Published in final edited form as:

Chem Biol Interact. 2008 September 25; 175(1-3): 83–87. doi:10.1016/j.cbi.2008.04.024.

An Albumin-Butyrylcholinesterase for Cocaine Toxicity and Addiction:

Catalytic and Pharmacokinetic Properties

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Abstract

Butyrylcholinesterase (BChE, EC 3.1.1.8) is important in human cocaine metabolism despite its limited ability to hydrolyze this drug. Efforts to improve the catalytic efficiency of this enzyme have led to a quadruple mutant cocaine hydrolase, “CocH”, that in animal models of addiction appears promising for treatment of overdose and relapse. We incorporated the CocH mutations into a BChE-albumin fusion protein, “Albu-CocH”, and evaluated the pharmacokinetics of the enzyme after i.v. injection in rats. As assessed from the time course of cocaine hydrolyzing activity in plasma, Albu-CocH redistributed into extracellular fluid (16% of estimated total body water) with a $t_{1/2}$ of 0.66 hr and it underwent elimination with a $t_{1/2}$ of 8 hr. These results indicate that the enzyme has ample stability for short-term applications and may be suitable for longer-term treatment as well. Present data also confirm the markedly enhanced power of Albu-CocH for cocaine hydrolysis and they support the view that Albu-CocH might prove valuable in treating phenomena associated with cocaine abuse.

INTRODUCTION

Human plasma butyrylcholinesterase (BChE, EC 3.1.1.8) has been recognized for quite some time as a major contributor to cocaine metabolism and detoxification [10], and experiments with rodents have shown that large doses of native BChE offer modest protection against cocaine toxicity [5,14]. Such findings encouraged several groups, including our own, to begin engineering BChE for improved ability to hydrolyze cocaine [17,18,20,21]. “CocE”, a double mutant (A328W/Y332A) developed in our laboratory in light of computer-based analysis of cocaine docking to BChE, was found to blunt drug-induced hyper-locomotion and pressor effects [9,21]. This enzyme, as well as “AME” a still more powerful mutant discovered by Pancook et al [18], were likewise effective when transduced in vivo with adenoviral vector [8]. Similar mutagenesis efforts have now culminated in “CocH”, which Pan et al [17] designed to minimize the free energy of the cocaine-BChE transition state complex. This enzyme incorporates the A328W mutation in CocE, the S287G mutation in AME, a Y332G mutation homologous to those in CocE and AME, and a unique mutation, A199S. Because CocH may be an optimally efficient cocaine hydrolase and potentially suitable for treatment of cocaine overdose, we recently undertook to produce and characterize a new version of this enzyme that would be readily manufactured and likely to exhibit favorable pharmacokinetics. With that aim we fused CocH at its C-terminus with human serum albumin as a stabilizing excipient. In work just reported elsewhere [3] we have found that this fusion protein, “Albu-CocH”, rescues rats from cocaine toxicity and selectively suppresses a critical type of drug-seeking behavior. A molecule with such therapeutic promise deserves further attention with regard to its preclinical

pharmacology, including its distribution and stability after administration to animals. Here we present basic data regarding the enzymatic properties of Albu-CocH and its pharmacokinetics in rats.

METHODS AND PROCEDURES

Animals

Animals were handled according to the Principles of Laboratory Animal Care (National Research Council, 2003) in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care, under IACUC protocol A9306 (Mayo Clinic). Male and female Wistar rats (200–300 g) were obtained from Harlan Sprague-Dawley (Madison WI). Cocaine and enzyme were administered through the tail vein with a rinse of isotonic NaCl (total injection volume ~ 1.5 ml). Blood samples (100–300 μ l) were taken from the femoral vein, with care not to exceed a total of 0.7 ml in a 24 hr period. Tissues were obtained after euthanasia with sodium pentobarbital (250 mg/kg, i.p.) followed by intra-aortic perfusion with ~ 150 ml of isotonic NaCl.

Drug, reagents, and enzymes

Drugs were prepared in 0.9% NaCl (saline). Non-radioactive cocaine HCl was from Mallinckrodt (St. Louis MO), while 3H-cocaine (50 Ci/mmol) was from Dupont NEN, Boston MS). Di-isopropylfluorophosphate (DFP) and sodium pentobarbital were from Sigma-Aldrich (St. Louis MO). “Pansorbin” was purchased from (Calbiochem-EMD Biosciences, La Jolla CA). Albu-CocH is a C-terminally truncated (E1-V529) and mutant (A199S, S287G, A328W, Y332G) form of BChE (accession number gi:116353), fused to the N-terminus of human serum albumin (gi:28592). This monomeric protein was expressed in Chinese hamster ovary cells stably transfected with the gene for Albu-CocH. The clonal cell line was adapted for suspension and serum-free growth in a bioreactor and was grown for 10 days prior to harvest of the conditioned culture media. Protein was initially captured on Blue Sepharose and further purified using DEAE Sepharose followed by Q-HP Sepharose ion exchange chromatography (all chromatography resins from GE Healthcare). HPLC was performed using a TSK gel G300SWXL on a 7.8mm $\times$ 30cm SEC column (Tosoh Biosciences). N-terminal sequencing was performed on a 494 cLC Protein Sequencer (Applied Biosystems). For active site titration, each batch of enzyme was incubated 24 hr with varying amounts of the irreversible inhibitor, DFP, followed by determination of residual esterase activity as previously described [21].

Enzyme assays

Blood collected into heparin-treated tubes was centrifuged (10 min at 8,000 g) to obtain plasma. Brains were homogenized in 10 volumes of 10 mM sodium phosphate, pH 7.4 with 0.5% Tween-20, and centrifuged as above. Cocaine hydrolase activity in duplicate 50- μ l supernatant aliquots was assayed by incubating 30 min with ³H cocaine (18 μ M, except for substrate kinetics) and measuring liberated ³H- benzoic acid after partition or extraction into toluene-based fluor for scintillation counting [4]. A related procedure was used to determine levels of ³H-cocaine and benzoic acid, as previously described [9]. Hydrolysis of ³H-acetylcholine was measured by the assay of Johnson and Russell [11].

Antibody detection

Pansorbin cells were incubated for 1 hr at 37° in buffer (50 mM sodium phosphate pH 7.4 plus 0.1% bovine serum albumin) with a saturating amount of rabbit anti-rat IgG (Sigma). Other Pansorbin cells were incubated first with rabbit anti-mouse IgG (Chemicon) and then with mouse anti-rat IgM (Sigma), which bound to the solid-phase through the high-affinity rabbit immunoglobulin (direct binding of mouse IgG may be poor). After centrifugation (1500 $\times$ g

10 min) and 2 rinses in 1 vol of buffer, 100- μ l aliquots were further incubated for 1 hr with 100- μ l samples of rat plasma to adsorb any rat IgG or IgM. The cells were then rinsed again and, after a final exposure to 5 ng of purified Albu-CocH in buffer and one last centrifugation, aliquots of both the supernatant and the resuspended pellet fractions were assayed for cocaine hydrolase activity.

Enzyme kinetics, pharmacokinetics, and statistics

Enzyme concentration-time profiles in plasma were analyzed with Sigma Plot 4.1 (Jandel Scientific, Temecula CA), fitting the data to a bi-exponential decay curve that estimated rate constants and half-lives for redistribution and terminal elimination. Steady state volume of distribution was estimated by extrapolation of the terminal elimination phase curve to zero time. Substrate kinetics data (V = velocity, S = substrate concentration) were analyzed by direct fit to the Michaelis-Menten equation: $V = V_{\max} * (S / (S + K_m))$. Substrate concentration varied from 0.5 to 100 μ M for cocaine, and from 20 to 333 μ M for acetylcholine. Enzyme activities were determined in units of μ mol/min, and k_{cat} was evaluated from data obtained at concentrations below those at which substrate activation occurs (i.e., not accounting for the “b value”). SEM from means of independent replicate determinations was used throughout as the measure of variation; $p < 0.05$ was considered statistically significant.

RESULTS

Conditioned medium containing approximately 750mg/L Albu-CocH was purified to > 98% purity based on Western blot and SEC-HPLC analysis (Fig 1). Amino acid sequencing revealed that greater than 95% of the product contained the predicted N-terminus, while active site titration indicated that most of the material was enzymatically active. The migration of the single band resolved by gel electrophoresis was in line with the predicted molecular weight of 126,051. Assay of this enzyme with ^3H -cocaine revealed a high catalytic efficiency (k_{cat} / K_m), which was 1000 times greater than that of wild-type human BChE with the same substrate (Table 1). This large increase was not accompanied by enhanced capacity to hydrolyze acetylcholine. In fact Albu-CocH with acetylcholine as substrate (specific activity, 440 ± 40 units/mg) exhibited a K_m 30% higher than that of wild type BChE and a k_{cat} 10% lower.

In order to establish that Albu-CocH was adequately stable for future therapeutic applications, the enzyme was injected i.v. into male Wistar rats. The extremely low background of cocaine hydrolyzing activity in plasma and tissues of untreated rats enabled us to use time-dependent changes in activity to determine the kinetics of Albu-CocH redistribution and elimination. Figure 2 illustrates the plasma time course in 4 rats given enzyme at 10 mg/kg (essentially identical results were obtained from 4 other rats given a 100-fold lower dose). A rapid drop occurred during the first 1 - 2 hr after enzyme delivery followed by a much slower decline over the next three days. Non-linear least squares fitting to a double exponential decay model (see Methods) indicated that the first phase, “redistribution”, had an apparent $t_{1/2}$ of 0.66 ± 0.14 hr while the second phase, “elimination”, had an apparent $t_{1/2}$ of 8 ± 0.5 hr. The apparent volume of distribution, calculated by extrapolating the elimination phase back to zero time, was 33 ml, or approximately 16% of total body water.

Because Albu-CocH was developed as a means of modifying cocaine’s neurobehavioral actions, we tested the possibility that the protein could redistribute into the central nervous system. Four rats were given i.v. injections of the enzyme, and brains were harvested 12-24 hr later, after perfusion with isotonic NaCl to remove blood-borne material (see Methods). The results showed that cocaine hydrolase activity in brain, per gram wet weight, was only 1.3 ± 0.12 % of the activity in a comparable 1 ml sample of plasma. Hence, redistribution into this tissue must be slow and inefficient at best.

Further pharmacokinetics experiments were carried out to determine whether the half-life of Albu-CocH would remain stable in rats given repeated injections, or if it would decline, owing to the appearance of antibodies. Two rats were given repeated i.v. injections of the enzyme, 3 mg/kg, on days zero, 7, 15, and 28. Starting after the third and fourth injections, repeated blood samples were obtained over a 22 hr period to establish the rate at which cocaine hydrolase activity was lost from plasma. Fig. 2 shows the results from these two animals alongside those from naive rats receiving their first injection of Albu-CocH. The rates of enzyme decay were normal in one rat but accelerated in the other at both 15 and 28 days. To follow up this observation, specific immunoadsorption tests for anti-BChE IgG or IgM antibodies were performed on plasma samples collected from the same two rats on days 7, 14, and 21. Solid-phase mouse anti-rat IgM and rabbit anti-rat IgG were used to adsorb possible anti-CocH antibodies, which were then quantitated by their ability to bind an Albu-CocH standard (see Methods, "Antibody detection"). This test gave no signal for IgM antibodies in either treated rat at any time, nor for IgG antibodies on days 7 and 14. However, at day 21 a signal for IgG antibodies (binding 59% of the Albu-CocH standard) was recorded in the plasma of the rat that had shown accelerated loss of plasma enzyme activity.

DISCUSSION

Albu-CocH has an impressive ability to hydrolyze cocaine. The observed 1000-fold increase in catalytic efficiency of this fusion protein, as compared to wild type BChE, was more than twice the 450-fold rise reported by Pan et al [17] for the un-fused mutant. Further work is needed to determine if this difference reflects the albumin fusion or, as seems equally likely, variations in assay methodology. In any event, Albu-CocH compares favorably with the cocaine esterase produced by the bacterium *Rhodococcus* sp., MB1 [2], which was recently investigated for its potential to ameliorate cocaine toxicity in rats [7]. The bacterial enzyme has been reported to have a k_{cat} of 470 min⁻¹, and a K_m of 0.64 μ M [24], but catalytic efficiency, measured as k_{cat}/K_m , is actually 75% higher in the mutated BChE.

Mammalian enzymes are far more stable in vivo than bacterial ones. Bacterial cocaine esterase injected into rats has a plasma half-life of only about 15 min [7], as compared with 8 hr for Albu-CocH. Our present data indicate, however, that the elimination kinetics include a more rapid initial phase. We observed a similar dual exponential decay curve with a preparation of the predecessor enzyme, CocE, which had also been expressed in CHO cells [9]. Some of the quick early decline may reflect a true removal of incompletely glycosylated forms. The remainder of the initial loss probably reflects escape of protein from the vascular system and redistribution into extracellular water. We detected insignificant amounts of Albu-CocH in brain, where tight junctions do not allow large polypeptides to pass. However, we previously observed uptake of circulating CocE into rat heart, liver, lung, spleen, and skeletal muscle [9]. Furthermore the present value for the apparent volume of distribution, 16% of body water, is in line with values ranging from 14% to 18% in a classical study on human patients treated with native human BChE [16]. Since Albu-CocH may therefore have access to peripheral tissues, it is reassuring to note that it is less efficient than native BChE in hydrolyzing acetylcholine. This feature, together with the abundant presence of the native enzyme makes it unlikely that treatments with Albu-CocH would disturb cholinergic neurotransmission.

In vivo stability is a critical issue for proteins that are candidates for long-term therapeutic applications, and multiple factors come into play. One is antigenicity. There is evidence that bacterial cocaine esterase evokes antibody responses that reduce effective levels of the enzyme when doses are given repeatedly to mice [12]. Our present data suggest that a similar but probably weaker response follows repeated dosing of rats with Albu-CocH. Neither of these results is directly relevant to the treatment of human beings. We continue to expect that the administration of a homospecific construct assembled from human serum albumin and a human

plasma protein with minor modifications would be tolerated by our own immune system. Nonetheless, as would be the case for any protein-based therapeutic, the possibility that Albu-CocH might be antigenic in humans is an issue that will require further attention.

Other factors that affect enzyme stability *in vivo* include the extent and nature of glycosylation [13]. In the case of BChE, tetrameric forms are much longer-lived than monomers [19]. A typical mammalian expression culture system generates primarily monomeric BChE, but a high proportion of tetramers can be obtained when the proline-rich-attachment domain (PRAD subunit) is co-expressed [1]. Fusion with human serum albumin was explored here as an alternative method of increasing protein stability that may lend itself better to large-scale production [23]. The 8-hr half-life of monomeric Albu-CocH in the present experiments is shorter than that of native tetrameric BChE but not greatly below what we have observed with largely tetrameric preparations of the double mutant, CocE [22]. Using allometric scaling guided by experience with other albumin fusion proteins [15] one can extrapolate potential half-lives ranging from 1 to several days in humans. It is possible that the half-life of Albu-CocH could be further increased by post-translational modifications such as polyethylene glycosylation [6]. Meanwhile, the upper end of the predicted range might allow once-weekly administration for sustained therapy.

The prime therapeutic goals for an optimal cocaine hydrolase are rescue from cocaine overdose and treatment of cocaine addiction. Recent results from our laboratory [3] indicate that Albu-CocH is a realistic candidate for such purposes. In a dose of 3 mg/kg, *i.v.*, this protein prevented signs of cocaine toxicity when administered to rats before a typically fatal cocaine challenge (100 mg/kg, *i.p.*). Consistent with the present finding of an 8-hr plasma half-life, a dose of 10 mg/kg offered full protection for up to 12 hr. Albu-CocH not only protected rats, it also rescued them from cocaine seizures, and it selectively abolished cocaine-induced “reinstatement” of drug-seeking behavior in “abstinent” animals that formerly self-administered this drug.

In conclusion, we argue that available animal data are already sufficient to indicate that a cocaine hydrolase with properties like those of Albu-CocH deserves serious consideration for the emergency treatment of cocaine overdose. If a safe and convenient method for long-term delivery could be established, such enzymes might also have a role to play in the therapy of cocaine abuse, especially in dealing with the highly problematic issue of addiction-relapse after successful withdrawal. Much effort will be required to reach that goal, but it appears well worth pursuing.

ACKNOWLEDGEMENTS

The research in this article was supported by a research grant from the Minnesota Partnership for Medical Genomics and Biotechnology and by grant R01-DA023979-01 from NIDA/NIH. Additional funds were provided by Cogenesys, Inc., Rockville MD, which generated the material under study. Four of the authors (DL, RS, QZ, and MS) are employees of Cogenesys.

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Figure 1.

Purification and titration of Albu-CocH. **A)** Coomassie-Blue stained SDS electrophoresis gel of final product (M = markers, R = sample under reducing conditions, NR = sample under non-reducing conditions). The high apparent purity was confirmed by N-terminal sequencing and HPLC analysis (data not shown). **B)** Active site titration. Residual BChE activity was reduced in linear fashion after overnight incubation with increasing sub-stoichiometric amounts of the irreversible organophosphate cholinesterase inhibitor, di-isopropylfluorophosphate (DFP). The X-axis intercept calculated for this typical batch (one of three) indicates approximately 7.7 pmol of active site serine residues (the putative DFP target). The amount of enzyme protein was 1.3 μ g, or 10.3 pmol calculated from the theoretical MW of 126,100. This titration outcome

is compatible with the gel and sequencing data, and it indicates that the majority of the enzyme is catalytically active.

Figure 2.

Stability of Albu-CocH in vivo. Time course of plasma cocaine hydrolase activity in rats injected at zero-time with Albu-CocH, 10 mg/kg i.v. The data from 4 naïve rats (filled circles), fitted to a double exponential decay equation, indicated a redistribution phase with a half-life ($t_{1/2r}$) of 0.66 hr and an elimination half-life ($t_{1/2e}$) of 8 hr. Dashed lines show the log-linear regression lines calculated by fitting these data to a bi-exponential decay curve. Other data are from two rats given Albu-CocH for a final time after three previous injections (4, 3, and 2 weeks earlier). In one of these animals (open circles), enzyme clearance was normal. In the other rat (gray stars) the terminal elimination phase was greatly accelerated. A binding assay indicated anti-Albu-CocH IgG antibodies in the plasma of this rat (not shown).

Enzyme kinetics of wild type human BChE (WT) and BChE-derived cocaine hydrolases with various substrates. Units for K_m are μM ; units for k_{cat} are min^{-1} . Note that native BChE has higher k_{cat} for acetylcholine than butyrylthiocholine (thio-esters are disfavored)

Table 1

	cocaine		butyrylthiocholine		acetylcholine	
	K_m	k_{cat}	K_m	k_{cat}	K_m	k_{cat}
WT	3 ± 0.2	4 ± 0.1	42	25,000	150 ± 50	61200
CocE ^a	18	154	29	16,700	--- ^c	--- ^c
AME ^b	20	620	--- ^c	--- ^c	--- ^c	--- ^c
Albu-Coch	2.1 ± 0.1	2700 ± 190	--- ^c	--- ^c	200 ± 17	56000

^aData from Sun et al., 2002.

^bData from Pancook, 2003 and Gao et al., 2004.

^cNot determined