ser	Phe 525	Phe	Pro	ГЛа
Val	Leu 530	Glu	Met	Thr	Gly	Asn 535	Ile	Asp	Glu	Ala	Glu 540	Trp	Glu	Trp	ГЛа
Ala 545	Gly	Phe	His	Arg	Trp 550	Asn	Asn	Tyr	Met	Met 555	Asp	Trp	Гла	Asn	Gln 560
Phe	Asn	Asb	Tyr	Thr 565	Ser	Lys	Lys	Glu	Ser 570	Сүз	Val	Gly	Leu		

What is claimed is:

1. An isolated nucleic acid molecule comprising a nucleic acid sequence which encodes a butyrylcholinesterase variant peptide, said nucleic acid sequence comprising SEQ ID NO: 1.

2. An isolated nucleic acid molecule comprising a nucleic acid sequence which encodes a butyrylcholinesterase variant peptide comprising the amino acid sequence of SEQ ID NO: 2.

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