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Evaluation of the Hydrolytic Activity of a Long-Acting Mutant Bacterial Cocaine in the Presence of Commonly Co-Administered Drugs

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Abstract

Background—Cocaine toxicity is a prevalent problem in the United States for which there is currently no FDA-approved pharmacotherapy. We have developed a bacterial cocaine esterase (CocE) towards this indication. A thermostabilized mutant of CocE (DM-CocE) retains the hydrolytic activity of the wild-type esterase, rapidly hydrolyzing cocaine into the inactive metabolites ecgonine methyl ester and benzoic acid, and can prevent cocaine toxicities in rodent and non-human primate models. To advance DM-CocE towards clinical use, we examine here how the hydrolytic activity of DM-CocE is altered by some drugs commonly co-administered with cocaine.

Methods—We employed a spectrophotometric cocaine hydrolysis assay to evaluate whether pharmacologically relevant doses of alcohol, nicotine, morphine, phencyclidine, ketamine, methamphetamine, naltrexone, naloxone, or midazolam would alter the Michaelis-Menten kinetics of DM-CocE for cocaine. Mass spectrometry was used to evaluate interaction with diazepam as this drug interferes with the absorbance spectra of cocaine critical for the spectrophotometric assay.

Results—Alcohol, nicotine, morphine, phencyclidine, ketamine, methamphetamine, naltrexone, naloxone, and midazolam did not alter cocaine hydrolysis by DM-CocE. However, diazepam

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Contributors

Author Brim designed and wrote the study protocol, performed experiments, analyzed and interpreted data and wrote the first draft of the paper for publication. Author Noon performed and analyzed mass spectrometry experiments. Authors Nichols and Narasimhan purified and prepared enzyme. Authors Woods and Sunahara contributed to study design, data analysis and editing of the manuscript.

Conflict of Interest

Authors Brim, Narasimhan, Woods, and Sunahara are authors on patent PCT/US2008, “Thermostabilization of Proteins”. Authors Woods and Sunahara have consulted for Reckitt Benckiser Corporation. All other authors declare no other conflict of interest.

Author Disclosures

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significantly slowed DM-CocE cocaine hydrolysis at very high concentrations, most likely through interaction of the phenyl ring of the benzodiazepine with the active site of DM-CocE.

Conclusions—DM-CocE does not display significant drug interactions, with the exception of diazepam, which may warrant further study as DM-CocE progresses toward a clinically used pharmacotherapy for cocaine toxicity. Alternate benzodiazepines, e.g., midazolam could be used to avoid this potential interaction.

Keywords

Cocaine; Cocaine Esterase; Cocaine Toxicity; Benzodiazepines

1. Introduction

Cocaine is a psychotropic and addictive alkaloid derived from leaves of the South American shrub *Erythroxylon coca*. In the United States, 1.6 million people use cocaine (Substance Abuse and Mental Health Services Administration, 2010), resulting in more than 500,000 emergency room visits each year related to cocaine toxicity (US Department of Health and Human Services).

Because cocaine blocks several targets (including sodium channels and monoamine transporters) and causes a wide range of symptoms, it has been difficult to develop a pharmacotherapy to treat cocaine toxicity, and there is currently no FDA-approved therapy for this indication. Cocaine toxicity is currently treated only by controlling symptoms and the selection of drugs is often difficult and controversial (Boehrer et al., 1993; Canning et al., 2010; Freye and Levy, 2009; Gupta et al., 2010; Rangel et al., 2010; Sand et al., 1991; Schrank, 1992). To this end, we have developed a bacterial enzyme, cocaine esterase (CocE), as a potential treatment for this indication.

CocE is a 65 kDa (130 kDa dimer) serine hydrolase originally isolated from the *Rhodococcus sp.* MB1 soil bacterium (Bresler et al., 2000; Narasimhan et al., 2010; Turner et al., 2002). CocE hydrolyzes cocaine into the inactive products ecgonine methyl ester and benzoic acid, the same reaction catalyzed by human serum butyrylcholinesterase, but with a rate nearly 1000 times faster than the endogenous enzyme (CocE $V_{\max}=2300 \text{ min}^{-1}$) (Bresler et al., 2000; Cooper et al., 2006; Gao et al., 2009; Larsen et al., 2002; Turner et al., 2002). Initial *in vivo* studies demonstrated that the rapid conversion of cocaine to its inactive metabolites by wild-type CocE prevented or reversed cocaine-induced toxicities in animal models, including cardiovascular and neurological effects (Jutkiewicz et al., 2009; Ko et al., 2007; Wood et al., 2010). These studies served as proof that the pharmacokinetic approach we have employed with CocE may be an effective clinical strategy for the treatment of cocaine toxicity when given by emergency medical personnel.

Although the effects of wild-type CocE were promising, this enzyme is thermally unstable at 37°C (Cooper et al., 2006; Gao et al., 2009; Narasimhan et al., 2010). To this end, we have added thermostabilizing mutations to CocE (T172R and G173Q, DM-CocE) allowing for a prolonged duration of action as compared to wild-type and preservation of the hydrolytic capacity and potency of the enzyme both *in vitro* and *in vivo* (Collins et al., 2011; Collins et al., In Press; Gao et al., 2009; Narasimhan et al., 2010). The kinetics of cocaine hydrolysis by wild-type CocE (as well as various thermostable mutants) have classified in both physiological buffer (Gao et al., 2009; Narasimhan et al., 2010) and human plasma (Brim et al., 2010; Cooper et al., 2006); however, these studies examined hydrolytic activity only in presence of cocaine.

It is well known that cocaine is generally co-abused with other drugs. Therefore, we sought to investigate whether the hydrolytic ability of DM-CocE for cocaine would be preserved in the presence of commonly co-administered drugs. By investigating this, we will gain insight into the specificity of the stable DM-CocE mutant as a potential clinical therapy for cocaine toxicity.

2. Methods

2.1. Production and purification of DM-CocE

A BioFlow 3000 bioreactor (New Brunswick, NJ) was prepared with 10 L of Terrific Broth (TB; Maniatis, Benton Dickinson) with kanamycin (50 µg/mL). For the inoculum, BL21 cells transformed with pET24b plasmid containing DM-CocE were grown in 250 mL TB containing kanamycin (50 µg/mL) at 37°C while shaking until the culture reached log phase growth (about 8 hours). In this growth phase, the bioreactor was inoculated with the starter culture and allowed to grow at 37°C until the culture reached an OD₆₀₀ of 5. The bioreactor was then cooled to 18°C and induced by adding IPTG (1 mM final concentration). After 16 hours of induction, the culture was harvested by centrifugation (4500 × g, 20 minutes).

Cell paste from 5 liters of a fermentor run was resuspended in PBS pH 7.4 and passed through a French Press 2–3× to lyse the cells (maximum pressure approximately 1100 psi). The lysate was clarified by centrifugation (100,000 × g, Beckman Coulter XL-100K ultracentrifuge, Ti45 rotor). Clarified lysate was diluted with an equal volume of Q-buffer A (20mM Hepes pH 8.0) to a final volume of 1000 mL.

The dilution (500 mL) was passed onto a 450 mL Q Sepharose HP column attached to Pharmacia P-500 pumps and a Pharmacia LCC 501 FPLC controller at room temperature. The column was washed with 1000 mL Q-buffer A followed by a wash gradient set to reach 50% Q-buffer B (20mM Hepes pH 8.0 + 1M NaCl) within 1000 mL. Protein was eluted using a gradient from 50 to 100% Q-buffer B over 2250 mL. Fraction collection began after the washes were completed using a Pharmacia LKB Frac 100 fraction collector. The entire run was carried out at a flow rate of 10 mL/min. The DM-CocE protein was monitored by running 5 µl of each fraction on an 8% SDS PAGE Gel and staining with Coomassie Brilliant Blue.

Fractions from this separation were pooled and adjusted to 1M ammonium sulfate (final volume 525 mL). A 450 mL Phenyl-Sepharose column was washed with 1000 mL of PS-Buffer A (Buffer A: 50mM Na PO₄, pH 7 + 1M AS). An additional wash step was performed to increase PS-Buffer B (Buffer B: 50mM NaPO₄, pH 7) concentration to 30% within two column volumes (1000 mL). A gradient with PS buffers was run from 30% to 100% PS Buffer-B over 5 column volumes (2250 mL). The presence and purity of DM-CocE were checked by running 5 µl of each fraction on an 8% SDS PAGE gel.

Fractions from the Phenyl-Sepharose column were pooled and extracted twice with triton X 114. The twice-extracted protein was then diluted with Q-buffer A and this was loaded on to a second Q-Sepharose HP column (450mL). Chromatography was performed as described above.

2.2. Drug Interaction Studies

2.2.1. Spectrophotometric—Drugs were tested for interaction using a cocaine hydrolysis assay (Turner et al., 2002). Drugs chosen for testing were screened for absorbance at 240nm, to ensure no overlap between the drug tested and cocaine. Only diazepam had an absorbance spectra overlapping cocaine and was therefore tested using mass spectrometry.

DM-CocE (50 ng/mL) was incubated with 2× the concentration of drug tested in Table 1 in PBS 10 minutes at room temperature before addition to cocaine of equal volume (25 ng/mL final CocE concentration). Each experiment contained a no-drug vehicle, a DM-CocE control and 3 different drug concentrations, each in duplicate. Michaelis-Menten kinetic parameters were obtained from curve fits by Graph Pad Prism software. Separate experiments were performed to obtain triplicate data. Significance of a drug interaction was determined by one-way ANOVA with Dunnet's post-test between V_{max} , K_m , and catalytic efficiency of the cocaine-alone and cocaine-drug conditions.

2.2.2. Mass Spectrometry—To test the interaction between diazepam and DM-CocE, diazepam (at the concentrations listed in Table 1) was added to human plasma samples (obtained from the University of Michigan Hospital Blood Bank) at 37°C containing 8 μ M cocaine. Midazolam was used as a control to test the equivalence of the assays. Diazepam vehicle was tested at the volume used for the “3×” interaction point. No CocE and no drug controls were also run.

DM-CocE (0.003 μ M final concentration) was added to the samples at time $t=0$. Aliquots of plasma (20 μ L) were taken from the 1 mL sample at time $t=20$ sec, 40 sec, 1 min, 2 min, and 5 min. These aliquots were immediately placed into a solution of 66 μ L of acetonitrile, 4 μ L of saturated (1M) sodium fluoride, and 10 μ L deuterated cocaine (as an internal standard) to prevent further cocaine metabolism and to prepare samples for LC/MSMS. Samples were centrifuged at 13,000 rpm for 45 minutes and the supernatant was collected. The extract was concentrated on a Savant Speed Vac to remove acetonitrile. Extracted dried samples were reconstituted and further diluted 1–20 times depending on the time point.

2.3. Mass Spectral Analysis

2.3.1. Preparation of calibration standards for mass spectrometry—Calibration standards of cocaine (3.16 μ M–0.0043 μ M) were prepared in human plasma (obtained from the University of Michigan Hospital Blood Bank). All standards were stored at -80°C until sample preparation. Calibration standards were prepared with every set of experimental samples. Calibration stocks (20 μ L) were extracted with 68 μ L ACN, 4 μ L 1 M NaF, and 2 μ L of 2.5 μ M of deuterium-labeled cocaine as described above. Calibration standards were reconstituted to 100 μ L resulting in final internal standard concentrations of 50 nM.

2.3.2. Mass spectrometry of cocaine—LC-MS/MS analysis was performed on a Prominence HPLC system (Shimadzu, Kyoto, Japan) interfaced directly to the Turbo Ionspray source of an API 3000 triple quadrupole mass spectrometer (PE Sciex, Toronto, ON, Canada). Separation was achieved with a Phenomenex Synergi Hydro RP column (50 $\times$ 2.0mm i.d., 4 μ m packing) maintained at 40°C using a binary gradient and a flow rate of 0.15 mL/min. Solvent A was water and Solvent B was acetonitrile; both solvents were modified with 0.1% formic acid (v/v). The gradient program was as follows: 20% B at 0 min, 100% B at 3 min, hold 100% B for 1 min, return to initial conditions in 2 min and re-equilibrate at 20% B for 9 min.

Positive ions were generated in the source using purified air for the source gases under the following conditions and settings: Turbo ESI gas, 7.0 L/min; nebulizer (NEB) setting, 6; ion spray voltage (IS), 4200 V; source gas temperature (TEM), 300 °C; declustering potential (DP), 75 V; focusing potential (FP), 160 V; entrance potential (EP), 10 V. Nitrogen was used as the curtain gas (CUR) at a setting of 8. Mass analysis was performed by single reaction monitoring (SRM) with 300 ms dwell times. Nitrogen served as the collision gas (CAD=8). The collision energy (CE) was set to 32 eV (lab frame) with a collision cell exit potential (CXP) of 12 V. Precursor/product-ion pairs for the SRM transitions were m/z 304.2

→ 182.2 for cocaine and m/z 307.2 → 185.2 for d3-cocaine. Analyst software (version 1.4.2; MDS Sciex, Toronto, ON, Canada) was used for instrument control, data acquisition, and quantitative analysis. Calibration curves were constructed from the standard samples using the ratio of cocaine to the deuterium-labeled internal standard (d3-cocaine) and a least squares linear regression analysis with $1/x$ weighting.

3. Results

3.1 Drug-DM-CocE Interactions

Commonly co-administered drugs were tested for inhibition of DM-CocE at the concentrations shown in Table 1. These drugs were chosen to be a representative sample of the drugs most commonly abused with cocaine and the first line treatments for cocaine overdose (Freye and Levy, 2009; Schrank, 1992). These concentrations represent 1, 3 and 10× the pharmacologically relevant serum concentrations in humans. The 1× drug level represents the typical serum concentration observed in drug abusers or the peak serum concentration after dosing by a healthcare professionals (Couper and Logan, 2004; Ferrari et al., 1998; Karch, 2001; Midazolam Hydrochloride [Package Insert] Lake Forest; Narcan (R) [Package Insert]. Chadds Ford; Ngo et al., 2008). All drugs in this assay were first tested for absorbance at 240 nm to assure that they would not interfere with the absorbance spectrum of cocaine (only diazepam absorbed at 240nm and was thus analyzed using LC-MS/MS (Figure 1B)). The Michaelis-Menten kinetics of cocaine hydrolysis by DM-CocE was not altered by any drug tested in the spectrophotometric assay (Table 1). Figure 1A illustrates typical results for the drugs tested using morphine and DM-CocE.

3.2 Inhibition of DM-CocE by diazepam

Diazepam and midazolam were tested for inhibition of DM-CocE-mediated cocaine hydrolysis using LC-MS/MS (Figure 1B). Cocaine concentrations were followed over time after the addition of DM-CocE and one of the benzodiazepines. Midazolam, at 10× the normal serum concentrations achieved in medical settings, did not interfere with cocaine hydrolysis by DM-CocE, confirming the results of the *in vitro* assay. In contrast, diazepam, at concentrations 3 and 10× the peak serum levels achieved with patient sedation, did significantly slow the hydrolysis of cocaine by DM-CocE. The addition of fluorine on the benzoyl ring of midazolam or its larger molecular weight may explain the difference in effect on DM-CocE between these two structurally similar benzodiazepines (Figure 1C); however, the mechanism of diazepam inhibition has yet to be elucidated.

4. Discussion

We have demonstrated that DM-CocE is able to hydrolyze cocaine in the presence of all drugs tested in this study, with reduced ability in the presence of very high concentrations of diazepam. Although this is not a comprehensive list of drugs that are co-administered with cocaine, we sought a sample of the most likely candidates with diversity in structure and class. A more exhaustive list of drugs would be warranted if DM-CocE continues to progress toward the clinic.

The interaction with diazepam was dose-dependent, and only seen with concentrations higher than what would be used by medical personnel or persons abusing diazepam (Couper and Logan, 2004). Additionally, this study was designed with very low DM-CocE concentrations (25 ng/mL spectrophotometric assay, 195 ng/mL mass spectrometry assay) to increase the likelihood of detecting interactions. These DM-CocE concentrations are below what we predict will be minimally necessary clinically, based on previous studies examining the effects of DM-CocE on cocaine's toxic effects in Sprague Dawley rats (Collins et al., In

Press) and cardiovascular effects in Rhesus monkeys (Collins et al., 2011); extrapolated to a 70 kg man, this dose would elicit a blood concentration of about 4.5 µg/mL, almost 25 times higher than the concentrations tested here. Thus, we can predict mathematically that the ratio of diazepam to DM-CocE concentration studied here is not likely to exist in a clinical scenario: however, a potential interaction with diazepam may warrant investigation. A potential interaction could also be easily avoided by using another benzodiazepine, e.g., midazolam, for sedation.

This study reveals that drug interactions can occur with enzymes engineered to be specific. The mechanism of diazepam inhibition is unclear, as we were not able to obtain Michaelis-Menten kinetics for this drug with our spectrophotometric assay. We predict that the inhibition is most likely due to competition of the phenyl rings of cocaine and diazepam for binding to the active site of the DM-CocE, increasing the K_m of the enzyme for cocaine. Investigations into drug-interactions are warranted for engineered human butyrylcholinesterases that have increased hydrolytic activity against cocaine (Brimijoin et al., 2008; Gao and Brimijoin, 2004; Gao et al., 2008; Lynch et al., 1997; Mattes et al., 1997; Pan et al., 2005; Xue et al., 2011), as wild type butyrylcholinesterase metabolizes several drugs besides cocaine such as succinylcholine, procaine, and mivacurium, and may have a higher propensity for drug interactions. The data presented here demonstrating inhibition of DM-CocE only at very high diazepam concentrations and not with other drugs tested, further strengthen the pre-clinical evidence that DM-CocE may be an effective treatment for cocaine toxicity in humans.

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Figure 1. Cocaine hydrolysis by DM-CocE is affected by diazepam but no other drug tested

Using the spectrophotometric assay, all drugs were assessed for interaction with CocE by adding a physiological (or higher) concentration of drug to a 50 ng/mL DM-CocE solution for 10 minutes at room temperature. The hydrolysis assay was also run in the presence of drug. **A.** Morphine does not inhibit cocaine hydrolysis by cocaine at any concentration tested. The effect of each concentration of morphine on each Michaelis-Menten parameter of CocE was assessed by one-way ANOVA as presented in Table 1. Michaelis-Menten kinetics of DM-CocE in the presence of each drug tested are summarized in Table 1. **B** Diazepam, but not midazolam slow cocaine hydrolysis by DM-CocE. The addition of 3 nM CocE to human plasma spiked with 8 μ M cocaine caused rapid cocaine hydrolysis (blue). Neither diazepam vehicle (purple) or midazolam (pink) had an effect on the rate of cocaine hydrolysis by DM-CocE ($p>0.05$ at all time points, significance assessed by 2-way ANOVA using Bonferroni posttests ($F(6, 60)=7.37$)). Significant interactions were seen with 1 $\times$ (red, $p<0.01$ at 5 minutes), 3 $\times$ (orange, $p<0.001$ at 2 and 5 minutes), and 10 $\times$ diazepam (yellow, $p<0.01$ at 1 minute, $p<0.001$ at 2 and 5 minutes). **C** The chemical structures of cocaine and the benzodiazepines tested in thus study. The structures reveal the larger size and fluorination of midazolam as compared to diazepam.

Table 1

Drug Concentrations and Tested Interactions with DM-CocE.

	Pharmacological Blood Concentrations	$V_{\max}$ (min^{-1})	K_m (μM)	Catalytic Efficiency ($\text{min}^{-1}\mu\text{M}^{-1}$)	Significant Interaction
Alcohol	0 mg/dL	2027	16.8	120.3	ns
	80 mg/dL	2093	18.9	110.3	
	240 mg/dL	2150	20.9	102.9	
	800 mg/dL	2041	20.3	105.9	
Nicotine	0 μM	1993	16.8	118.8	ns
	0.12 μM	1926	16.8	118.5	
	0.36 μM	2006	19.8	107.2	
	1.2 μM	1938	15.0	129.3	
Morphine	0 μM	1972	17.2	114.7	ns
	0.18 μM	2122	20.5	103.8	
	0.54 μM	1974	18.0	111.6	
	1.8 μM	2042	16.7	122.9	
Phencyclidine	0 μM	2092	18.0	116.2	ns
	0.41 μM	1969	16.8	118.0	
	1.23 μM	2072	19.1	106.3	
	4.1 μM	2164	19.8	109.2	
Ketamine	0 μM	2078	19.5	107.9	ns
	0.35 μM	1947	20.0	100.2	
	1.05 μM	1834	19.2	96.7	
	3.5 μM	1762	15.0	119.6	
Methamphetamine	0 μM	2410	21.3	117.4	ns
	6.7 μM	2123	19.1	112.9	
	20.1 μM	2208	18.4	117.9	
	67 μM	2299	21.3	116.3	
Naltrexone	0 μM	1978	16.2	122.5	ns
	25 nM	1851	14.3	129.6	
	75 nM	1936	14.5	133.6	

	Pharmacological Blood Concentrations	V_{max} (min^{-1})	K_m (μM)	Catalytic Efficiency ($\text{min}^{-1}\mu\text{M}^{-1}$)	Significant Interaction
	250 nM	1957	13.1	152.2	
Naloxone	0 μM	2145	19.7	109.4	ns
	1 μM	2067	20.0	102.6	
	3 μM	1716	17.5	98.9	
	10 μM	1866	17.6	106.0	
Midazolam	0 μM	2098	18.5	112.7	ns
	0.64 μM	2457	23.8	100.0	
	1.9 μM	1943	18.9	102.8	
	6.4 μM	2109	19.6	107.0	

ns: No significant interaction found. V_{max} and K_m were determined for three separate experiments. Significance was determined by conducting a one-way ANOVA with Dunnett's post-test between the 0 μM control and increasing drug conditions for V_{max} . K_m and catalytic efficiency individually.