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TITLE: Repeated administration of a mutant cocaine esterase: Effects on plasma cocaine levels, cocaine-induced cardiovascular activity, and immune responses in rhesus monkeys

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Running Title: Repeat dosing with DM CocE in primates

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Abbreviations: BChE: butyrylcholinesterase; CocE: cocaine esterase; DM CocE: double mutant cocaine esterase; ED: emergency department; HR: heart rate; IV: intravenous; IM: intramuscular; MAP: mean arterial pressure; PBS: phosphate-buffered saline
ABSTRACT:

Previous studies have demonstrated the capacity of a long-acting mutant form of a naturally occurring bacterial cocaine esterase (DM CocE) to antagonize the reinforcing, discriminative, convulsant, and lethal effects of cocaine in rodents, and to reverse the increases in mean arterial pressure (MAP) and heart rate (HR) produced by cocaine in rhesus monkeys. This study was aimed at characterizing the immunologic responses to repeated dosing with DM CocE, and determining if the development of anti-CocE antibodies altered the capacity of DM CocE to reduce plasma cocaine levels and ameliorate the cardiovascular effects of cocaine in rhesus monkeys. Under control conditions, intravenous administration of cocaine (3 mg/kg) resulted in a rapid increase in the plasma concentration of cocaine (n=2), as well as long-lasting increases in MAP and HR (n=3). Administration of DM CocE (0.32mg/kg;IV), 10 min after cocaine resulted in a rapid hydrolysis of cocaine, with plasma levels below detection limits within 5 to 8 min. Elevations in MAP and HR were significantly reduced within 25 and 50 min of DM CocE administration, respectively. Although slight (10-fold) increases in anti-CocE antibodies were observed following the fourth administration of DM CocE, these antibodies did not alter the capacity of DM CocE to reduce plasma cocaine levels, or to ameliorate cocaine's cardiovascular effects. Anti-CocE titers were transient and generally dissipated within eight weeks. Together, these results suggest that highly efficient cocaine esterases, such as DM CocE, may provide a novel and effective therapeutic for the treatment of acute cocaine intoxication in humans.
INTRODUCTION:

Cocaine abuse remains a significant public health problem with an estimated 15-19 million individuals using cocaine within the past year worldwide (United Nations Office on Drugs and Crime, 2010). In the United States alone there are an estimated 1.5 million current cocaine users, with approximately 1700 people trying cocaine for the first time each day (Substance Abuse and Mental Health Services Administration, 2011). Although moderate doses of cocaine are often associated with “pleasurable” effects, large doses of cocaine can produce a variety of adverse effects including anxiety, convulsion, delirium, hypothermia, and chest pain, the latter of which results from cocaine-induced increases in mean arterial pressure (MAP) and heart rate (HR) (Glauser & Queen, 2007; Olson et al., 1994). These large-dose effects of cocaine account for the majority of all illicit drug-related emergency department (ED) visits in the United States, with recent estimates suggesting that cocaine-related ED cases are more than twice as common as those involving heroin use, and four-times as common as those involving other stimulants, such as methamphetamine (Substance Abuse and Mental Health Services Administration, 2011).

Despite longstanding efforts to identify small molecules capable of selectively inhibiting the reinforcing and/or toxic effects of cocaine (e.g., Dackis and O’Brien, 2003; Grabowski et al., 2004; Tanda et al., 2009; Vocci et al., 2005), there are currently no United States Food and Drug Administration (USFDA) approved medications for the treatment of cocaine abuse or toxicity. Recently a significant effort has been directed towards the development of cocaine-specific enzymes capable of reducing the reinforcing and/or toxic effects of cocaine by dramatically altering its pharmacokinetics. In both human and non-human primates, cocaine is naturally metabolized by butyrylcholinesterase (BChE) to the inactive metabolites eegonine methyl ester and benzoic acid, with an elimination half-life of ~45 min (Mello et al., 2002; Mendelson et al., 1999). Through a series of site-directed mutagenesis studies, Zhan and colleagues identified mutant BChEs capable of hydrolyzing cocaine approximately 450- to 2000-
times faster than native BChE (Pan et al, 2005; Zheng et al., 2008). In rats and mice, these mutant BChEs effectively reduce the cardiovascular, lethal, and abuse-related effects of cocaine (Brimijoin et al., 2008; Carroll et al., 2011; Xue et al., 2010; Zheng et al., 2008), suggesting that such enzymes may provide a viable strategy for treating cocaine toxicity and abuse in humans.

In a parallel series of studies, a highly efficient bacterial cocaine esterase (CocE) \( (k_{cat}/K_m \sim 800\text{-fold greater than BChE: Larsen et al., 2002; Turner et al., 2002}) \) was extensively evaluated as an alternative to BChE, which is often difficult to purify or produce in the laboratory (e.g., Huang et al., 2007). Although the wild-type (wt) form of CocE dose-dependently protect mice and rats against the cardiovascular, convulsant, and lethal effects of cocaine in rats and mice, it is rapidly inactivated at body temperature with a half-life of \( \sim 15 \text{ min} \) (Cooper et al., 2006; Jutkiewicz et al., 2009; Ko et al., 2007; 2009; Wood et al., 2010). Site-directed mutations have improved thermostability and resulted in an equally efficient mutant CocE \( (T172R/G173Q \text{ CocE; RQ \text{ CocE; DM \text{ CocE}}}) \) that retains some activity \textit{in vitro} and \textit{in vivo} for over 4 h (Collins et al., 2009; Gao et al., 2009; Narasimhan et al., 2010). In addition to rapidly hydrolyzing circulating cocaine in rats and monkeys (Brim et al., 2011a; 2012), DM CocE is capable of dose-dependently inhibiting the cardiovascular, convulsant, lethal, and reinforcing effects of cocaine in rats and rhesus monkeys (Collins et al, 2009; 2011a; 2011b).

While these findings suggest that highly efficient cocaine hydrolyzing enzymes, such as CocE, may provide a valuable therapeutic option for the treatment of acute cocaine toxicity, it is important to note that the repeated administration of wt form of the bacterial CocE elicits potentially neutralizing immune responses in mice (Ko et al., 2007; 2009). Although increases in anti-CocE antibodies were observed during an initial dose-response study of DM CocE in rhesus monkeys (Collins et al., 2011a), the immunologic potential of DM CocE has yet to be systematically evaluated in any species. Thus, the current studies characterized the capacity of DM CocE to stimulate the development of anti-CocE antibodies, and evaluated the potential for these anti-CocE antibodies to neutralize the effectiveness of DM CocE in reducing plasma
cocaine levels and ameliorating the cardiovascular effects of cocaine during four, bi-weekly trials in rhesus monkeys.

METHODS:

Subjects: Three adult male (BE, BL and CA), and two adult female (UR and KY) rhesus monkeys (Macaca mulatta) were used in these studies. All monkeys were singly housed in stainless steel monkey cages in an environmentally controlled room (temperature 21 ± 3°C, relative humidity 30-70%, 10-15 air changes per hour) under a 12-hour light:dark cycle with lights on at 07:00. The monkeys’ diet consisted of 20-50 Lab Fiber Plus Monkey Diet Chows (Lab Diet; PMI Nutrition International, LLC; Brentwood, MO), fresh fruit, and free access to water, and health checks were performed daily to ensure that all monkeys remained healthy. All experimental procedures were approved by the University of Michigan Committee on the Use and Care of Animals and performed in accordance with the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the National Institutes of Health.

Effectiveness of DM CocE to reduce plasma cocaine concentrations following repeated dosing: One male (BE) and female (UR) adult rhesus monkey, trained for arm-restraint chairs, were used to evaluate the time course of the plasma levels of cocaine produced by an intravenous infusion of 3 mg/kg cocaine. Each monkey was tested with this dose of cocaine five times (1x with phosphate-buffered saline [PBS] and 4x w/DM CocE) with 14 days separating each administration. Once seated in the restraint chairs, each monkey had an acute catheter placed in the saphenous vein to allow for the administration of cocaine (3 mg/kg at t=0 min) and 0.32 mg/kg DM-CocE or PBS, with each infusion followed by a 3 ml flush with physiologic saline to clear the catheter and to ensure the entire dose had been delivered. For both monkeys PBS was delivered 10 min post-cocaine, however, due to onset of preconvulsant behaviors (e.g., tremor, drooling), BE received DM CocE at the 1min post-cocaine time point rather than the 10
min time point used for UR. These catheters also allowed for serial collection of blood samples taken 2 min before cocaine, as well as 1, 8, 15, 30, 60, 90, and 120 min after cocaine administration. The pre-cocaine blood sample was used to determine both anti-CocE titer levels, as well as baseline cocaine concentrations in the plasma, whereas samples collected at later time points were only used to determine plasma concentrations of cocaine. For this reason, the pre-cocaine sample was split so that 0.5 ml of blood was allowed to clot at room temperature prior to collection of serum, with the remainder of the sample (~2 ml) treated identically to the samples taken at later time points. Each sample was transferred into tubes containing EDTA (5 ml; BD Vacutainer K2EDTA Plus Blood Collection; BD Biosciences) and 1/10 volume of 1 M NaF to prevent clotting and eliminate further cocaine metabolism, respectively. Samples were centrifuged at 4000 RPM for 5 minutes at 4 °C, and transferred to 2 ml cryovials prior to being stored at -80 °C. Portions of the data from these studies (PBS and DM CocE 1st trial conditions) were published elsewhere (Brim et al., 2012).

**Sample preparation and mass spectral analysis of DM CocE-mediated cocaine**

**Hydrolysis:** Plasma fractions from each blood sample (50 – 200 µl) were added to 570 µl of acetonitrile, 20 µl of 1 M NaF, and 2 µl of internal standard solution containing 750 nM deuterium-labeled norcocaine, cocaine, benzoylecgonine, and ecgonine methyl ester (Cerilliant Corporation). Cocaine metabolites were included as internal standards to maintain consistency between this study and other in vivo cocaine metabolite studies (Brim et al., 2011a; 2012). Samples were vortexed for 30 s and centrifuged at 25,000 relative centrifugal force at 20°C for 30 min. The supernatant was removed and added to a clean microcentrifuge tube. Samples were centrifuged a second time under 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 analysis.
Mass spectral analysis was performed at the University of Michigan Biomedical Mass Spectrometry Facility as previously described (Brim et al., 2012). Briefly, 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. In order to achieve cocaine concentrations within the limits of quantification, samples were diluted further (varying along the time course) with 10 mM ammonium formate, pH 4.6/acetonitrile (97:3; v/v) and 50 nM internal standards. Samples were vortexed for 30 s, then centrifuged at 13,600 relative centrifugal force for 20 min. Aliquots of the supernatants were transferred to polypropylene autosampler vials for analysis within 12 h. LC-MS/MS was performed on a Prominence HPLC system (Shimadzu) interfaced directly to the Turbo Ionspray source of an API 3000 triple quadrupole mass spectrometer (PerkinElmerSciex Instruments). Separation was achieved with a Thermo Fisher Scientific Hypersil Gold column (50 X 2.1 mm i.d.; 1.9-μm packing) maintained at 45°C using a binary gradient and a flow rate of 0.45 ml/min. The injection volume was 4 μl, and 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. The sample tray was cooled to 10°C to prevent sample degradation, and each analysis was completed within 15 min.

Analyst software (version 1.4.2; MDS Sciex) was used for instrument control, data acquisition, and quantitative analysis. Calibration curves were constructed from the standard samples. The ratio of the peak area of cocaine to the corresponding deuterium-labeled internal standards was plotted as a function of the analyte concentration normalized to the internal standard concentration. Calibration curves were generated by using a least-squares linear regression analysis with 1/x weighting. Calibration standards for cocaine (4.0 – 0.03 μM) were prepared in commercial plasma from untreated animals (Valley Biomedical, Winchester VA).
standards were stored at -80°C and prepared fresh for each set of experimental samples. Twenty microliters of each calibration stock was extracted with 68 μl of acetonitrile, 4 μl of 1 M NaF, and 8 μl of internal standard and prepared identically to the samples described above. Calibration standards were reconstituted to 100 μl, resulting in final internal standard concentrations of 50 nM.

**Surgical preparation:** Three of the monkeys (BL, CA and KY) were implanted with radiotelemetric probes (D70-PCT; DSI Inc., St. Paul, MN) to allow for the collection of cardiovascular measures, as well as an indwelling venous catheter to allow for drug delivery. Prior to surgery, monkeys were anesthetized with ketamine (10.0 mg/kg; IM) and placed on a heating pad set to maintain the animal's body temperature at approximately 37°C. Monkeys were prepared by shaving the hair along the right flank, above the femoral artery on the right leg, as well as the left and just above the zyphoid process and to the right of the right clavicle. All areas were scrubbed with alternating betadine/alcohol swabs and small incisions were made to allow for the implantation of the telemetric probe. A pocket was teased out to allow for placement of the probe, and the blood pressure catheter was tunneled to and implanted in the femoral artery to allow for arterial pressure measures. Electrocardiographic leads were tunneled to the incisions above the zyphoid process, and clavicle, and sutured to the muscle. All incisions were closed with 5-0 Ethilon® suture, and monkeys were allowed 5-7 days to recover from surgery prior to implantation of an indwelling catheter in a previously unused vein (i.e., jugular or femoral vein). Monkeys were shaved between the scapula, and above the vein to be catheterized, and areas were scrubbed with alternating betadine/alcohol swabs. Small incisions were made between the scapula and above the vein to be catheterized, and upon implantation the catheter was tunneled to and exited from the incision between the scapula. These monkeys were then fitted with mesh jackets, and attached to a steal tether on a swivel to allow for unrestrained movement.
in the animals home cage. A recovery period of at least 7 days was provided prior to experimentation. Catheters were flushed daily with 3 ml of saline to ensure catheter patency.

Effectiveness of DM CocE to ameliorate cocaine-induced changes in MAP, HR, core body temperature, and locomotor activity following repeated dosing: Three adult rhesus monkeys, 2 males (BL and CA) and 1 female (KY) were used to evaluate the cardiovascular effects of intravenous cocaine (3 mg/kg). Each monkey was tested with this dose of cocaine six times, with each test separated by 14 days to reduce the possibility for the development of tolerance to the cardiovascular effects of cocaine, and to increase the possibility for observing the development of anti-CocE antibodies. Each dose of cocaine was followed immediately by a 5 ml saline flush to ensure the entire dose was delivered. Experimental treatments (PBS or 0.32 mg/kg DM CocE) were administered intravenously, 10 min after cocaine, and were similarly followed by a 5 ml saline flush. PBS served as the vehicle control, and was always evaluated during the first and last test sessions (i.e., sessions 1 and 6) to allow for the development of tolerance or sensitization to cocaine’s effects to be observed. The effects of 0.32 mg/kg; IV DM CocE were always evaluated during the intervening 4 test sessions (i.e., sessions 2, 3, 4 and 5). Test sessions were performed between 13:00 and 17:00, with real-time measures of mean arterial pressure (MAP), heart rate (HR), core body temperature, and locomotor activity collected at 1 s intervals for at least 45 min before, and 120 min after cocaine administration. In order to determine if monkeys were developing anti-DM CocE antibodies, serum samples were collected from each monkey at 2-week intervals throughout the course of the study, beginning with the initial cocaine Vs. PBS condition. During weeks in which monkeys were tested 2 ml of blood was collected via the saphenous vein 24 hours prior to the test session. Blood samples were collected without preservatives and stored at room temperature for 60 minutes prior to centrifugation at 4000 RPM for 5 minutes at 4 °C. Serum was then
collected and pipetted into 2 ml cryovials and stored at -80 °C until being assayed for anti-CocE antibody titer determinations.

**Immunologic determinations:** In order to determine if monkeys were developing anti-DM CocE antibodies, a direct ELISA specific for anti-CocE antibodies was set up using a standard protocol. CocE was used (1 g/ml) to coat a 96-well micro-titer plate using borate buffered saline (1.5M NaCl, 0.5M H$_3$BO$_3$, 1.0M NaOH) to resuspend CocE (50 μl/well). The coating plates were left overnight at 4 °C. The coating buffer was removed the following morning and the plates blocked with 2% normal goat serum in phosphate buffered saline for 1 h at 37 °C and washed three times. Serum from the various monkeys was serially diluted in 50 μl of phosphate buffered saline in the wells in a range of 102 to 107 and run in duplicate. The plates were covered and incubated for 1 hr at 37 °C. Subsequently, the plates were washed three times and 50 μl/well of goat anti-mouse IgG peroxidase labeled antibody diluted 1:400. The plates were then washed three times and 100 μl peroxidase substrate solution (OPD dissolved citrate/phosphate buffer) was added to each well. After a 5-10 minute incubation (based upon color development in the positive controls), the reaction was stopped using 3M H$_2$SO$_4$ (50 μl/well). The plates were read at 490 nm and titer was determined by the highest dilution that showed increases over background absorbance.

**Drugs:** Cocaine HCl was obtained from Mallinckrodt Inc. (St. Louis, MO), and dissolved in 0.9% sterile saline to a concentration of 10.0 mg/ml and administered on a mg/kg basis over 30 seconds. DM CocE (T172R/G173Q CocE) was prepared as previously described (Brim et al., 2012) and stored at -80 °C until needed. Endotoxin levels for these preparations were assessed using an end-point Limulus Amebocyte Lysate (LAL) assay (Charles River) as per manufacturers specifications, and were less than 30 EU/ml (~0.2 ng/ml or less than 2 EU/kg at
the 0.32 mg/kg dose of DM CocE). Prior to administration, DM CocE (5.0 mg/ml) was thawed on ice and administered on a mg/kg basis over a period of 10 seconds.

**Data analysis:** Real-time measures of mean arterial pressure (MAP), heart rate (HR), body temperature (°C), and locomotor activity were collected at 1 s intervals, beginning at least 45 min before cocaine administration. Baseline measures for each of the parameters represent the mean ± standard error of the mean (S.E.M.) of the 15 min preceding each infusion for each measure with the exception of locomotor activity which represents the total locomotor activity observed during the 10 min period immediately preceding the infusion of cocaine. Cardiovascular and physiologic parameters were collected for at least 120 min after cocaine infusion, and the effects of cocaine, or CocE, on each of the parameters are reported as the change from baseline for each 5 min block of time (mean of 5 min block - mean of 15 min prior to infusion). Locomotor activity was summed over 10 minute blocks, and reported as the total locomotor counts / 10 min. Two-way ANOVA with repeated measures and post-hoc Bonferroni tests were used to determine if DM CocE administration produced significant alterations in the cardiovascular or physiologic effects of 3 mg/kg; IV cocaine for each 5-minute bin over the 120-minute period following cocaine infusion, and to determine if the effectiveness of DM CocE was altered with repeated administration. Plasma cocaine levels are reported as the mean ± S.E.M. concentration of cocaine (ng/ml) for each time point. Two-way ANOVA with repeated measures and post-hoc Bonferroni tests were used to determine if DM CocE administration significantly plasma cocaine concentrations associated with 3 mg/kg; IV cocaine at each time point, and to determine if the effectiveness of DM CocE was altered with repeated administration. Physiologic and locomotor data are also presented as the mean ± S.E.M. (change in MAP, HR and TEMP) or total (total locomotor activity) for the 110 min period after DM CocE (or PBS) administration. Significant differences among conditions were determined by one-way ANOVA with repeated measures and post-hoc Bonferroni tests. Immunologic data are expressed as the mean ±
S.E.M. change from baseline (pre CocE) in the anti-CocE antibody titer (using a log 10 dilution). One-way ANOVA with repeated measures and post-hoc Dunnett’s tests were used to determine if there was a significant effect of DM CocE administration on anti-CocE titer levels over time.

RESULTS:

Effectiveness of DM CocE to reduce plasma cocaine concentrations following repeated dosing: As shown in Figure 1, IV administration of 3 mg/kg cocaine, followed 10 min later by PBS, resulted in large increases plasma cocaine concentrations, with cocaine concentrations of $9115.8 \pm 227.5$, and $2106.8 \pm 459.6$ ng/ml plasma at the 8 and 15 min time point, respectively. Assuming the high concentrations of cocaine obtained at the 8 min time point were due to the bolus of cocaine not being fully distributed, or contamination of the sample with residual cocaine in the sampling catheter, cocaine exhibited normal first-order kinetics with an elimination half-life of $t_{1/2} = 50.7$ min.

Although the experimental design called for DM CocE to be administered 10 min after cocaine, the timing of DM CocE administrations had to be adjusted due to differences in the sensitivities of the monkeys to the large dose effects of cocaine (BE exhibited tremor, sialorrhea, and decreased respiration shortly after the bolus administration of 3 mg/kg; IV cocaine). For this reason, BE received DM CocE 1 min after cocaine, whereas UR received DM CocE 10 min after cocaine. Despite this difference, the administration of DM CocE (0.32 mg/kg; IV) significantly reduced (Treatment: $[F(4,20)=71.4; p<0.001]$; Time: $[F(4,20)=5.8; p<0.05]$), the plasma cocaine concentrations to below the limits of quantification within the first 5 to 7 min after administration. Although cocaine levels remained below quantification limits at the 30 min time point, a slight rise in plasma cocaine concentrations was observed over the second hour of sampling during the first two tests with DM CocE (Figure 1; inset panel). There were no significant differences observed when the plasma levels of cocaine were compared across the
four DM CocE test sessions. Data from the PBS and DM CocE 1st trial conditions has been published elsewhere (Brim et al., 2012).

**Effectiveness of DM CocE to ameliorate cocaine-induced changes in MAP, HR, core body temperature, and locomotor activity following repeated dosing:** Figure 2 depicts the cardiovascular effects of 3 mg/kg IV cocaine during six bi-weekly sessions in which PBS or 0.32 mg/kg; IV DM CocE were administered 10 min after cocaine. Although cocaine produced rapid increases in MAP that peaked (69.2 ± 2.7 mmHg above baseline) within the first 5-10 min of cocaine administration, the cocaine-induced increases in HR were slower to develop with peak increases (73.7 ± 9.1 BPM) in HR typically observed within 80 min of cocaine administration.

As shown in Figure 2 (top left panel) administration of 0.32 mg/kg; IV DM CocE 10 min after cocaine resulted in a significant (Treatment: [F(4,176)=135.0; p<0.001]; Time: [F(4,176)=4.6; p<0.001]), and rapid amelioration of cocaine’s hypertensive effects, with significant reductions in MAP observed within 25 to 35 min of DM CocE administration. Cocaine-induced increases in MAP returned to baseline within 30 min of DM CocE administration where they remained for the remainder of the 120 min session. As shown in Figure 2 (top right panel), DM CocE also significantly reduced [F(5,10)=14.1; p<0.001] the mean change in MAP over the final 110 min of each of the four repeated trials with 0.32 mg/kg; IV DM CocE. Importantly, the effectiveness of DM CocE to reduce the hypertensive effects of cocaine did not change across the four trails.

Administration of 0.32 mg/kg DM CocE produced a similar and significant reduction (Treatment: [F(4,176)=153.9; p<0.001]; Time: n.s.) in the tachycardic effects of cocaine when evaluated across 5 min blocks of time. Although significant reductions in cocaine’s HR-stimulating effects were not observed until 45 to 50 min after DM CocE administration, it is important to note that these effects correspond to the portion of the sessions when the
effects of cocaine’s tachycardic effects were largest. As was observed with MAP, DM CocE significantly reduced the mean change in HR observed over the final 110 min of the session \([F(5,10)=41.7; p<0.001]\). Although, there were no significant differences in the effectiveness of DM CocE to ameliorate the tachycardic effects of cocaine across the four, bi-weekly tests, cocaine did appear to produce a slightly larger mean HR response during the fourth trial. Although it is possible that this may represent a loss of activity, it is important to note that HR responses appeared to spike at the 40 min time point before decreasing to near baseline like levels. There were no significant differences between the MAP or HR stimulating effects of cocaine during the PBS test sessions that were conducted before and after DM CocE tests.

In addition to the cardiovascular effects described above, the IV administration of 3 mg/kg cocaine also stimulated locomotor activity and produced a modest increase in core body temperature (Figure 3). Not only was the administration of DM CocE (0.32 mg/kg; IV) 10 min after cocaine effective at reducing cocaine’s hyperthermic effects when evaluated over individual 5 min bins (Treatment: \([F(4,176)=44.0; p<0.001]\); Time: n.s.), but a similar reduction in the mean change in body temperature was also observed over the final 110 min of the session \([F(5,10)=5.6; p<0.05]\). Although there were no significant differences in the body temperature responses observed across the four DM CocE trials, the mean temperatures obtained during the third and fourth trials with DM CocE were significantly lower than those obtained in the PBS condition (\(p<0.01\) for both), suggesting that the administration of DM CocE may have produced a mild hypothermia. Finally, the administration of 0.32 mg/kg; IV DM CocE was similarly effective at reducing the locomotor stimulatory effects of 3 mg/kg cocaine. Not only was DM CocE effective at reducing cocaine-stimulated activity when evaluated across 10 min bins (Treatment: \([F(4,88)=15.4; p<0.001]\); Time: n.s.), but DM CocE also reduced the locomotor effects of cocaine when they were summed across the final 110 min of the session \([F(5,10)=3.7; p<0.05]\). Although DM CocE appeared to produce a consistent inhibition of cocaine’s locomotor
stimulatory effects, post-hoc tests failed to reveal any significant differences among the treatments.

**Immunologic effects of repeated dosing with DM CocE:** Figure 4 shows the effects of repeated administration of 0.32 mg/kg; IV DM CocE on the development, and subsequent disappearance, of anti-CocE antibodies for each of the five monkeys. Although the first three administrations of DM CocE failed to increase anti-CocE titers in four of the five monkeys, each of the five monkeys displayed some level of immunologic response following the fourth trial, with a mean increase in anti-CocE titer of ~10-fold. The strongest response (~1000-fold increase in anti-CocE titer) was observed in KY, the female monkey that participated in the cardiovascular portion of the study, whereas the weakest responses (~3-fold increase in anti-CocE titer) was observed in monkeys BL and CA, the two male monkeys that participated in the cardiovascular portion of the study. Although the administration of DM CocE tended to result in a gradual increase in anti-CocE titer over the course of the four trials, these effects failed to reach significance when the data were grouped. Just as anti-CocE titers gradually increased with the repeated administration of DM CocE, discontinuation of treatment led to a gradual decline in anti-CocE titer levels, with four out of five monkeys showing little or no immunologic response by 8 weeks after treatment.

**DISCUSSION:**

We have previously described the capacity of DM CocE to rapidly hydrolyze cocaine, both in vitro and in vivo, as well as the effectiveness of DM CocE to ameliorate the reinforcing, cardiovascular, convulsant, and lethal effects of cocaine in a variety of species including mice, rats, and rhesus monkeys (Brim et al., 2010; 2011a; 2012; Collins et al., 2009; 2011a; 2011b; Gao et al., 2009; Narasimhan et al., 2010). The current studies systematically evaluated (1) the immunologic responses to repeated doses of DM CocE, (2) the effectiveness of repeated doses
of DM CocE to reduce circulating concentrations of cocaine, and (3) the effectiveness of repeated doses of DM CocE to ameliorate the cardiovascular and psychomotor stimulatory effects of cocaine in rhesus monkeys during four bi-weekly trials in which 0.32 mg/kg DM CocE was administered as a 10-min post-treatment to an IV bolus of 3 mg/kg cocaine. There were five main findings. First, DM CocE greatly accelerated the elimination of cocaine from the circulation, with plasma concentrations of cocaine reduced below detectable limits within 5-7 min. Second, DM CocE significantly reduced cocaine-induced increases in MAP and HR, with baseline-like cardiovascular responses generally recovered within 25-30 min. Third, DM CocE significantly reduced the locomotor stimulatory effects of the 3 mg/kg dose of cocaine. Fourth, repeated dosing with DM CocE stimulated the development of anti-CocE antibodies, however, these effects were relatively mild with increases in titer levels of 10-fold or less observed in four of five monkeys. Fifth, there was no evidence to suggest that anti-CocE antibodies were capable of neutralizing the effectiveness of DM CocE to hydrolyze cocaine, or to ameliorate the cardiovascular and locomotor stimulatory effects of cocaine in any of the rhesus monkeys.

Finally, when taken together with the results of previous studies, these findings strongly suggest that highly efficient bacterial cocaine esterases, such as DM CocE, should provide a safe and effective method to rapidly eliminate the symptoms of acute cocaine intoxication in humans, even if an individual requires multiple treatments over a relatively short period of time.

Although it has been difficult to establish a clear dose-response function for the toxic effects of cocaine in humans, blood levels obtained from patients in the ED, and from cocaine overdose fatalities (Finkle and McCloskey, 1978; Karch et al., 1998; Koehler et al, 2005; Blaho et al., 2000) suggest that cocaine concentrations at the time of ED admission may well exceed 1000 ng/ml. Similarly high concentrations of cocaine were observed in the current studies, with a single IV dose of 3 mg/kg cocaine, during the PBS condition, resulting in plasma cocaine levels in excess of 2000 ng/ml during the first 15 min, and in excess of 1000 ng/ml for the entirety of the first 60 min after cocaine administration. In addition to confirming previous
estimates of cocaine’s elimination half-life (~51 min in the current studies) in rhesus monkeys (Mello et al., 2002), these data also provide further validation for using the 3 mg/kg IV dose of cocaine as a rhesus monkey model of acute cocaine intoxication. Although concerns for the well-being of one of the monkeys (BE) precluded our ability to administer DM CocE at the same post-treatment time in both monkeys, these studies clearly demonstrate the effectiveness of 0.32 mg/kg DM CocE to rapidly eliminate high concentrations of cocaine from the circulation. Moreover, these findings extend previous reports (Brim et al., 2011a; 2012) by demonstrating that the hydrolytic effects of DM CocE are not affected by repeated administrations with DM CocE.

In addition to the increased blood levels of cocaine, IV administration of 3 mg/kg cocaine also produced a variety of cardiovascular and psychomotor stimulatory effects, including rapid increases in MAP that gradually decreased over the 2 hr period, as well as a more gradual increases in HR and locomotor activity that tended to peak during the second 60 min of observation. A close correspondence was observed between the elevations in MAP and the plasma concentrations of cocaine produced by 3 mg/kg IV cocaine. Given that blood levels of cocaine are also closely linked to the cardiovascular stimulation and cocaine-associated chest pain that is commonly reported with cocaine-related ED cases (Brody et al., 1990; Foltin and Fischman, 1991; Javaid et al., 1978; Mittleman et al., 1999), these findings provide further validation of the current model of acute cocaine intoxication in rhesus monkeys. Treatment with 0.32 mg/kg DM CocE, 10 min after cocaine, resulted in a rapid amelioration of the hypertensive effects of cocaine and virtually eliminated the increases in HR and locomotor activity that were observed during the second hour of the PBS conditions. In addition to confirming the results of our initial dose-response studies with DM CocE in rhesus monkeys (Collins et al., 2011a), these studies provide the first demonstration that the effectiveness of DM CocE to ameliorate the cardiovascular and psychomotor stimulatory effects of cocaine does not change with repeated administrations.
That DM CocE retained its effectiveness across multiple administrations is important for several reasons. First, in mice, the repeated administration of wt CocE has been shown to stimulate the production of anti-CocE antibodies and reduce the effectiveness of wt CocE to protect against cocaine-induced lethality (Ko et al., 2007; 2009). Unlike the relatively large increases observed in these early studies (≥1000-fold), DM CocE failed to stimulate large increases in anti-CocE titers, with four out of five monkeys displaying increases of 10-fold or less. The current studies are also in agreement with previous findings in which minimal increases in titers were observed in rhesus monkeys treated with three doses of DM CocE (0.32, 1.0, and 3.2 mg/kg) at one-week intervals (Collins et al., 2011a). Importantly, in both studies the increased titer levels were transient, with baseline anti-CocE titer levels observed within 6-10 weeks of the final treatment.

Although it is possible that species differences contributed to the milder immune response observed in the current and past studies (Collins et al., 2011a), that DM CocE failed to elicit strong immune responses also suggests that the immunogenic potential of CocE is not necessarily due to its bacterial origins (Bresler et al., 2000). Rather, it seems likely that the comparatively strong immune potential of wt CocE resulted from a contamination of earlier formulations with higher levels of endotoxin; a consequence of using a gram negative, \textit{e. coli} expression system. Even though endotoxin levels were not reported in the previous studies with wt CocE, that the current formulation of DM CocE (used herein and, Collins et al., 2011a) required extensive purification to reduce endotoxin to levels below the 5 EU/kg limit (current formulation was 2 EU/kg) imposed by the United States Pharmacopoeia and the USFDA (Malyala & Singh, 2008), suggests that endotoxin likely enhanced the immune responses observed with wt CocE. Nevertheless, studies in mice suggest that any potentially neutralizing effect of anti-CocE antibodies could be easily surmounted by larger doses of CocE (Ko et al., 2009).
When taken together with previous reports (Brim et al., 2011a; 2011b; 2012; Collins et al., 2009; 2011a; 2011b), the results of the current studies suggest that DM CocE would provide distinct advantages over benzodiazepines, the current standard of care for acute cocaine intoxication (McCord et al., 2008). Unlike the symptomatic relief provided by the anxiolytic and/or analgesic properties of benzodiazepines, highly efficient cocaine hydrolases aim to treat cocaine-associated chest pain and/or psychiatric symptoms by rapidly eliminating the underlying cause of these symptoms, that being cocaine. In addition to ameliorating the cardiovascular and psychostimulant effects of cocaine, therapeutics such as DM CocE would also limit the direct, cardiotoxic effects of cocaine, such as myocardial ischemia, left ventricular hypertrophy, and dilated cardiomyopathy (Hollander, 1995; Hollander and Henry, 2006), thereby reducing the patient’s risk of future heart failure. Moreover, rapidly eliminating cocaine would have the added benefit of reducing, or even eliminating, the need for lengthy observational periods to allow patients to naturally clear cocaine from their system, thus reducing the cost of treatment and freeing up ED resources for other cases.

In conclusion, the results of the current studies clearly demonstrate that even though DM CocE is mildly antigenic in rhesus monkeys, it retained is effectiveness to hydrolyze cocaine and ameliorate the cardiovascular and psychostimulant effects of cocaine across each of four, bi-weekly tests. Although these findings suggest that immunologic responses to repeated administrations of DM CocE are not likely to impact its therapeutic effectiveness, recent attempts to improve the duration and further reduce the immunogenicity of CocE have resulted in a polyethylene glycol modified mutant form of CocE (PEG-CCRQ CocE) capable of reducing the reinforcing, cardiovascular, convulsant, and lethal effects of cocaine for up to 48 hrs without eliciting increases in anti-CocE antibodies (Narasimhan et al., 2011; Collins et al., 2012). Importantly, even though self-administration studies in rats (Collins et al., 2009; 2012) suggest that human cocaine abusers would be able to surmount the protective effects of CocE (by ingesting approximately 10-times more cocaine), the capacity of CocE to rapidly metabolize
large doses of cocaine (>100 mg/kg; IV self-administered over 90 min) would likely protect these individuals from the adverse effects that would otherwise be associated with such large doses of cocaine. When taken together with the results of previous studies demonstrating the effectiveness of DM CocE to protect against and/or reverse acute cocaine toxicity in mice, rats and rhesus monkeys the current findings strongly suggest that highly efficient cocaine hydrolases, such as DM CocE, could provide a safe and effective option for the treatment of acute cocaine intoxication in humans.
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Participated in research design: Collins, Brim, Noon, Narasimhan, Lukacs, Sunahara, Woods, Ko

Conducted experiments: Collins, Brim, Noon

Contributed new reagents of analytic tools: Noon, Narasimhan

Performed data analysis: Collins, Brim, Noon

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Portions of this work were presented previously:


Potential Conflicts of Interest:

Woods and Sunahara have served as consultants to Reckitt Benckiser Pharmaceuticals (Richmond, VA). Brim, Narasimhan, Ko, Woods, and Sunahara are listed as inventors on patent number PCT/US2008/069659 for DM CocE.

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FIGURE LEGENDS:

Figure 1)
Plasma concentrations of cocaine produced by IV doses of 3 mg/kg cocaine during five bi-weekly trials in two rhesus monkeys. Plasma cocaine concentrations were assessed by mass spectrometry ~2 min before cocaine (Baseline; BL), and at 8, 15, 30, 60, 90 and 120 min after the administration of cocaine. Open circles represent the mean ± SEM plasma concentrations of cocaine when PBS was administered 10 min after 3 mg/kg cocaine. Plasma concentrations of cocaine during the first (shaded, inverted triangles), second (open, inverted triangles), third (shaded triangles), and fourth (shaded triangles) trials in which 0.32 mg/kg DM CocE was administered as a post-treatment to 3 mg/kg cocaine. Data from the 8 min time point were excluded due to differences in the post-treatment times (UR, 10 min post-treatment; BE, 1 min post-treatment). Inset panel shows plasma cocaine concentrations during the four DM CocE conditions plotted on a y-axis with a smaller range of concentrations. ***, p<0.001. Significant differences in plasma cocaine concentrations were determined by two-way ANOVA with repeated measures and post-hoc Bonferroni tests.

Figure 2)
Effects of repeated doses of DM CocE (0.32 mg/kg; IV) or PBS on the cocaine-induced changes in mean arterial pressure (MAP; top panels) and heart rate (HR; bottom panels) when administered 10 min after an IV dose of 3 mg/kg cocaine (n=3) during six bi-weekly trials. Left panels) Data points represent the mean ± SEM change from baseline for MAP or HR over successive 5-min blocks of time during sessions in which 3 mg/kg cocaine was followed by PBS (open circles), or 0.32 mg/kg DM CocE (triangles). Right panels) Data represent the mean ± SEM change from baseline for MAP or HR over the entire 110 min after the administration of PBS (black bars) or 0.32 mg/kg DM CocE (open bars). **, p<0.001; ***, p<0.001. Significant
differences in mean change in MAP or HR among conditions were determined by one-way ANOVA with repeated measures with post hoc Bonferroni tests.

**Figure 3)**
Effects of repeated doses of DM CocE (0.32 mg/kg; IV) or PBS on the cocaine-induced changes in core body temperature (°C; top panels) and locomotor activity (bottom panels) when administered 10 min after an IV dose of 3 mg/kg cocaine (n=3) during six bi-weekly trials. Left panels) Data points represent the mean ± SEM change from baseline for TEMP or sum of locomotor activity over successive 5-min blocks of time during sessions in which 3 mg/kg cocaine was followed by PBS (open circles), or 0.32 mg/kg DM CocE (triangles). Right panels) Data represent the mean ± SEM change from baseline for TEMP or sum of locomotor activity over the entire 110 min after the administration of PBS (black bars) or 0.32 mg/kg DM CocE (open bars).

**Figure 4)**
Time course for the development of anti-CocE antibody titers following the repeated administration of 0.32 mg/kg DM CocE. Serum samples were collected 24 h before each of 6 bi-weekly trials (PBS and DM CocE), as well as 4 and 8 wks after the final DM CocE administration. Symbols represent anti-CocE antibody titers for each of the five monkeys. Solid line represents the mean ± SEM of the anti-CocE antibody titers (n=5).
Figure 1.

<table>
<thead>
<tr>
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<th>Post-Treatment to 3 mg/kg IV Cocaine</th>
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<tr>
<td>○ PBS</td>
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<tr>
<td>△ 0.32 DM CocE (3rd Trial)</td>
<td>△ 0.32 DM CocE (4th Trial)</td>
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Plasma Cocaine Concentration (ng/ml) vs. Time Since Cocaine (min)
Figure 2.

10 minute Post-Treatment to 3 mg/kg IV Cocaine

- PBS
- 0.32 DM CocE (1st Trial)
- 0.32 DM CocE (3rd Trial)
- 0.32 DM CocE (2nd Trial)
- 0.32 DM CocE (4th Trial)

**Figure 2A**

**MAP**

Δ MAP (mmHg)

Time (min)

**Figure 2B**

Mean Δ MAP (110 min)

**Figure 2C**

**HR**

Δ HR (BPM)

Time (min)

**Figure 2D**

Mean Δ HR (110 min)
Figure 3.

10 minute Post-Treatment to 3 mg/kg IV Cocaine

- **PBS**
- **0.32 DM CocE (1st Trial)**
- **0.32 DM CocE (2nd Trial)**
- **0.32 DM CocE (3rd Trial)**
- **0.32 DM CocE (4th Trial)**

**Δ Core Body Temp °C**

**Time (min)**

**Mean Δ Temp (°C)**

(110 min)

**Locomotor Counts / 10 min**

**Total Locomotor Activity (110 min)**

**Time (min)**
Figure 4.

Δ Anti-CocE Titer (Log 10 Dilution)

- BE
- UR
- BL
- CA
- KY

Mean±SEM

DM CocE Trial

BL DM1 DM2 DM3 DM4 4 wks Post 8 wks Post