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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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#### Related U.S. Application Data

- (60) Continuation-in-part of application No. 12/192,394, filed on Aug. 15, 2008, 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

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(56) References Cited

U.S. PATENT DOCUMENTS

7,438,904 B1 \* 10/2008 Zhan et al. ...... 424/94.6

\* cited by examiner

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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 continuation-in-part of co-pending U.S. patent application Ser. No. 12/192,394 filed Aug. 15, 2008, 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 (N1H). The government has <sup>20</sup> 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 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).

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

$$H_3C$$
 $H$ 
 $OCH_3$ 
 $+$ 
 $H_2O$ 
 $BChE$ 
 $O=C$ 
 $(-)$ -cocaine

-continued

H<sub>3</sub>C

H

OCH<sub>3</sub>

$$+$$

H<sub>2</sub>O

BChE

OCH<sub>3</sub>
 $+$ 

OCH<sub>3</sub>

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 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:

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 <sup>10</sup> 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, 15 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 amino acid substitutions, as compared to wild type BChE: A199S, F227A, P285K, S287G, A328W, and Y332G.

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standing 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 butyryl-cholinesterase (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 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 Polypeptide Variants and Associated SEQ ID NOs													
Variant .			Amino	Acid Subs	titution			Catalytic Efficiency $(k_{cal}/K_M)$ against $(-)$ -cocaine $(Approximate Fold)$	Nucleic Acid SEQ ID	Amino Acid SEQ ID			
Number	199	227	285	286	287	328	332	Increase) <sup>a</sup>	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	_	S287Q	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			

<sup>&</sup>lt;sup>a</sup>The approximate ratio of the  $k_{caf}/K_M$  value for the BChE mutant to that for the wild-type BChE against (–)-cocaine.

## 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 under-

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 prodtots, 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 5 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 10 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 15 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 some or all of the cocaine hydrolysis activity, i.e., the catalytic efficiency for (-)-cocaine, of the particular BChE polypep- 20 tide 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 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 25 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 polypeptide variant" is inclusive of functional fragments wherein one or more residues from 1 to 67 and/or one or more 30 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, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, and 32) can be formulated in a pharmaceutical composition along with a 35 suitable pharmaceutical carrier known to one skilled in the 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, 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 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 50 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 that this amount will vary greatly depending on the nature of cocaine consumed, e.g., injected or inhaled, and the condition 55 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 60 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

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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 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 ng plasmid DNA per each well. Cells were incubated at 37° C. in a  $\rm CO_2$  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.* 5 *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.).

In brief, to initiate reactions, 100 nCi of [<sup>3</sup>H]-(-)-cocaine was mixed with 100 µl of culture medium. Reactions pro- 10 ceeded 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 while ensuring a positive charge on the residual cocaine. [3H]benzoic acid was extracted by 1 ml of toluene and mea- 15 sured 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) 20 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 Screening of Transition States", J. Am. Chem. Soc. 2008, 130, 12148-12155.

The catalytic efficiency ( $k_{cal}/K_M$ ) 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

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

 $^a{\rm The}$  approximate ratio of the  ${\rm k}_{cat}/{\rm K}_M$  value for the BChE mutant to that for the wild-type ~50 BChE against (–)-cocaine.

The catalytic efficiencies ( $k_{cat}/K_M$ ) of the BChE polypeptide variants were found to be between about  $6.74\times10^8$  and  $3.72\times10^9~M^{-1}~min^{-1}$ , which is about 740 to about 4080 times the  $k_{cat}/K_M$  value ( $9.11\times10^5~M^{-1}~min^{-1}$ ) of the wild-type 55 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.* 60 *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 65 9.5. The diluent buffer (EIA buffer) was potassium phosphate monobasic/potassium phosphate monobydrate buffer, pH

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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 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

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 ±10%, in some embodiments ±5%, in some embodiments ±1%, 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 reference

## SEQUENCE LISTING

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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

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Glu	Ala	Pro	Gly	Asn 165	Met	Gly	Leu	Phe	Asp 170	Gln	Gln	Leu	Ala	Leu 175	Gln
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1680

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1080

1380

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<211> LET < 212> TYI < 213> ORG < 212> TYI < 213> ORG < 220> FEZ < 223> OTH < 400> SEG < 210 Asp # 1	NGTH: ! PE: PR: GANISM ATURE: HER INI QUENCE Asp II Thr Va. 20 Gln Pro 35 Lys Tr	FORMATE SORMATE SORMAT	Ile Gly Leu Asp	Ala Gly Gly Ile 55	ant Thr Thr Arg 40 Trp	of h Lys Val 25 Leu Asn	Asn Gl 10 Thr Al Arg Ph	y Lys a Phe e Lys r Lys 60 y Phe	Leu Lys 45	Gly 30 Pro Ala	15 Ile Gln Asn	Pro Ser Ser	
<pre>&lt;211&gt; LET &lt;212&gt; TYI &lt;213&gt; ORC &lt;220&gt; FEA &lt;223&gt; OTH &lt;400&gt; SEQ Glu Asp A 1 Asn Leu Tyr Ala (</pre>	NGTH: !PE: PR:GANISM ATURE: PR:GANISM ATURE: PR:GANISM ATURE: PR:GANISM ASP II:  COURNCE Asp II:  COURNCE Asp II:  COURNCE Thr Va: COURNCE Thr	From Pro	Ile Gly Leu Asp	Ala Gly Gly Ile 55 Gln	ant Thr Thr Arg 40 Trp Ser	of h Lys Val 25 Leu Asn	Asn Gl 10 Thr Al Arg Ph Ala Th Pro Gl 75	y Lys a Phe e Lys r Lys 60 y Phe	Leu Lys 45 Tyr	Gly 30 Pro Ala Gly	15 Ile Gln Asn Ser	Pro Ser Ser Glu 80	
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Asn Tyr Arg Val Gly Ala Leu Gly Phe Leu Ala Leu Pro Gly Asn Pro

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_	$\alpha$	٦r	ı 🛨	٦	n	11	ρ,	$^{\circ}$

145					150					155					160
145					150					155					100
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Glu	Thr	Glu	Ile 260	Ile	ГÀв	Cys	Leu	Arg 265	Asn	Lys	Asp	Pro	Gln 270	Glu	Ile
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Ile	Leu 370	Phe	His	Tyr	Thr	Asp 375	Trp	Val	Asp	Asp	Gln 380	Arg	Pro	Glu	Asn
Tyr 385	Arg	Glu	Ala	Leu	Gly 390	Asp	Val	Val	Gly	395	Tyr	Asn	Phe	Ile	Cys 400
Pro	Ala	Leu	Glu	Phe 405	Thr	ГÀа	ГЛа	Phe	Ser 410	Glu	Trp	Gly	Asn	Asn 415	Ala
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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
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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	Cys	Arg 520	Phe	Trp	Thr	Ser	Phe 525	Phe	Pro	Lys
Val	Leu 530	Glu	Met	Thr	Gly	Asn 535	Ile	Asp	Glu	Ala	Glu 540	Trp	Glu	Trp	Lys
Ala 545	Gly	Phe	His	Arg	Trp 550	Asn	Asn	Tyr	Met	Met 555	Asp	Trp	Lys	Asn	Gln 560
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                                                                     180
tatgcaaatt cttgctgtca gaacatagat caaagttttc caggcttcca tggatcagag
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                                                                    1020
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<210> SEQ ID NO 29

<sup>&</sup>lt;211> LENGTH: 574

<sup>&</sup>lt;212> TYPE: PRT

<sup>&</sup>lt;213 > ORGANISM: Artificial Sequence

<sup>&</sup>lt;220> FEATURE:

<sup>&</sup>lt;223> OTHER INFORMATION: mutant of human BChE

<sup>&</sup>lt;400> SEOUENCE: 30

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Tyr	Ala	Gln 35	Pro	Pro	Leu	Gly	Arg 40	Leu	Arg	Phe	Lys	Lys 45	Pro	Gln	Ser
Leu	Thr 50	Lys	Trp	Ser	Asp	Ile 55	Trp	Asn	Ala	Thr	60 Fåa	Tyr	Ala	Asn	Ser
65 65	Cha	Gln	Asn	Ile	Asp 70	Gln	Ser	Phe	Pro	Gly 75	Phe	His	Gly	Ser	Glu 80
Met	Trp	Asn	Pro	Asn 85	Thr	Asp	Leu	Ser	Glu 90	Asp	Cys	Leu	Tyr	Leu 95	Asn
Val	Trp	Ile	Pro 100	Ala	Pro	Lys	Pro	Lys 105	Asn	Ala	Thr	Val	Leu 110	Ile	Trp
Ile	Tyr	Gly 115	Gly	Gly	Phe	Gln	Thr 120	Gly	Thr	Ser	Ser	Leu 125	His	Val	Tyr
Asp	Gly 130	Lys	Phe	Leu	Ala	Arg 135	Val	Glu	Arg	Val	Ile 140	Val	Val	Ser	Met
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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
Thr	Leu	Phe 195	Gly	Glu	Ser	Ser	Gly 200	Ala	Ala	Ser	Val	Ser 205	Leu	His	Leu
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Gly 225	Ser	Phe	Asn	Ala	Pro 230	Trp	Ala	Val	Thr	Ser 235	Leu	Tyr	Glu	Ala	Arg 240
Asn	Arg	Thr	Leu	Asn 245	Leu	Ala	Lys	Leu	Thr 250	Gly	Cys	Ser	Arg	Glu 255	Asn
Glu	Thr	Glu	Ile 260	Ile	Lys	Cys	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	Pro 285	Met	Gly	Val
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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
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Ile	Leu 370	Phe	His	Tyr	Thr	Asp 375	Trp	Val	Asp	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	Lys	Lys	Phe	Ser 410	Glu	Trp	Gly	Asn	Asn 415	Ala
Phe	Phe	Tyr	Tyr	Phe	Glu	His	Arg	Ser	Ser	Lys	Leu	Pro	Trp	Pro	Glu

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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 Phe Lys Ser Thr 485 490 495
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
Val Leu Glu Met Thr Gly Asn Ile Asp Glu Ala Glu Trp Glu Trp Lys 530 535 540
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gtagtgtcaa tgaactatag ggtgggtgcc ctaggattct tagctttgcc aggaaatcct 480
gaggetecag ggaacatggg tttatttgat caacagttgg etetteagtg ggtteaaaaa 540
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Asn	Leu	Thr	Val 20	Phe	Gly	Gly	Thr	Val 25	Thr	Ala	Phe	Leu	Gly 30	Ile	Pro	
Tyr	Ala	Gln 35	Pro	Pro	Leu	Gly	Arg 40	Leu	Arg	Phe	Lys	Lys 45	Pro	Gln	Ser	
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Сув 65	Cys	Gln	Asn	Ile	Asp 70	Gln	Ser	Phe	Pro	Gly 75	Phe	His	Gly	Ser	Glu 80	
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Val	Trp	Ile	Pro 100	Ala	Pro	Lys	Pro	Lys 105	Asn	Ala	Thr	Val	Leu 110	Ile	Trp	
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Thr	Leu	Phe 195	Gly	Glu	Ser	Ser	Gly 200	Ala	Ala	Ser	Val	Ser 205	Leu	His	Leu	
Leu	Ser 210	Pro	Gly	Ser	His	Ser 215	Leu	Phe	Thr	Arg	Ala 220	Ile	Leu	Gln	Ser	
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Val	Asn	Lys	Asp	Glu 325	Gly	Thr	Trp	Phe	Leu 330	Val	Gly	Gly	Ala	Pro 335	Gly
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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	Ala	Leu	Glu	Phe 405	Thr	Lys	Lys	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
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Arg 465	Ser	Ile	Val	Tàa	Arg 470		Ala	Asn	Phe	Ala 475	ГÀа	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	Cys	Arg 520	Phe	Trp	Thr	Ser	Phe 525	Phe	Pro	Lys
Val	Leu 530	Glu	Met	Thr	Gly	Asn 535	Ile	Asp	Glu	Ala	Glu 540	Trp	Glu	Trp	Lys
Ala 545	Gly	Phe	His	Arg	Trp 550	Asn	Asn	Tyr	Met	Met 555	Asp	Trp	Lys	Asn	Gln 560
Phe	Asn	Asp	Tyr	Thr 565		Lys	Lys	Glu	Ser 570	Cys	Val	Gly	Leu		

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What is claimed is:

- 1. A butyrylcholinesterase polypeptide variant comprising the amino acid sequence of SEQ ID NO: 2.
- **2**. A pharmaceutical composition comprising a butyrylcholinesterase polypeptide variant comprising the amino acid 55 sequence of SEQ ID NO: 2 and a suitable pharmaceutical carrier.
- 3. A method of treating a cocaine-induced condition comprising administering to an individual an effective amount of a 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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