Drug and Alcohol Dependence 202 (2019) 168–171

Contents lists available at ScienceDirect

Drug and Alcohol Dependence

journal homepage: www.elsevier.com/locate/drugalcdep

Evaluation of the cholinesterase activity of a potential therapeutic cocaine esterase for cocaine overdose

Shurong Hou[1], Yun Zhang[1], Yao Zhu, Chao Zhang, Yichao Kong, Xiaoling Chen, Rong Chen, Xiaopu Yin, Tian Xie*, Xiabin Chen*

Key Laboratory of Elemene Class Anti-Cancer Chinese Medicine of Zhejiang Province, Engineering Laboratory of Development and Application of Traditional Chinese Medicine from Zhejiang Province, Holistic Integrative Pharmacy Institutes (HIPI), School of Medicine, Hangzhou Normal University. 2318 Yuhangtang Rd, Hangzhou, Zhejiang 311121, China

ARTICLE INFO

Keywords:
Cocaine esterase
Enzyme therapy
Cocaine overdose
Acetylcholine

ABSTRACT

Background: Cocaine is a commonly abused drug and there is no approved medication specifically to treat its addiction or overdose. Bacterial cocaine esterase (CocE)-derived RBP-8000 is currently under clinical development for cocaine overdose treatment. It is proven to be effective for human use to accelerate cocaine metabolism into physiologically inactive products. Besides cocaine, RBP-8000 may hydrolyze the neurotransmitter acetylcholine (ACh), however, no study has reported its cholinesterase activity. The present study aims to examine RBP-8000's cholinesterase activity and substrate selectivity to address the potential concern that this enzyme therapy might produce cholinergic side-effects.

Methods: Both computational modeling and experimental kinetic analysis were carried out to characterize the potential cholinesterase activity of RBP-8000. Substrates interacting with RBP-8000 were modeled for their enzyme-substrate binding complexes. *In vitro* enzymatic kinetic parameters were measured using Ellman's colorimetric assay and analyzed by Michaelis–Menten kinetics.

Results: It is the first demonstration that RBP-8000 catalyzes the hydrolysis of acetylthiocholine (ATC). However, its catalytic efficiency (k_{cat}/K_M) against ATC is 1000-fold and 5000-fold lower than it against cocaine at 25 °C and 37 °C, respectively, suggesting RBP-8000 has the desired substrate selectivity for cocaine over ACh.

Conclusion: Given the fact that clinically relevant dose of RBP-8000 displays insignificant cholinesterase activity relative to endogenous cholinesterases in human, administration of RBP-8000 is unlikely to produce any significant cholinergic side-effects. This study provides supplemental evidences in support of further development of RBP-8000 towards a clinically used pharmacotherapy for cocaine overdose.

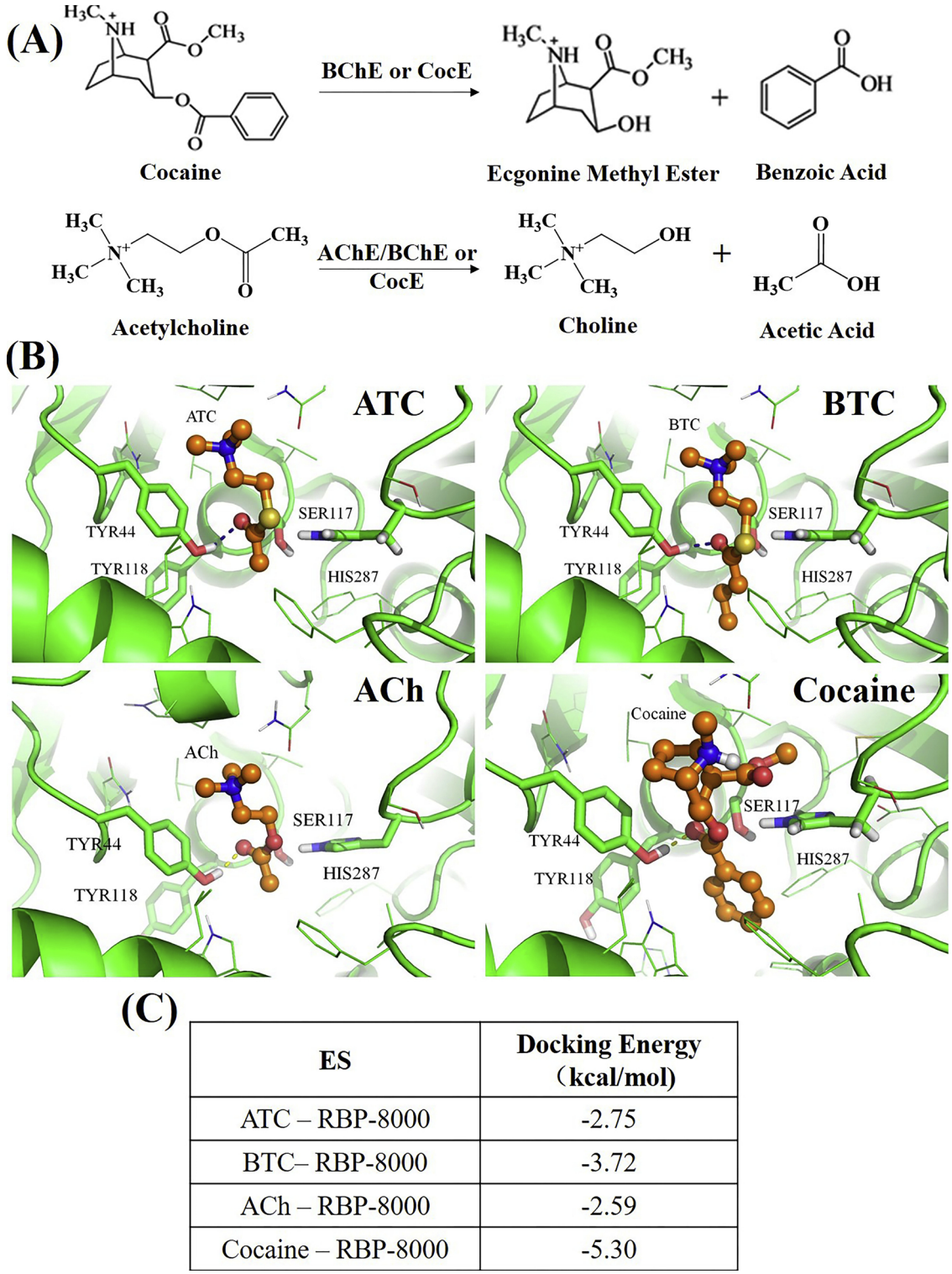
1. Introduction

Cocaine abuse is a worldwide public health problem, especially in North America, South America, Oceania, and Western and Central Europe (UNODC, 2018). Take the United States as an example, ~2.3% of population aged 16–64 years use cocaine, resulting in over 500,000 cocaine-related emergency department (ED) visits annually (SAMHSA, 2013). Cocaine-induced toxicity results from its multiple physiological effects in central nervous system (CNS) and cardiovascular system (Heard et al., 2008). The acute toxicity from cocaine overdose could lead to lethal events, including cardiac arrest or seizures, and followed respiratory failure (Brody et al., 1990). Current emergence treatment for cocaine overdose follows the standard protocol, *i.e.* initial administration of an anticonvulsant agent diazepam, followed by interventions to control other significant symptoms presented (McCord et al., 2008). However, there is no approved medication specific for cocaine intoxication.

Enzyme therapy using an efficient cocaine-metabolizing enzyme has been recognized as the most promising approach for cocaine overdose treatment (Narasimhan et al., 2012; Rogers et al., 2005; Zheng and Zhan, 2011, 2012). By directly targeting cocaine in the body, these enzymes would rapidly metabolize cocaine into physiologically inactive ecgonine methyl ester and benzoic acid, thus eliminating cocaine-induced physiological effects and acute toxicity. Recent efforts have led to the discovery of two potential therapeutic enzymes including a thermally stable mutant (T172R/G173Q) of bacterial cocaine esterase

* Corresponding authors.
E-mail addresses: xbs@dljg.sina.net (T. Xie), xch226@hznu.edu.cn (X. Chen).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.drugalcdep.2019.04.034
Received 28 November 2018; Received in revised form 6 April 2019; Accepted 30 April 2019
Available online 19 July 2019



(C)

ES	Docking Energy (kcal/mol)
ATC – RBP-8000	-2.75
BTC– RBP-8000	-3.72
ACh – RBP-8000	-2.59
Cocaine – RBP-8000	-5.30

Fig. 1. (A) Metabolic pathway of cocaine and acetylcholine in human and animals with metabolic enzymes including BChE, AChE and exogenous CocE. (B) The energy-minimized ES structures for ATC, BTC, ACh and cocaine interacting with RBP-8000. (C) Docking energy for the binding of ATC, BTC, ACh and cocaine with RBP-8000.

(CocE), known as RBP-8000 (Collins et al., 2011; Howell et al., 2014; Nasser et al., 2014) and a high-activity mutant (A199S/S287G/A328W/Y332G) of human butyrylcholinesterase (BChE), known as CocH1 (Cohen-Barak et al., 2015; Pan et al., 2005; Xue et al., 2011; Zhang et al., 2017). The randomized, double-blind, placebo-controlled trial of RBP-8000 in cocaine abusers demonstrated its effectiveness for accelerating cocaine metabolism in humans. In the presence of RBP-8000, cocaine plasma concentration was decreased by 90% within 2 min; cocaine-induced cardiovascular effects were significantly reduced compared to placebo. In addition, its pharmacokinetics profiles (Geometric mean $t_{1/2}$ ~2 h; 100–200 mg i.v. infusion: C_{max} = 50.6–69.2 μg/mL at 7.5–10 min) and observed negative immune response also strongly favor the continued development of RBP-8000 as a pharmacotherapy for cocaine overdose (Nasser et al., 2014).

An ideal enzyme therapy for cocaine overdose should not only be highly effective in cocaine metabolism but also has the desired selectivity. Given that the essential neurotransmitter ACh and cocaine both contain a cationic nitrogen and reactive ester function group, as shown in Fig. 1A, cocaine-metabolizing enzymes might also hydrolyze ACh (Hou et al., 2013; Rogers et al., 2006). In fact, BChE-derived CocH1 has been extensively investigated for its catalytic properties against cocaine and ACh, showing that CocH1 could quickly break down both cocaine and ACh (Hou et al., 2013).

It is well known that ACh regulates a variety of functions of cholinergic neurons within both the CNS and peripheral nervous system (PNS) and plays a critical role in the signal of muscle movement and learning and memory formation (English and Jones, 2012; Masson and Lockridge, 2010). It is metabolized by both BChE and acetylcholinesterase (AChE) in human body. Therefore, administration of exogenous therapeutic enzymes with cholinesterase activity might affect the cholinergic nervous system. Since RBP-8000 is currently under clinical development for cocaine overdose treatment, it must be carefully examined for its potential adverse clinical consequences. However, to the best of our knowledge, no study has ever characterized RBP-8000 for its cholinesterase activity. In this study, we sought to investigate whether RBP-8000 has the desired substrate selectivity for cocaine over ACh, which will give insights into the possibility of RBP-8000 to produce cholinergic side-effects.

2. Methods

2.1. Molecular modeling

Various substrates including cocaine, ACh, ATC and butyrylthiocholine (BTC) interacting with RBP-8000 were modeled for their enzyme-substrate binding complexes (denoted as ES). Autodock Vina was employed to perform the molecular docking studies. Autodock Tools was used to process the structure of RBP-8000 (PDB ID: 312F) by removing the crystal waters and other molecules. The default setting was used for the docking. The docking box size was set to 25*25*25 (Å). Top 20 conformations of each docking would be saved. The finally obtained ES binding structures were the ones with the lowest binding free energies.

2.2. Expression and purification of RBP-8000

The cDNA encoding 6 × His-tagged RBP-8000 was synthesized by Generay Biotechnology (Shanghai, China), and cloned into a bacterial expression vector, pET-22b(+). RBP-8000 was expressed in *E. coli* BL-21 (DE3) cells. Enzyme expression was induced with 1 mM IPTG (Sigma-Aldrich) for ~15 h at 18 °C. Cells were pelleted, resuspended in 50 mM Tris−HCl (pH 8.0), 150 mM NaCl buffer and lysed using a French press (Thermo Fisher Scientific). The enzyme was then purified using Talon metal affinity resin (Takara). The eluted fractions were concentrated using an Amicon Ultra-30 K centrifuge (Millipore). The enzyme concentration was determined using a Bradford Assay kit (Sangon Biotech).

2.3. In vitro characterization of RBP-8000

The catalytic activities of the RBP-8000 against ATC and BTC were determined by Ellman's colorimetric assay (Worek et al., 2012) using a Multiskan FC Microplate Reader (Thermo Fisher Scientific). The reaction rates of the enzymatic hydrolysis of ATC or BTC at various initial substrate concentrations were measured in triplicate at 25 °C and 37 °C by recording the time-dependent absorption at 450 nm in the presence of 1 mM dithiobisnitrobenzoic acid (DTNB) in 0.1 M potassium phosphate buffer (pH 7.4). The Michaelis-Menten kinetic analysis was performed by using Prism 5 (GraphPad Software Inc.) to determine the V_{max} and K_M values.

3. Results

3.1. Molecular modeling

Directed by the reaction mechanism, molecular modeling provides insights on how RBP-8000 interacts with its substrate in the ES structures. The interaction of various substrates with RBP-8000 is depicted in Fig. 1B with key residues labeled. According to the modeling, either substrate including ATC, BTC, ACh or cocaine forms a hydrogen bond with TYR44 of RBP-8000 in the ES structure, which will later help to stabilize the transition state structure during the enzyme hydrolysis reaction.

Although all the substrates share the same binding mode by forming a hydrogen bond with RBP-8000 in the ES structure, there is a difference in their binding energies. As shown in Fig. 1C, the calculated docking energies for cocaine-RBP-8000, BTC-RBP-8000, ATC-RBP-8000 and ACh-RBP-8000 are −5.30, −3.72, −2.75, −2.59 kcal/mol, respectively. The lower the binding energy, the more stable the ES complex structure. Therefore, the binding rank between RBP-8000 and these substrates is as follows: cocaine > BTC > ATC ˜ ACh, which is consistent with their *in vitro* kinetic parameters.

3.2. Kinetic parameters

Based on the computational insights, *in vitro* experimental tests were performed to characterize the enzymatic kinetics of RBP-8000 at both 25 °C and 37 °C. The kinetic parameters summarized in Table 1 clearly shows that RBP-8000 is able to catalyze the hydrolysis of ATC, but its catalytic efficiency against ATC is 1000-fold and 5000-fold lower than it against cocaine at 25 °C and 37 °C, respectively. As for CocH1, there is no significant difference in the catalytic activity between the hydrolysis of ACh and cocaine.

4. Discussion

In the present study, RBP-8000 was examined for its cholinesterase activity and substrate selectivity through computational modeling and *in vitro* experimental tests. The calculated docking energy in molecular modeling suggests, as expected the binding of RBP-8000 with ACh is similar to its binding with ATC, and its binding with cocaine is much stronger than its binding with ACh or ATC. According to the *in vitro* kinetic data, RBP-8000 is able to catalyze the hydrolysis of ATC, but its catalytic efficiency against ATC is 1000-fold and 5000-fold lower than it against cocaine at 25 °C and 37 °C, respectively. Therefore, both the

Table 1
Kinetic parameters determined for ATC, BTC and cocaine hydrolysis catalyzed by RBP-8000 and CocH1.

Enzyme (Temperature)	Substrate	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M ($M^{-1} min^{-1}$)	RCE[a]
RBP-8000 (25 °C)	Cocaine[b]	1432	14.2	1.0×10^8	1
	ATC	578	6030.0	9.6×10^4	0.001
	BTC	7985	327.6	2.4×10^7	0.24
RBP-8000 (37 °C)	Cocaine[c]	2600	2.9	9.0×10^8	1
	ATC	839	3809.0	2.2×10^5	0.0002
	BTC	12771	293.4	4.4×10^7	0.049
CocH1[d] (25 °C)	Cocaine	3060	3.1	9.9×10^8	1
	ATC	7880	21.0	3.8×10^8	0.38
	BTC	14400	5.3	2.7×10^9	2.75

[a] RCE refers to the relative catalytic efficiency (k_{cat}/K_M), *i.e.* the ratio of the k_{cat}/K_M value of the enzyme against ATC or BTC to that against cocaine.
[b] Data for RBP-8000 against cocaine at 25 °C from reference Brim et al. (2011).
[c] Data for RBP-8000 against cocaine at 37 °C from reference Fang et al. (2014).
[d] Data for CocH1 from reference Hou et al. (2013).

molecular modeling and experimental kinetic analysis have consistently revealed RBP-8000 has the desired substrate selectivity for cocaine over ATC or ACh. In contrast, the observed substrate selectivity for cocaine over ATC on RBP-8000 was not observed on the other therapeutic candidate CocH1.

In addition, the cholinesterase activity (k_{cat}/K_M) of RBP-8000 is ~4000-fold less than that of CocH1 or wide-type BChE (for ATC hydrolysis: $k_{cat} = 20{,}200\,min^{-1}$, $K_M = 33\,\mu M$, $k_{cat}/K_M = 6.1 \times 10^8\,M^{-1}\,min^{-1}$) (Hou et al., 2013), and ~80000-fold less than AChE (for ACh hydrolysis: $k_{cat} = 702{,}000\,min^{-1}$, $K_M = 90\,\mu M$, $k_{cat}/K_M = 7.8 \times 10^9\,M^{-1}\,min^{-1}$) (Wolfenden and Yuan, 2011). As determined in the human clinical study, the mean peak plasma concentration of RBP-8000 was 1.06 μM (69.15 mg/L) for i.v. infusion of 200 mg enzyme, an effective dose for cocaine intoxification treatment (Nasser et al., 2014). The molar concentrations of AChE and BChE in blood are roughly similar, averaged ~0.07 μM (Bartels et al., 2000; Li et al., 2000). Thus, the cholinesterase activity of the clinically relevant dose of RBP-8000 for cocaine overdose treatment would be ~5800-fold lower than that of the endogenous AChE and BChE in blood, regardless of the densely packed AChE in peripheral cholinergic synapses. Therefore, a clinically relevant dose of RBP-8000 displays insignificant cholinesterase activity relative to endogenous cholinesterases. For these reasons, administration of exogenous RBP-8000 for cocaine overdose treatment is unlikely to produce any significant cholinergic side-effects, and thus would not result in muscle weakness in peripheral tissues. These findings provide the supplemental evidence in support for clinical development of RBP-8000 for cocaine overdose treatment.

5. Conclusion

This is the first report of cholinesterase activity of a potential therapeutic enzyme RBP-8000 for cocaine overdose treatment. Both the computational and experimental kinetic analysis have consistently revealed that RBP-8000 has the desired substrate selectivity for cocaine over ACh, and its insignificant cholinesterase activity relative to endogenous cholinesterases in human, suggests RBP-8000 is unlikely to produce any significant cholinergic side-effects. Along with RBP-8000's positive clinical profiles, this study provides additional support in continued development of RBP-8000 as an enzyme therapy for cocaine overdose.

Role of funding source

This work was supported by the National Science Foundation of China (Grant No. 81803417, 81730108, 21606062), Key Project of Zhejiang Province Ministry of Science and Technology (Grant No. 2015C03055), and National Science Foundation of Zhejiang (Grant No. LQ15C020003).

Contributors

S. Hou designed the study, performed experiments, interpreted data, and wrote and revised the manuscript for publication. Y. Zhang performed the experiments, generated data and analyzed data. Y. Zhu, C. Zhang, Y. Kong and X. Chen provided help in the protocol design, experiment execution and method validation. R. Chen and X. Yin provided with study reagents and instrumentation. T. Xie contributed to project guidance and acquisition of the financial support. X. Chen contributed to study design, data analysis, and interpretation of results, editing of the manuscript and funding acquisition. All authors have approved the final article for publication.

Additional information

All the authors declare that RBP-8000 is held by Indivior, that there was no support from the holder of the patent for the product for the research done, and that RBP-8000 was not supplied by a company.

Declaration of Competing Interest

All the authors declare that there are no conflicts of interest.

References

Bartels, C.F., Xie, W., Miller-Lindholm, A.K., Schopfer, L.M., Lockridge, O., 2000. Determination of the DNA sequences of acetylcholinesterase and butyrylcholinesterase from cat and demonstration of the existence of both in cat plasma. Biochem. Pharmacol. 60, 479–487.

Brim, R.L., Noon, K.R., Collins, G.T., Nichols, J., Narasimhan, D., Sunahara, R.K., Woods, J.H., 2011. The ability of bacterial cocaine esterase to hydrolyze cocaine metabolites and their simultaneous quantification using high-performance liquid chromatography-tandem mass spectrometry. Mol. Pharmacol. 80, 1119–1127.

Brody, S.L., Slovis, C.M., Wrenn, K.D., 1990. Cocaine-related medical problems: consecutive series of 233 patients. Am. J. Med. 88, 325–331.

Cohen-Barak, O., Wildeman, J., van de Wetering, J., Hettinga, J., Schuilenga-Hut, P., Gross, A., Clark, S., Bassan, M., Gilgun-Sherki, Y., Mendzelevski, B., Spiegelstein, O., 2015. Safety, pharmacokinetics, and pharmacodynamics of TV-1380, a novel mutated butyrylcholinesterase treatment for cocaine addiction, after single and multiple intramuscular injections in healthy subjects. J. Clin. Pharmacol. 55, 573–583.

Collins, G.T., Zaks, M.E., Cunningham, A.R., St. Clair, C., Nichols, J., Narasimhan, D., Ko, M.-C., Sunahara, R.K., Woods, J.H., 2011. Effects of a long-acting mutant bacterial cocaine esterase on acute cocaine toxicity in rats. Drug Alcohol Depend. 118, 158–165.

English, B.A., Jones, C.K., 2012. Chapter 14 - cholinergic neurotransmission. In: Robertson, D., Biaggioni, I., Burnstock, G., Low, P.A., Paton, J.F.R. (Eds.), Primer on the Autonomic Nervous System (Third Edition). Academic Press, San Diego, pp. 71–74.

Fang, L., Chow, K.M., Hou, S., Xue, L., Chen, X., Rodgers, D.W., Zheng, F., Zhan, C.-G., 2014. Rational design, preparation, and characterization of a therapeutic enzyme mutant with improved stability and function for cocaine detoxification. ACS Chem. Biol. 9, 1764–1772.

Heard, K., Palmer, R., Zahniser, N.R., 2008. Mechanisms of acute cocaine toxicity. Open Pharmacol. J. 2, 70–78.

Hou, S., Xue, L., Yang, W., Fang, L., Zheng, F., Zhan, C.-G., 2013. Substrate selectivity of high-activity mutants of human butyrylcholinesterase. Org. Biomol. Chem. 11, 7477–7485.

Howell, L.L., Nye, J.A., Stehouwer, J.S., Voll, R.J., Mun, J., Narasimhan, D., Nichols, J., Sunahara, R., Goodman, M.M., Carroll, F.I., Woods, J.H., 2014. A thermostable bacterial cocaine esterase rapidly eliminates cocaine from brain in nonhuman primates. Transl. Psychiatry 4, e407.

Li, B., Stribley, J.A., Ticu, A., Xie, W., Schopfer, L.M., Hammond, P., Brimijoin, S., Hinrichs, S.H., Lockridge, O., 2000. Abundant tissue butyrylcholinesterase and its possible function in the acetylcholinesterase knockout mouse. J. Neurochem. 75, 1320–1331.

Masson, P., Lockridge, O., 2010. Butyrylcholinesterase for protection from organophosphorus poisons: catalytic complexities and hysteretic behavior. Arch. Biochem. Biophys. 494, 107–120.

McCord, J., Jneid, H., Hollander Judd, E., de Lemos James, A., Cercek, B., Hsue, P., Gibler, W.B., Ohman, E.M., Drew, B., Philippides, G., Newby, L.K., 2008. Management of cocaine-associated chest pain and myocardial infarction. Circulation 117, 1897–1907.

Narasimhan, D., Woods, J.H., Sunahara, R.K., 2012. Bacterial cocaine esterase: a protein-based therapy for cocaine overdose and addiction. Future Med. Chem. 4, 137–150.

Nasser, A.F., Fudala, P.J., Zheng, B., Liu, Y., Heidbreder, C., 2014. A randomized, double-blind, placebo-controlled trial of RBP-8000 in cocaine abusers: Pharmacokinetic profile of RBP-8000 and cocaine and effects of RBP-8000 on cocaine-induced physiological effects. J. Addict. Dis. 33, 289–302.

Pan, Y., Gao, D., Yang, W., Cho, H., Yang, G., Tai, H.-H., Zhan, C.-G., 2005. Computational redesign of human butyrylcholinesterase for anticocaine medication. Proc. Natl. Acad. Sci. U. S. A. 102, 16656–16661.

Rogers, C.J., Eubanks, L.M., Dickerson, T.J., Janda, K.D., 2006. Unexpected acetylcholinesterase activity of cocaine esterases. J. Am. Chem. Soc. 128, 15364–15365.

Rogers, C.J., Mee, J.M., Kaufmann, G.F., Dickerson, T.J., Janda, K.D., 2005. Towards cocaine esterase therapeutics. J. Am. Chem. Soc. 127, 10016–10017.

SAMHSA, 2013. The DAWN Report: Highlights of the 2011 Drug Abuse Warning Network (DAWN) Findings on Drug-related Emergency Department Visits. Accessed on March 20 2019. https://www.samhsa.gov/data/report/dawn-report-highlights-2011-drug-abuse-warning-network-dawn-findings-drug-related-emergency.

UNODC, 2018. World Drug Report 2018. Accessed on March 20 2019. https://www.unodc.org/wdr2018/.

Wolfenden, R., Yuan, Y., 2011. The "neutral" hydrolysis of simple carboxylic esters in water and the rate enhancements produced by acetylcholinesterase and other carboxylic acid esterases. J. Am. Chem. Soc. 133, 13821–13823.

Worek, F., Eyer, P., Thiermann, H., 2012. Determination of acetylcholinesterase activity by the Ellman assay: a versatile tool for in vitro research on medical countermeasures against organophosphate poisoning. Drug Test. Anal. 4, 282–291.

Xue, L., Ko, M.-C., Tong, M., Yang, W., Hou, S., Fang, L., Liu, J., Zheng, F., Woods, J.H., Tai, H.-H., Zhan, C.-G., 2011. Design, preparation, and characterization of high-activity mutants of human butyrylcholinesterase specific for detoxification of cocaine. Mol. Pharmacol. 79, 290–297.

Zhang, T., Zheng, X., Zhou, Z., Chen, X., Jin, Z., Deng, J., Zhan, C.-G., Zheng, F., 2017. Clinical potential of an enzyme-based novel therapy for cocaine overdose. Sci. Rep. 7, 15303.

Zheng, F., Zhan, C.-G., 2011. Enzyme-therapy approaches for the treatment of drug overdose and addiction. Future Med. Chem. 3, 9–13.

Zheng, F., Zhan, C.-G., 2012. Are pharmacokinetic approaches feasible for treatment of cocaine addiction and overdose? Future Med. Chem. 4, 125–128.