JPET Fast Forward. Published on October 11, 2011 as DOI: 10.1124/jpet.111.186049

Title Page

The Fate of Bacterial Cocaine Esterase (CocE): An *in vivo* Study of CocE-Mediated Cocaine Hydrolysis, CocE Pharmacokinetics, and CocE Elimination

Remy L. Brim, Kathleen R. Noon, Gregory T. Collins, Aron Stein, Joseph Nichols, Diwa Narasimhan, Mei-Chuan Ko, James H. Woods, Roger K. Sunahara

Department of Pharmacology
University of Michigan
1301 Medical Science Research Building III
1150 West Medical Center Drive
Ann Arbor, MI 48109-0600
(RLB, JN, DN, MCK, JHW, RKS)

Innovation Center
Medical College of Wisconsin
8701 Watertown Plank Road
Milwaukee, WI 53226
(KRN, current)

Alcohol and Drug Abuse Research Center
McLean Hospital/Harvard Medical School
115 Mill St
Belmont, MA 02478
(GTC, current)

Stein Consulting
3525 Del Mar Heights Rd., No. 552
San Diego CA 92130
(AS)

JPET Fast Forward. Published on October 11, 2011 as DOI: 10.1124/jpet.111.186049
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title Page

Running Title: *In vivo* action and elimination of bacterial cocaine esterase

Corresponding Author: Roger K Sunahara
University of Michigan
1301 Medical Science Research Building III
1150 West Medical Center Drive
Ann Arbor, MI 48109-0600
Phone: (734) 647-6377
Fax: (734) 763-4450
Email: sunahara@umich.edu

Text pages: 31

Tables: 1

Figures: 7

References: 42

Words in the Abstract: 249

Words in the Introduction: 681

Words in the Discussion: 1495

Abbreviations: CocE: cocaine esterase; DM-CocE: T172R/G173Q-CocE; WT: wild-type; cpm: counts per minute; PTT: phenylmethanesulfonyl fluoride, tosyl-l-lysine-chloromethyl ketone and tosyl-l-phenylalanine chloromethyl ketone

Section Assignment: Drug Development and Translational Medicine

JPET Fast Forward. Published on October 11, 2011 as DOI: 10.1124/jpet.111.186049
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract:

Cocaine abuse and toxicity remain widespread problems in the United States. Currently cocaine toxicity is treated only symptomatically, as there is no FDA-approved pharmacotherapy for this indication. To address the unmet need, a stabilized mutant of bacterial cocaine esterase (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 μg/kg effectively eliminated 4 mg/kg cocaine within 2 minutes in both male and female rats as measured by mass spectrometry. We expanded on these findings by using a pharmacologically 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 30-60 minutes. Elimination of DM-CocE was quantified using [^{35}S] labeling of the enzyme and was found to have a half-life of 2.1 hours in rats. Minor urinary output of DM-CocE is also observed. Immunohistochemistry, western blotting, and radiography were all 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 towards clinical use.

Introduction

Cocaine abuse is a widespread problem in the United States, with over 1.5 million people currently abusing the drug (Substance Abuse and Mental Health Services Administration, 2010). 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 (Schrank, 1992; Freye and Levy, 2009). Toxicity is primarily caused by cocaine's blockade of cardiac sodium channels, in addition to the high levels of norepinephrine that accumulate during monoamine transporter blockade (Schrank, 1992; Freye and Levy, 2009). Each year, over 500,000 emergency department visits are caused by cocaine toxicity (US Department of Health and Human Services). The abuse of cocaine and its toxicity contribute to the estimated loss of $180.8 billion from the US economy each year due to 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 alpha-beta 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).

JPET Fast Forward. Published on October 11, 2011 as DOI: 10.1124/jpet.111.186049
This article has not been copyedited and formatted. The final version may differ from this version.

However, wild type CocE is limited by thermal instability, exhibits an activity half-life of about 10 minutes *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), 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 dose and pharmacologically relevant doses of DM-CocE in both male and female Sprague Dawley rats and Rhesus monkeys using mass spectrometry. To complement these data, the pharmacokinetics of DM-CocE are determined using [^{35}S]-labeling and the distribution and elimination are assessed by immunologic and radiologic techniques. The work presented here represents a significant advance towards understanding the way that live biological systems will

handle exogenous CocE protein, and help us understand how to further stabilize it in the circulation.

Methods

Production and Purification of RQ-Cocaine Esterase: A BioFlow 3000 bioreactor (New Brunswick, NJ) was prepared with 10 L of Terrific Broth (Maniatis, BentonDickinson) 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 OD_{600} of 5. Once the target turbidity was reached, the bioreactor was cooled to 18°C and then induced by adding IPTG for a final concentration of 1 mM. After 16 hours of induction, the culture was harvested by spinning down the broth at 4500 x *g* for 20 minutes. 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 twice or three times to lyse the cells. The maximum pressure for lysis is approximately 1100 psi. The lysate was clarified by spinning at 100,000 x g in an ultracentrifuge (Beckman Coulter XL-100K ultracentrifuge) using the rotor type Ti45. Clarified lysate was diluted by adding an equal volume of Q-buffer A (20mM 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 Q-buffer A followed by a gradient set to reach 50% Q-buffer B (20mM Hepes pH 8.0 + 1M NaCl) within 1000 mL. Flow-through and washes were saved for analysis. DM-CocE was eluted from the column using a gradient from 50 to 100% Q-buffer B over 5 column volumes (2250 mL). Fractions (25 mL) were collected at a

JPET Fast Forward. Published on October 11, 2011 as DOI: 10.1124/jpet.111.186049
This article has not been copyedited and formatted. The final version may differ from this version.

flow rate of 10 mL/min. DM-CocE was monitored by running 5 µl of each fraction on an 8% SDS PAGE Gel and staining with Coomassie Brilliant Blue stain according to established protocols.

Fractions from the first Q Sepharose HP separation were pooled and adjusted to 1M ammonium sulfate (final volume 525 mL). Samples were applied to a 450 mL Phenyl-Sepharose column pre-equilibrated with PS-Buffer A (Buffer A: 50mM Na Phos, pH 7 + 1M AS). 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: 50mM Na Phos, pH 7) over two column volumes (~1000 mL). DM-CocE was eluted with 30% to 100% PS Buffer-B over 5 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 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 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 (450mL). Chromatography was performed as described above.

Production and purification of [^{35}S]-DM-CocE: DM-CocE contains 11 methionines (Met1, 13, 16, 113, 141, 323, 350, 483, 507, 521, 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®) were transformed with a pET24b plasmid containing cDNA for histidine-tagged DM-CocE. A transformed clone was selected and confirmed by DNA sequencing.

A 5 mL starter culture in LB medium containing ampicillin with B834-pET24b cells was incubated for 12 hr at 37°C. The 5 mL culture was then used to inoculate 25 mL LB medium containing ampicillin and culture was grown to at 37°C to OD_{600}=0.8. The culture was spun at

JPET Fast Forward. Published on October 11, 2011 as DOI: 10.1124/jpet.111.186049
This article has not been copyedited and formatted. The final version may differ from this version.

4000 g 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 M9 medium containing ampicillin and grown at 37°C for 30 min. DM-CocE expression was induced with 1 mM IPTG, 10 µg/mL methionine and 1 mCi of [^{35}S]-methionine and grown at 18°C overnight.

After induction cells were spun and resuspended in 3 mL Tris-HCl buffered saline, pH 7.4 (TBS) containing 3 µg/mL of both leupeptin and lima bean trypsin inhibitor (LS) as well as and lysozyme 50 µg/mL. To initiate cell lysis, cells were incubated for 20 min at room temperature with gentle mixing and then snap frozen with liquid N_2. Lysate was thawed and added to 4 mL B-PER® Bacterial Protein Extraction Reagent (Thermo Scientific) per gram cell pellet containing 8 µg/mL DNase 1 and 1 mM $MgCl_2$. Lysate was incubated 20 min at room temperature with gentle mixing and centrifuged at 13,000 rpm for 30 min to remove cellular debris.

[^{35}S]-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 TBS+LS, 15 mL TBS+LS + 300 mM NaCl and 15 mL 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 using Coomassie brilliant blue stain.

Fractions containing [^{35}S]-DM-CocE were combined, diluted 4x in 50 mM Tris-HCl, pH 8.0, and loaded onto the Q-Sepharose Column. The column was washed with 5 mL 50 mM Tris-HCl, pH 8.0, 5 mL 50 mM Tris-HCl, pH 8.0 + 300 mM NaCl and eluted with 0.5 mL step gradient with 50 mM Tris-HCl, pH 8.0 + each 325-475 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 3mm blotting paper using a Bio-Rad model 483 slab dryer for 4 hr at 80°C. Dried gels were exposed to film for 8-12 hr at -4 ° C to assess purity of samples (Supplemental Figure 1). Fractions from

the Q-Sepharose column containing [^{35}S]-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, 250-350g, obtained from Harlan Sprague Dawley, Indianapolis) were maintained on a temperature controlled environment with a 12-hr light cycle, on at 7 AM. Rats were housed in Plexiglas cages (49 cm long x 23 cm wide x 21 cm high) with cob bedding and 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 hour light cycle beginning at 7 AM. Monkeys were fed 20-50 Lab Fiber Plus Monkey Diet Chows (Lab Diet; PMI Nutrition International, LLC; Brentwood, MO) per day, 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, and all experiments were additionally approved by the University of Michigan Committee on the Use and Care of Animals (UCUCA).

Animal preparation for determination of* in vivo *rates of cocaine hydrolysis:

For the 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 37ºC. 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 µg/kg, based on *in vitro* results) or vehicle control were administered intravenously via one catheter. Two minutes post DM-CocE or vehicle administration, rats were given a dose of cocaine (4 mg/kg) intravenously through the second catheter. Blood samples (approximately 200 µL/sample) were collected from the first catheter at 30 seconds, 1, 2, and 5 minutes post cocaine administration. Blood samples were immediately placed into 70 µL of EDTA solution containing 50 µL 50mM EDTA and 20 µL 1M 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).

For the 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 runs 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 49 cm long x 23 cm wide x 21 cm high Plexiglas cages 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 second 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 minutes. Blood was sampled (200 µL) at t= 2, 5, 9, 10:45, 12, 15, 20, 40 and 60 minutes and immediately added to 5 µL of 500 mM EDTA and 20 µL of 1M NaF to prevent coagulation and further cocaine hydrolysis, respectively. Plasma was collected by centrifugation at 4,000 rpm in a bench top microcentrifuge, flash frozen in liquid nitrogen and stored at -80°C until preparation for mass spectrometry.

JPET Fast Forward. Published on October 11, 2011 as DOI: 10.1124/jpet.111.186049
This article has not been copyedited and formatted. The final version may differ from this version.

For the 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 time t=0) and DM-CocE or PBS (0.32 mg/kg at time t=10 minutes) were administered through acute intravenous catheters. Blood samples were taken at time t=0, 8, 15, 30, 60, 90 and 120 minutes from the saphenous vein. Blood was immediately collected into tubes containing EDTA (BD Vacutainer K_2EDTA Plus Blood Collection, 5 mL) and 1/10 volume 1M 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 mL human plasma samples obtained from the University of Michigan Hospital Blood Bank were spiked with 8 µM cocaine. Plasma samples containing cocaine were incubated at 37°C for 5 minutes. After warming the plasma, DM-CocE was added. Twenty µL aliquots of plasma were taken from the 1 mL sample at time 20 and 40 seconds, 1, 2, and 5 minutes after DM-CocE addition. These aliquots were immediately placed into 80 µL of acetonitrile solution containing 4 µl of saturated (1M) NaF to prevent further cocaine metabolism along with 10 µL of internal standard (deuterated cocaine, Cerilliant, Round Rock), and then immediately prepared for LC-MS/MS.

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

JPET Fast Forward. Published on October 11, 2011 as DOI: 10.1124/jpet.111.186049
This article has not been copyedited and formatted. The final version may differ from this version.

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 x 2.0mm i.d., 4 µm packing) maintained at 40ºC 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 minutes. Ten µL aliquots were injected onto the column and the sample tray was cooled to 10ºC to prevent sample degradation.

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), 300ºC; 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 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) was added to 570 µL ACN, 20 µL 1M NaF, and

JPET Fast Forward. Published on October 11, 2011 as DOI: 10.1124/jpet.111.186049
This article has not been copyedited and formatted. The final version may differ from this version.

2µL of internal standard solution containing 750 nM deuterium-labeled norcocaine, cocaine, benzoylecgonine and ecgonine methyl ester (Cerilliant, Round Rock). Cocaine metabolites were included as internal standards to maintain consistency between this study and *in vivo* cocaine metabolite studies to be published elsewhere (Brim et al., 2011). Samples were vortexed for 30 seconds and centrifuged at 25,000 rcf at 20°C for 30 minutes. The supernatant was removed and added to a clean microcentrifuge tube. Samples were centrifuged a second time 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 a 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 seconds, then centrifuged at 13,600 rcf for 20 minutes. Aliquots of the supernatants were transferred to polypropylene autosampler vials for analysis within 12 hours.

LC-MS/MS 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 Thermo Hypersil Gold column (50 x 2.1mm i.d., 1.9 µm packing) maintained at 45ºC 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

JPET Fast Forward. Published on October 11, 2011 as DOI: 10.1124/jpet.111.186049
This article has not been copyedited and formatted. The final version may differ from this version.

in 15 minutes. The sample tray was cooled to 10 ºC to prevent sample degradation.

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 setting, 15; ion spray voltage, 3000 V; source gas temperature 400 ºC. Compound-specific ionization parameters were optimized for cocaine, each metabolite, and the internal standards as shown in Table A1.1. 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 (CAD=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 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. 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 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 µM-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 µL each calibration stock was extracted with 68 µL ACN, 4 µL 1M NaF, and 8 µL of internal standard as described above. Calibration standards were reconstituted to 100 µL resulting in

JPET Fast Forward. Published on October 11, 2011 as DOI: 10.1124/jpet.111.186049
This article has not been copyedited and formatted. The final version may differ from this version.

final internal standard concentrations of 50 nM.

Serum and urine collection from conscious rats: Male Sprague Dawley rats (300-350 g) (Harlan Laboratories, Indianapolis) were implanted with indwelling jugular catheters. Briefly, 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 hours. Blood samples (100 µL) were taken from the jugular catheter at the same time as urine collection and the volume was replaced with 100 µL saline. Serum was collected from blood samples using BD 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- or [^{35}S]-DM- CocE in urine and serum was determined by Western blotting. Urine (120 µL) was de-salted using Protein Desalting Spin Columns (Thermo Scientific). De-salted urines were concentrated to a volume of 30 µL. A portion of the desalted sample (15 µL) was loaded onto a 10% SDS-PAGE gel in loading buffer containing β-mercaptoethanol.

Serum samples from animals administrated DM- or [^{35}S]-DM-CocE were assessed for total protein concentration using a Bradford assay and 25 µg 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 minutes. Additionally, some samples were

JPET Fast Forward. Published on October 11, 2011 as DOI: 10.1124/jpet.111.186049
This article has not been copyedited and formatted. The final version may differ from this version.

treated with protease inhibitors (phenylmethanesulfonyl fluoride, tosyl-l-lysine-chloromethyl ketone and tosyl-l-phenylalanine chloromethyl ketone; PTT).

The same Western blotting procedure was used on both urine and serum samples from animals receiving RQ- or [^{35}S]-DM-CocE. Gels were run at 200 volts for 40 minutes. Protein was transferred into a PVDF membrane at 60 volts for 1.5 hours. Membranes were blocked in Blotto for 1 hour before the addition of rabbit anti-CocE primary antibody (generously produced and supplied by New England Peptide) at a concentration of 1:4,000 diluted in Blotto. Primary antibody was incubated overnight shaking at 4°C. Membranes were washed 3 x for 15 minutes in PBS containing 0.1% Tween (w/v) (PBS-T) before the addition of goat anti-rabbit-HRP secondary antibody diluted 1:30,000 in Blotto. Secondary antibody was incubated for 1 hour at room temperature. Blots were washed 3x for 15 minutes in PBS-T and exposed to ECL reagent for one minute. Finally, blots were exposed on Kodak Film.

Blotting membranes containing samples from [^{35}S]-DM-CocE treated animals were allowed to stand for 6 hours after ECL 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) according to package instructions. Urine measures from normal rats was compared to urine measures from DM-CocE treated animals to ensure that readings outside the specified normal range were due to a species difference between rats and humans, not an effect of DM-CocE.

Determination of glomerular filtration: Glomerular filtration rates of normal and unilaterally nephrecotmized animals were determined using an adapted method (Qi et al., 2004). Briefly, a 5% FITC-Inulin (Sigma Chemical) solution was made in 0.9% sodium chloride (saline, Hospira)

JPET Fast Forward. Published on October 11, 2011 as DOI: 10.1124/jpet.111.186049
This article has not been copyedited and formatted. The final version may differ from this version.

by boiling for 2 minutes. Solution was dialyzed overnight in 1000 mL saline using a dialysis membrane (1000 Da cutoff, Spectra/Por® 6, Spectrum Laboratories). Solution was sterilized by filtration before injection. FITC-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 minutes after injection. Samples were buffered by diluting 1:25 in 500 mM HEPES, pH 7.4. Buffered sample (50 µL) was added in duplicate to a 96 well microplate, and fluorescence was determined using a SpectraMax M5 (Molecular Devices) with 485 nm excitation and 538 nm emission.

Fluorescence per µL of serum was plotted and fitted to a 2-phase decay model using GraphPad Prism Software. Glomerular filtration rate was calculated using equation (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 and α and β are the decay constants for the fast and slow decay phases.

(1) $$GFR = \frac{I}{\left(\frac{A}{\alpha}+\frac{B}{\beta}\right)}$$

Calculation of pharmacokinetic parameters: Before injection of [^{35}S]-DM-CocE (8 mg/kg, 18 µCi/kg) to male Sprague Dawley rats, the total counts per minute (cpm) were assessed to determine the specific activity of each radioactive dose. To assess the radioactive content in the blood, 3 µL of each sample was taken at t=3, 16, 20, 30, 45, 60, and 90 minutes, as well as 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 18, 19, and 29 hours. Urine samples were collected after every urination. Samples were analyzed by a Packard 1900 TR liquid scintillation analyzer in 4 mL of CryoScint ES (MP Biomedicals). From the radioactive content, the concentration of CocE (determined using equation 2) in the blood was plotted against time using Prism software.

JPET Fast Forward. Published on October 11, 2011 as DOI: 10.1124/jpet.111.186049
This article has not been copyedited and formatted. The final version may differ from this version.

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

Area under the curve (AUC) analysis was carried out by the Prism software and clearance (Cl) was determined using equation 3 (Goodman et al., 1996). 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 using equation 4 (Goodman et al., 1996). Half-life ($t_{1/2}$) in the blood was then determined using equation 5 (Goodman et al., 1996).

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

(4) $$V_d\ (mL) = \frac{Dose\ (\mu g)}{Cmax\ (\mu g/mL)}$$

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

Estimations of these parameters were initially made 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 minutes after CocE injection was assumed to be 100% of this C_{max}. Each subsequent density was expressed as a percentage of the 5-minute time point and multiplied by the C_{max}. Half-life was determined by a one-phase decay model fit of the estimated concentrations by GraphPad 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 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 mL 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 37% Formaldehyde, 4 g sodium phosphate monobasic, 6.5 g sodium phosphate dibasic, and volume to 1 L) for immunohistochemistry.

Preparation of organs and histology:

Immunoperoxidase: Organs were fixed in 10% buffered formalin overnight at room temperature. Dehydration was completed by 3x 10-minute washes in 30% ethanol, followed sequentially by 3x 10 minute washes in 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 µm and heated for 20 minutes at 65°C. Slides were deparaffinized in xylenes, 3 changes of 2 minutes each. Slides were then rehydrated through graduated washes of 2 minutes each, ending with tap water (100% alcohol, 95% alcohol, 70% alcohol, water). Epitope retrieval was completed by incubating slides for 30 minutes in 80°C citrate buffer (pH 6.0). Slides were cooled for 10 minutes followed by a 10-minute running water wash.

Staining was performed on the DAKO AutoStainer 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 minutes. After 3x PBS buffer rinse, EnVision Plus Rabbit detection kit was used as the secondary antibody for 30 minutes. Slides were exposed to chromagen for 5 minutes before hematoxylin counter-stain for two minutes. Slides were rinsed with water and dehydrated in gradual alcohol rinses. Three changes of xylenes for 2 minutes each were followed by addition of the coverslip. Slides were stored at room temperature until imaging using a Nokia 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 using Adobe Photoshop CS2.

Fluorescence: Organ sections were prepared as described above. Non-specific binding was reduced by blocking slides with 10% goat serum for 1 hour. 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 3x for 10 minutes in PBS. Slides were exposed to secondary goat anti-rabbit Dylight-594 antibody (Jackson ImmunoResearch Laboratories) at a 1:1000 dilution in 10% goat serum for 1 hour. Coverslips were placed onto slides using ProLong Gold (Invitrogen). Slides were stored in the dark at room temperature until imaging with a Leica 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 using Image J (NIH) software and statistics performed with GraphPad Prism software. Images were adjusted equally for contrast and brightness using Adobe Photoshop CS2.

Autoradiography: Organs sections were prepared for freezing by dehydration in 10% sucrose for 6 hours and 30% sucrose overnight. Organs were frozen on dry ice in Optimal Cutting Temperature Compound (O.C.T, Tissue Tek® Sakura) and stored at -80°C until sections were cut from the blocks. Frozen section were cut on a microtome at -20°C to 30 µm 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 µM cocaine, the maximum peak plasma concentration achieved in an anesthetized Sprague Dawley rat after a 4 mg/kg bolus intravenous injection. Low

JPET Fast Forward. Published on October 11, 2011 as DOI: 10.1124/jpet.111.186049
This article has not been copyedited and formatted. The final version may differ from this version.

concentrations of DM-CocE (0.3-10 nM) were chosen in order 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-minute 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 minutes (Figure 1A). The 3 nM concentration of DM-CocE was able to eliminate approximately 50% of the cocaine within 1 minute of addition.

To determine the *in vivo* effect of low dose DM-CocE, and to 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 μg/kg DM-CocE, corresponding to the *in vitro* concentrations of 3 nM and 10 nM, respectively (based on a 350 g rat with a blood volume 7% that of the body weight). DM-CocE (13.6 μg/kg) significantly reduced cocaine plasma concentrations at the 30-second ($p<0.05$) and 2-minute ($p<0.01$) time points (Figure 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 minutes post-injection. DM-CocE at a dose of 50.5 μg/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 minutes post- injection. 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 Figure 2).

JPET Fast Forward. Published on October 11, 2011 as DOI: 10.1124/jpet.111.186049
This article has not been copyedited and formatted. The final version may differ from this version.

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 minutes 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 causing cause tonic-clonic convulsions (Collins et al., 2011a; Collins et al., 2011b).

In rats, cocaine (5.6 mg/kg) produced transient tonic-clonic convulsions and initial plasma cocaine levels ranging from 7-27 μM (Figure 2A). The cocaine concentration in rats receiving only the PBS control 10 minutes after cocaine fell to 0.1-1.7 μM over the 1-hour time period due to the endogenous metabolism of cocaine. In contrast, animals receiving DM-CocE 10 minutes after cocaine had unquantifiable cocaine concentrations within 45 seconds. Interestingly, between the 20- and 60-minute 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 are to be reported elsewhere. Time courses of DM-CocE administration differed between these two animals due to their sensitivity to the high dose of cocaine. In the female monkey, administration of 3 mg/kg cocaine yielded a plasma concentration between 4.2-30.8 μM, a range similar to the rats (Figure 2B). After administration of DM-CocE at t=10 minutes, all cocaine was hydrolyzed to below quantifiable levels within 5 minutes. Although technical limitations resulted in the monkey blood sample being taken longer after DM-CocE administration compared to 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 minute time point, suggesting that this rise is not a species-specific phenomenon.

JPET Fast Forward. Published on October 11, 2011 as DOI: 10.1124/jpet.111.186049
This article has not been copyedited and formatted. The final version may differ from this version.

The male monkey was tested on a different dosing regimen, as DM-CocE was given only 1 minute after cocaine (Figure 2C). Even at this short time point when cocaine not been fully distributed, DM-CocE still eliminated blood cocaine concentrations to below quantifiable levels by 8 minutes (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 minutes, blood concentrations of cocaine begin to slowly rise. The final cocaine 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 plasma after a DM-CocE administration that initially appeared 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, $t_{1/2}$ @ 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 hours, 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, [^{35}S]-methionine was used to metabolically label the protein. The pharmacokinetic parameters of DM-CocE determined from monitoring [^{35}S] in the blood are presented in Table 1. These parameters were determined using curve fit and AUC analysis by Prism software over 10 hours (the time to plateau-phase) post-CocE injection. The volume of distribution (V_d) was calculated using the C_{max} and total dose of CocE (equation 4, methods). The V_d was not significantly different than the total blood volume of a rat (7% body weight), confirming assumptions based on the molecular weight (65 kDa monomer, 130 kDa

JPET Fast Forward. Published on October 11, 2011 as DOI: 10.1124/jpet.111.186049
This article has not been copyedited and formatted. The final version may differ from this version.

dimer) that CocE does not reside outside the bloodstream. Although the V_d was slightly higher than the estimated blood volume, this is most likely due to underestimation of the blood volume using percentage body weight and not due to protein binding of DM-CocE, since the amount of [^{35}S] radioactivity (in counts per minute, cpm) in each sample was determined using a whole blood sample.

The concentration of DM-CocE in blood over time in the rats (calculated using equation 2, methods) is illustrated graphically in Figure 3A. The concentration of DM-CocE peaked immediately after injection, due to the 100% bioavailability of intravenous delivery. Over the first 4 hours after injection, there was a slow, but steady decrease in concentration to approximately 40 µg/mL/kg DM-CocE (see Figure 3A insert). For the remainder of the time course, the concentration of DM-CocE remained relatively constant. After 29 hours, 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) is strongly visible on the radiograph (Figure 3B) at 0.5, 1 and 2 hours after injection. However at later time points, only small amounts of this band are 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 in order 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

JPET Fast Forward. Published on October 11, 2011 as DOI: 10.1124/jpet.111.186049
This article has not been copyedited and formatted. The final version may differ from this version.

minutes after DM-CocE injection, and followed by serum taken at 1, 2, 4, 6, 8, and 12 hours. Full length DM-CocE is seen in these western blots from serums taken up to 6 hours after DM-CocE injection (Figure 3C). To confirm that smaller fragments of DM-CocE are not present in the serum samples and not 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 5 chemical cleavage products of DM-CocE (Supplemental Figure 3), confirming that the anti-CocE antibody can recognize fragments of DM-CocE, but that these products are not exclusive to animal samples, as 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 that the radioactivity analyzed in the blood at the early time points, corresponds to full length DM-CocE (Figure 3D).

Urine samples were analyzed by scintillation counting and 10% of the total cpm injected was recovered in the urine over 29 hours. This cumulative elimination is illustrated in Figure 4A. Twenty-five percent of the total radioactivity eliminated was within the first 4 hours post-injection. 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 hours after DM-CocE injection contained full-length and some smaller molecular weight fragments of DM-CocE (Figure 4B). Dipstick measures of glucose, bilirubin, ketone, specific gravity, blood, pH, protein, urobilinogen, nitrate and leukocytes in the urine did not differ between animals receiving DM-CocE and PBS vehicle (data not shown).

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 hours after administration. However, after this time, more diffuse radioactivity across a wide range of molecular weights was seen on autoradiographs. Additionally after the amount of radioactivity in the serum stabilized to a constant level, the

JPET Fast Forward. Published on October 11, 2011 as DOI: 10.1124/jpet.111.186049
This article has not been copyedited and formatted. The final version may differ from this version.

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 non-specific 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 (Figure 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 (Figure 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, as 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 due to 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

JPET Fast Forward. Published on October 11, 2011 as DOI: 10.1124/jpet.111.186049
This article has not been copyedited and formatted. The final version may differ from this version.

indwelling jugular catheter. At each time point after DM-CocE injection (0.5, 1, 2, 4, 8, 12, 24 hours) three rats were sacrificed and kidneys were prepared for immunohistochemistry. DM-CocE reached maximum accumulation levels in the papilla 2 hours after DM-CocE injection and reached near baseline levels by 4 hours (representative images in Figure 6A). After 12 to 24 hours post- injection, there are no longer significant amounts of DM-CocE immunoreactivity compared to saline (Figure 6B).

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 whether 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 to normal animals (2619 µL/min compared to 6030 µL/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 to 0.21 mL/min) and the $t_{1/2}$ of CocE was slightly higher (156 compared to 125 minutes) than normal animals. These differences suggest that the kidneys are most likely assisting with the clearance of CocE, but that glomerular 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-hour 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 µm) 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] (Figure 7B). Immunohistochemical analysis on the kidney and liver from rats treated with DM-CocE 24 hours prior reveals no DM-CocE immunoreactivity (Figure 7A).

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 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 minutes) novel to the cocaine metabolism literature. The very low doses of DM-CocE (13.6, 50.5 µg/kg) builds upon previous work demonstrating the high catalytic efficiency of the enzyme. These results demonstrate that very low doses can enhance the elimination of cocaine and that the minimally effective dose *in vivo* is only half a log dose higher than it is *in vitro*. This near equivalence is somewhat surprising due to 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, no significant differences were observed in DM-CocE's ability to hydrolyze cocaine in males in

JPET Fast Forward. Published on October 11, 2011 as DOI: 10.1124/jpet.111.186049
This article has not been copyedited and formatted. The final version may differ from this version.

comparison to females, in either rats or monkeys (Collins et al. 2011a; Collins et al. 2011b). Additionally, 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 and L169K/G173Q-CocE have short serum half-lives in mice (2.3 and 2.2 hours respectively, estimated by western blotting) and that 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 hours) and by [^{35}S]-labeling (2.1 hours). The similar half-lives of DM- 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 consentient with the clinical half-life of several biologic protein enzyme drugs of similar size currently on the US drug market such as agalsidase (Fabrazyme®, Genzyme, 100 kDa, $t_{1/2}$=45-102 minutes (Fabrazyme (R) [Package Insert]. Cambridge)), denileukin difitox, (Ontak®, Eisai, 58 kDa, $t_{1/2}$=70-80 minutes (Ontak (R) [Package Insert]. Woodcliff Lake)) and laronidase (Aldurazyme®, Genzyme, 83kDa, $t_{1/2}$=1.5-3.6 hrs (Aldurazyme (R) [Package Insert]. Cambridge)). 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 about 10% of DM-CocE (or its fragments) were eliminated in the urine over 29 hours. Western blot analysis confirmed that full length CocE is present in the urine as a full length protein for the first 2 hours after injection. Additionally, 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; expected, when considering DM-

CocE's presence in the urine. This reactivity was time dependant and reversible, as no significant reactivity was seen 24 hours after DM-CocE administration.

These results are somewhat surprising considering that DM-CocE is a 65 kDa protein that exists as a non-covalent 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). Recently 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, both by 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 hours or 24 hours after injection. However, serum proteins of larger and smaller size than DM-CocE were labeled with [^{35}S] 4-18 hours after [^{35}S]-DM-CocE injection (Figure 3C) and perfused organs taken from these [^{35}S]-DM-CocE treated animals were completely metabolically labeled (Figure 7) after 29 hours. 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 immunohistochemisty data (Figures 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 μmol/L methionine) (Greco, 2009) 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 like methionine (Verrey, 2003; Broer, 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^+-dependant, symport (McGivan and Pastor-Anglada, 1994; Broer, 2008)), ASC-type (Na^+-dependant, antiport (Kanai and Hediger, 2004; Broer, 2008)) and B^o-type (Na^+- or Ca^{2+}-dependant, symport (Broer, 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, as it could be administered to a patient, act rapidly on cocaine, and then 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; Ko et al., 2009) and in monkey (Collins et al., 2011a; Collins 2011c). Rapid elimination also prevents the accumulation of large aggregates, which may cause damage to capillary beds or organs like the lung and kidney (supported by our data that indicates no full-length protein 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 (Collins et al., 2009)), the rapid elimination needs to be prevented to ensure a long serum half-life. This is feasible using established techniques such as PEGylation (the chemical modification of proteins with polyethylene gycol chains), a process that has

successfully been used to extend the half-life of FDA-approved biologics such as L-asparaginase (extension from 8-30 hours (Elspar (R) [Package Insert] West Point) to 3.2-5.7 days (Oncaspar (R) [Package Insert] New Jersey)) and filgrastim (extension from 3.5 hours (Neupogen (R) [Package Insert] Thousand Oaks) to 15-80 hours (Neulasta (R) [Package Insert]. Thousand Oaks)). These increases are due to 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 hours to more than 48 hours (Narasimhan et al., 2011). Although these are dramatic increases, we believe that an addiction therapy needs remain in the circulation at least one week, as 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, our laboratory is 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.

Acknowledgements

We thank Davina Barron and Alyssa Cunningham, for their excellent technical assistance and Dr. Donald Landry for his insightful conversations and suggestions regarding the renal elimination of DM-CocE. We acknowledge Sebastien Fournier at LAB Research Inc for overseeing DM-CocE administration and preparation of histological samples used to determine

the organ distribution of DM-CocE (Figure 5). Thank you to Dr. R. D. Davenport for providing us with human plasma samples, and Asim Beg for use of the Leica DMI6000 B microscope.

Author Contributions

Participated in research design: Brim, R.L., Noon, K.R., Collins, G.T., Stein, A., Ko, M.C., Sunahara, R.K., Woods, J.H.

Conducted experiments: Brim, R.L., Noon, K.R., Collins, G.T.

Performed data analysis: Brim, R.L., Noon, K.R.

Contributed new reagents or analytic tools: Narasimhan, D., Nichols, J.

Wrote or contributed to the writing of the manuscript: Brim, R.L., Sunahara, R.K., Woods, J.H.

References

Aldurazyme (R) [Package Insert] Cambridge, MA: Genzyme Corporation; 2003.

Bresler MM, Rosser SJ, Basran A and Bruce NC (2000) Gene cloning and nucleotide sequencing and properties of a cocaine esterase from Rhodococcus sp. strain MB1. *Appl Environ Microbiol* **66**:904-908.

Brim RL, Nance MR, Youngstrom DW, Narasimhan D, Zhan CG, Tesmer JJ, Sunahara RK and Woods JH (2010) A thermally stable form of bacterial cocaine esterase: a potential therapeutic agent for treatment of cocaine abuse. *Mol pharmacol* **77**:593-600.

Brim RL, Noon KR, Collins GT, Nichols J, Narasimhan D, Sunahara RK and Woods JH (2011) The Ability of Bacterial Cocaine Esterase to Hydrolyze Cocaine Metabolites and their Simultaneous Quantification Using High Performance Liquid Chromatography-Tandem Mass Spectrometry. *Mol pharmacol* doi:10.1124/mol.111.074534

Broer S (2008) Amino acid transport across mammalian intestinal and renal epithelia. *Physiol Rev* **88**:249-286.

Collins GT, Brim RL, Narasimhan D, Ko MC, Sunahara RK, Zhan CG and Woods JH (2009) Cocaine esterase prevents cocaine-induced toxicity and the ongoing intravenous self-administration of cocaine in rats. *J Pharmacol Exp Ther* **331**:445-455.

Collins GT, Carey KA, Narasimhan D, Nichols J, Berlin AA, Lukacs NW, Sunahara RK, Woods JH and Ko MC (2011a) Amelioration of the Cardiovascular Effects of Cocaine in Rhesus Monkeys by a Long-Acting Mutant Form of Cocaine Esterase. *Neuropsychopharmacology* **36(5)**:1047-1059.

Collins GT, Zaks ME, Cunningham AR, St. Clair C, Nichols J, Narasimhan D, Ko MC, Sunahara RK and Woods JH (2011b) Effects of a long-acting mutant bacterial cocaine esterase on acute cocaine toxicity in rats. *Drug Alcohol Depend* doi:10.1016/j.drugalcdep.2011.03.015.

Collins GT, Brim RL, Noon KR, Narasimhan D., Lukacs NW, Sunahara RK, Woods JH and Ko MC (2011c) "Repeated administration of a long acting mutant cocaine esterase: Effects on cardiovascular activity, plasma cocaine levels and immune responses in rhesus monkeys", American Society of Pharmacology and Experimental Therapeutics Annual Meeting, Washington DC.

Comper WD, Haraldsson B and Deen WM (2008) Resolved: normal glomeruli filter nephrotic levels of albumin. *J Am Soc Nephrol* **19**:427-432.

Cooper ZD, Narasimhan D, Sunahara RK, Mierzejewski P, Jutkiewicz EM, Larsen NA, Wilson IA, Landry DW and Woods JH (2006) Rapid and robust protection against cocaine-induced lethality in rats by the bacterial cocaine esterase. *Mol pharmacol* **70**:1885-1891.

del Amo EM, Urtti A and Yliperttula M (2008) Pharmacokinetic role of L-type amino acid transporters LAT1 and LAT2. *Eur J Pharm Sci* **35**:161-174.

Elspar (R) [Package Insert] West Point, PA: Merck & Co; 2000.

Fabrazyme (R) [Package Insert] Cambridge, MA: Genzyme Corporation; 2006.

Freye E and Levy J (2009) *Pharmacology and Abuse of Cocaine, Amphetamines, Ecstasy and Related Designer Drugs: A Comprehensive Review on Their Mode of Action, Treatment of Abuse and Intoxication*. Springer, Doedrecht.

Gao D, Narasimhan DL, Macdonald J, Brim R, Ko MC, Landry DW, Woods JH, Sunahara RK and Zhan CG (2009) Thermostable variants of cocaine esterase for long-time protection against cocaine toxicity. *Mol pharmacol* **75**:318-323.

Goodman L, Gilman A, Hardman J and Limbird L (1996) *Goodman and Gilman's the pharmacological basis of therapeutics*. McGraw-Hill, Health Professions Division.

Greco FA (2009) Medline Plus, in *Plasma Amino Acids*, US National Library of Medicine and the National Institutes of Health.

Haraldsson B, Nystrom J and Deen WM (2008) Properties of the glomerular barrier and mechanisms of proteinuria. *Physiol Rev* **88**:451-487.

Jutkiewicz EM, Baladi MG, Cooper ZD, Narasimhan D, Sunahara RK and Woods JH (2009) A bacterial cocaine esterase protects against cocaine-induced epileptogenic activity and lethality. *Ann Emerg Med* **54**:409-420.

Kanai Y and Hediger MA (2004) The glutamate/neutral amino acid transporter family SLC1: molecular, physiological and pharmacological aspects. *Pflugers Arch* **447**:469-479.

Ko MC, Bowen LD, Narasimhan D, Berlin AA, Lukacs NW, Sunahara RK, Cooper ZD and Woods JH (2007) Cocaine esterase: interactions with cocaine and immune responses in mice. *J Pharmacol Exp Ther* **320**:926-933.

Ko MC, Narasimhan D, Berlin AA, Lukacs NW, Sunahara RK and Woods JH (2009) Effects of cocaine esterase following its repeated administration with cocaine in mice. *Drug Alcohol Depend* **101**:202-209.

Larsen NA, Turner JM, Stevens J, Rosser SJ, Basran A, Lerner RA, Bruce NC and Wilson IA (2002) Crystal structure of a bacterial cocaine esterase. *Nat Struct Biol* **9**:17-21.

McGivan JD and Pastor-Anglada M (1994) Regulatory and molecular aspects of mammalian amino acid transport. *Biochem J* **299 (Pt 2)**:321-334.

Narasimhan D, Collins G, Nance MA, Nichols J, Chan J, Edwald E, Ko MC, Woods JH, Tesmer JJ and Sunahara RK (2011) Subunit stabilization and PEGylation of cocaine esterase improves *in vivo* residence time. *Mol pharmacol* doi:10.1124/mol.111.074997.

Narasimhan D, Nance MR, Gao D, Ko MC, Macdonald J, Tamburi P, Yoon D, Landry DM, Woods JH, Zhan CG, Tesmer JJ and Sunahara RK (2010) Structural analysis of thermostabilizing mutations of cocaine esterase. *Protein Eng Des Sel* **23**:537-547.

Neulasta (R) [Package Insert] Thousand Oaks, CA: Amgen; 2002.

Neupogen (R) [Package Insert] Thousand Oaks, CA: Amgen; 2010.

Office of National Drug Control Policy (2004) The Economic Costs of Drug Abuse in the United States, 1992-2002. Washington, DC.

Oncaspar (R) [Package Insert] New Jersey, Enzon Pharmaceuticals, 2006.

Ontak (R) [Package Insert] Woodcliff Lake, NJ: Eisai Inc.; 1999.

Park JB, Kwon YM, Lee TY, Brim R, Ko MC, Sunahara RK, Woods JH and Yang VC (2010) PEGylation of bacterial cocaine esterase for protection against protease digestion and immunogenicity. *J Control Release* **142**:174-179.

Qi Z, Whitt I, Mehta A, Jin J, Zhao M, Harris RC, Fogo AB and Breyer MD (2004) Serial determination of glomerular filtration rate in conscious mice using FITC-inulin clearance. *Am J Physiol Renal Physiol* **286**:F590-596.

Sarav M, Wang Y, Hack BK, Chang A, Jensen M, Bao L and Quigg RJ (2009) Renal FcRn reclaims albumin but facilitates elimination of IgG. *J Am Soc Nephrol* **20**:1941-1952.

Schrank K (1992) Cocaine-Related Emergency Department Presentations, in *Acute Cocaine Intoxication: Current Methods of Treatment* (Sorer, H. ed.), National Institutes of Health, Rockville, MD.

Stitzer ML, Polk T, Bowles S and Kosten T (2010) Drug users' adherence to a 6-month vaccination protocol: effects of motivational incentives. *Drug Alcohol Depend* **107**:76-79.

Substance Abuse and Mental Health Services Administration. (2009). *Results from the 2008 National Survey on Drug Use and Health: National Findings* (Office of Applied Studies, NSDUH Series H-36, HHS Publication No. SMA 09-4434). Rockville, MD.

Tang L and Meibohm B (2006) Pharmacokinetcs of Peptides and Proteins, in *Pharmacokinetics and Pharmacodynamics of Biotech Drugs: Principles and Case Studeis in Drug Development* (Meibohm, B. ed.) pp 17-44, Wiley-VCH, Weinheim.

Turner JM, Larsen NA, Basran A, Barbas CF, 3rd, Bruce NC, Wilson IA and Lerner RA (2002) Biochemical characterization and structural analysis of a highly proficient cocaine esterase. *Biochemistry* **41**:12297-12307.

Substance Abuse and Mental Health Services Administration, Office of Applied Studies. *Drug Abuse Warning Network, 2003: Interim National Estimates of Drug-Related Emergency*

Department Visits. DAWN Series D-26, DHHS Publication No. (SMA) 04-3972. Rockville, MD, 2004

Verrey F (2003) System L: heteromeric exchangers of large, neutral amino acids involved in directional transport. *Pflugers Arch* **445**:529-533.

Wood SK, Narasimhan D, Cooper Z, Sunahara RK and Woods JH (2010) Prevention and reversal by cocaine esterase of cocaine-induced cardiovascular effects in rats. *Drug Alcohol Depend* **106**:219-229.

Footnotes

a) This work was supported by the National Institutes of Health [Grants DA028086-01, DA021416, DA023213, GM007767]; and Reckitt Benckiser. The contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH, NIGMS, or Reckitt Benckiser.

b) Financial Disclosure: James H. Woods and Roger K. Sunahara have been consultants to Reckitt Benckiser Pharmaceuticals. Remy Brim, Diwa Narasimhan, Mei-Chuan Ko, James H Woods and Roger K Sunahara are inventors on patent number PCT/US2008/069659 for DM-CocE.

c) Collins, G.T, Brim, R.L., Noon, K.R., Narasimhan, D., Lukacs, N.W., Sunahara, R.K., Woods, J.H., Ko, M.C.. “Repeated administration of a long acting mutant cocaine esterase: Effects on cardiovascular activity, plasma cocaine levels and immune responses in rhesus monkeys” , American Society of Pharmacology and Experimental Therapeutics Annual Meeting. Washington DC, April 2011

Brim, R.L., Noon, K.R., Stein, A., Nance, M.R., Nichols, J., Narasimhan, D., Sunahara, R.K., Woods, J.H.. “Quantification of rapid in vivo cocaine hydrolysis by bacterial cocaine esterase”, American Society of Pharmacology and Experimental Therapeutics Annual Meeting. Washington DC, April 2011

Brim, R.L., Investigations into the therapeutic potential of a bacterial cocaine esterase for the treatment of cocaine toxicity and cocaine abuse, Thesis in Pharmacology. 2011, University of Michigan: Ann Arbor.

Brim, R.L., Sunahara, R.K., Woods, J.H. “Pharmacokinetic properties of a bacterial cocaine esterase to be used as a treatment against cocaine-induced toxicities”, The College on Problems of Drug Dependence Annual Meeting. Scottsdale AZ, June 2010.

d) Please send reprint requests to:

Roger K Sunahara

University of Michigan

1301 Medical Science Research Building III

1150 West Medical Center Drive

Ann Arbor, MI 48109-0600

Phone: (734) 647-6377

Fax: (734) 763-4450

Email: sunahara@umich.edu

e) [1] Department of Pharmacology

University of Michigan

1301 Medical Science Research Building III

1150 West Medical Center Drive

Ann Arbor, MI 48109-0600

Phone: (734) 647-6377

Fax: (734) 763-4450

(RLB, JN, DN, RKS, JHW)

[2] Innovation Center

Medical College of Wisconsin

8701 Watertown Plank Road

Milwaukee, WI 53226

(KRN)

[3] Alcohol and Drug Abuse Research Center

McLean Hospital/Harvard Medical School

115 Mill St

Belmont, MA 02478

(GTC, current)

[4] Stein Consulting

3525 Del Mar Heights Rd., No. 552

San Diego CA 92130

(AS)

JPET Fast Forward. Published on October 11, 2011 as DOI: 10.1124/jpet.111.186049
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for Figures

Figure 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 μM cocaine at time=0. After 5 minutes at 37°C, DM-CocE was added at plasma the given concentrations and aliquots were taken and all hydrolysis stopped at the given points. Aliquots were assessed for 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 μg/kg DM-CocE followed 2 minutes 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.

Figure 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 minutes. PBS data is the average data from all three rats. DM-CocE data is separated by individual rat. DM-CocE rapidly removes cocaine to below the limit of quantification within 45 seconds **B** A female rhesus monkey 2 was administered 3.2 mg/kg cocaine intravenously at time 0 and DM-CocE or PBS at time 10 minutes. 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 fall to below the 30 nM limit of quantification, At times later than 30 minutes, low concentrations of cocaine appear in the plasma, suggesting that the elimination of DM-CocE from the serum allows very low amounts of cocaine to diffuse back into the blood from other body compartments. Monkey F exhibits the same pattern of cocaine elimination as the rat, suggesting no effect of species or repeat dosing of DM-CocE in the non-human primate. **C** A male rhesus monkey 1 was administered 3.2 mg/kg cocaine intravenously at time 0 and DM-

JPET Fast Forward. Published on October 11, 2011 as DOI: 10.1124/jpet.111.186049
This article has not been copyedited and formatted. The final version may differ from this version.

CocE or PBS at time 1-minute. Dosing regimen and sample analysis was conducted as described above for the female monkey. Like the rat and the female, cocaine reappears at higher concentrations by 40 minutes after cocaine injection.

Figure 3. Elimination of [^{35}S]-DM-CocE from the blood **A** Concentration of DM-CocE in the serum of rats (n=3) as measured by [^{35}S]-methionine labeling over time. Eight mg/kg [^{35}S]-DM-CocE (18 µCi/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 using the specific activity of each radioactive dose and plotted against time. DM-CocE is eliminated rapidly over the first 4 hours after injection, until reaching a plateau state (insert). Both normal and unilaterally nephrectomized animals are shown. **B** Representative autoradiograph of [^{35}S] in serum over time from rats administered 8 mg/kg [^{35}S]-DM-CocE. Male Sprague Dawley rats were administered 8 mg/kg [^{35}S]-DM-CocE at time 0. At 0.5,1, 2, 4, 6, 8, 12 and 18 hours post 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 anti-body. The immunolabled 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 using densitrometry of the 65 kDa band. Raw values are plotted against time.

Figure 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=Y0 + (Plateau-Y0)*(1-exp(-K*x))). Data plotted are SEM **B** Western blot analysis of the presence of DM-CocE in urine. Samples were added to SDS and BME loading buffer and immediately loaded onto 10 % SDS-PAGE gels DM-CocE (40 ng) was loaded as a positive control, de-salted urine alone as a negative control and 40 ng DM-CocE spiked into urine to control for protein recovery from urine desalting. Urine was collected at the times shown after CocE injection and was loaded in a time-dependant order.

Figure 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). 6 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 counter-stained with hematoxylin (blue) and DM-CocE reactivity 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.

Figure 6. Immunohistochemical analysis of DM-CocE accumulation 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 image (DIC) shows the area of the papilla that the florescence image highlights (TXR). 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 hours, after which there is a rapid decline. At 24 hours, DM-CocE reactivity is no longer

JPET Fast Forward. Published on October 11, 2011 as DOI: 10.1124/jpet.111.186049
This article has not been copyedited and formatted. The final version may differ from this version.

seen. (* One-way ANOVA F(7, 16)=2.47 Dunnetts Multiple Comparison Test $p<0.05$, data plotted as SEM)

Figure 7. Radiography and immunohistochemistry of organs 24 hours 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 hours after administration, respectively. Organ slices were subjected to immunohistochemistry (**A**) or radiography (**B**), respectively, and representative images are shown here. No DM-CocE reactivity is detected with the anti-CocE antibody, however evenly distributed and very dense radioactivity was detected in all organ slices.

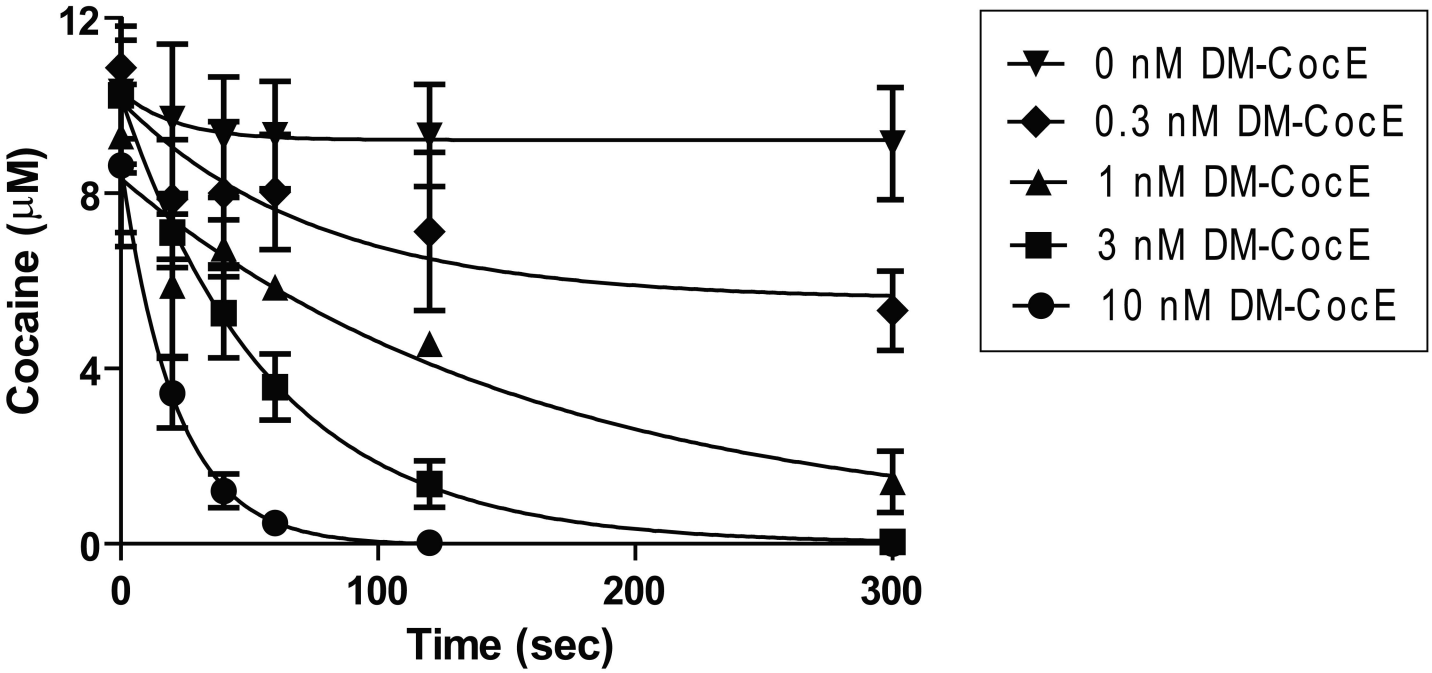
JPET Fast Forward. Published on October 11, 2011 as DOI: 10.1124/jpet.111.186049
This article has not been copyedited and formatted. The final version may differ from this version.

	Western Blot Estimation	Normal (mean +/- SD)	Unilateral Nephrectomy (mean +/- SD)
GFR (µL/min)		6030 (+/- 106.4)	2610 (+/- 53.4) ***
Urine Output (total 29 hrs)		13.0 (+/- 1.8)	15.2 (+/- 6.3) n/s
Weight		343.3 (+/- 31.75)	302.0 (+/- 10.82) n/s
AUC (µg/mL/min)		14,461 (+/- 7445)	14,932 (+/- 606.8) n/s
Clearance (mL/min)	0.12	0.21 (+/- 0.07)	0.16 (+/- 0.01) n/s
V_d (mL)	24.6 mL	38.1 (+/- 11.4)	36.6 (+/- 5.4) n/s
$T_{1/2}$ (min)	144	125 (+/- 6.8)	156 (+/- 19.8) n/s

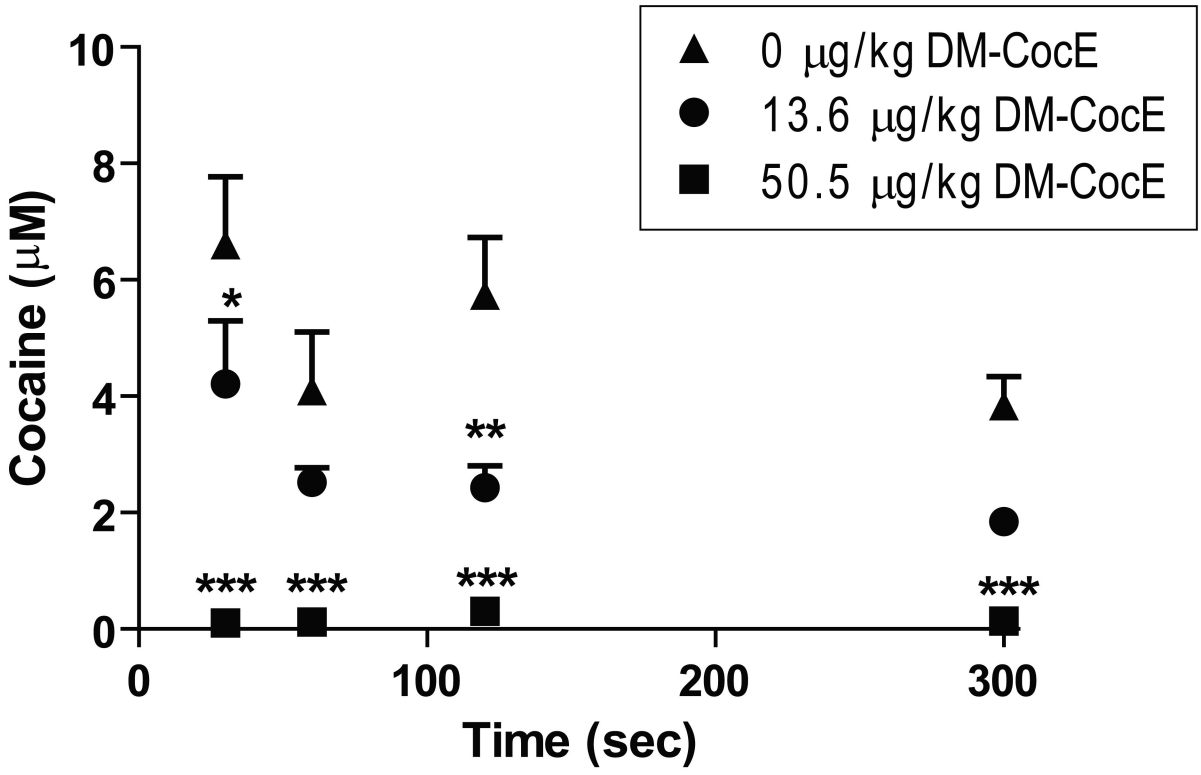
Table 1. Pharmacokinetic Parameters of DM-CocE. Each condition n=3. ***p<0.001 Students T-test compared to normal. n/s p>0.05 Students T-test compared to normal.

Figure 1

A


12
8
4
0
Cocaine (μM)
0
100
200
300
Time (sec)
0 nM DM-CocE
0.3 nM DM-CocE
1 nM DM-CocE
3 nM DM-CocE
10 nM DM-CocE

B


10
8
6
4
2
0
Cocaine (μM)
*
**
***
***
***
***
0
100
200
300
Time (sec)
0 μg/kg DM-CocE
13.6 μg/kg DM-CocE
50.5 μg/kg DM-CocE

JPET Fast Forward. Published on October 11, 2011 as DOI: 10.1124/jpet.111.186049

Figure 2

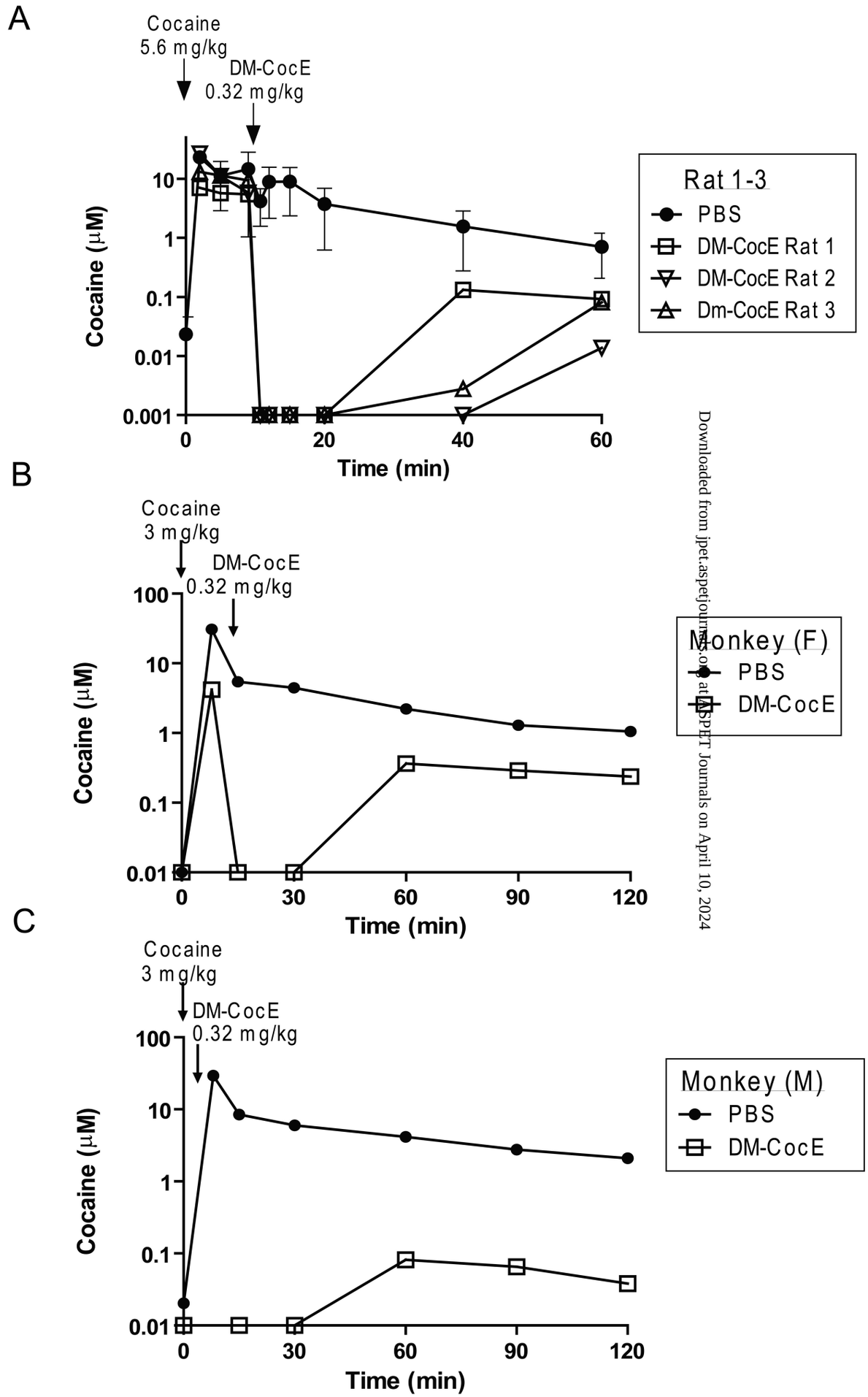
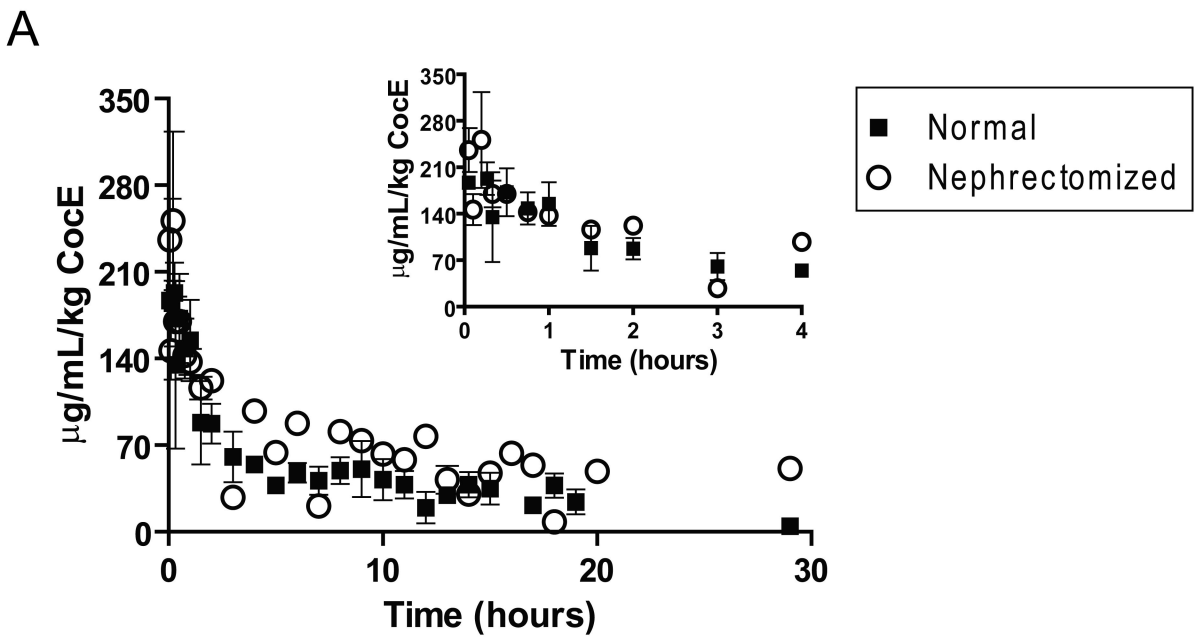
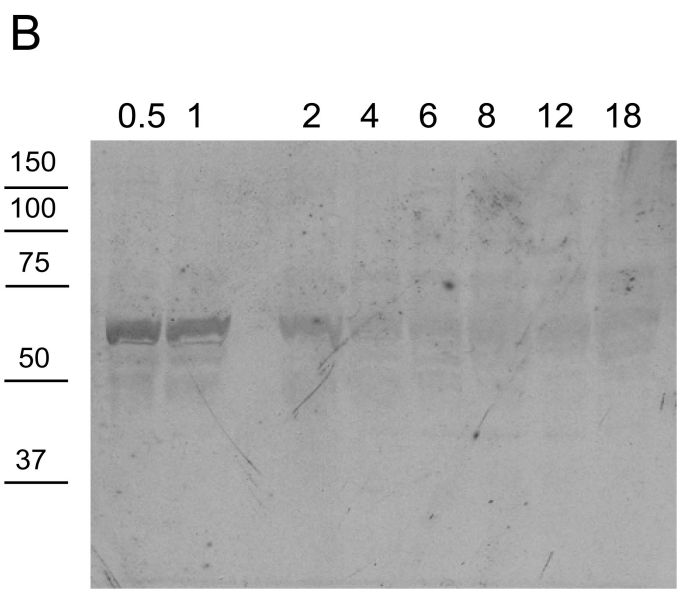
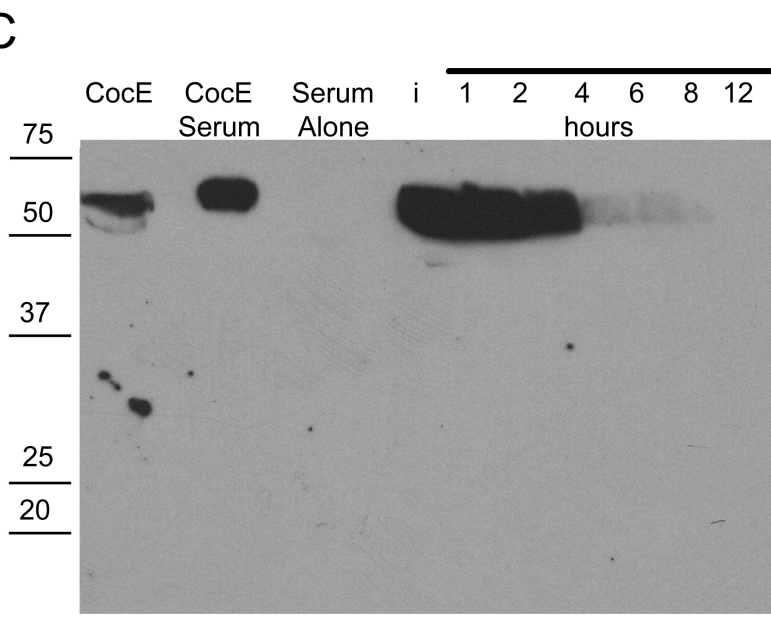


Figure 3


A
μg/mL/kg CocE
Time (hours)
Normal
Nephrectomized


B
0.5 1 2 4 6 8 12 18
150
100
75
50
37


C
CocE
CocE Serum
Serum Alone
i
1 2 4 6 8 12
hours
75
50
37
25
20

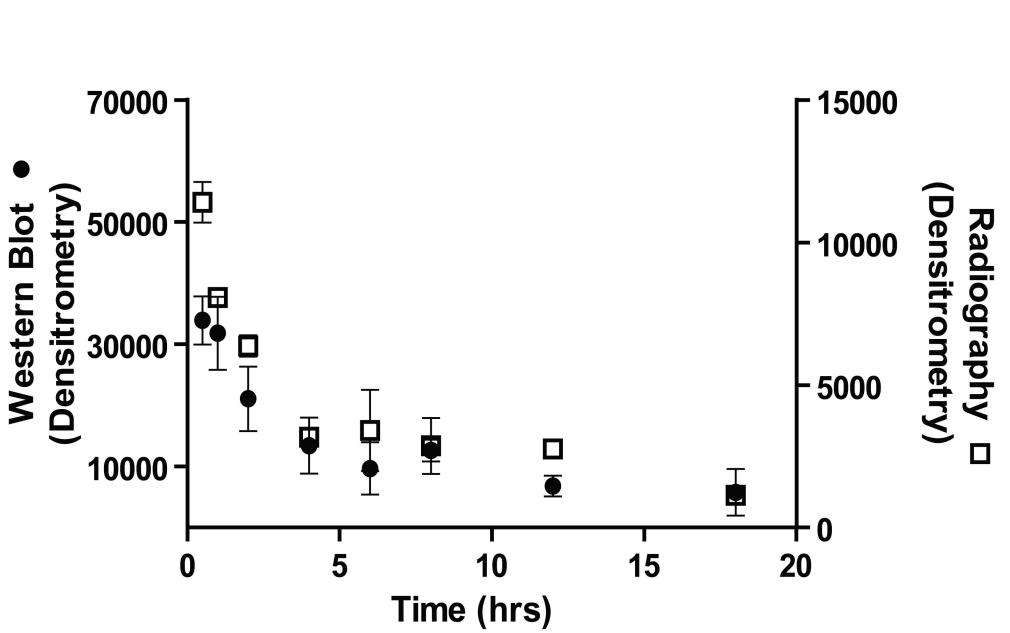
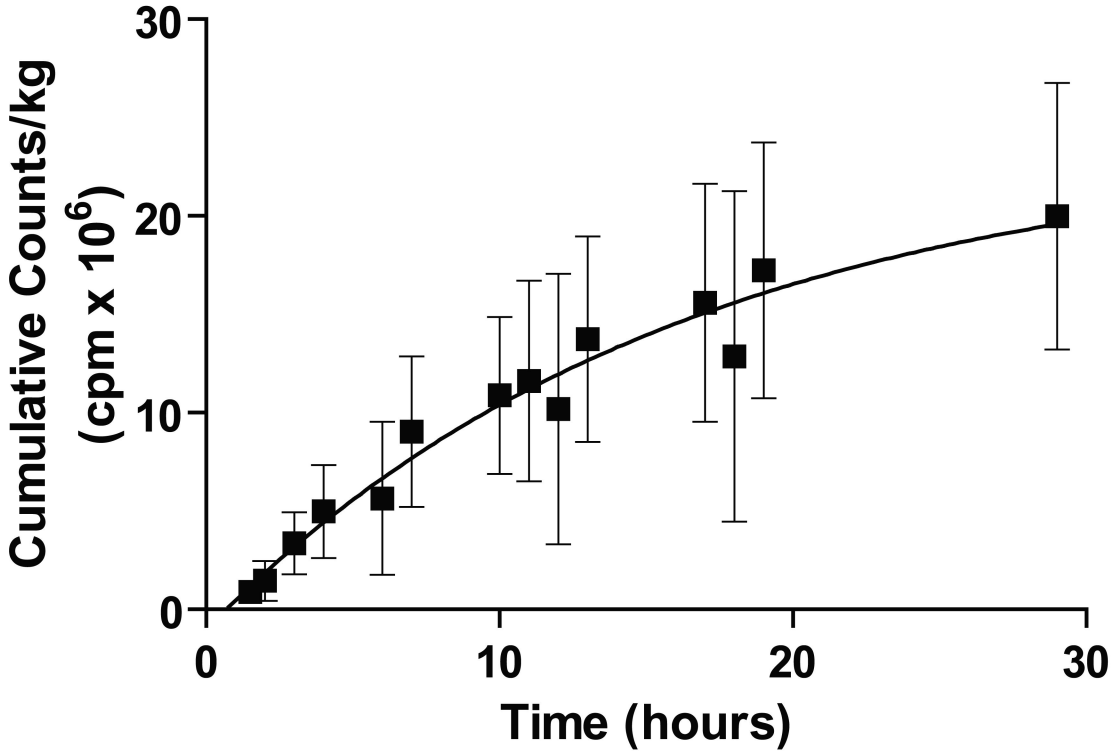

D
Western Blot ● (Densitometry)
Radiography □ (Densitometry)
Time (hrs)

Figure 4

A


Cumulative Counts/kg
(cpm x 10^6)
30
20
10
0
0
10
20
30
Time (hours)

B

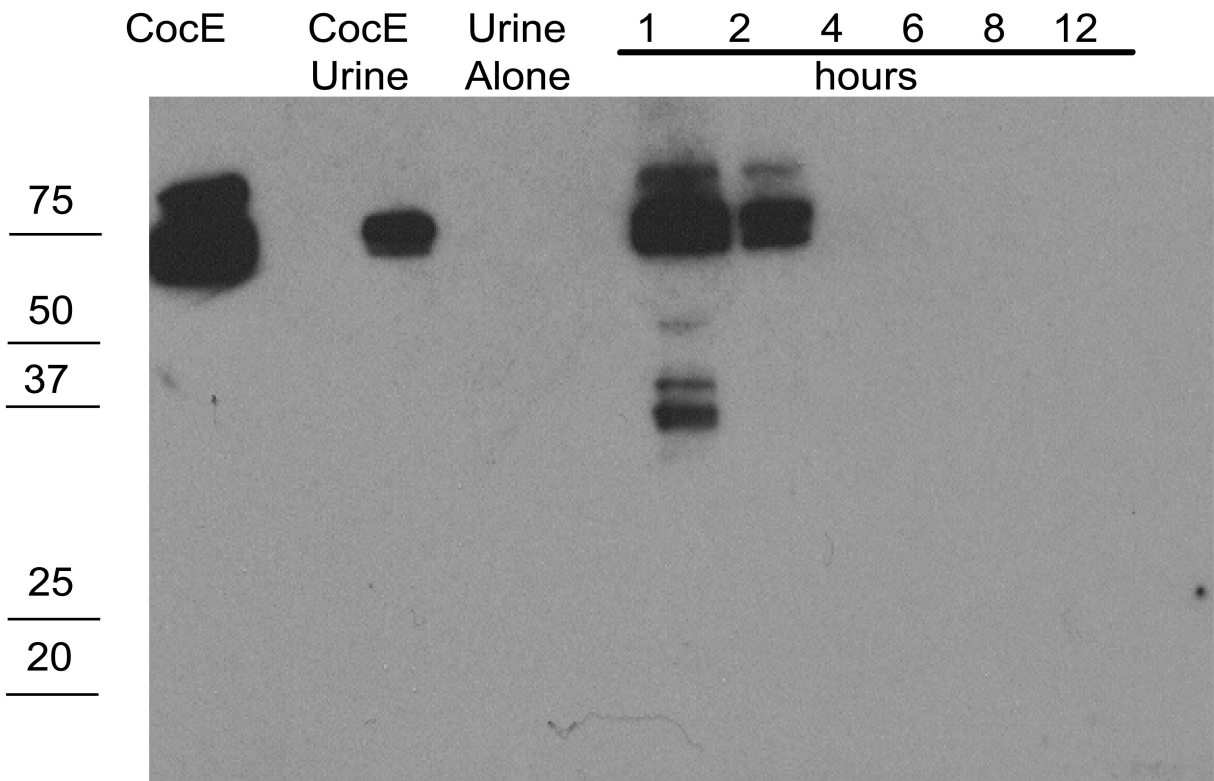

CocE
CocE
Urine
Urine
Alone
1
2
4
6
8
12
hours
75
50
37
25
20

Figure 5

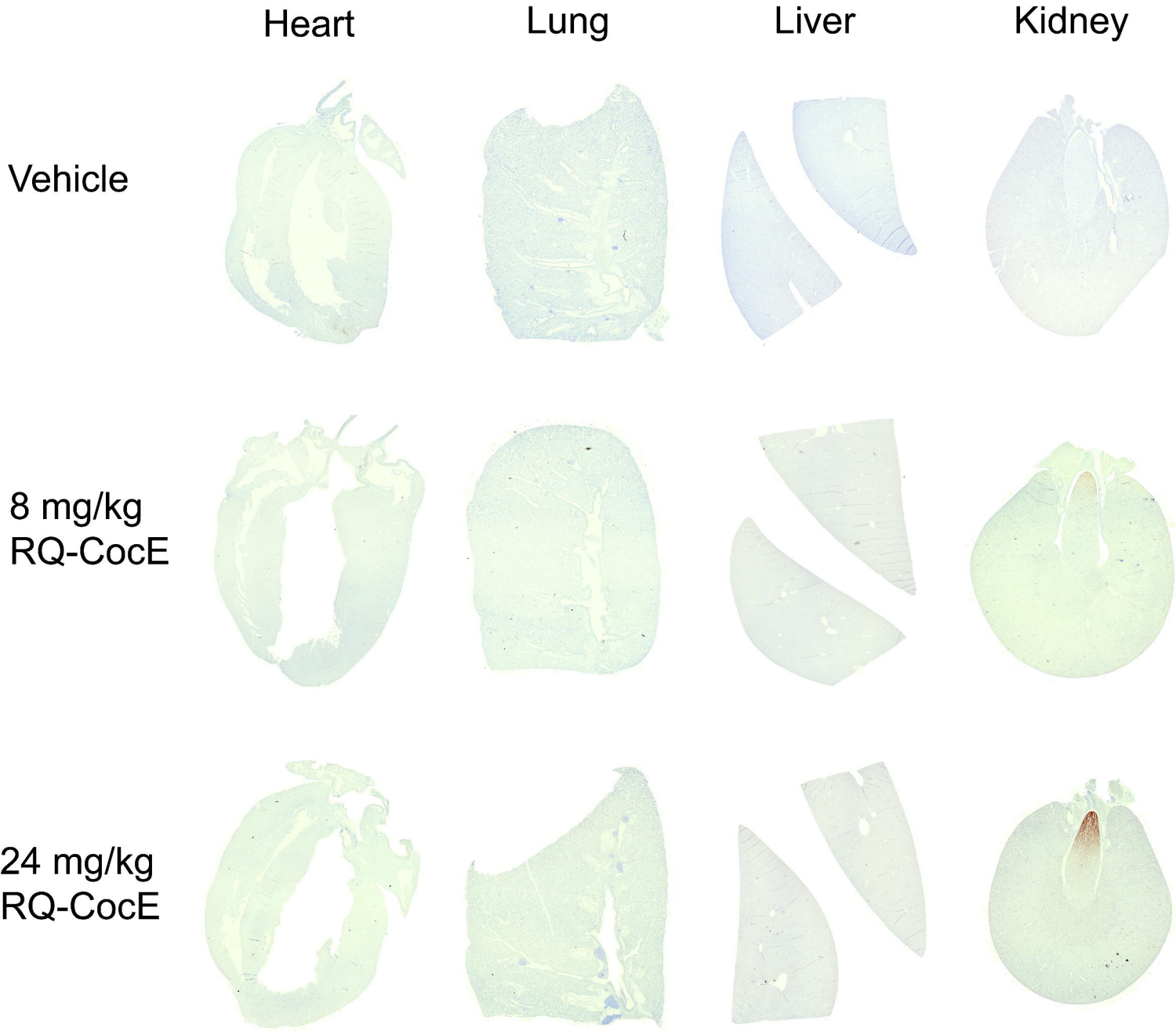
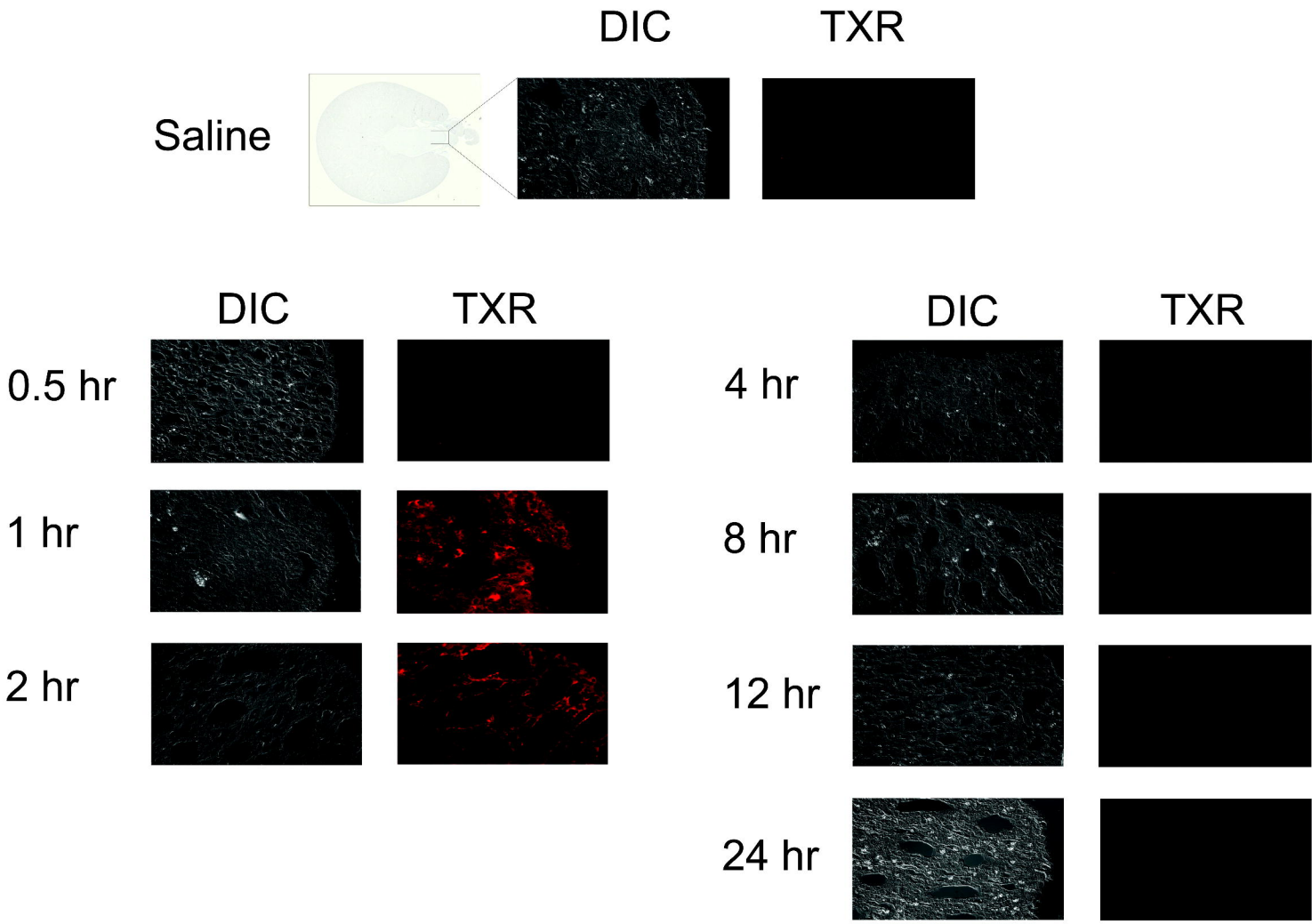

Heart
Lung
Liver
Kidney
Vehicle
8 mg/kg
RQ-CocE
24 mg/kg
RQ-CocE

Figure 6

A


DIC
TXR
Saline
DIC
TXR
0.5 hr
1 hr
2 hr
DIC
TXR
4 hr
8 hr
12 hr
24 hr

B

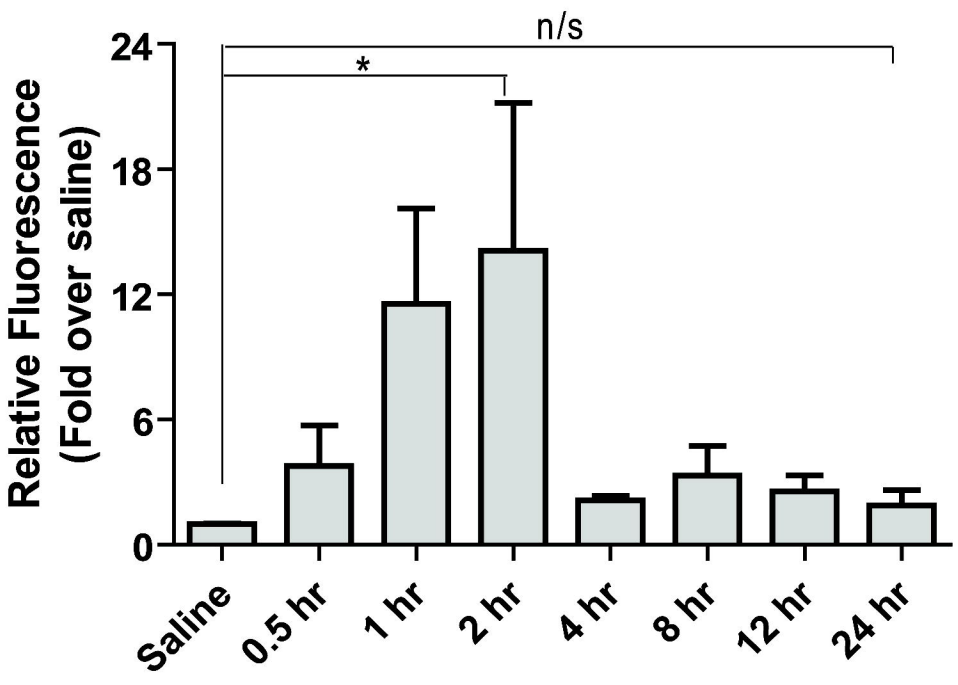
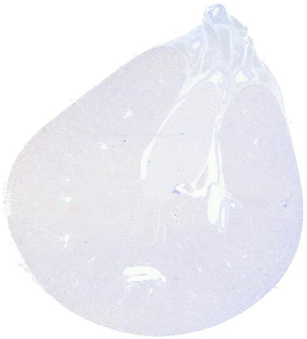

n/s
*
Relative Fluorescence
(Fold over saline)
24
18
12
6
0
Saline
0.5 hr
1 hr
2 hr
4 hr
8 hr
12 hr
24 hr

Figure 7

A

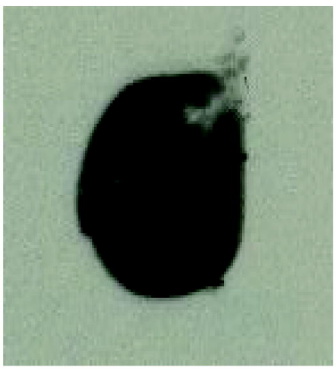
Kidney



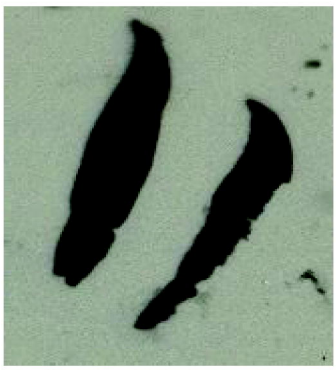
Liver

B

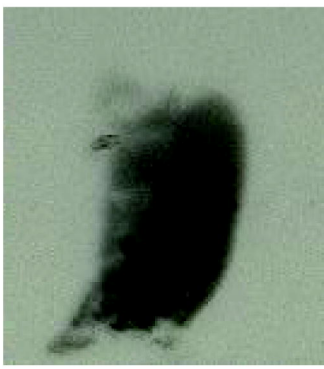
Kidney



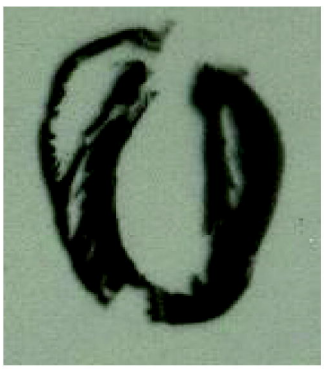
Liver



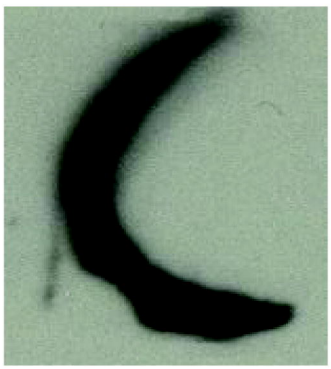
Lung



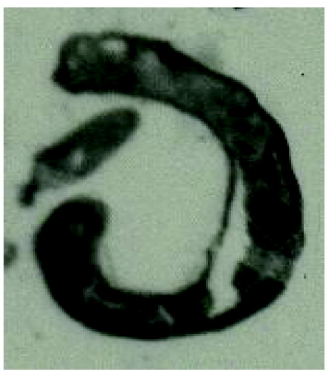
Heart



Spleen



S. Intestine



Stomach