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Development of a Long-acting Fc-fused Cocaine Hydrolase with Improved Yield of Protein Expression

Xiabin Chen, Jing Deng, Xirong Zheng, Jinling Zhang, Ziyuan Zhou, Huimei Wei, Chang-Guo Zhan*, and Fang Zheng*

Molecular Modeling and Biopharmaceutical Center and Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, 789 South Limestone Street, Lexington, KY 40536

Abstract

Human butyrylcholinesterase (BChE) is known as a safe and effective protein for detoxification of organophosphorus (OP) nerve agents. Its rationally designed mutants with considerably improved catalytic activity against cocaine, known as cocaine hydrolases (CocHs), are recognized as the most promising drug candidates for the treatment of cocaine abuse. However, it is a grand challenge to efficiently produce active recombinant BChE and CocHs with a sufficiently long biological half-life. In the present study, starting from a promising CocH, known as CocH3 (*i.e.* A199S/F227A/S287G/A328W/Y332G mutant of human BChE), which has a ~2000-fold improved catalytic activity against cocaine compared to wild-type BChE, we designed an N-terminal fusion protein, Fc(M3)-(PAPAP)₂-CocH3, which was constructed by fusing Fc of human IgG1 to the N-terminal of CocH3 and further optimized by inserting a linker between the two protein domains. Without lowering the enzyme activity, Fc(M3)-(PAPAP)₂-CocH3 expressed in Chinese hamster ovary (CHO) cells has not only a long biological half-life of 105 ± 7 hours in rats, but also a high yield of protein expression. Particularly, Fc(M3)-(PAPAP)₂-CocH3 has a ~21-fold increased protein expression yield in CHO cells compared to CocH3 under the same experimental conditions. Given the observations that Fc(M3)-(PAPAP)₂-CocH3 has not only a high catalytic activity against cocaine and a long biological half-life, but also a high yield of protein expression, this new protein entity reported in this study would be a more promising candidate for therapeutic treatment of cocaine overdose and addiction.

Keywords

Protein engineering; protein production; butyrylcholinesterase; cocaine hydrolase

Correspondence to: Chang-Guo Zhan, Ph.D., Director, Molecular Modeling and Biopharmaceutical Center (MMBC), Director, Chemoinformatics and Drug Design Core of CPRI, Endowed College of Pharmacy Professor in Pharmaceutical Sciences, Professor, Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, 789 South Limestone Street, Lexington, KY 40536, Phone: 859-323-3943, FAX: 859-257-7585, zhan@uky.edu. *To whom correspondence should be addressed. zhan@uky.edu (C.-G.Z.) or fzhen2@email.uky.edu (F.Z.).

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Additional Information

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INTRODUCTION

Human plasma butyrylcholinesterase (BChE) has a long history of clinical application, without any adverse events reported [1]. Two clinical trials (NCT00333515 and NCT00333528) of BChE protein were performed by Baxter Healthcare Corporation, showing that recombinant human BChE is also safe for use in humans. It has been well known that BChE can intercept and destroy the organophosphorus (OP) nerve poisons before they reach their target – acetylcholinesterase (AChE) [1–3]. Thus, administration of BChE is recognized as an effective and safe medication for the prevention of organophosphorus (OP) nerve agent toxicity [3–6]. Because of the stoichiometric binding of BChE with OP nerve agent, a large amount of BChE protein is required to achieve its nerve protective effects *in vivo*. Thus, without an efficient BChE expression method, the clinical application of BChE is severely impeded by its actual availability, since the quantity of BChE protein purified from human plasma is very limited. Hence, it is highly desired to develop methods that can be used to efficiently produce BChE in a large-scale for further preclinical and clinical development.

Another driven force to solve this protein production problem comes from the potential application of mutant BChE for treatment of cocaine abuse. BChE is a major metabolic enzyme that catalyzes the hydrolysis of cocaine to produce biologically inactive metabolites. Unfortunately, the catalytic efficiency (k_{cat}/K_M) of wild-type BChE against naturally occurring (-)-cocaine is too low ($k_{\text{cat}} = 4.1 \text{ min}^{-1}$ and $K_M = 4.5 \text{ }\mu\text{M}$) [7] to be effective for accelerating cocaine metabolism. Through structure and mechanism based computational design and wet experimental tests, a series of human BChE mutants with significantly improved catalytic efficiency against cocaine have been designed and discovered [7–12]. These BChE mutants have been recognized as *true* cocaine hydrolases (CocHs) in literature [8] when they have at least 1,000-fold improved catalytic efficiency against (-)-cocaine compared to wild-type human BChE [9–12]. The CocH-based approach has been recognized as a truly promising strategy for treatment of cocaine overdose and addiction [8, 13–15]. Thus, it is critical for further preclinical and clinical development towards the actual use of a BChE mutant in clinical practice to improve the protein production efficiency of the BChE and its mutants.

In fact, extensive efforts have been made to improve the protein production, with the goal to economically produce recombinant human BChE or BChE mutants. Expression in bacteria is recognized as the most economical method for producing recombinant proteins, but wild-type BChE expressed in bacteria cannot fold appropriately to become an active enzyme [16]. BChE proteins expressed in silkworm and insect cells were proven to be active [17, 18], but their pharmacokinetic profiles have not been characterized. Transgenic plants and animals were also generated to produce BChE or CocHs with a significantly improved efficiency, but the proteins produced usually have significantly shorter biological half-lives [19–22]. The short biological half-life is mainly explained by possibly incomplete post-translational modification causing the BChE or CocH to be taken up by asialo receptors in the liver [1]. Compared to all the expression systems above, CHO (Chinese-hamster ovary) cells provide more consistently proper protein post-translational modification [23]. Considering that the improper post-translational modification would not only shorten the protein's biological

half-life, but also increase the risk of immunogenicity as an improper glycan structure might cause the protein to be recognized as an immunogen [1], CHO cells might be the most appropriate system to produce the desirably safe and effective BChE (or BChE mutant) with a relatively long biological half-life. However, the biological half-life of the recombinant BChE or mutant produced in CHO [19–22, 24, 25] is still much shorter than that of native BChE. For example, Coch3 produced in CHO cells has a biological half-life of 7.3 hr in rats, which is considerably longer than that (~13 min) of Coch3 expressed in plants [19, 24], but it is still much shorter than that (43 hr) of native BChE [26]. In addition, the low expression yield of BChE or its mutant in CHO cells is another major problem.

The present study was aimed to develop a fusion protein entity of Coch3 which has not only a long biological half-life, but also a significantly improved yield of protein production. Our design was based on a few considerations. First, using a protein that is normally expressed in a high level as the N-terminal fusion partner to improve the expression of protein of interest [27, 28]. The N-terminal fusion partner may “fool” the cellular process to express the fusion protein at a high level [28]. Second, human IgG has a very long biological half-life ($t_{1/2}$). The fragment crystallizable (Fc) region of IgG binds to the neonatal Fc receptor (FcRn) in the acidic environment of the endosome and later is transported to the cell surface where, upon exposure to a neutral pH, IgG is released back into the main bloodstream [29]. In addition, IgG is the most common type of antibody found in the circulation, and can be expressed in CHO cells with a yield of more than 1 g/L [30]. We aimed to design a long-acting Coch form which has not only a prolonged biological half-life without affecting the catalytic activity, but also an improved expression level in CHO cells. For this purpose, starting from Coch3 (the A199S/F227A/S287G/A328W/Y332G mutant [9] of human BChE), a IL-2 signal peptide followed by Fc(M3) (the A1V/D142E/L144M mutant [31] of Fc) which was fused with the N-terminal of Coch3. Then the tetramerization domain (amino-acid residues 530 to 574) of Coch3 was deleted to minimize the possibility of affecting the correct folding of Fc(M3) or Coch3. On the other hand, the presence of Fc(M3) might break the tetramer structure, resulting in a long and flexible peptide, which could be proteolyzed easily. In addition, according to computational modeling (Yuan, Zheng, and Zhan et al., unpublished data), directly fusing Fc(M3) with the N-terminal of Coch3 could affect the entrance of substrate to the active site of Coch3, thus affecting the catalytic activity of Coch3. Hence, several types of linkers were selected and inserted between Fc(M3) and Coch3. In this way, various Fc(M3)-linker-Coch3 entities were prepared and tested for their catalytic activity against cocaine, protein expression yields in CHO cells, and pharmacokinetic profile (for the most promising entity), leading to identification of a promising Fc(M3)-linker-Coch3 entity, as discussed below.

MATERIALS AND METHODS

Materials

Q5® Site-Directed Mutagenesis Kit was ordered from New England Biolabs (Ipswich, MA). All oligonucleotides were synthesized by Eurofins MWG Operon (Huntsville, AL). Chinese Hamster Ovary-suspension (CHO-S) cells, FreeStyle™ CHO Expression Medium, Fetal Bovine Serum (FBS), 4-12% Tris-Glycine Mini Protein Gel, and SimpleBlue SafeStain were

obtained from Invitrogen (Grand Island, NY). TransIT-PRO[®] Transfection Kit was purchased from Mirus (Madison, WI). The rmp Protein A Sepharose Fast Flow was from GE Healthcare Life Sciences (Pittsburgh, PA). (–)-Cocaine was provided by the National Institute on Drug Abuse (NIDA) Drug Supply Program (Bethesda, MD); and [³H](–)-Cocaine (50 Ci/mmol) was obtained from PerkinElmer (Waltham, Massachusetts). All other materials were from Sigma-Aldrich (St Louis, MO) or Thermo Fisher Scientific (Waltham, MA).

Preparation of gene fusion constructs in pCMV-MCS

Q5[®] Site-Directed Mutagenesis Kit was used to introduce each linker between Fc(M3) and Coch3. The pCMV-Fc(M3)-Coch3, constructed in our previous study [32] to encode N-terminal Fc-fused Coch3 without a linker, was used as the template. PCR reactions with Q5 hot start high-fidelity DNA polymerase along with primers listed in Table 1 were utilized to create insertions. Then 1 µl of each PCR product was incubated with Kinase-Ligase-DpnI enzyme mix for 15 minutes at room temperature. These steps allowed for rapid circulation of the PCR product and removal of the template DNA. 5 µl of final product was added to 50 µl of chemically-competent *E. coli* cells for transformation. All obtained plasmid encoding different Fc-fused Coch3 were confirmed by DNA sequencing.

Expression and purification

CHO-S cells were grown under the condition of 37°C and 8% CO₂ in a humidified atmosphere. Once cells grown to a density of $\sim 1.0 \times 10^6$ cells/ml, cells were transfected with plasmids encoding various proteins using TransIT-PRO[®] transfection kit. The culture medium was harvested 7 days after transfection. Enzyme secreted in the culture medium was purified by protein A affinity chromatography described previously [31]. Briefly, pre-equilibrated rmp Protein A Sepharose Fast Flow was mixed with cell-free medium, and incubated overnight at 6°C with occasional stirring. Then the suspension was packed in a column, washed with 20 mM Tris-HCl (pH 7.4), and eluted by adjustment of salt concentration and pH. The eluate was concentrated and dialyzed in storage buffer (50 mM HEPES, 20% sorbitol, 1 M glycine, pH 7.4). Purified proteins were analyzed by native PAGE electrophoresis.

In vitro activity assay against (–)-cocaine

A radiometric assay based on toluene extraction of [³H](–)-cocaine labeled on its benzene ring was used to determine the catalytic activity of proteins [9, 11, 33]. Reactions were initiated by adding 150 µl enzyme solution (100 mM phosphate buffer, pH 7.4) to 50 µl [³H](–)-cocaine solution with varying concentration. Then 200 µl of 0.1 M HCl was added to stop each reaction and neutralize 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. Catalytic rate constant (k_{cat}) and Michaelis–Menten constant (K_m) were determined by fitting the substrate concentration-dependent data using Michaelis–Menten kinetics.

Determination of relative expression level of proteins

Cells were grown in 12-well plates to a density of $\sim 1.0 \times 10^6$ cells/ml. Then cells were transfected with plasmids encoding different proteins using the same method described above. The test was tripled for each protein, occupying 3 out of 12 wells in a plate. Medium was collected from each well 3 days post the transfection. Cells were removed by centrifuge at 4000 rpm for 15 min, and the catalytic activity of each sample against cocaine was determined using radiometric assay described above. Protein concentration was calculated by dividing the catalytic activity by the k_{cat} (determined by using the aforementioned purified protein) for each specific protein.

Determination of Biological half-life in Rats

Male Sprague-Darley rats (220–250 g) were ordered from Harlan (Harlan, Indianapolis, IN), and housed initially as one or two rats per cage. All rats were allowed ad libitum access to food and water and maintained on a 12 h light/12 h dark cycle, with the lights on at 8:00 a.m. at a room temperature of 21–22°C. Experiments were performed in a same colony room in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. The animal procedure was approved by the IACUC (Institutional Animal Care and Use Committee) as part of the animal protocol 2010-0722 on June 21, 2016 at the University of Kentucky. Rats were injected with the purified Fc(M3)-(PAPAP)₂-CocH3 protein *via* tail vein (0.075 mg/kg). Blood samples were collected from saphenous vein puncture. Approximately 100 μ l blood was collected by using heparin-treated capillary tube at various time points after enzyme administration. Collected samples were centrifuged for 15 min at a speed of 5000 *g* to separate the plasma, which was kept at 4°C before analysis. Radiometric assay using 100 μ M (–)-cocaine was carried out to measure the active enzyme concentration in plasma.

RESULTS AND DISCUSSION

Optimization of Fc-fused CocH3 entity with a linker

Four different linkers, including a flexible linker (GGGSGGGG) and three rigid linkers (EAAAK, PAPAP, and PAPAPPAPAP), were utilized in this study. Previous studies reported in literature indicated that a linker similar to these could separate carrier protein and functional protein effectively and lead to improved biological activity of the fusion proteins with a linker. The four fusion proteins (see Fig. 1) with various linkers were then expressed, purified, and characterized for their catalytic activity against cocaine by performing the sensitive radiometric assay using [³H](–)-cocaine with various concentrations. All of the fusion proteins have a size ~ 210 kDa (Fig. 2), which is consistent with the expected dimeric structure with disulfide bonds formed on the Fc part. CocH3, which was produced predominantly in a tetramer form in our previous study [9] has a k_{cat} of 5700 min^{–1}; however, the fusion protein, Fc(M3)-CocH3, which was constructed without any linker in our previous study [32], exhibited only $\sim 30\%$ catalytic activity against cocaine as compared to the corresponding unfused CocH3.

To optimize the construct of Fc(M3)-CocH3, we used the above mentioned four linkers to eliminate the negative effects of N-terminal Fc portion on the catalytic activity of C-terminal

CochH3. As seen in Table 2 and Fig. 3, with the insertion of a flexible linker (G3S)₂, CochH3(529)-(G3S)₂-Fc(M3) has a k_{cat} of 3579 min⁻¹ which was 2-fold higher than that of the fusion protein without a linker. However, the k_{cat} value of CochH3(529)-(G3S)₂-Fc(M3) was still lower than that of the corresponding unfused CochH3 (3579 min⁻¹ compared to 5700 min⁻¹). Nevertheless, Fc(M3)-EAAAK-CochH3, Fc(M3)-PAPAP-CochH3, and Fc(M3)-(PAPAP)₂-CochH3 all have a catalytic activity comparable to the k_{cat} of the unfused CochH3. The k_{cat} values of Fc(M3)-EAAAK-CochH3, Fc(M3)-PAPAP-CochH3, and Fc(M3)-(PAPAP)₂-CochH3 are 5287, 6078, and 5270 min⁻¹, respectively. As a rigid linker used in these fusion proteins provides enough space between the Fc(M3) and CochH3 domains, it is likely that these two protein domains can be fold correctly without affecting each other in terms of their functions, including the entrance to active site of CochH3 for cocaine. We did not test the use of any further longer linker in this study for a couple of reasons. First, the currently designed linker could provide enough space for the two protein domains, thus fusing the Fc(M3) is not expected to affect the catalytic activity of CochH3 against cocaine. Further, a further longer linker between CochH3 and Fc could be proteolyzed easily and, thus, could shorten the biological half-life of the fusion protein [31].

Effects of the linker on the expression of Fc(M3)-fused CochH3 protein

Fc(M3)-EAAAK-CochH3, Fc(M3)-PAPAP-CochH3, and Fc(M3)-(PAPAP)₂-CochH3 were further expressed together with Fc(M3)-CochH3 and the unfused CochH3 for comparison of relevant protein expression levels. All five proteins were expressed in the same plate under the same conditions at the same time. Cells in each well transfected using the same method and cultured under the same conditions after the transfection. All media were collected 3 days after the transfection. Protein expression level in each well was determined using the radiometric assay using 100 μM [³H](–)-cocaine. As seen in Table 3, the expression of the unfused CochH3 was 0.5 mg/L 3 days after the transient transfection. Usually, inserting the Fc portion at the N-terminal of the target protein could significantly improve the protein expression level. In this study, directly fusing Fc to the N-terminal of CochH3 increased the protein expression level by ~2-fold. However, as Fc(M3)-CochH3 protein had only ~30% catalytic activity against cocaine as compared to the unfused CochH3 [32]. As Fc(M3) domain sterically interferes with the CochH3 domain activity and lowers its catalytic activity against cocaine, it is also possible that this steric interference affects the efficiency of the protein folding. Therefore, an appropriate linker capable of avoiding such steric interference may not only improve the catalytic activity against cocaine, but also increase the protein expression level. As shown in Table 3, the protein expression yields of Fc(M3)-EAAAK-CochH3 and Fc(M3)-PAPAP-CochH3 was 4.8, and 5.2 mg/L, respectively. Linkers EAAAK, and PAPAP improved the yield of Fc(M3)-CochH3 protein expression by ~9 and ~10 folds, respectively. Among all fusion proteins constructed in this study, Fc(M3)-(PAPAP)₂-CochH3 has the highest protein expression yield. The linker (PAPAPPAPAP) increased the yield of protein expression by ~10 fold compared to the corresponding fusion protein without a linker. Further, compared to the corresponding unfused protein (CochH3), Fc(M3)-(PAPAP)₂-CochH3 had a ~21-fold improved yield of protein expression.

It should be pointed out that the transient expression method (with the protein expression within only three days) in this study was used only for the purpose of comparing the relative

expression levels of various fusion proteins and unfused protein under the same conditions. So, the key results of this study are the relative protein expression levels, rather than the absolute protein expression levels. The absolute protein expression levels are expected to significantly increase when the stable CHO cell lines are developed and used to express the same proteins; of course, development of a stable cell line is a very time-consuming process. For example, using a lentivirus-based repeated-transduction method which was established in our previous study [24], the protein expression yield of the unfused Coch3 reached ~10 mg/L in a flask-based culture. Thus, one would reasonably expect that an appropriately developed stable CHO cell line might be able to express ~200 mg/L Fc(M3)-(PAPAP)₂-Coch3 protein by using the same lentivirus-based repeated-transduction method. The protein expression yield could be improved further by optimizing of the culture conditions, such as cell density, medium, and culture temperature.

Biological half-life of Fc(M3)-(PAPAP)₂-Coch3 in Rats

Pharmacokinetic testing was carried out to determine biological half-life of Fc(M3)-(PAPAP)₂-Coch3. Rats (n = 3) were administered IV with 0.075 mg/kg of the purified protein. The blood was collected at 1 hr, 4 hr, 8 hr, 12 hr, 1 day and once each day within 14 days after the enzyme injection. Depicted in Fig. 4 is the time course of the percentage of the enzyme activity remained after enzyme injection. The time-dependent data were fitted to a double-exponential equation ($[E]_t = Ae^{-k_1t} + Be^{-k_2t}$) by using the GraphPad Prism 6 software. For comparison, summarized in Table 4 are the biological half-lives (in mice/rats) of the native BChE (purified from human plasma) and recombinant BChE, BChE mutant, and Coch3-Fc(M3) produced using various methods. The biological half-life of Fc(M3)-(PAPAP)₂-Coch3 is 105 ± 7 hr, which is ~6.6-fold longer than the biological half-life of recombinant BChE or mutant produced using the same method, ~39-fold longer than the half-life of BChE produced in transgenic goat, and ~525-fold longer than the BChE mutant produced in transgenic plant. Even compared to native human BChE, Fc(M3)-(PAPAP)₂-Coch3 protein produced in CHO cells has a ~2.4-fold prolonged biological half-life. In our previously reported study [31], we demonstrated that a novel Coch form, *i.e.* a C-terminal Fc-fused Coch3, known as Coch3-Fc(M3), had a biological half-life of 107 ± 6 hr in rats. But Coch3-Fc(M3) was expressed at a yield of ~2.1 mg/L in CHO cells under the similar conditions. The Fc(M3)-(PAPAP)₂-Coch3 reported in the current study has a similarly long half-life (105 ± 7 hr vs 107 ± 6 hr) compared to Coch3-Fc(M3) but with a significantly improved protein expression yield.

Our previously reported study [31] demonstrated that a single injection of Coch-Fc(M3) was able to accelerate cocaine metabolism in rats after 20 days and, thus, block cocaine-induced physiological and toxic effects for a long period [31]. The Coch3-Fc(M3) protein was expected to allow dosing once every 2-4 wk, or longer, for treating cocaine addiction in humans. Given the facts that Fc(M3)-(PAPAP)₂-Coch3 has the similarly long biological half-life in rats and same catalytic activity against cocaine, it is reasonable to expect that Fc(M3)-(PAPAP)₂-Coch3 may also be able to provide the similar efficacy and duration for the cocaine addiction treatment.

CONCLUSION

It has been a grand challenge to efficiently express BChE or its mutants with both a long biological half-life comparable to the native BChE purified from human plasma and a high yield of protein expression. In this study, we have demonstrated that Fc(M3)-(PAPAP)₂-CocH3 has not only a long biological half-life (*i.e.* $\sim 105 \pm 7$ hr in rats), but also a ~ 21 -fold improved yield of protein expression compared to CocH3. The same strategy used in the present study could also be applied to efficient production of wild-type BChE with both an extended biological half-life and improved yield of protein expression. So, Fc(M3)-(PAPAP)₂-CocH3 may serve a more economically favorable therapeutic candidate for treatment of cocaine overdose and addiction in comparison with CocH3-Fc(M3). The insights obtained in this study may also be valuable in guiding development of other fusion proteins used for different therapeutic purposes, such as treatment and prevention of OP nerve agent exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Research Highlights

- CocH3 fusion with Fc of IgG1 has improved the biological half-life.
- A linker between Fc(M3) and CocH3 can improve protein expression yield.
- A rigid linker is more effective than a flexible linker in protein expression.
- The new fusion protein entity is a more promising therapeutic candidate.

Figure 1.

The four Fc-fused protein with various linkers in comparison with the non-linked Fc(M3)-Coch3. The small grey box represents the IL-2 secretion signal peptide; the black box represents the sequence of Fc(M3); and the white box represents the truncated Coch3.

Figure 2. Coomassie-Blue stained native electrophoresis gel of the purified fusion proteins, showing that the native structures of all fusion proteins exist in a dimer. L3: Fc(M3)-G₆S-CocH3.

Figure 3.
Kinetic data obtained *in vitro* for (-)-cocaine hydrolysis catalyzed by the fusion proteins.
The reaction rate is represented in $\mu\text{M min}^{-1}$ per nM enzyme.

Figure 4. Time-dependent concentration of Fc(M3)-(PAPAP)₂-CocH₃ in the plasma of rats after IV administration of the enzyme (0.075 mg/kg) determined in triplicate.

Table 1.

Primers for inserting various linkers

Linker	Primer name	Primer sequence
EAAAK	EAAAK-F	5'-G TCT CCG GGT AAA GAG GCT GCC GCC AAG GAA GAT GAC ATC A-3'
	EAAAK-R	5'-CTT GGC GGC AGC CTC TTT ACC CGG AGA CAG GGA GAG-3'
PAPAP	PAPAP-F	5'-G TCT CCG GGT AAA CCT GCT CCA GCC CCG GAA GAT GAC ATC A-3'
	PAPAP-R	5'-CGG GGC TGG AGC AGG TTT ACC CGG AGA CAG GGA GAG-3'
GGSGGGS	(G3S) ₂ -F	5'-G TCT CCG GGT AAA GGT GGA GGT TCC GGT GGA GGT TCC GAA GAT GAC ATC A-3'
	(G3S) ₂ -R	5'- GGA ACC TCC ACC GGA ACC TCC ACC TTT ACC CGG AGA CAG GGA GAG-3'
PAPAPPAPAP	(PAPAP) ₂ -F	5'-G TCT CCG GGT AAA CCT GCT CCA GCC CCG CCT GCT CCA GCC CCG GAA GAT GAC ATC A-3'
	(PAPAP) ₂ -R	5'- CGG GGC TGG AGC AGG CGG GGC TGG AGC AGG TTT ACC CGG AGA CAG GGA GAG-3'

Table 2.

Kinetic parameters determined for (–)-cocaine hydrolysis catalysed by the fusion proteins

Protein #	Enzyme	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_M ($\text{min}^{-1} \text{M}^{-1}$)
1 *	Fc(M3)CocH3 (529)	3.9 ± 0.5	1835 ± 61	4.7×10^8
2 *	Fc(M3)-G ₆ S-CocH3	4.4 ± 0.6	5694 ± 238	1.3×10^9
3	Fc(M3)-EAAAK-CocH3(529)	4.3 ± 0.4	5684 ± 148	1.3×10^9
4	Fc(M3)-PAPAP-CocH3(529)	4.2 ± 0.5	6078 ± 202	1.4×10^9
5	Fc(M3)-(G ₃ S) ₂ -CocH3(529)	3.7 ± 0.4	3579 ± 104	9.6×10^8
6	Fc(M3)-(PAPAP) ₂ -CocH3(529)	4.5 ± 0.5	5666 ± 148	1.3×10^9

* The k_{cat} and K_M of the enzymes against (–)-cocaine were reported in ref.[32]

Table 3.

Transient expression levels of fusion proteins, in comparison of CocH3

Enzyme	Expression level (mg/L)	Ratio
CocH3	0.5 ± 0.1	1
Fc(M3)-CocH3	1.1 ± 0.2	2
Fc(M3)-EAAAK-CocH3	4.8 ± 0.6	9
Fc(M3)-PAPAP-CocH3	5.2 ± 0.6	10
Fc(M3)-(PAPAP) ₂ -CocH3	10.9 ± 1.1	21

Table 4.

Summary of biological half-life of BChE or mutants in mice or rats

Protein form	<i>In vivo</i> half-life (h)
BChE purified from human plasma	43 ^a
BChE produced in transgenic goat	2.7 ^b
BChE mutant produced in transgenic plant	0.2 ^c
BChE mutant produced in CHO cells	7.3 ^d
Coch3-Fc(M3) produced in CHO cells	107 ^e
Fc(M3)-(PAPAP) ₂ -Coch3 produced in CHO cells	105

^a Biological half-life of enzyme was reported in ref.[26].^b Biological half-life of enzyme was reported in ref.[21].^c Biological half-life of enzyme was reported in ref.[19].^d Data from ref.[24].^e Data from ref.[31].