The Fate of Bacterial Cocaine Esterase (CocE): An In Vivo Study of CocE-Mediated Cocaine Hydrolysis, CocE Pharmacokinetics, and CocE Elimination^[S]

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ABSTRACT

Cocaine abuse and toxicity remain widespread problems in the United States. Currently cocaine toxicity is treated only symptomatically, because there is no Food and Drug Administration-approved pharmacotherapy for this indication. To address the unmet need, a stabilized mutant of bacterial cocaine esterase [T172R/G173Q-CocE (DM-CocE)], which hydrolyzes cocaine into inactive metabolites and has low immunogenic potential, has been developed and previously tested in animal models of cocaine toxicity. Here, we document the rapid cocaine hydrolysis by low doses of DM-CocE in vitro and in vivo, as well as the pharmacokinetics and distribution of the DM-CocE protein in rats. DM-CocE at 50.5 μ g/kg effectively eliminated 4 mg/kg cocaine within 2 min in both male and female rats as measured by mass spectrometry. We expanded on these findings by using a phar-

macologically relevant dose of DM-CocE (0.32 mg/kg) in rats and monkeys to hydrolyze convulsant doses of cocaine. DM-CocE reduced cocaine to below detection limits rapidly after injection; however, elimination of DM-CocE resulted in peripheral cocaine redistribution by 30 to 60 min. Elimination of DM-CocE was quantified by using [³⁵S] labeling of the enzyme and was found to have a half-life of 2.1 h in rats. Minor urinary output of DM-CocE was also observed. Immunohistochemistry, Western blotting, and radiography all were used to elucidate the mechanism of DM-CocE elimination, rapid proteolysis, and recycling of amino acids into all tissues. This rapid elimination of DM-CocE is a desirable property of a therapeutic for cocaine toxicity and should reduce the likelihood of immunogenic or adverse reactions as DM-CocE moves toward clinical use.

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Introduction

Cocaine abuse is a widespread problem in the United States, with more than 1.5 million people currently abusing the drug (Substance Abuse and Mental Health Services Administration, 2011a). This natural alkaloid, derived from the South American shrub *Erythroxylon coca*, elicits its powerful euphoric and addicting effects by blocking monoamine transporters in the brain, resulting in increased levels of endogenous catecholamines. In addition to causing its euphoric effects, cocaine is toxic at high doses, causing convulsions, severe hypertension, ventricular fibrillation, hyperthermia, and even death (Schrunk, 1992; Freye and Levy, 2009). Toxicity is caused primarily by cocaine's blockade of cardiac sodium channels, in addition to the high levels of norepinephrine that accumulate during monoamine transporter blockade (Schrunk, 1992; Freye and Levy, 2009). Each year, more than 500,000 emergency department visits are caused by cocaine toxicity (Substance Abuse and Mental Health Services Administration, 2011b). The abuse of cocaine and its toxicity contribute to the estimated loss of \$180.8 billion from the American economy each year because of decreased productivity and medical expenses related to drug use (Office of National Drug Control Policy, 2004).

There is currently no Food and Drug Administration-approved pharmacotherapy to treat either cocaine abuse or cocaine toxicity. To this end, we have been developing a bacterial cocaine esterase (CocE) into a suitable therapy for the abuse-related and toxic effects of cocaine in humans. CocE is an α - β serine hydrolase originating from the bacterium *Rhodococcus* sp. strain MB1 found in the soil surrounding the coca plant (Bresler et al., 2000). CocE is the most efficient natural cocaine hydrolase known and converts cocaine into the physiologically inactive products ecgonine methyl ester and benzoic acid (Bresler et al., 2000; Larsen et al., 2002; Turner et al., 2002). Wild-type CocE has been described extensively in rodent models and was found to reverse the cardiovascular, neurological, and toxic effects of cocaine (Cooper et al., 2006; Ko et al., 2007; Jutkiewicz et al., 2009; Wood et al., 2010). However, wild-type CocE is limited by thermal instability and exhibits an activity half-life of approximately 10 min in vitro when incubated at 37°C (Cooper et al., 2006; Gao et al., 2009; Narasimhan et al., 2010). In vivo assays demonstrated that the thermal instability of wild-type CocE limited the duration of CocE's protection against cocaine's toxic and reinforcing effects (Ko et al., 2007; Collins et al., 2009; Gao et al., 2009; Narasimhan et al., 2010).

Through the addition of thermostabilizing mutations [T172R, G173Q, L169K, T172R/G173Q (Ko et al., 2007; Collins et al., 2009; Gao et al., 2009; Narasimhan et al., 2010) and L169K/G173Q (Brim et al., 2010)] the activity half-life in vitro and the in vivo duration of action of CocE was improved while maintaining or improving the catalytic efficiency of the enzyme. These improvements allowed for proof-of-concept studies that revealed T172R/G173Q-CocE (DM-CocE) could block the reinforcing effects of cocaine, while preserving CocE's ability to block the toxic effects of the drug (Collins et al., 2009). DM-CocE has been studied in both rodents and monkeys, and we now possess a potential therapeutic for cocaine toxicity that is worthy of pharmacodynamic and pharmacokinetic studies.

The rapid rate of cocaine hydrolysis by CocE and various thermostable mutants in vitro has been extensively documented (Turner et al., 2002; Cooper et al., 2006; Gao et al., 2009; Brim et al., 2010; Narasimhan et al., 2010), and in vivo studies using toxic doses of cocaine and large doses of CocE have demonstrated the reversal or prevention of cocaine-related behaviors and toxicities. However, a study directly measuring cocaine elimination by CocE in vivo has not been performed. Herein, we describe the rate of cocaine hydrolysis by both low doses and pharmacologically relevant doses of DM-CocE in both male and female Sprague-Dawley rats and rhesus monkeys by using mass spectrometry. To complement these data, the pharmacokinetics of DM-CocE were determined by using [³⁵S] labeling, and the distribution and elimination were assessed by immunologic and radiologic techniques. The work presented here represents a significant advance toward understanding the way that live biological systems will handle exogenous CocE protein and help us to understand how to further stabilize it in the circulation.

Materials and Methods

Production and Purification of RQ-Cocaine Esterase

A BioFlow 3000 bioreactor (New Brunswick Scientific, Edison, NJ) was prepared with 10 liters of Terrific Broth (Maniatis; BD Biosciences, San Jose, CA) with kanamycin (50 μ g/ml). For the inoculum, BL21 cells transformed with pET24b plasmid containing DM-CocE were grown in 250 ml of Terrific Broth containing kanamycin (50 μ g/ml) at 37°C while shaking until the culture reached log-phase growth (approximately 8 h). 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. Once the target turbidity was reached, the bioreactor was cooled to 18°C and then induced by adding isopropyl β -D-thiogalactoside for a final concentration of 1 mM. After 16 h of induction, the culture was harvested by spinning down the broth at 4500g for 20 min. The resulting pellet was stored at -80°C.

Cell paste from 5 liters of a fermentor run was resuspended in phosphate-buffered saline, pH 7.4. The resuspended paste was passed through a French press two or three times to lyse the cells. The maximum pressure for lysis was approximately 1100 psi. The lysate was clarified by spinning at 100,000g in an ultracentrifuge (XL-100K ultracentrifuge; Beckman Coulter, Fullerton, CA) using the rotor type Ti45. Clarified lysate was diluted by adding an equal volume of Q-buffer A (20 mM HEPES, pH 8.0) to a final volume of 1000 ml.

The clarified lysate (500 ml) was passed onto a 450-ml Q-Sepharose HP column at room temperature. The column was washed with 1000 ml of Q-buffer A followed by a gradient set to reach 50% Q-buffer B (20 mM HEPES, pH 8.0 + 1 M NaCl) within 1000 ml. Flow-through and washes were saved for analysis. DM-CocE was eluted from the column by using a gradient from 50 to 100% Q-buffer B over five column volumes (2250 ml). Fractions (25 ml) were collected at a flow rate of 10 ml/min. DM-CocE was monitored by running 5 μ l of each fraction on an 8% SDS/PAGE Gel and staining with Coomassie Brilliant Blue according to established protocols.

Fractions from the first Q-Sepharose HP separation were pooled and adjusted to 1 M ammonium sulfate (final volume 525 ml). Samples were applied to a 450-ml phenyl-Sepharose column pre-equilibrated with PS-buffer A (buffer A: 50 mM Na Phos, pH 7 + 1 M ammonium sulfate). The column was washed with 1000 ml of PS-buffer A followed by an additional wash in the form of a linear 30% PS-buffer B (buffer B: 50 mM Na Phos, pH 7) over two column volumes (~1000 ml). DM-CocE was eluted with 30 to 100% PS buffer-B over five column volumes (2250 ml). Fractions (25 ml) were collected over the elution gradient. The presence and purity of DM-CocE were checked by running 5 μ l of each fraction on an 8% SDS/PAGE gel.

Fractions from the phenyl-sepharose column were pooled and extracted twice with Triton X-114 to remove endotoxin. The twice-extracted protein was then diluted with Q-buffer A, and this was loaded on to a second Q-Sepharose HP column (450 ml). Chromatography was performed as described above.

Production and Purification of [³⁵S]DM-CocE

DM-CocE contains 11 methionines (Met 1, 13, 16, 113, 141, 323, 350, 483, 507, 521, and 550), all well distributed throughout the molecule to assure that large fragments of DM-CocE would retain a radioactive residue. B834 (DE3) methionine-autotrophic competent cells (Novagen, Madison, WI) were transformed with a pET24b plasmid containing cDNA for histidine-tagged DM-CocE. A transformed clone was selected and confirmed by DNA sequencing.

ABBREVIATIONS: CocE, cocaine esterase; DM-CocE, T172R/G173Q-CocE; PAGE, polyacrylamide gel electrophoresis; PS, phenyl-Sepharose; LB, lysogeny broth; TBS, Tris-HCl-buffered saline; LS, lima bean trypsin inhibitor; Ni-NTA, nickel-nitriloacetic acid; LC, liquid chromatography; MS, mass spectrometry; PBS, phosphate-buffered saline; SRM, single reaction monitoring; PVDF, polyvinylidene difluoride; AUC, area under the curve; PEG, polyethylene glycol; DIC, differential interference contrast; TXR, Texas Red.

A 5-ml starter culture in lysogeny broth (LB) medium containing ampicillin with B834-pET24b cells was incubated for 12 h at 37°C. The 5-ml culture was then used to inoculate 25 ml of LB medium containing ampicillin, and culture was grown at 37°C to OD₆₀₀ of 0.8. The culture was spun at 4000g for 5 min and resuspended in 10 ml of M9 minimal medium containing ampicillin. This wash step was repeated to remove LB medium. The final resuspension was added to 15 ml of M9 medium containing ampicillin and grown at 37°C for 30 min. DM-CocE expression was induced with 1 mM isopropyl β -D-thiogalactoside, 10 μ g/ml methionine, and 1 mCi of [³⁵S]methionine and grown at 18°C overnight.

After induction cells were spun and resuspended in 3 ml of Tris-HCl-buffered saline, pH 7.4 (TBS) containing 3 μ g/ml of both leupeptin and lima bean trypsin inhibitor (LS) and lysozyme (50 μ g/ml). To initiate cell lysis, cells were incubated for 20 min at room temperature with gentle mixing and then snap-frozen with liquid N₂. Lysate was thawed and added to 4 ml of B-PER Bacterial Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA) per gram of cell pellet containing 8 μ g/ml DNase 1 and 1 mM MgCl₂. Lysate was incubated 20 min at room temperature with gentle mixing and centrifuged at 13,000 rpm for 30 min to remove cellular debris.

[³⁵S]DM-CocE was purified away from cellular lysate using a 3-ml nickel-nitriloacetic acid (Ni-NTA) column and a 1-ml Q-Sepharose column. Lysate was loaded onto the Ni-NTA column and washed with 15 ml of TBS + LS, 15 ml of TBS + LS + 300 mM NaCl, and 15 ml of TBS + LS + 10 mM imidazole. DM-CocE was eluted with 1.5-ml fractions of TBS + LS + 150 mM imidazole. Fractions were analyzed by SDS/PAGE and visualized by using Coomassie Brilliant Blue stain.

Fractions containing [³⁵S]DM-CocE were combined, diluted four times in 50 mM Tris-HCl, pH 8.0, and loaded onto the Q-Sepharose column. The column was washed with 5 ml of 50 mM Tris-HCl, pH 8.0 and 5 ml of 50 mM Tris-HCl, pH 8.0 + 300 mM NaCl and eluted with a 0.5-ml step gradient with 50 mM Tris-HCl, pH 8.0 + 325 to 475 mM NaCl in 25-mM increments. Fractions were resolved by SDS/PAGE.

Gels from both Ni-NTA and Q-Sepharose columns were dried under vacuum onto 3-mm blotting paper by using a slab dryer (model 483; Bio-Rad Laboratories, Hercules, CA) for 4 h at 80°C. Dried gels were exposed to film for 8 to 12 h at -4°C to assess the purity of samples (Supplemental Fig. 1). Fractions from the Q-Sepharose column containing [³⁵S]DM-CocE were combined, dialyzed into sterile saline to remove excess salt, and stored at 4°C until injection.

Animal Housing and Welfare

Male and female Sprague-Dawley rats (normal and unilaterally nephrectomized; 250 to 350 g; obtained from Harlan, Indianapolis, IN) were maintained on a temperature-controlled environment with a 12-h light cycle, on at 7:00 AM. Rats were housed in Plexiglas cages (49 cm long $\times$ 23 cm wide $\times$ 21 cm high) with cob bedding, and they had ad libitum access to food and water. Rats were allowed to acclimate to the room for at least 5 days before any surgical procedures were performed.

One adult male and one adult female rhesus monkey (*Macaca mulatta*), both fitted with indwelling catheters in the jugular or femoral vein, were used for this study. Monkeys were singly housed in stainless-steel caging in a temperature-controlled environment with a 12-h light cycle beginning at 7:00 AM. Monkeys were fed 20 to 50 Lab Fiber Plus Monkey Diet Chows (Lab Diet; PMI Nutrition International, Brentwood, MO) per day and fresh fruit and had free access to water.

All procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* from the National Institutes of Health (Institute of Laboratory Animal Resources, 1996), and all experiments were approved by the University of Michigan Committee on the Use and Care of Animals.

Animal Preparation for Determination of In Vivo Rates of Cocaine Hydrolysis

Dose Dependence Study (Rats). Animals were administered 3.5% isoflurane anesthesia and 100% oxygen through a rat anesthesia mask. Animals were placed on a heating pad set to maintain the animal's body temperature at approximately 37°C. Body temperature was monitored throughout the experiment via a rectal thermometer. The region of the animals' left and right legs were shaved and sterilized with alcohol and betadine. The femoral veins were exposed by incision and by peeling away connective tissue. A catheter was inserted and tied in place with sterile silk suture. After the catheter was in place it was flushed with saline. Anesthesia was continued and doses of DM-CocE (13.6 or 50.5 μ g/kg, based on in vitro results) or vehicle control were administered intravenously via one catheter. Two minutes after DM-CocE or vehicle administration, rats were given a dose of cocaine (4 mg/kg) intravenously through the second catheter. Blood samples (approximately 200 μ l/sample) were collected from the first catheter at 30 s and 1, 2, and 5 min after cocaine administration. Blood samples were immediately placed into 70 μ l of EDTA solution containing 50 μ l of 50 mM EDTA and 20 μ l of 1 M NaF. Samples were stored on ice and spun at 1200 rpm in a bench-top microcentrifuge to separate plasma. Plasma was transferred to a clean low retention tube and prepared for LC/MS (see *Cocaine Quantification*).

Species Comparison Study (Rats). Rats were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) for catheter implantation into both femoral veins as described above. Catheters were threaded under the skin and attached to stainless-steel tubing that ran through a metal tether plate sutured to the back muscle. Animals were allowed 5 days after surgery to recover from the procedure.

On the day of the experiment, rats were placed into Plexiglas cages (49 cm long $\times$ 23 cm wide $\times$ 21 cm high) containing cob bedding. A long piece of catheter tubing was run out of the cage from the stainless-steel tubing of the indwelling catheter so that rats could be infused through one catheter, and blood could be drawn from the other catheter without handling. At time $t = 0$, rats were given a 10-s bolus of 5.6 mg/kg cocaine followed later by a bolus of either DM-CocE (0.32 mg/kg) or PBS at $t = 10$ min. Blood was sampled (200 μ l) at $t = 2, 5, 9, 10.75, 12, 15, 20, 40$, and 60 min and immediately added to 5 μ l of 500 mM EDTA and 20 μ l of 1 M NaF to prevent coagulation and further cocaine hydrolysis, respectively. Plasma was collected by centrifugation at 4000 rpm in a bench top microcentrifuge, flash-frozen in liquid nitrogen, and stored at -80°C until preparation for mass spectrometry.

Species Comparison Study (Monkeys). A male and female rhesus monkey trained for arm-restraint chairs were used for this study. Cocaine (3 mg/kg at $t = 0$) and DM-CocE or PBS (0.32 mg/kg at $t = 10$ min) were administered through acute intravenous catheters. Blood samples were taken at $t = 0, 8, 15, 30, 60, 90$, and 120 min from the saphenous vein. Blood was immediately collected into tubes containing EDTA (5 ml; BD Vacutainer K₂EDTA Plus Blood Collection; BD Biosciences) and 1/10 volume of 1 M NaF to prevent clotting and eliminate further cocaine metabolism, respectively.

Sample Preparation and Mass Spectral Analysis of DM-CocE-Mediated Cocaine Hydrolysis In Vitro and In Vivo

In Vitro Rates of Cocaine Hydrolysis by DM-CocE. One milliliter of human plasma samples obtained from the University of Michigan Hospital Blood Bank was spiked with 8 μ M cocaine. Plasma samples containing cocaine were incubated at 37°C for 5 min. After warming the plasma, DM-CocE was added. Twenty-microliter aliquots of plasma were taken from the 1-ml sample at 20 and 40 s and 1, 2, and 5 min after DM-CocE addition. These aliquots were immediately placed into 80 μ l of acetonitrile solution containing 4 μ l of saturated (1 M) NaF to prevent further cocaine metabolism along with 10 μ l of internal standard (deuterated cocaine;

Cerilliant Corporation, Round Rock, TX), and then immediately prepared for LC-MS/MS.

Quantification of Cocaine. Plasma collected from animals (as described above) was extracted with 3× volume of 100% acetonitrile containing NaF and 10 µl of internal standard, then immediately prepared for LC-MS/MS. All samples (in vitro and in vivo) were then centrifuged at 13,000 rpm for 30 min, and the supernatant was collected into a clean low-retention microcentrifuge tube. Extracts were dried on a Savant Speed Vac (Thermo Fisher Scientific) to remove acetonitrile then frozen at −80°C for 1 week or less until analysis was performed. Samples were subsequently reconstituted in water and further diluted 10 to 100 times depending on the sample time point. LC-MS/MS was performed at the University of Michigan Biomedical Mass Spectrometry Facility.

LC-MS/MS analysis was performed on a Prominence HPLC system (Shimadzu, Kyoto, Japan) interfaced directly to the Turbo Ion-spray source of an API 3000 triple quadrupole mass spectrometer (PerkinElmerSciex Instruments, Toronto, ON, Canada). Separation was achieved with a Phenomenex (Torrance, CA) Synergi Hydro RP column (50 × 2.0 mm i.d.; 4-µm packing) maintained at 40°C by 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. Each assay was completed in 15 min. Ten-microliter aliquots were injected onto the column, and the sample tray was cooled to 10°C to prevent sample degradation.

Positive ions were generated in the source by using purified air for the source gases under the following conditions and settings: turbo electrospray ionization gas, 7.0 l/min; nebulizer setting, 6; ion spray voltage, 4200 V; source gas temperature, 300°C; declustering potential, 75 V; focusing potential, 160 V; and entrance potential, 10 V. Nitrogen was used as the curtain gas 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 (collisionally activated dissociation = 8). The collision energy was set to 32 eV (lab frame) with a collision cell exit potential 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 deuterated cocaine. Analyst software (version 1.4.2; MDS Sciex, Concord, ON, Canada) was used for instrument control, data acquisition, and quantitative analysis. Calibration curves were constructed from the standard samples by using the ratio of cocaine to the deuterium-labeled cocaine internal standard and a least-squares linear regression analysis with $1/x$ weighting.

Determination of Cocaine Concentrations across Species by Mass Spectrometry

Plasma fractions from each blood sample (50–200 µl) were added to 570 µl of acetonitrile, 20 µl of 1 M NaF, and 2 µl of internal standard solution containing 750 nM deuterium-labeled norcocaine, cocaine, benzoylecgonine, and ecgonine methyl ester (Cerilliant Corporation). Cocaine metabolites were included as internal standards to maintain consistency between this study and other in vivo cocaine metabolite studies (Brim et al., 2011). Samples were vortexed for 30 s and centrifuged at 25,000 relative centrifugal force at 20°C for 30 min. The supernatant was removed and added to a clean microcentrifuge tube. Samples were centrifuged a second time by using the same conditions, and the supernatants were again transferred to clean tubes. Samples were evaporated to dryness in a vacuum centrifuge and stored at −80°C until analyzed within 1 week of preparation.

Mass spectral analysis was performed at the University of Michigan Biomedical Mass Spectrometry Facility. For mass spectrometry, the dried samples were reconstituted with 30 µl of 10 mM ammonium formate, pH 4.6/acetonitrile (97:3; v/v) to yield a 50 nM final concentration of each internal standard. To achieve concentrations of

cocaine within the limits of quantification, samples were diluted further (varying along the time course) with 10 mM ammonium formate, pH 4.6/acetonitrile (97:3; v/v) and 50 nM internal standards. Samples were vortexed for 30 s, then centrifuged at 13,600 relative centrifugal force for 20 min. Aliquots of the supernatants were transferred to polypropylene autosampler vials for analysis within 12 h.

LC-MS/MS was performed on a Prominence HPLC system (Shimadzu) interfaced directly to the Turbo Ion-spray source of an API 3000 triple quadrupole mass spectrometer (PerkinElmerSciex Instruments). Separation was achieved with a Thermo Fisher Scientific Hypersil Gold column (50 × 2.1 mm i.d.; 1.9-µm packing) maintained at 45°C using a binary gradient and a flow rate of 0.45 ml/min. The injection volume was 4 µl. The flow was split approximately 1 to 3.5 so that 0.13 ml/min was directed into the ionization source. Solvent A was 10 mM ammonium formate, pH 4.6, and solvent B was acetonitrile. The gradient program was as follows: 2% B at 0 min, hold 2% B for 1 min, 18% B at 2 min, 40% B at 10 min, 100% B at 11 min, 2% B at 12 min, and re-equilibrate at 2% B for 3 min. Each analysis was completed in 15 min. The sample tray was cooled to 10°C to prevent sample degradation.

Positive ions were generated in the source by using purified air for the source gases under the following conditions and settings: Turbo electrospray ionization gas, 7.0 l/min; nebulizer setting, 15; ion spray voltage, 3000 V; and source gas temperature, 400°C. Compound-specific ionization parameters were optimized for cocaine, each metabolite, and the internal standards. Nitrogen was used as the curtain gas at a setting of 12. Mass analysis was performed by single reaction monitoring with 100-ms dwell times. Nitrogen served as the collision gas (collisionally activated dissociation = 12). 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 deuterated cocaine. Analyst software (version 1.4.2; MDS Sciex) was used for instrument control, data acquisition, and quantitative analysis. Calibration curves were constructed from the standard samples. The ratio of the peak area of cocaine to the corresponding deuterium-labeled internal standards was plotted as a function of the analyte concentration normalized to the internal standard concentration. Calibration curves were generated by using a least-squares linear regression analysis with $1/x$ weighting.

Preparation of Calibration Standards for Mass Spectrometry

For the in vitro and in vivo comparison, calibration standards of cocaine (3.16–0.0043 µM) were prepared in human plasma (obtained from the University of Michigan Hospital Blood Bank) and commercial rat plasma from untreated Sprague-Dawley rats (Valley Biomedical, Winchester, VA). For the species comparison, calibration standards of cocaine, benzoylecgonine, ecgonine methyl ester (4.0–0.0313 µM), and norcocaine (0.4–0.00313 µM) were prepared in plasma from untreated Sprague-Dawley rats. All standards were stored at −80°C until sample preparation. The respective calibration standards were prepared with every set of experimental samples. Twenty microliters of each calibration stock was extracted with 68 µl of acetonitrile, 4 µl of 1 M NaF, and 8 µl of internal standard as described above. Calibration standards were reconstituted to 100 µl, resulting in final internal standard concentrations of 50 nM.

Serum and Urine Collection from Conscious Rats

Male Sprague-Dawley rats (300–350 g) (Harlan) were implanted with indwelling jugular catheters. In brief, the rats were anesthetized with 90 mg/kg ketamine and 10 mg/kg xylazine. Tubing was inserted into the jugular vein of the neck. Catheters were threaded under the skin and out the back.

On test day, rats were placed into cages fitted with a wire mesh bottom that allowed the passage of urine and feces through to the floor of the cage and did not permit animal access to excrements.

After administration of either DM-CocE or [35 S]DM-CocE, urine was collected from the cage once each hour after DM-CocE administration for a total of 12 h. Blood samples (100 μ L) were taken from the jugular catheter at the same time as urine collection, and the volume was replaced with 100 μ L of saline. Serum was collected from blood samples by using BD Biosciences Microtainer centrifuge tubes. Both serum samples and urine samples were aliquoted and flash-frozen with liquid nitrogen. Samples were stored at -80°C until analysis.

Western Blotting and Autoradiography

The presence of DM-CocE or [35 S]DM-CocE in urine and serum was determined by Western blotting. Urine (120 μ L) was desalted by using Protein Desalting Spin Columns (Thermo Fisher Scientific). Desalted urines were concentrated to a volume of 30 μ L. A portion of the desalted sample (15 μ L) was loaded onto a 10% SDS/PAGE gel in loading buffer containing β -mercaptoethanol.

Serum samples from animals administered DM-CocE or [35 S]DM-CocE were assessed for total protein concentration by using a Bradford assay, and 25 μ g of total protein was loaded onto a 10% SDS/PAGE gel in loading buffer containing β -mercaptoethanol. To assess the capacity of the CocE antibody to recognize smaller fragments of DM-CocE, samples were incubated at 100°C in the loading buffer described above for 10 min. In addition, some samples were treated with protease inhibitors (phenylmethanesulfonyl fluoride, tosyl-L-lysine-chloromethyl ketone, and tosyl-L-phenylalanine chloromethyl ketone).

The same Western blotting procedure was used on both urine and serum samples from animals receiving DM-CocE or [35 S]DM-CocE. Gels were run at 200 V for 40 min. Protein was transferred into a PVDF membrane at 60 V for 1.5 h. Membranes were blocked in Blotto for 1 h before the addition of rabbit anti-CocE primary antibody (generously produced and supplied by New England Peptide, Gardner, MA) at a concentration of 1:4000 diluted in Blotto. Primary antibody was incubated overnight shaking at 4°C . Membranes were washed three times for 15 min in PBS containing 0.1% Tween (w/v) before the addition of goat anti-rabbit-horseradish peroxidase secondary antibody diluted 1:30,000 in Blotto. Secondary antibody was incubated for 1 h at room temperature. Blots were washed three times for 15 min in PBS containing 0.1% Tween (w/v) and exposed to enhanced chemiluminescence reagent for 1 min. Finally, blots were exposed on Kodak film (Eastman Kodak, Rochester, NY).

Blotting membranes containing samples from [35 S]DM-CocE-treated animals were allowed to stand for 6 h after enhanced chemiluminescence exposure to ensure total completion of the light-emitting enzymatic reaction. These membranes were then exposed to film in cassettes containing intensifier screens for 2 weeks at room temperature to assess the radioactive contents of the membranes.

Urinalysis

Urinalysis was performed with 10SG Urine Reagent Strips (Fisherbrand; Thermo Fisher Scientific) according to package instructions. Urine measures from normal rats was compared with urine measures from DM-CocE-treated animals to ensure that readings outside of the specified normal range were caused by a species difference between rats and humans, not by an effect of DM-CocE.

Determination of Glomerular Filtration

Glomerular filtration rates of normal and unilaterally nephrectomized animals were determined by using an adapted method (Qi et al., 2004). In brief, a 5% fluorescein isothiocyanate-inulin (Sigma, St. Louis, MO) solution was made in 0.9% sodium chloride (saline; Hospira, Lake Forest, IL) by boiling for 2 min. Solution was dialyzed overnight in 1000 ml of saline by using a dialysis membrane (1000-Da cutoff; Spectra/Por 6; Spectrum Laboratories, Rancho Dominguez, CA). Solution was sterilized by filtration before injection. Fluorescein isothiocyanate-inulin solution (40 mg/kg) was injected intravenously through indwelling jugular catheters. Serum

samples were taken at 2, 5, 8, 11, 15, 20, 40, 60, 20, 120, 180, and 240 min after injection. Samples were buffered by diluting 1:25 in 500 mM HEPES, pH 7.4. Buffered sample (50 μ L) was added in duplicate to a 96-well microplate, and fluorescence was determined by using a SpectraMax M5 (Molecular Devices, Sunnyvale, CA) with 485-nm excitation and 538-nm emission.

Fluorescence per microliter of serum was plotted and fitted to a two-phase decay model by using Prism software (GraphPad Software, Inc. San Diego, CA). Glomerular filtration rate (GRF) was calculated by using eq. 1 where I is the amount of total fluorescence delivered by the bolus injection, A and B are the y-intercept values of the fast and slow decay rates, respectively, and α and β are the decay constants for the fast and slow decay phases, respectively.

$$\text{GRF} = \frac{1}{\left(\frac{A}{\alpha} + \frac{B}{\beta}\right)} \quad (1)$$

Calculation of Pharmacokinetic Parameters

Before injection of [35 S]DM-CocE (8 mg/kg, 18 $\mu\text{Ci/kg}$) to male Sprague-Dawley rats, the total cpm were assessed to determine the specific activity of each radioactive dose. To assess the radioactive content in the blood, 3 μ L of each sample was taken at $t = 3, 16, 20, 30, 45, 60$, and 90 min, as well as at 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 18, 19, and 29 h. Urine samples were collected after every urination. Samples were analyzed by a Packard 1900 TR liquid scintillation analyzer (PerkinElmer Life and Analytical Sciences, Waltham, MA) in 4 ml of CryoScint ES (MP Biomedicals, Solon, OH). From the radioactive content, the concentration of CocE (determined by using eq. 2) in the blood was plotted against time by using Prism software.

$$\frac{\mu\text{g}}{\text{mL}} \text{ CocE} = \frac{\text{cpm}}{\mu\text{L}} + \frac{\text{total cpm injected}}{\text{total ug CocE injected}} \quad (2)$$

Area under the curve (AUC) analysis was carried out by using Prism software, and clearance (Cl) was determined by using eq. 3 (Goodman et al., 1996).

$$\text{Cl} = \frac{\text{Dose } (\mu\text{g})}{\text{AUC } (\mu\text{g/mL/min})} \quad (3)$$

C_{max} was determined from the y-intercept of a log plot of the concentration data, and volume of distribution (V_d) was subsequently calculated by using eq. 4 (Goodman et al., 1996).

$$V_d \text{ (mL)} = \frac{\text{Dose } (\mu\text{g})}{C_{\text{max}} \text{ } (\mu\text{g/mL})} \quad (4)$$

Half-life ($t_{1/2}$) in the blood was then determined by using eq. 5 (Goodman et al., 1996).

$$t_{1/2} \text{ (min)} = \frac{0.693}{\text{Cl (mL/min)}} \times V_d \text{ (mL)} \quad (5)$$

Estimations of these parameters were initially made by using densitometry from Western blots. The C_{max} was assumed to be the total amount of CocE injected divided by the blood volume of each animal (7% total body weight). Density from the serum sample taken 5 min after CocE injection was assumed to be 100% of this C_{max} . Each subsequent density was expressed as a percentage of the 5-min time point and multiplied by the C_{max} . Half-life was determined by a one-phase decay model fit of the estimated concentrations by using Prism software.

Collection of Organs

Male Sprague-Dawley rats were deeply anesthetized with sodium pentobarbital (75 mg/kg). After animals no longer responded to paw pressure, an incision was made in the abdomen, and the sternum

was cut to reveal the heart and lungs. The right atrium was cut, and an 18.5-gauge needle was inserted into the left ventricle. Sixty milliliters of saline was delivered through the left ventricle. Complete perfusion was determined by no red color remaining in the heart or any lobe of the liver. Organs were removed and placed into 10% buffered formalin (100 ml of 37% formaldehyde, 4 g of sodium phosphate monobasic, 6.5 g of sodium phosphate dibasic, and volume to 1 liter) for immunohistochemistry.

Preparation of Organs and Histology

Immunoperoxidase. Organs were fixed in 10% buffered formalin overnight at room temperature. Dehydration was completed by 3×10 -min washes in 30% ethanol, followed sequentially by 3×10 -min washes at both 50 and 70%. Slide preparation and staining was performed at the University of Michigan Comprehensive Cancer Center Tissue Core. Organs were paraffinized and stored at room temperature until sections were cut from the blocks. Paraffin sections were cut on a microtome to $5 \mu\text{m}$ and heated for 20 min at 65°C . Slides were deparaffinized in xylenes, three changes of 2 min each. Slides were then rehydrated through graduated washes of 2 min each, ending with tap water (100% alcohol, 95% alcohol, 70% alcohol, and water). Epitope retrieval was completed by incubating slides for 30 min in 80°C citrate buffer, pH 6.0. Slides were cooled for 10 min followed by a 10-min running water wash.

Staining was performed on the Dako AutoStainer (Dako North America, Inc., Carpinteria, CA) at room temperature. Tissues were blocked with peroxidase before the addition of primary rabbit anti-CocE antibody at a dilution of 1:1000 for 30 min. After $3 \times$ PBS buffer rinse, an EnVision Plus Rabbit detection kit (Dako North America, Inc.) was used as the secondary antibody for 30 min. Slides were exposed to chromagen for 5 min before hematoxylin counter stain for 2 min. Slides were rinsed with water and dehydrated in gradual alcohol rinses. Three changes of xylenes for 2 min each were followed by addition of the coverslip. Slides were stored at room temperature until imaging by using a Nokia (Espoo, Finland) slide scanner at the Microscopy and Imaging Laboratory at the University of Michigan. Images were adjusted for contrast and brightness, and the background was removed by using Photoshop CS2 (Adobe Systems, San Jose, CA).

Fluorescence. Organ sections were prepared as described above. Nonspecific binding was reduced by blocking slides with 10% goat serum for 1 h. Primary rabbit anti-CocE antibody (produced and supplied by New England Peptide) was added onto slides at a dilution of 1:1000 in 10% goat serum and incubated overnight at 4°C . Tissues were washed three times for 10 min in PBS. Slides were exposed to secondary goat anti-rabbit Dylight-594 antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) at a 1:1000 dilution in 10% goat serum for 1 h. Coverslips were placed onto slides using ProLong Gold (Invitrogen, Carlsbad, CA). Slides were stored in the dark at room temperature until imaging with a Leica (Wetzlar, Germany) DMI6000 B microscope. Exposures were adjusted so that the maximal pixel intensities of each time-course set were at least half saturation. Fluorescence was quantified by using Image J software (National Institutes of Health, Bethesda, MD), and statistics were performed with Prism software. Images were adjusted equally for contrast and brightness by using Adobe Photoshop CS2.

Autoradiography. Organs sections were prepared for freezing by dehydration in 10% sucrose for 6 h and 30% sucrose overnight. Organs were frozen on dry ice in Optimal Cutting Temperature Compound (Tissue Tek; Sakura, Torrance, CA) and stored at -80°C until sections were cut from the blocks. Frozen sections were cut on a microtome at -20°C to $30 \mu\text{m}$ and fixed to glass slides. To image, slides were exposed to Kodak film at room temperature for 3 weeks.

Results

DM-CocE-Mediated Cocaine Hydrolysis. Cocaine hydrolysis by DM-CocE in vitro in human plasma was assessed

to establish a baseline for in vivo experiments. Plasma was spiked with $8 \mu\text{M}$ cocaine, the maximum peak plasma concentration achieved in an anesthetized Sprague-Dawley rat after a 4 mg/kg bolus intravenous injection. Low concentrations of DM-CocE (0.3–10 nM) were chosen to ensure slow cocaine hydrolysis that would allow differentiation between DM-CocE concentrations.

The addition of PBS (vehicle for DM-CocE) to cocaine-spiked plasma did not result in any reduction of cocaine concentration over the 5-min time course, demonstrating that the plasma was void of any endogenous rapid cocaine-metabolizing enzymes. The addition of increasing concentrations of DM-CocE was able to increase the amount of cocaine hydrolyzed over the time course in a dose-dependent manner, with 0.3 nM DM-CocE creating a slight reduction in the cocaine concentration and 10 nM eliminating cocaine to levels below the limit of quantification within 2 min (Fig. 1A). The 3 nM concentration of DM-CocE was able to eliminate approximately 50% of the cocaine within 1 min of addition.

To determine the in vivo effect of low-dose DM-CocE and explore any effect of sex on DM-CocE-mediated cocaine hydrolysis, male and female Sprague-Dawley rats were used to test the magnitude of cocaine hydrolysis by 13.6 or 50.5 $\mu\text{g/kg}$ DM-CocE, corresponding to the in vitro concentrations of 3 and 10 nM, respectively (based on a 350-g rat with a blood

Fig. 1. In vitro and in vivo cocaine hydrolysis by DM-CocE. A, in vitro DM-CocE dose response analysis. Human samples were spiked with $8 \mu\text{M}$ cocaine at $t = 0$. After 5 min at 37°C , DM-CocE was added to plasma at the given concentrations, aliquots were taken, and all hydrolysis was stopped at the given points. Aliquots were assessed for the remaining cocaine concentration by LC/MS analysis. B, in vivo assessment of cocaine hydrolysis by DM-CocE. Sprague-Dawley rats were intravenously administered 0, 13.6, or 50.5 $\mu\text{g/kg}$ DM-CocE followed 2 min later by an intravenous administration of 4 mg/kg cocaine. Blood samples were taken at the times after cocaine injection shown, hydrolysis activity was stopped, and samples were evaluated by LC/MS for cocaine concentration. Significance was assessed by two-way analysis of variance with Bonferroni posttests ($F(6, 60) = 7.37$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

volume 7% that of the body weight). DM-CocE (13.6 $\mu\text{g/kg}$) significantly reduced cocaine plasma concentrations at the 30-s ($p < 0.05$) and 2-min ($p < 0.01$) time points (Fig. 1B). This dose was found to eliminate the initial spike in cocaine concentration seen in the untreated animals; however, it was not able to completely clear all cocaine by 5 min after injection. DM-CocE at a dose of 50.5 $\mu\text{g/kg}$ significantly reduced cocaine concentrations at all time points ($p < 0.001$), eliminated the initial cocaine concentration spike, and eliminated cocaine to a level below the detection level by 5 min postinjection. Analysis of the data collected from the male and female rats used in this paradigm revealed that the sex of the animals tested did not have a statistically significant impact on overall cocaine concentration (Supplemental Fig. 2).

The experimental paradigms described above all used minimally effective concentrations of DM-CocE. We sought to characterize the hydrolytic effects of a more clinically relevant dose of DM-CocE (0.32 mg/kg) administered 10 min after a physiologically equivalent dose of cocaine in rats (5.6 mg/kg) and monkeys (3 mg/kg). These cocaine doses produce equivalent increases in mean arterial pressure as well as cause tonic-clonic convulsions (Collins et al., 2011b,c).

In rats, cocaine (5.6 mg/kg) produced transient tonic-clonic convulsions and initial plasma cocaine levels ranging from 7 to 27 μM (Fig. 2A). The cocaine concentration in rats receiving only the PBS control 10 min after cocaine fell to 0.1 to 1.7 μM over the 1-h time period because of the endogenous metabolism of cocaine. In contrast, animals receiving DM-CocE 10 min after cocaine had unquantifiable cocaine concentrations within 45 s. It is noteworthy that between the 20- and 60-min time points, the cocaine concentrations began a statistically insignificant, but repeatable and reliable, rise into the lower limits of quantification.

To assess this same paradigm in a higher species, two rhesus monkeys were chosen for this study. The results reported here are those from the first of four trials of this paradigm in each monkey to assess immunogenic effects of DM-CocE, the results of which are outside the scope of this study and will be reported elsewhere. Time courses of DM-CocE administration differed between these two animals because of their sensitivity to the high dose of cocaine. In the female monkey, administration of 3 mg/kg cocaine yielded a plasma concentration between 4.2 and 30.8 μM , a range similar to the rats (Fig. 2B). After administration of DM-CocE at $t = 10$ min, all cocaine was hydrolyzed to below quantifiable levels within 5 min. Although technical limitations resulted in the monkey blood sample being taken longer after DM-CocE administration compared with the rat, it nonetheless demonstrates the rapid hydrolysis of cocaine in the blood. Like the rat, the cocaine concentrations began to slowly rise after the 30-min time point, suggesting that this rise is not a species-specific phenomenon.

The male monkey was tested on a different dosing regimen, as DM-CocE was given only 1 min after cocaine (Fig. 2C). Even at this short time point when cocaine had not been fully distributed DM-CocE still eliminated blood cocaine concentrations to below quantifiable levels by 8 min (the first sample taken after CocE injection). The male monkey also showed results consistent with those from the rats and female monkey, in that after 30 min blood concentrations of cocaine begin to slowly rise. The final co-

Fig. 2. DM-CocE hydrolysis of high-dose cocaine across species. A, figure adapted from Brim et al. (2011). Three male Sprague-Dawley rats were administered 5.6 mg/kg cocaine (a physiologically equivalent dose to 3.2 mg/kg in the rhesus monkey) at time 0 and DM-CocE or PBS at time 10 min. PBS data are the average data from all three rats. DM-CocE data are separated by individual rats. DM-CocE rapidly removed cocaine to below the limit of quantification within 45 s. B, a female rhesus monkey was administered 3.2 mg/kg cocaine intravenously at time 0 and DM-CocE or PBS at time 10 min. Plasma cocaine concentrations were assessed at the given time points by mass spectrometry. Administration regimen was performed once every two weeks for 6 weeks. After DM-CocE administration, cocaine levels fell below the 30-nM limit of quantification. At times later than 30 min, low concentrations of cocaine appeared in the plasma, suggesting that the elimination of DM-CocE from the serum allowed very low amounts of cocaine to diffuse back into the blood from other body compartments. Monkey F exhibited the same pattern of cocaine elimination as the rat, suggesting no effect of species or repeat dosing of DM-CocE in the nonhuman primate. C, a male rhesus monkey was administered 3.2 mg/kg cocaine intravenously at time 0 and DM-CocE or PBS at time 1 min. Dosing regimen and sample analysis were conducted as described above for the female monkey. Like the rat and the female, cocaine reappeared at higher concentrations by 40 min after cocaine injection.

caine concentrations at the end of the observation period were below the discriminable threshold in both species.

Pharmacokinetics of DM-CocE. In both the rat and the monkey, very small amounts of cocaine reappeared in the

TABLE 1
Pharmacokinetic parameters of DM-CocE
Each condition is *n* = 3. Mean ± S.D. is in parentheses.

	Western Blot Estimation	Normal	Unilateral Nephrectomy
Glomerular filtration rate, $\mu\text{L}/\text{min}$		6030 (106.4)	2610 (53.4)*
Urine output, total 29 h		13.0 (1.8)	15.2 (6.3) <i>n/s</i>
Weight		343.3 (31.75)	302.0 (10.82) <i>n/s</i>
AUC, $\mu\text{g}/\text{mL}/\text{min}$		14,461 (7445)	14,932 (606.8) <i>n/s</i>
Clearance, mL/min	0.12	0.21 (0.07)	0.16 (0.01) <i>n/s</i>
V_d , mL	24.6	38.1 (11.4)	36.6 (5.4) <i>n/s</i>
$t_{1/2}$, min	144	125 (6.8)	156 (19.8) <i>n/s</i>

*, *p* < 0.001 Student's *t* test compared with normal.
n/s, *p* > 0.05 Student's *t* test compared with normal.

plasma after a DM-CocE administration that initially seemed to clear all cocaine. We hypothesized that if DM-CocE was eliminated from the circulation (causing a reduction in the rate of cocaine hydrolysis) then very low concentrations of cocaine from outside the circulation would diffuse back into the bloodstream. This is supported by previous work demonstrating high doses of both wild-type and a thermostable CocE mutant (L169K/G173Q-CocE; $t_{1/2}$ at 37°C in vitro = 2.9 days) were rapidly eliminated from the serum of mice with an estimated half-life of 2.1 and 2.2 h, respectively, based on Western blot analysis (Brim et al., 2010). If this elimination also occurs with DM-CocE at the lower dose used in this study, it would explain the small increase in cocaine concentration observed.

To accurately determine the elimination profile of DM-CocE and attempt to elucidate the mechanism of observed elimination, [³⁵S]methionine was used to metabolically label the protein. The pharmacokinetic parameters of DM-CocE determined from monitoring [³⁵S] in the blood are presented in Table 1. These parameters were determined by using curve fit and AUC analysis by Prism software over 10 h (the time to plateau-phase) post-CocE injection. The volume of distribution (V_d) was calculated by using the C_{max} and total dose of CocE (eq. 4). The V_d was not significantly different from the total blood volume of a rat (7% body weight), confirming assumptions based on the molecular mass (65-kDa monomer; 130-kDa dimer) that CocE does not reside outside the bloodstream. Although the V_d was slightly higher than

Fig. 3. Elimination of [³⁵S]DM-CocE from the blood. A, concentration of DM-CocE in the serum of rats (*n* = 3) as measured by [³⁵S]methionine labeling over time. [³⁵S]DM-CocE (8 mg/kg; 18 $\mu\text{Ci}/\text{kg}$) was administered intravenously through an indwelling jugular catheter at time 0. Blood samples (3 μL) were taken and assessed for radioactive content by scintillation counting. The concentration of DM-CocE was calculated by using the specific activity of each radioactive dose and plotted against time. DM-CocE was eliminated rapidly over the first 4 h after injection, until reaching a plateau state (inset). Both normal and unilaterally nephrectomized animals are shown. B, representative autoradiograph of [³⁵S] in serum over time from rats administered 8 mg/kg [³⁵S]DM-CocE. Male Sprague-Dawley rats were administered 8 mg/kg [³⁵S]DM-CocE at time 0. At 0.5, 1, 2, 4, 6, 8, 12, and 18 h after DM-CocE injection, serum samples were taken. Total serum protein (25 μg) from each time point was loaded onto a 10% SDS/PAGE gel. Gel was transferred onto a PVDF membrane, and membrane was exposed to film for 2 weeks. C, representative Western blot of DM-CocE in serum from rats administered 8 mg/kg DM-CocE. Serum protein was handled as described above, but membrane was subjected to Western blotting with an anti-CocE antibody. The immunolabeled protein was visualized with chemiluminescence and film. D, comparison of the 65-kDa band of both the Western blots (*n* = 3) and autoradiograph (*n* = 3). Both the chemiluminescent and radiographic data were assessed by using densitometry of the 65-kDa band. Raw values are plotted against time.

the estimated blood volume, this was most likely caused by underestimation of the blood volume using percentage body weight and not by protein binding of DM-CocE, because the amount of [35 S] radioactivity (in cpm) in each sample was determined by using a whole blood sample.

The concentration of DM-CocE in blood over time in the rats (calculated using eq. 2) is illustrated graphically in Fig. 3A. The concentration of DM-CocE peaked immediately after injection, because of the 100% bioavailability of intravenous delivery. Over the first 4 h after injection, there was a slow, but steady, decrease in concentration to approximately 40 μ g/ml/kg DM-CocE (see Fig. 3A inset). For the remainder of the time course, the concentration of DM-CocE remained relatively constant. After 29 h, only 10% of the total radioactivity injected remained in the blood. To determine whether the measured radioactivity represents full-length DM-CocE, or smaller fragments in the circulation, further analysis was conducted.

Serum from [35 S]DM-CocE-treated animals was separated by SDS/PAGE and transferred to a PVDF membrane. Autoradiography was performed on these membranes to visualize the size specificity of the radioactive signal. A band corresponding to the size of full-length DM-CocE (65 kDa) was strongly visible on the radiograph (Fig. 3B) at 0.5, 1, and 2 h after injection. However, at later time points, only small amounts of this band were visible, and more radioactive labeling of higher and lower molecular weight species was seen, suggesting that DM-CocE may be recycled.

Western blot analysis was also performed on serum samples from rats administered unlabeled 8 mg/kg DM-CocE IV to ensure that the 65-kDa band represented DM-CocE at all time points. The initial concentration after bolus injection was assessed by serum taken 5 min after DM-CocE injection and followed by serum taken at 1, 2, 4, 6, 8, and 12 h. Full-length DM-CocE was seen in these Western blots from serums taken up to 6 h after DM-CocE injection (Fig. 3C). To confirm that smaller fragments of DM-CocE were not present in the serum samples and that the antibody cannot recognize smaller fragments of DM-CocE, serum samples were boiled under denaturing and reducing conditions to create chemical cleavages. Western blot analysis of these samples revealed five chemical cleavage products of DM-CocE (Supplemental Fig. 3), confirming that the anti-CocE antibody can recognize fragments of DM-CocE, but that these products are not exclusive to animal samples, because control DM-CocE also revealed these cleavages. A comparison between radiograph and Western blot also revealed that these methods of analysis were highly correlated, and the radioactivity analyzed in the blood at the early time points corresponds to full-length DM-CocE (Fig. 3D).

Urine samples were analyzed by scintillation counting, and 10% of the total cpm injected was recovered in the urine over 29 h. This cumulative elimination is illustrated in Fig. 4A. Twenty-five percent of the total radioactivity eliminated was within the first 4 h postinjection. Urine samples were also collected from the animals administered 8 mg/kg unlabeled DM-CocE for analysis by Western blot. Urine samples taken 1 and 2 h after DM-CocE injection contained full-length and some smaller molecular weight fragments of DM-CocE (Fig. 4B). Dipstick measures of glucose, bilirubin, ketone, specific gravity, blood, pH, protein, urobilinogen, nitrate, and leuko-

Fig. 4. Appearance of [35 S]DM-CocE in the urine. A, cumulative urine accumulation of [35 S] radioactivity. After [35 S]DM-CocE administration to rats ($n = 3$), urine was collected and assessed for radioactive content by scintillation counting. Cumulative counts per minute per kilogram are shown against time and fit to a one-phase association model [$Y = Y_0 + (\text{plateau} - Y_0) \times (1 - \exp(-K \times x))$]. Data plotted are S.E.M. B, Western blot analysis of the presence of DM-CocE in urine. Samples were added to SDS and β -mercaptoethanol loading buffer and immediately loaded onto 10% SDS/PAGE gels. DM-CocE (40 ng) was loaded as a positive control, desalted urine alone was a negative control, and 40 ng DM-CocE spiked into urine was to control for protein recovery from urine desalting. Urine was collected at the times shown after CocE injection and was loaded in a time-dependent order.

cytes in the urine did not differ between animals receiving DM-CocE and PBS vehicle (data not shown).

The Presence of DM-CocE in Major Organs. Analysis of serum samples by Western blotting and autoradiography revealed that radioactivity (in cpm) was a good indicator of full-length DM-CocE during the first 4 h after administration. However, after this time, more diffuse radioactivity across a wide range of molecular weights was seen on autoradiographs. In addition, after the amount of radioactivity in the serum stabilized to a constant level, the cumulative amount of radioactivity eliminated in the urine continued to rise, indicating that the radiolabel must be sequestered in another compartment. We hypothesized that DM-CocE could undergo specific or nonspecific receptor-mediated endocytosis, uptake into major organs, or rapid degradation to produce recycled amino acids for cellular pathways. To this end, we performed immunohistochemical analysis on the heart, lung, liver, and kidney of male Sprague-Dawley rats.

Rats were administered DM-CocE (8 mg/kg) into the tail vein every day for 14 days, or 24 mg/kg every 4 days for 14 days. Six hours after the final DM-CocE injection, rats were

sacrificed and organs were collected for immunohistochemistry and pathological analysis. No significant pathological difference was found between the organs of saline- and DM-CocE-treated rats (data not shown). Upon immunohistological analysis, no DM-CocE was detected in the heart, liver, or lung. However, significant DM-CocE was detected in the kidney, specifically localized to the renal papilla (Fig. 5). Papillae accumulation was dose-dependent, with the kidneys from the 8 mg/kg-treated animals showing much less immunoreactivity than the kidneys from the 24 mg/kg-treated animals (Fig. 5). The immunological findings were consistent between individual animals in each group. These data are consistent with the [35 S]DM-CocE labeling data that revealed significant amounts of radioactivity in the urine, because the papilla is the last portion of the kidney structure the renal filtrate contacts before entering the renal pelvis and exiting the body via the ureters. It is unclear from this experimental design whether the reactivity seen here is caused by normal elimination pathways or an accumulation of DM-CocE over multiple doses.

Time Course of Renal Accumulation and Clearance.

To examine whether DM-CocE might be accumulating in the papilla, a potentially serious phenomenon, a time course of DM-CocE accumulation in the renal papilla was performed by fluorescence immunohistochemistry. Sprague-Dawley rats were given either saline or 8 mg/kg DM-CocE intravenously through an indwelling jugular catheter. At each time point after DM-CocE injection (0.5, 1, 2, 4, 8, 12, and 24 h) three rats were sacrificed and kidneys were prepared for immunohistochemistry. DM-CocE reached maximum accumulation levels in the papilla 2 h after DM-CocE injection and reached near baseline levels by 4 h (representative im-

ages in Fig. 6A). After 12 to 24 h postinjection, there are no longer significant amounts of DM-CocE immunoreactivity compared with saline (Fig. 6B).

The Effect of Unilateral Nephrectomy. To test whether the kidneys play a major role in the rapid elimination of full or fragmented CocE from the circulation, or urinary elimination of CocE is only secondary to another means of elimination, [35 S]DM-CocE was given to unilaterally nephrectomized rats with a reduced glomerular filtration rate compared with normal animals (2619 μ l/min compared with 6030 μ l/min, respectively; $p < 0.001$). Assessment of the pharmacokinetics of [35 S]DM-CocE in these animals yielded parameters that were not statistically significantly different from the normal animals (Table 1). Figure 3A illustrates that the patterns of elimination from the blood are not significantly different in the nephrectomized animals. Although no statistically significant differences between groups were observed, the clearance rate in the nephrectomized animals was slightly lower (0.16 compared with 0.21 ml/min) and the $t_{1/2}$ of CocE was slightly higher (156 compared with 125 min) than normal animals. These differences suggest that the kidneys are most likely assisting with the clearance of CocE, but that glomer-

Fig. 5. Immunohistochemical analysis of DM-CocE distribution in perfused organs from Sprague-Dawley rats. Sprague-Dawley rats received intravenous administration of the DM-CocE (8 mg/kg) or vehicle once daily for 14 days ($n = 3$ each) or DM-CocE (24 mg/kg) once every 4 days for 14 days ($n = 3$). Six hours after the final dose of DM-CocE, rats were sacrificed and perfused, with organs fixed and embedded in paraffin for immunohistochemical analysis. Sections were counterstained with hematoxylin (blue), and DM-CocE reactivity was indicated by brown precipitate formed by diaminobenzadine. Positive DM-CocE reactivity is dose-dependently seen at the tip of the renal papilla. Images are from one representative animal from each group.

Fig. 6. Immunohistochemical analysis of DM-CocE accumulation in the renal papilla. A, Sprague-Dawley rats ($n = 3$) were administered 8 mg/kg DM-CocE intravenously. At the times shown after injection, rats were sacrificed and perfused with saline, and kidneys were fixed and embedded in paraffin. Immunohistochemistry was performed on kidney sections. Representative images from each time point are shown. Each differential interference contrast (DIC) image shows the area of the papilla that the fluorescence image (TXR) highlights. The overall area of the papilla that the images are taken from is exemplified by the saline sample. B, analysis of kidneys from three animals at each time point reveals peak DM-CocE accumulation at 2 h, after which there is a rapid decline. At 24 h, DM-CocE reactivity is no longer seen. *, one-way analysis of variance; $F_{7, 16} = 2.47$; Dunnett's Multiple Comparison Test, $p < 0.05$. Data are plotted as S.E.M. n/s, not significant.

ular filtration of either full-length or fragmented CocE is not the predominant mechanism of elimination. Although this could be predicted by the findings that only 10% of the total cpm injected was found in the urine, the observation that nephrectomy had no significant effect on the 10% of DM-CocE eliminated in the urine definitively demonstrates the minor role of renal clearance.

Uptake of Radioactivity into Organs. To evaluate the fate of the unrecovered 80% of radioactivity administered (10% remained in the blood, and 10% was recovered in the urine), animals receiving [35 S]DM-CocE were analyzed post-mortem. [35 S]DM-CocE-treated animals were sacrificed after the 29-h observation period and thoroughly perfused to remove all radioactivity in the vascular circulation so that the major organs could be analyzed by autoradiography. Slices (30 μ m) of heart, lung, liver, kidney, spleen, stomach, and small intestine were exposed to film for 3 weeks. All tissues examined were labeled with [35 S] (Fig. 7B). Immunohistochemical analysis on the kidney and liver from rats treated with DM-CocE 24 h prior revealed no DM-CocE immunoreactivity (Fig. 7A).

Fig. 7. Radiography and immunohistochemistry of organs 24 h post-DM-CocE injection. Rats administered either DM-CocE or [35 S]DM-CocE ($n = 3$) were sacrificed and perfused with saline 29 or 24 h after administration, respectively. Organ slices were subjected to immunohistochemistry (A) or radiography (B), and representative images are shown. No DM-CocE reactivity was detected with the anti-CocE antibody; however, evenly distributed and very dense radioactivity was detected in all organ slices.

Discussion

This study explored the biologic fate of DM-CocE, both its ability to hydrolyze cocaine once injected and its elimination. The pharmacodynamics of DM-CocE were assessed by using the disappearance of cocaine as a marker for DM-CocE activity. In vivo cocaine hydrolysis by DM-CocE was measurable and was similar to the in vitro time course of degradation. The in vivo experiment was performed over a very short time course (5 min) novel to the cocaine metabolism literature. The very low doses of DM-CocE (13.6 and 50.5 μ g/kg) builds on previous work demonstrating the high catalytic efficiency of the enzyme. These results demonstrate that very low doses can enhance the elimination of cocaine and the minimally effective dose in vivo is only half a log dose higher than it is in vitro. This near equivalence is somewhat surprising because of the constant redistribution of cocaine that must take place across biological membranes to reach the enzyme in the bloodstream, a barrier not present in controlled in vitro experiments. This can be used as a guide to determine appropriate starting doses for clinical safety trials.

This is also the first study to directly compare DM-CocE's effectiveness across species and the third study to test DM-CocE across sexes. Consistent with the findings of Collins et al. (2011b,c), no significant differences were observed in DM-CocE's ability to hydrolyze cocaine in males in comparison to females, in either rats or monkeys. In addition, the data presented here confirm that DM-CocE's ability to hydrolyze cocaine is not affected by the species in which it is tested.

We have previously shown that wild-type CocE and L169K/G173Q-CocE have short serum half-lives in mice (2.3 and 2.2 h, respectively, estimated by Western blotting) and this limits their duration of action in vivo (Brim et al., 2010). We have significantly expanded on this work to describe the pharmacokinetics of DM-CocE and the mechanism of DM-CocE's rapid elimination. Half-life of DM-CocE was determined by both Western blotting (2.4 h) and [35 S] labeling (2.1 h). The similar half-lives of DM-CocE and [35 S]DM CocE demonstrate that the radioactive labeling process did not affect the elimination of the enzyme and validates the Western blot method used in previous studies as an accurate indicator of half-life.

The short in vivo half-life of DM-CocE is consistent with the clinical half-life of several biologic protein enzyme drugs of similar size currently on the American drug market such as agalsidase (Fabrazyme; Genzyme, Cambridge, MA; 100 kDa; $t_{1/2} = 45$ –102 min), denileukin difitox (Ontak; Eisai, Woodcliff Lake, NJ; 58 kDa, $t_{1/2} = 70$ –80 min), and laronidase (Aldurazyme; Genzyme; 83 kDa, $t_{1/2} = 1.5$ –3.6 h). These proteins are subject to rapid elimination or degradation, presumably through receptor-mediated endocytosis and rapid breakdown in the liver (Tang and Meibohm, 2006). We sought to understand the mechanism of elimination of DM-CocE from the circulation. [35 S] labeling of DM-CocE demonstrated that approximately 10% of DM-CocE (or its fragments) was eliminated in the urine over 29 h. Western blot analysis confirmed that full-length CocE is present in the urine as a full-length protein for the first 2 h after injection. In addition, DM-CocE immunoreactivity was detected in the renal papilla, the last portion of the kidney the renal filtrate flows through before entering the renal pelvis and leaving the body through the ureters and bladder; this was expected,

considering DM-CocE's presence in the urine. This reactivity was time-dependent and reversible, because no significant reactivity was seen 24 h after DM-CocE administration.

These results are somewhat surprising considering that DM-CocE is a 65-kDa protein that exists as a noncovalent dimer of approximately 130 kDa. The urinary elimination mechanism thus seems unlikely in a healthy subject if one considers the long existing dogma that the exclusion size for glomerular filtration is approximately 65 kDa, the size of serum albumin (Haraldsson et al., 2008). This supposition has been called into question by advances in two-photon microscopy and recombinant technology. Several studies suggest that albumin does in fact readily cross through the glomerulus to the proximal tubules but then is taken back into the blood or degraded into small fragments before reaching the urine (Comper et al., 2008; Sarav et al., 2009). These data have been met with some resistance; however, in light of our data with DM-CocE, this seems to be a plausible mechanism of the urinary exclusion of albumin. If albumin is filtered through the glomerulus, then other large proteins, endogenous and exogenous, will also most likely be filtered. The endogenous proteins have mechanisms in place to rapidly remove them from the filtrate, by both reuptake and degradation. However, an exogenous protein, such as DM-CocE, would have no such mechanisms and would be expelled through the urine.

DM-CocE's presence in the urine was, however, minor, and no epitope-containing DM-CocE was detected in the major organs at 6 h or 24 h after injection. However, serum proteins of larger and smaller size than DM-CocE were labeled with [35 S] 4–18 h after [35 S]DM-CocE injection (Fig. 3C), and perfused organs taken from these [35 S]DM-CocE-treated animals were completely metabolically labeled (Fig. 7) after 29 h. Though it is possible that some residual blood may have been left behind after perfusion the concentration of radiolabel in the blood at the time of perfusion was too low to be visualized by autoradiography. These data taken as whole with the immunohistochemistry data (Figs. 5 and 6) suggest that the primary elimination of DM-CocE occurs through rapid proteolysis in the serum and amino acid uptake.

Steady-state concentrations of amino acids (16–30 μ M methionine) (<http://www.nlm.nih.gov/medlineplus/ency/article/003361.htm>) are maintained by consent uptake and efflux from cells (especially liver and muscle) through various high-affinity transporters such as the L-type transport system, which possess broad tissue distribution and high affinity for large neutral amino acids such as methionine (Verrey, 2003; Bröer, 2008; del Amo et al., 2008). Such distribution is in good agreement with the autoradiographic data from [35 S]methionine-treated rats demonstrating complete and ubiquitous labeling of the major organs. The A-type [Na^+ -dependent symport (McGivan and Pastor-Anglada, 1994; Bröer, 2008)], ASC-type [Na^+ -dependent antiport (Kanai and Hediger, 2004; Bröer, 2008)], and B o -type [Na^+ - or Ca^{2+} -dependent symport (Bröer, 2008)] systems may also play roles in the rapid uptake of [35 S]methionine from DM-CocE.

The rapid breakdown of DM-CocE is an advantageous quality for its potential use as a cocaine toxicity treatment, because it could be administered to a patient, act rapidly on cocaine, and then be eliminated within hours. The rapid elimination should reduce the likelihood of an immune response in humans, consistent with the lack of major immune

response seen with CocE in mice (Ko et al., 2007, 2009) and monkey (Collins et al., 2011a,b). Rapid elimination also prevents the accumulation of large aggregates, which may cause damage to capillary beds or organs such as the lung and kidney (supported by our data that indicate no full-length protein is evident in the major organs).

However, if CocE is to be used as a treatment for cocaine abuse [the proof of concept established by Collins et al. (2009)], the rapid elimination needs to be prevented to ensure a long serum half-life. This is feasible by using established techniques such as PEGylation (the chemical modification of proteins with polyethylene glycol chains), a process that has successfully been used to extend the half-life of Food and Drug Administration-approved biologics such as L-asparaginase [extension from 8–30 h (Elspar; Merck, West Point, PA) to 3.2–5.7 days (Oncaspar; Enzon Pharmaceuticals, Inc., Bridgewater, NJ)] and filgrastim [extension from 3.5 h; Neupogen, Amgen, Thousand Oaks, CA) to 15–80 h (Neulasta; Amgen)]. These increases are caused by an increase in hydrodynamic volume protecting the proteins from proteolysis and glomerular filtration (Tang and Meibohm, 2006).

PEGylation has been successfully used to extend the serum half-life of CocE in initial preclinical laboratory models. PEGylation prevents DM-CocE proteolysis by trypsin in vitro (Park et al., 2010), and in vivo studies with PEGylated S4C/S10C/T172R/G173Q-CocE demonstrate extended duration of protection against cocaine-induced lethality in rats from less than 24 h to more than 48 h (Narasimhan et al., 2011). Although these are dramatic increases, we believe that an addiction therapy needs to remain in the circulation for at least 1 week, because drug abusers showed good compliance to once-weekly clinic visits when participating in a motivational incentive program to receive a vaccination regimen (Stitzer et al., 2010). To achieve this duration of protection, we are currently investigating alternate PEGylation sites and elucidating the sites of proteolysis. Overall, this study is the first account of the pharmacokinetics and dynamics of DM-CocE and gives insight into the ability of bacterial enzymes to become feasible drug products.

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Authorship Contributions

Participated in research design: Brim, Noon, Collins, Stein, Ko, Woods, and Sunahara.

Conducted experiments: Brim, Noon, and Collins.

Contributed new reagents or analytic tools: Nichols and Narasimhan.

Performed data analysis: Brim and Noon.

Wrote or contributed to the writing of the manuscript: Brim, Woods, and Sunahara.

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