Chemico-Biological Interactions 293 (2018) 107–114

Contents lists available at ScienceDirect

Chemico-Biological Interactions

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

Kinetic characterization of cholinesterases and a therapeutically valuable cocaine hydrolase for their catalytic activities against heroin and its metabolite 6-monoacetylmorphine

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A B S T R A C T

As the most popularly abused one of opioids, heroin is actually a prodrug. In the body, heroin is hydrolyzed/activated to 6-monoacetylmorphine (6-MAM) first and then to morphine to produce its toxic and physiological effects. It has been known that heroin hydrolysis to 6-MAM and morphine is accelerated by cholinesterases, including acetylcholinesterase (AChE) and/or butyrylcholinesterase (BChE). However, there has been controversy over the specific catalytic activities and functional significance of the cholinesterases, which requires for the more careful kinetic characterization under the same experimental conditions. Here we report the kinetic characterization of AChE, BChE, and a therapeutically promising cocaine hydrolase (CocH1) for heroin and 6-MAM hydrolyses under the same experimental conditions. It has been demonstrated that AChE and BChE have similar k_{cat} values (2100 and 1840 min^{-1}, respectively) against heroin, but with a large difference in K_M (2170 and 120 μM, respectively). Both AChE and BChE can catalyze 6-MAM hydrolysis to morphine, with relatively lower catalytic efficiency compared to the heroin hydrolysis. CocH1 can also catalyze hydrolysis of heroin (k_{cat} = 2150 min^{-1} and K_M = 245 μM) and 6-MAM (k_{cat} = 0.223 min^{-1} and K_M = 292 μM), with relatively larger K_M values and lower catalytic efficiency compared to BChE. Notably, the K_M values of CocH1 against both heroin and 6-MAM are all much larger than previously reported maximum serum heroin and 6-MAM concentrations observed in heroin users, implying that the heroin use along with cocaine will not drastically affect the catalytic activity of CocH1 against cocaine in the CocH1-based enzyme therapy for cocaine abuse.

1. Introduction

Heroin (3,6-diacetylmorphine) is one of the drugs most commonly co-abused by cocaine-dependent individuals [1–6]. The concurrent use of cocaine and heroin has received increasing clinical attentions because it not only causes more serious morbid psychopathology [7,8] and poor addiction treatment outcomes [9,10], but also considerably increases the risk of severe drug overdose which ends in death [11]. In our previous studies, we designed and discovered high-activity butyrylcholinesterase (BChE) mutants, also known as cocaine hydrolases (CocHs), that can rapidly convert naturally occurring biologically active (−)-cocaine to physiologically inactive metabolites ecgonine methyl ester (EME) and benzoic acid. In particular, the first one of our designed CocHs (denoted as CocH1), *i.e.* the A199S/S287G/A328W/Y332G mutant, demonstrated a ~1000-fold improved catalytic efficiency against (−)-cocaine compared with the wild-type BChE (k_{cat} = 4.1 min^{-1} and K_M = 4.5 μM) [12,13] and its effectiveness as an enzyme or gene therapy for cocaine abuse treatment without significant toxicity in animal experiments [14–17].

Further, CocH1 truncated after amino acid 529 was fused with human serum albumin (HSA) to prolong the biological half-life without changing the catalytic activity of CocH1 against cocaine [18]. The HSA-fused CocH1 (known as Albu-CocH, Albu-CocH1, AlbuBChE or TV-1380 in the literature) has been proven safe and promising for use in animals and humans in preclinical and clinical studies [19,20], but its actual therapeutic value for cocaine addiction treatment is still limited by the insufficiently high catalytic activity for cocaine hydrolysis and the insufficiently long biological half-life which is ~8 h in rats [18] or 43–77 h in humans [19]. Despite of the short biological half-life, the Phase II clinical trial using TV-1380 for cocaine addiction treatment was performed using a once-weekly dosing schedule. Due to the relatively short biological half-life, the Phase II clinical trial did not show

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https://doi.org/10.1016/j.cbi.2018.08.002
Received 26 April 2018; Received in revised form 24 July 2018; Accepted 3 August 2018
Available online 04 August 2018
0009-2797/

statistically significant efficacy of TV-1380 with the once-weekly dosing schedule for cocaine addiction treatment [21]. Nevertheless, it has been concluded that "*Although the continued development of TV-1380 appears unlikely, its promising clinical profile should embolden efforts to develop new enzyme products that are capable of delivering greater catabolic activity*" [21] in order to be effective with the desirable once-weekly dosing schedule for cocaine addiction treatment.

According to our most recently reported studies in various animal models of cocaine overdose treatment using clinically relevant timing for rescue studies, whereas a long-lasting CocH form is required for effective treatment of cocaine addiction using a weekly dosing schedule, Albu-CocH1 (or TV-1380) itself should be more appropriate for cocaine overdose treatment [22,23]. It has been demonstrated that the key to cocaine toxicity treatment is to accelerate cocaine metabolism. Once cocaine is completely converted to EME and benzoic acid, the toxicity of cocaine will be reversed for the subjects [22].

Considering the frequent use of cocaine in combination with heroin by addicts, a question is whether or not cocaine degradation by CocH1 is significantly inhibited by heroin or its metabolites 6-monoacetylmorphine (6-MAM) and morphine. In fact, heroin is quickly converted to 6-MAM and then more slowly to morphine in the circulating system [24–26]. Two cholinesterases, plasma BChE and erythrocyte acetylcholinesterase (AChE), are generally regarded as the principal enzymes involved in both the majority of 6-MAM formation and significant morphine production from heroin. It has been demonstrated that 6-MAM is the prime metabolite responsible for heroin's acute psychoactive effects (the rush) and intoxication, but the euphoria following the rush is more due to the stimulant effects of morphine produced from 6-MAM hydrolysis [27–30], indicating the importance of the rates of 6-MAM formation and degradation in the onset of heroin effects on the central nervous system. At heroin blood concentrations attainable *in vivo* $\leq$ 270 nM[24, 29, 31−32], ~80% of the total heroin hydrolysis in blood is accounted for plasma and erythrocyte cytosol where BChE and AChE are located, respectively [33–35]. *In vitro* enzyme kinetic studies using purified native human cholinesterases further demonstrated that BChE, rather than AChE, is mainly responsible for degradation of heroin to 6-MAM with a higher catalytic efficiency under first-order kinetics [36]. However, there has been controversy over the catalytic activity and functional significance of the cholinesterases (AChE and BChE) on the hydrolysis of 6-MAM to morphine [1,36,37], which makes it difficult to interpret their actual roles in 6-MAM degradation. In addition, the reported values of the kinetic parameters (k_{cat} and K_M) for BChE against heroin ranged from 12.9 to 540 min^{-1} and from 0.11 to 3.5 mM, respectively [1,36,37], requiring for the more careful kinetic characterization under the same experimental conditions.

In the present study, we kinetically compared CHO cell-expressed human recombinant AChE, BChE, and CocH1 with the aims to examine their catalytic efficiencies against heroin and 6-MAM and to assess the possible interaction between cocaine and heroin or 6-MAM in their hydrolysis reactions catalyzed by CocH1 in comparison with human enzymes AChE and BChE. The complete catalytic parameters obtained for AChE, BChE, and CocH1 against heroin and 6-MAM reveal how the abused drugs (cocaine and heroin) can possibly affect each other in terms of their hydrolysis reactions and detoxification under various conditions. The insights from the kinetic characterization will be valuable in guiding further development of novel enzyme therapies for the drug detoxification. In particular, concurrent use of heroin and cocaine is not expected to significantly affect the efficacy of CocH1 (or its fusion protein form TV-1380) in cocaine detoxification.

2. Materials and methods

2.1. Materials

Phusion DNA polymerases, restriction enzymes, and T4 DNA ligase were purchased from New England Biolabs. All oligonucleotides were purchased from Eurofins MWG Operon. Vector pCMV–MCS was obtained from Agilent Technologies. Chinese hamster ovary (CHO)–S cells and FreeStyle™ CHO Expression Medium, hypoxanthine/thymidine (HT) supplement, L-glutamine, 4–12% Tris-glycine Mini Protein Gel, and SimpleBlue SafeStain were purchased from Life Technologies (Carlsbad, CA). Reduction-modified protein (rmp) Protein A Sepharose Fast Flow was ordered from GE Healthcare Life Sciences (Pittsburgh, PA). Centrifugal filter units were ordered from Millipore (Burlington, MA). Heroin, 6-MAM, and morphine were provided by the National Institute on Drug Abuse (NIDA) Drug Supply Program. All other chemicals as well as the solvents used in high-performance liquid chromatography (HPLC), were of HPLC grade and purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Construction of mammalian expression plasmids

CocH1 truncated after amino acid 529 was fused with human serum albumin (HSA) for the extension of biological half-life [38]. For protein expression in mammalian cells, the cDNA for the CocH1 (the A199S/F227A/S287G/A328W mutant of human BChE) containing C-terminal HSA was generated and cloned in to pCMV-MCS in our previous studies [12,13,39]. Two expression plasmids, pCMV-BChE-Fc(WT), and pCMV-AChE-Fc(WT), were constructed as described previously [40]. Briefly, the C-terminal of truncated human enzyme (BChE or AChE) was genetically fused to the N-terminal of the Fc portion of wild-type human IgG (Fc(WT)) by overlapping extension PCR with Phusion DNA polymerase. Then, the PCR products were digested with restriction endonucleases Hind III and Bgl II. The gel purified PCR products were then ligated to the pCMV-MCS expression vector using T4 DNA ligase.

2.3. Protein expression and purification

CHO-S cells were incubated in FreeStyle CHO Expression Medium (Life Technologies) with 8 mM L-glutamine (Life Technologies) at 37 °C in a humidified atmosphere with 8% CO_2 and transfected with gene expression DNA constructs encoding the protein of interest using the TransIT-PRO Transfection Kit (Mirus Bio LLC, Madison, WI) when the number of the cells reached 1.0×10^6 cells/mL. The culture medium was harvested 6 days after transfection. The Fc-fused protein (BChE or AChE) secreted into the culture medium was purified by protein A affinity chromatography. After removing cells by centrifugation, the cell-free culture medium was mixed with rmp Protein A Sepharose Fast Flow (GE Healthcare Life Sciences) pre-equilibrated with 20 mM Tris·HCl (pH 7.4) and incubated for overnight at 6 °C with occasional stirring. Then, the suspension was packed in a column and washed with 5 column volume (CV) of 20 mM Tris·HCl (pH 7.4) until an OD_{280} < 0.02 was achieved; then the protein was eluted by adjustment of the pH and salt concentration. HSA-fused CocH1 was also expressed as described above. Using the AlbuPure matrix (Prometic Life Sciences Inc., Laval, Canada), CocH1-HSA was purified where the cell-free culture medium was loaded onto packed bed pre-equilibrated with 50 mM sodium acetate (pH 5.3), extensively washed with 8 CV of equilibration buffer. Then, the resin bound protein was eluted with 5 CV of 50 mM ammonium acetate, pH 7.4. For buffer exchange, the eluate was dialyzed in storage buffer (50 mM Hepes, 20% sorbitol, 1 M glycine, pH 7.4) by Millipore Centrifugal Filter Units. The entire purification process was performed in a cold room at 8 °C and the purified proteins were stored at −80 °C until the activity tests.

2.4. Enzyme activity assays

Enzymatic hydrolyses of heroin to 6-MAM and 6-MAM to morphine were tested under the following assay conditions. Incubations (50 µl final volume) contained purified enzyme and heroin or 6-monoacetylmorphine (6-MAM) in 0.1 M phosphate buffer, pH 7.4. All the

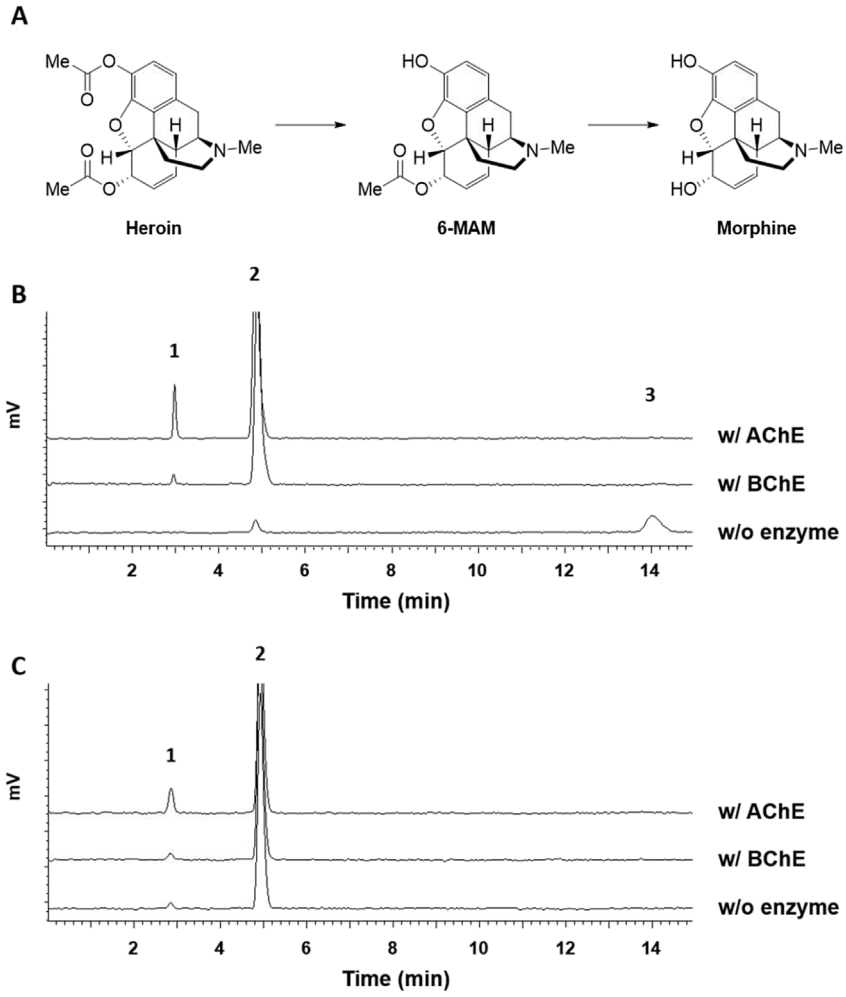

Fig. 1. Enzymatic activity of BChE and AChE on the hydrolysis of heroin and 6-MAM. Schematic presentation of heroin hydrolysis to morphine (A), and chromatograms for the deacetylation of heroin (B) and 6-MAM (C) in the presence or absence of BChE or AChE. Peak 1 (morphine) with retention time 3.8 min, peak 2 (6-MAM) with retention time 4.9 min and peak 3 (heroin) with retention time 14 min. The enzyme (AChE or BChE) was incubated with 1 mM substrate concentration at 4 μM designated enzyme at 37 °C for 25 min. Ten microliters of the incubation supernatant were injected onto HPLC for analysis.

activity assays were performed at 37 °C. For heroin hydrolysis, 0.02–2.5 mM heroin was incubated with 35 nM designated enzyme. For 6-MAM hydrolysis, 0.002–2 mM 6-MAM was incubated with 2 μM designated enzyme. The reaction time and concentration were adjusted such that no more than 10% of substrate was depleted during reaction. The reaction was terminated, and protein was precipitated by the addition of 100 μl of iced 50% acetonitrile/0.5 M hydrochloric acid, followed by 5 min centrifugation at 15,000 g. The resulting supernatants were subjected to reverse-phase HPLC (RP-HPLC) on a 5 μm C18 110 Å column (250 × 4.6 mm; Gemini) (Life Technologies) and RP-HPLC was performed using the mobile phase consisting of 20% acetonitrile in 0.1% TFA. The remaining substrate and resulting products were monitored by a fluorescence detector with an excitation wavelength of 230 nm and emission wavelength of 315 nm and by monitoring UV absorbance at 230 nm. The quantification was based on a standard curve prepared using an authentic standard compound.

2.5. In vitro hydrolysis of cocaine by CocH

Enzymatic hydrolysis of (−)-cocaine by CocH1 was tested under the following assay conditions. A sensitive radiometric assay using [^{3}H](−)-cocaine labelled on its benzene ring was employed to measure (−)-cocaine and benzoic acid, the product of the enzymatic (−)-cocaine hydrolysis [41]. Briefly, 100 nCi of [^{3}H](−)-cocaine was first mixed with heroin, 6-MAM, or water. CocH1 was then added to the mixture so that each incubation (200 μl final volume) finally contained 100 ng/ml CocH1 and 100 μM (−)-cocaine with or without 100 μM heroin or 6-MAM in 0.1 M phosphate buffer, pH 7.4. The enzymatic reactions occurred at 25 °C and stopped at varying time points by the addition of 200 μl of 0.1 M HCl. Since the reaction product benzoic acid is neutralized at strong acidic pH, whereas (−)-cocaine is protonated on its amino nitrogen, the radioactive product was extracted by 1 ml of toluene and measured by scintillation counting. Finally, the measured (−)-cocaine concentration-dependent radiometric data were analyzed to determine the residual concentrations of cocaine after the enzymatic reactions.

2.6. Molecular modelling

Heroin and 6-MAM binding with human AChE, BChE, and CocH1 were modelled by using our previously modelled structures of the same enzymes [42–45]. Our previous molecular dynamics (MD) simulations on the structures of enzyme-substrate complexes started from the X-Ray crystal structures deposited in the protein databank (PDB) (AChE: code 1B41; BChE: 2XQF and 1P0P). Molecular docking and subsequent optimization were carried out using a similar protocol described previously [44]. Briefly, the acetyl group of the substrate (heroin or 6-MAM) was positioned in the oxyanion hole (consisting of Gly116, Gly117, and Ala199 in BChE, or Gly121, Gly122, and Ala204 in AChE, or Gly116, Gly117, and Ser199 in CocH1), and the positively charged

amino-group of the substrate (heroin and 6-MAM) was placed in the choline-binding site near Trp82 in BChE and CocH1 or Trp86 in AChE. Finally, the binding models of heroin and 6-MAM in the corresponding enzyme-substrate complexes were optimized by performing the energy minimization.

2.7. Statistical analysis

Statistical analyses were performed with the GraphPad Prism 5.01 software (San Diego, CA). Comparisons of multiple factors were examined using the two-way ANOVA with post hoc analysis, allowing us to examine the significance of the difference in the *in vivo* activity data between each pair of the reaction systems. $p < 0.05$ was considered statistically significant.

3. Results & discussion

3.1. Morphine formation from heroin in the presence of recombinant human BChE and AChE

Two previously reported studies led to contradictory findings over the ability of BChE to catalyze hydrolysis of 6-MAM to morphine [1,36,37], which limits the interpretation of the data concerning the actual contributions of the enzymes to the drug metabolism to morphine in blood. In 1999, Salmon and his colleagues reported that only AChE, but not BChE, further hydrolyzes 6-MAM to morphine from heroin [36], but these findings are opposite to the previous observations of Kamendulis et al. showing the capability of BChE to catalyze 6-MAM into morphine (k_{cat} = 0.25 min^{-1} and K_M = 8.6 mM) [1]. Therefore, we first tested whether or not heroin is metabolized to 6-MAM and then eventually into morphine by recombinant human BChE or AChE. For each enzyme, 1 mM heroin was incubated with 4 μM enzyme. As shown in Fig. 1B, in the presence of either BChE or AChE, after 25 min of incubation, heroin has completely been converted to 6-MAM, and some 6-MAM has further been converted to morphine. Both BChE and AChE were highly active in metabolizing heroin to 6-MAM, but they were less active in further degrading 6-MAM to morphine. These results clearly show that like AChE, BChE is capable of hydrolyzing heroin to morphine eventually, which are in agreement with the findings of Kamendulis et al. [1], but unlike the observations of Salmon et al. [36]. We also observed that AChE produced more morphine than BChE in the given reaction condition, implying the relatively lower catalytic efficiency of BChE against 6-MAM.

3.2. Hydrolysis of free 6-MAM to morphine by human recombinant BChE and AChE

In a previous report by Salmon et al. [36], it was noted that AChE hydrolyzes 6-MAM only when 6-MAM is produced from heroin within its active site and free 6-MAM molecules only serve as an inhibitor for AChE [36]. This led us to examine whether free 6-MAM molecules can serve as a substrate for BChE or not. To address this question, the enzymatic activity of the enzyme (BChE or AChE) was studied using synthetic 6-MAM which we added to the reaction system. 1 mM 6-MAM was mixed and incubated with either 40 μM enzyme (BChE or AChE) under the incubation condition mentioned above. The results showed that direct incubation of 6-MAM with BChE produced the amount of morphine which is significantly larger than that in the control (without an enzyme), demonstrating that free 6-MAM molecules can serve as a substrate for BChE (Fig. 1C). Interestingly, AChE also converted synthetic 6-MAM to morphine in a significant amount comparable to that of morphine produced from heroin by AChE (Fig. 1B and C). Overall, these observations clearly indicate that both free heroin and 6-MAM molecules produced after heroin uptake in the circulatory system can be metabolized to morphine by both cholinesterases (AChE and BChE) in blood.

3.3. Kinetics of heroin hydrolysis by BChE, AChE, and CocH1

As a potential anti-cocaine medication, CocH1 has a considerably improved catalytic efficiency (k_{cat} = 3060 min^{-1}, K_M = 3.1 μM, and $k_{cat}/K_M = 9.9 \times 10^8\ min^{-1}\ M^{-1}$) compared to the wild-type BChE (k_{cat} = 4.1 min^{-1}, K_M = 4.5 μM, and $k_{cat}/K_M = 9.1 \times 10^5\ min^{-1}\ M^{-1}$) against (−)-cocaine. Thus, CocH1 may be used to effectively block the drug reward for a given dose of cocaine. Considering that CocH1 is developed from human BChE capable of metabolizing all of (−)-cocaine, heroin and its initial host metabolite 6-MAM, (−)-cocaine degradation by CocH1 can be affected by the drug-drug interaction with heroin or 6-MAM. Specifically, if heroin or 6-MAM can also be hydrolyzed by CocH1, we would like to know the K_M or the binding affinity (K_d) of the drug (heroin or 6-MAM) with CocH1 in order to estimate how heroin or 6-MAM could competitively inhibit CocH1 for its catalytic activity against (−)-cocaine. In principle, for a competitive inhibition of an enzyme, the inhibitory constant (K_i) value is equal to the corresponding K_d value ($K_i = K_d$). However, the K_d value can be different from the corresponding K_M value. Nevertheless, $K_d \approx K_M$ value under the well-known rapid equilibrium assumption [46] which is usually true for enzyme-substrate binding. Hence, we may reasonably use an experimentally measured K_M of CocH1 against heroin or 6-MAM to estimate the potential inhibitory activity of heroin or 6-MAM against CocH1-catalyzed hydrolysis of another substrate like (−)-cocaine when $K_i \approx K_M$.

In order to know whether heroin or 6-MAM can significantly inhibit CocH1-catalyzed hydrolysis of (−)-cocaine, we investigated kinetics of heroin degradation to 6-MAM by CocH1, BChE, and AChE. Under the experimental conditions generating the kinetic data depicted in Fig. 2, we only observed the metabolite 6-MAM, and there were no detectable levels of morphine, indicating that the enzyme activity for converting 6-MAM to morphine is much lower than that for converting heroin to 6-MAM. The obtained kinetic data are depicted in Fig. 2, and the kinetic

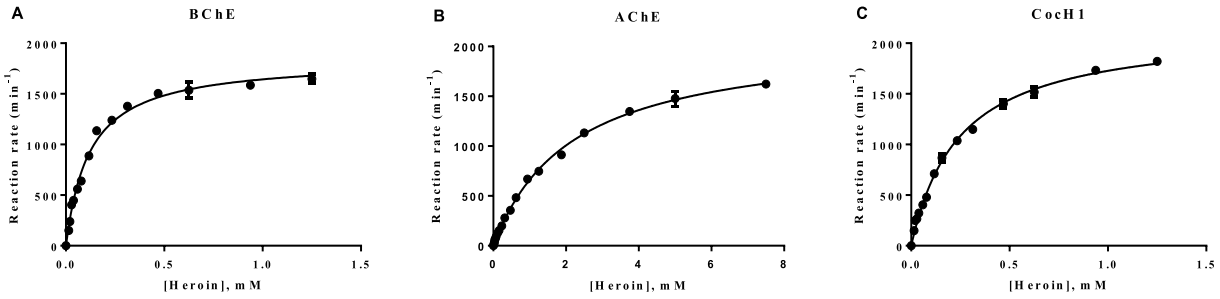

Fig. 2. Kinetic analysis of heroin hydrolysis by BChE, AChE, and CocH1. The hydrolysis of heroin to 6-MAM by BChE (A), AChE (B), and CocH1 (C) were determined at substrate concentrations of 0.015–1.25 mM (BChE and CocH1) or 0.015–7.5 mM (AChE). Kinetic parameters (k_{cat} and K_M) were determined by fitting the measured reaction rate data to the Michaelis-Menten kinetic equation using the Prism5.01 software. Each dot is the representative of the average of triplicates and its values are expressed as the mean ± standard deviation.

Table 1
Kinetic parameters of BChE, AChE, and CocH1 against heroin.

Enzyme	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M ($min^{-1} M^{-1}$)	RCE	R^2
BChE	1840 ± 24	120 ± 5	1.53×10^7	1	0.991
AChE	2100 ± 26	2170 ± 6	9.68×10^5	0.06	0.997
CocH1	2150 ± 31	245 ± 9	8.78×10^6	0.57	0.994

Table 2
Kinetic parameters of BChE, AChE, and CocH1 against 6-MAM.

Enzyme	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M ($min^{-1} M^{-1}$)	RCE	R^2
BChE	0.065 ± 0.001	24 ± 1.4	2.71×10^3	1	0.990
AChE	7.078 ± 0.141	259 ± 18	2.73×10^4	10.1	0.990
CocH1	0.223 ± 0.005	292 ± 22	0.764×10^3	0.282	0.987

parameters obtained are summarized in Table 1. As shown in Table 1, compared to BChE, CocH1 has a higher K_M value (245 μM compared to 120 μM) and a similar k_{cat} value (2150 min^{-1} compared to 1840 min^{-1}). The determined K_M of CocH1 against heroin is ~900-fold larger than the previously reported blood heroin concentrations attainable *in vivo* (≤0.27 μM) [24,29,31,32] and ~76-fold larger than its reported K_M value against (−)-cocaine (3.1 μM) [12,13]. Generally speaking, for a given inhibitor, when the K_i value is ~900-fold larger than the inhibitor concentration, the inhibitor can only decrease the enzyme activity by less than ~0.1%, suggesting that the blood heroin levels usually achieved by the heroin users are not expected to significantly change the enzymatic hydrolysis of (−)-cocaine by CocH1. Moreover, as one can see from the kinetic data in Table 1, AChE has ~18-fold larger K_M value (2170 μM) compared to that of BChE, but with a similar k_{cat} value (2100 min^{-1} compared to 1840 min^{-1}), against heroin. These data indicate that the major difference between wild-type AChE and BChE in the catalytic efficiency against heroin ($k_{cat}/K_M = 1.53 \times 10^7 min^{-1} M^{-1}$ for BChE *vs* $k_{cat}/K_M = 9.68 \times 10^5 min^{-1} M^{-1}$ for AChE) is mainly attributed to their difference in the binding affinity with heroin. Our kinetic data strongly support the argument [37,47] that plasma BChE is the prime enzyme responsible for the rapid enzymatic hydrolysis of the 3′-phenolic ester of heroin in the blood.

In this study, our experimental K_M value of BChE against heroin (120 μM) is consistent with the earlier K_M of 110 μM reported by Lockridge et al. [1,36,37] and cited by Salmon et al. [36], but quite different from the number of Kamendulis et al. (3.5 mM) [1,36,37]. The catalytic rate constant (k_{cat} = 1840 min^{-1}) determined is also substantially higher than the wide range (from 12.9 to 540 min^{-1}) reported by those research groups [1,36,37]. Moreover, the kinetic parameter values of AChE determined for the hydrolysis of heroin to 6-MAM (k_{cat} = 2100 min^{-1} and K_M = 2170 μM) are higher than the values (k_{cat} = 351 min^{-1} and K_M = 620 μM) reported by Salmon et al. [36]. It is likely that the differences in the catalytic parameters determined for the same enzymatic reactions are largely dependent on how enzymes are prepared if all of the kinetic assays are all in readily controlled experimental conditions. Generally, natural protein sources, especially from human or animal tissues, have the difficulty to meet the requirements for higher retention of functional properties including their enzymatic activities, mainly due to the complicated collection, treatment, storage, and extraction processes. These processes may affect the protein structure accompanied with a change (usually a decrease) in its binding affinity and activity, and result in inactivation or overall diminished enzymatic activity. Whereas the kinetic studies of Lockridge et al. [1,36,37], Salmon et al. [36], and Kamendulis et al. [1,36,37] were found on the use of natural BChE or AChE extracted from human blood samples, all the kinetic analysis in the present study were performed using the freshly expressed and purified BChE and AChE for comparison. In addition, another external factor affecting the enzymatic reaction kinetics is the temperature. The kinetic studies of Salmon et al. [36], and Kamendulis et al. [1,36,37] were performed at 37 °C, but that of Lockridge et al. [1,36,37] was accomplished at 25 °C. In this study, the same enzymatic kinetic analysis was carried out at 37 °C and the protein samples used this study are shown to have higher binding affinity and catalytic efficiency (reflected by the lower K_M and higher k_{cat}/K_M, respectively). These observations strongly suggest that the kinetic parameters determined in the present study are more likely to reasonably reflect the actual enzymatic activity of human BChE and AChE against heroin.

3.4. Kinetics of 6-MAM hydrolysis by BChE, AChE, and CocH1

As mentioned above, 6-MAM may also potentially inhibit CocH1-catalyzed cocaine hydrolysis because 6-MAM can also serve as a substrate for wild-type BChE (Fig. 1B and C). To access this possibility, we examined the kinetics of 6-MAM degradation to morphine by CocH1 as well as wild-type BChE and AChE. The catalytic parameters k_{cat} and K_M were determined for BChE against 6-MAM, and then were compared with those of AChE and CocH1 (Table 2 and Fig. 3). As seen in Table 2, the K_M value of CocH1 against 6-MAM was determined to be 292 μM which is ~94-fold-larger than the reported K_M value (3.1 μM) of CocH1 against (−)-cocaine. Given that the maximum serum concentration (C_{max}) of 6-MAM in humans has been reported to range from 5.2 to 17.5 μM after intravenous heroin administration [48–50], the K_M (292 μM) of CocH1 against 6-MAM is still ~16–56-fold larger than the C_{max} of 6-MAM achieved by heroin users. In comparison, the observed peak blood (−)-cocaine concentrations were ~3-fold higher than the K_M of CocH1 against (−)-cocaine. Overall, these data suggest that when both 6-MAM and (−)-cocaine reach their corresponding peak concentrations in the blood, CocH1-catalyzed (−)-cocaine hydrolysis can only be inhibited by 6-MAM for ~0.45–1.5%. The lower the 6-MAM concentration, the less the inhibition. The potential inhibition by 6-MAM would not be significant. Hence, CocH1 can still efficiently degrade (−)-cocaine at the 6-MAM concentrations usually achieved by heroin users. According to the kinetic data in Table 2, the determined k_{cat}/K_M value ($2.73 \times 10^4 min^{-1} M^{-1}$) of AChE against 6-MAM was approximately 10 times greater than that ($2.71 \times 10^3 min^{-1} M^{-1}$) of BChE against 6-MAM. Notably, these findings are in agreement with the findings of Kamendulis et al. [1,36,37] in that BChE catalyzes the hydrolysis of 6-MAM into morphine. However, our experimental K_M and k_{cat} values are much different from the corresponding kinetic parameters reported by Kamendulis et al. [1,36,37]. Our experimental K_M of 24 μM is considerably smaller than their K_M of 8.6 mM and our determined catalytic rate constant (k_{cat} = 0.065 min^{-1}) is also much different from the earlier k_{cat} of 0.25 min^{-1}. Accounting for all of the kinetic parameters, the catalytic efficiency ($k_{cat}/K_M = 2.71 \times 10^3 min^{-1} M^{-1}$) obtained for the same enzymatic hydrolysis in the present study is ~93-fold larger than that ($k_{cat}/K_M = 2.91 \times 10^1 min^{-1} M^{-1}$) reported by Kamendulis et al. [1,36,37]. As mentioned above, the differences in the catalytic parameters determined for the same enzymatic reaction seem to largely rely on how BChE is prepared for the kinetic assay. Compared to our kinetic analysis using the freshly expressed and purified BChE, the previous kinetic analysis by Kamendulis et al. [1,36,37] was based on the use of natural BChE extracted from human plasma samples.

Overall, all of our experimental kinetic data strongly suggest that BChE and AChE play distinct roles in heroin metabolism into morphine. BChE catalyzes hydrolysis of heroin to 6-MAM with a much higher catalytic efficiency than AChE. For the further degradation of 6-MAM to morphine, BChE has a relatively lower catalytic efficiency than AChE.

Further, we tested whether (−)-cocaine degradation by CocH1 will be affected by the drug-drug interaction when heroin or 6-MAM is present in the reaction system. According to the results obtained depicted in Fig. 4, (−)-cocaine degradation by CocH1 was not significantly changed in the presence of even an abnormally high

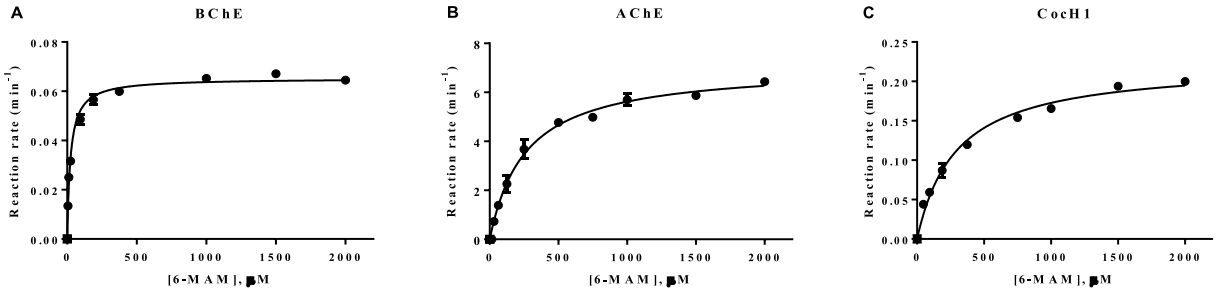

Fig. 3. Kinetic analysis of 6-MAM hydrolysis by BChE, AChE, and CocH1. The hydrolysis of heroin to 6-MAM by BChE (A), AChE (B), and CocH1 (C) were determined at substrate concentrations of 5–2000 μM. Kinetic parameters (k_{cat} and K_M) were determined by fitting the generated reaction rate data to the Michaelis-Menten kinetic equation using the Prism5.01 software. Each dot is the representative of the average of triplicates and its values are expressed as the mean ± standard deviation.

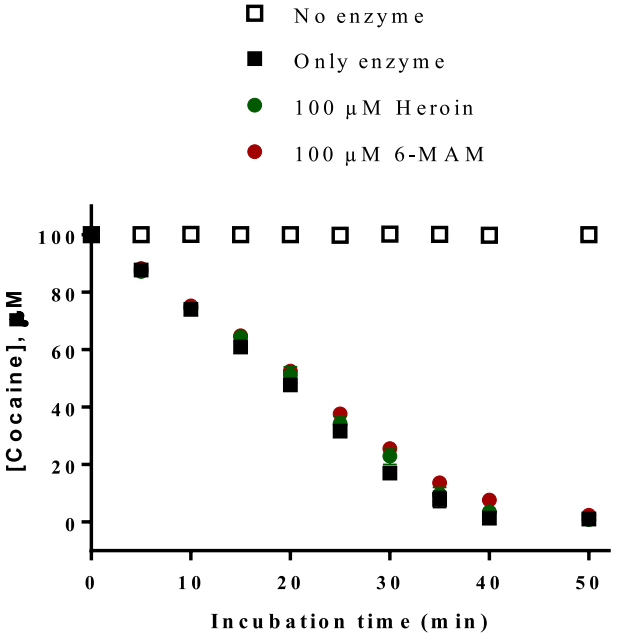

Fig. 4. (−)-Cocaine hydrolysis by CocH1. Both the enzyme (100 ng/ml) and (−)-cocaine (100 μM) were incubated together with 100 μM opioid (heroin or 6-MAM). Presented are the residual concentrations of cocaine *versus* time (○, no enzyme control; ■, only enzyme control, without opioid; ●, enzyme plus heroin; ●, enzyme plus 6-MAM). Cocaine concentrations were determined by using sensitive radiometric assays using [^{3}H](−)-cocaine. Results represent two independent experiments and the values are expressed as mean ± standard deviations.

concentration (100 μM) of heroin or 6-MAM.

3.5. Insights from molecular modeling

The reaction pathways of cholinesterase-catalyzed hydrolyses of heroin and 6-MAM were studied in our previous computational studies [44,45] through first-principles quantum mechanics and molecular mechanics-free energy (QM/MM-FE) simulations. The optimized reactant complexes (*e.g.* BChE complexed with heroin and 6-MAM, and AChE complexed with 6-MAM) obtained from our previous QM/MM studies show a couple of important enzyme-substrate interaction features. One is the interaction between the acetyl groups of heroin and 6-MAM and the oxyanion hole consisting of Gly116, Gly117, and Ala199 in BChE (corresponding to Gly121, Gly122, and Ala204 in AChE). The other is the interaction between the positively charged amino-groups of heroin and 6-MAM and the sidechain of Trp82 in BChE (corresponding to Trp86 in AChE). Guided by the aforementioned interactions, the substrate (heroin or 6-MAM) was docked into the active site of the enzyme (AChE, BChE, or CocH1). The molecular docking enabled us to understand how heroin may bind with human AChE, BChE, and CocH11 compared to 6-MAM binding with the corresponding enzymes. According to the enzyme-substrate binding structures obtained from molecular docking (as shown in Fig. 5), the binding mode for each enzyme (AChE, BChE, and CocH1) with heroin is similar to that with 6-MAM in terms of the overall hydrogen bonding with the oxyanion hole, particularly for the crucial interactions between the carbonyl oxygen of the substrate and the oxyanion hole of the enzyme. In particular, there are always two hydrogen bonds between the carbonyl oxygen of the substrate and backbone amide groups of the oxyanion hole residues (Gly120 and Gly121 of AChE corresponding to Gly116 and Gly117 of BChE and CocH11) no matter whether the substrate is heroin or 6-MAM. There are also two hydrogen bonds between the carbonyl oxygen of the substrate and the oxyanion hole of CocH1 (sidechain of Ser199 and backbone of Gly117), no matter whether the substrate is heroin or 6-MAM.

The modelling results depicted in Fig. 4 reveal that, no matter whether the enzyme is AChE, BChE, or CocH1, the acetyl-group of heroin forms stronger hydrogen bonds in the oxyanion hole compared to 6-MAM with the same enzyme. Hence, all of the enzymes concerned in the present study are expected to have a significantly better catalytic efficiency against heroin compared to 6-MAM. In particular, although a novel hydrogen bond from Ser199 of CocH1 is introduced to stabilize the acetyl-group of the substrate (heroin or 6-MAM), the hydrogen bond with Gly116 is lost compared to wild-type BChE. Therefore, CocH1 concerned in the present study is not expected to have a significantly improved catalytic efficiency against heroin or 6-MAM in comparison with BChE. The computational insight is supported by the measured kinetic parameters discussed above.

4. Conclusion

The catalytic activities of wild-type AChE, wild-type BChE, and CocH1 against heroin and 6-MAM have been characterized under the same experimental conditions for comparison. According to the determined kinetic parameters k_{cat} and K_M for all of these enzymatic reactions, wild-type AChE and BChE have similar k_{cat} values (k_{cat} = 2100 min^{-1} for AChE and k_{cat} = 1840 min^{-1} for BChE) against heroin. However, BChE has a ~16 fold-higher catalytic efficiency than AChE ($k_{cat}/K_M = 1.53 \times 10^7\ min^{-1}\ M^{-1}$ for BChE *vs* $k_{cat}/K_M = 9.67 \times 10^5\ min^{-1}\ M^{-1}$ for AChE), mainly because BChE has a ~18-fold stronger binding affinity with heroin compared to AChE ($K_d \approx K_M$ = 120 μM for BChE *vs* $K_d \approx K_M$ = 2170 μM for AChE). Besides, both AChE and BChE can catalyze 6-MAM hydrolysis to morphine, with relatively lower catalytic efficiency compared to the corresponding enzyme catalyzing heroin hydrolysis.

CocH1 can also catalyze hydrolysis of heroin (k_{cat} = 2150 min^{-1} and K_M = 245 μM) and 6-MAM (k_{cat} = 0.223 min^{-1} and K_M = 292 μM), with relatively larger K_M values and relatively lower

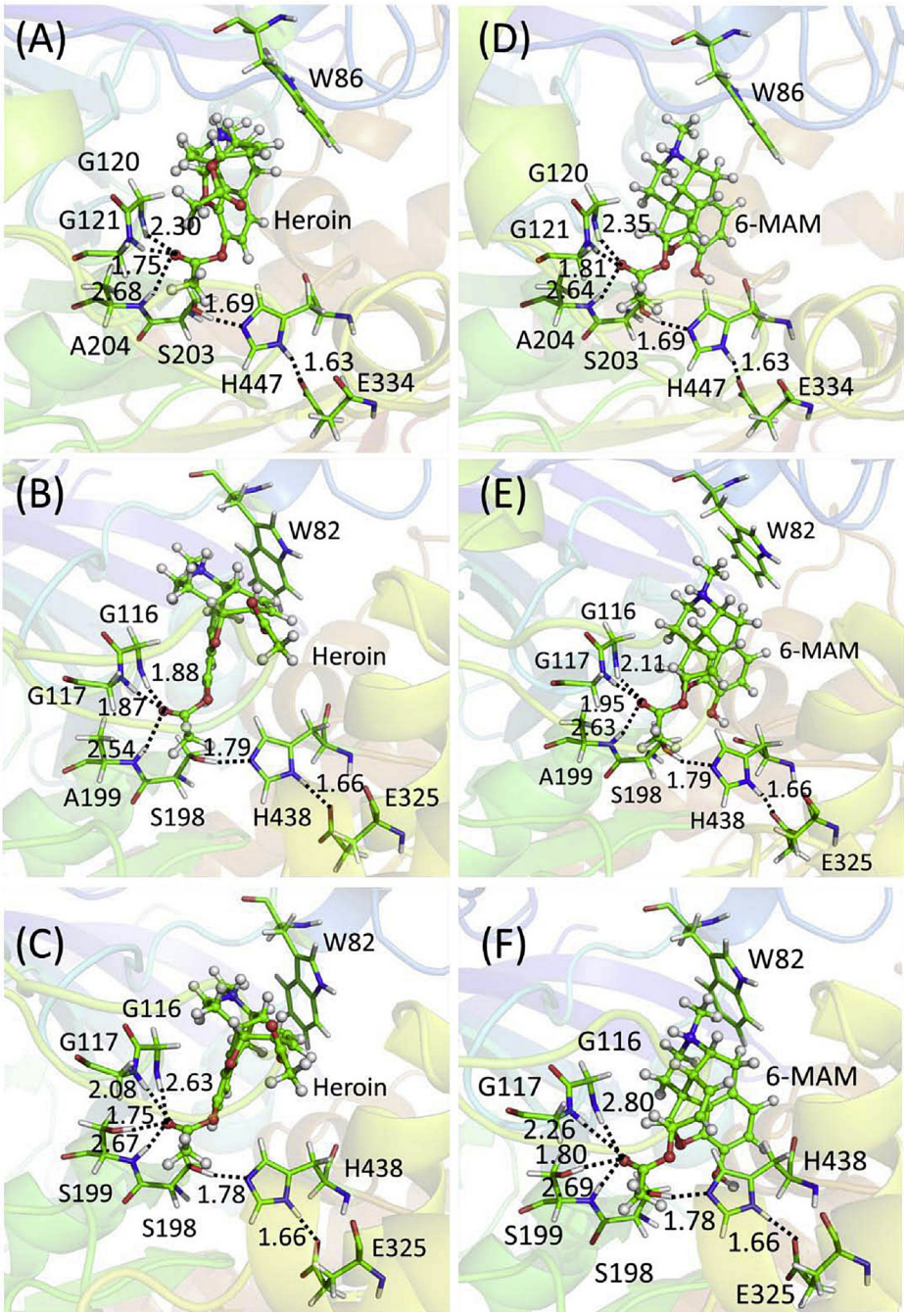

Fig. 5. Modeled structures of the AChE, BChE, and CocH1 (E14-3) binding with heroin and 6-MAM. (A) Wild-type AChE binding with heroin; (B) Wild-type BChE binding with heroin; (C) CocH1 binding with heroin; (D) Wild-type AChE binding with 6-MAM; (E) Wild-type BChE binding with 6-MAM; (F) CocH1 binding with 6-MAM.

catalytic efficiency compared to wild-type BChE. Notably, the K_M values of CocH1 against both heroin and 6-MAM are all much larger than previously reported maximum serum heroin and 6-MAM concentrations observed in heroin users, implying that the heroin use along with cocaine use will not significantly affect the catalytic activity of CocH1 against cocaine in the CocH1-based enzyme therapy for cocaine abuse.

Acknowledgements

This work was supported in part by the National Institutes of Health through the NIDA Translational Avant-Garde Award (UH2/UH3 DA041115) and R01 grants (R01 DA035552, R01 DA032910, R01 DA013930, and R01 DA025100) and the National Science Foundation through grant CHE-1111761. The authors also acknowledge the Computer Center at University of Kentucky for supercomputing time on a Dell X-series Cluster with 384 nodes or 4768 processors.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.cbi.2018.08.002.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.cbi.2018.08.002.

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