Molecular Pharmacology Fast Forward. Published on January 19, 2010 as doi:10.1124/mol.109.060806
This article has not been copyedited and formatted. The final version may differ from this version.

Title Page:

A Thermally Stable Form of Bacterial Cocaine Esterase: A Potential Therapeutic Agent for Treatment of Cocaine Abuse

Remy L. Brim, Mark R. Nance, Daniel W. Youngstrom, Diwahar Narasimhan, Chang-Guo Zhan, John J.G. Tesmer, Roger K. Sunahara, James H. Woods

Department of Pharmacology
University of Michigan Medical School
1301 Medical Sciences Research Building III
1150 W Medical Center Drive
Ann Arbor, MI 48109-0600
(RLB, DN, JJGT, RKS, JHW)

Life Sciences Institute
University of Michigan
210 Washtenaw Ave
Ann Arbor, MI 48109-2216
University of Michigan
Ann Arbor, MI
(MRN, JJGT)

Department of Biophysics
University of Michigan
4028 Chemistry Building
930 North University Avenue
Ann Arbor, MI 48109-1055
(DWY)

Department of Pharmaceutical Sciences
College of Pharmacy
University of Kentucky
725 Rose Street
Lexington, KT 40536
(CGZ)

Molecular Pharmacology Fast Forward. Published on January 19, 2010 as DOI: 10.1124/mol.109.060806
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title Page

Running Title: Characterization of a thermostable mutant cocaine esterase

Corresponding Author: James H. Woods

Department of Pharmacology

University of Michigan Medical School

1301 Medical Sciences Research Building III

1150 W Medical Center Drive

Ann Arbor, MI 48109-0600

734-764-9133 (phone)

734-764-7118 (fax)

jhwoods@umich.edu

Text pages: 27

Tables: 2

Figures: 5

References: 37

Words in the Abstract: 198

Words in the Introduction: 807

Words in the Discussion: 1282

Abbreviations: CocE: cocaine esterase; WT: wild-type; FR: fixed-ratio; TO: timeout, RMSD: root mean squared deviation

Molecular Pharmacology Fast Forward. Published on January 19, 2010 as DOI: 10.1124/mol.109.060806
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract:

Rhodococcal cocaine esterase (CocE) is an attractive potential treatment for both cocaine overdose and cocaine addiction. CocE directly degrades cocaine into inactive products, whereas traditional small-molecule approaches require blockade of the inhibitory-action of cocaine on a diverse array of monoamine transporters and ion channels. The usefulness of wild-type (wt) cocaine esterase, however, is hampered by its inactivation at 37°C. Herein we characterize the most thermostable form of this enzyme to date, CocE-L169K/G173Q. I*n vitro* kinetic analyses reveal that CocE-L169K/G173Q displays a half-life of 2.9 days at 37°C, representing a 340-fold improvement over wt and 15-fold greater than previously reported mutants. Crystallographic analyses of CocE-L169K/G173Q, determined at 1.6 Å resolution, suggest that stabilization involves enhanced domain-domain interactions involving van der Waals interactions and hydrogen bonding. *In vivo* rodent studies reveal that intravenous pre-treatment with CocE-L169K/G173Q in rats provides protection from cocaine-induced lethality for longer time periods prior to cocaine administration than wt CocE. Furthermore, intravenous administration (pre-treatment) of CocE-L169K/G173Q prevents self-administration of cocaine in a time-dependent manner. Termination of its *in vivo* effects appears to be dependent on, but not proportional to, its clearance from plasma as its half-life is approximately 2.3 hours and similar to that of wt CocE (2.2 hrs). Taken together these data suggest that CocE-L169K/G173Q possesses many of the properties of a biological therapeutic for treating cocaine abuse, but requires additional development to improve its serum half-life.

Molecular Pharmacology Fast Forward. Published on January 19, 2010 as DOI: 10.1124/mol.109.060806
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction:

Cocaine abuse affects over 1.7 million Americans (Substance Abuse and Mental Health Services Administration, 2007). Chronic use causes multiple health problems including heart arrhythmias, high blood pressure, liver necrosis, mental illness and death (Benowitz, 1993). The economic cost of health care and lost wages due to cocaine use accounts for a large portion of the $180.7 billion spent on drug abuse treatment in the United States (Office of National Drug Control Policy, 2004). Despite these tragic personal and financial costs, there is no FDA-approved pharmacotherapy for cocaine abuse.

Cocaine blocks monoamine transporters (Benowitz, 1993; Johanson and Fischman, 1989; Uhl et al., 2002) accounting for the drug's psychotropic effects. The enhanced adrenergic stimulation and direct blockade of sodium channel also leads to profound cardiac disturbances (Billman, 1990; Crumb and Clarkson, 1990). Inhibitors that block cocaine's action at dopamine and serotonin transporters (Carroll et al., 2006; Daniels et al., 2006; Negus et al., 2009; Rothman et al., 2007) cannot eliminate the cardiovascular effects of cocaine or enhance *in vivo* degradation.

An alternate pharmacological approach is to enhance cocaine metabolism. The endogenous enzyme butyrylcholinesterase (BchE) cleaves cocaine to physiologically inert products; ecgonine methyl ester and benzoic acid (V_{max} = 3.9 min^{-1}). Large doses of BchE can protect animals from cocaine-induced lethality (Browne et al., 1998; Gorelick, 1997; Hoffman et al., 1996; Lynch et al., 1997; Mattes et al., 1997). Albumin-fused engineered mutants of BchE displaying enhanced kinetics against cocaine (V_{max}=2,700 min^{-1}) (Duysen et al., 2002; Gao and Brimijoin, 2004; Xie et al., 1999) increase BchE's plasma half-life (Brimijoin et al., 2008; Gao et al., 2008) and block both the lethal effects of cocaine, and reinstatement of cocaine-seeking behavior in a rat cocaine self-administration model (Brimijoin et al., 2008). Commercial production of this enzyme, however, may be challenging due to its low expression levels in mammalian expression systems and complex post-translational processing.

Molecular Pharmacology Fast Forward. Published on January 19, 2010 as DOI: 10.1124/mol.109.060806
This article has not been copyedited and formatted. The final version may differ from this version.

Our approach to treating cocaine abuse and toxicity has been the use of a bacterial cocaine esterase (CocE). Isolated from the MB1 strain of *Rhodococcus* found in the Rhizosphere soil surrounding the coca plant, CocE has a high V_{max} towards cocaine (V_{max} =2,300 min^{-1}) (Gao et al., 2009) and produces the same products as BchE (Bresler et al., 2000). CocE has previously been shown to block cocaine-induced cardiac disturbance, neurological changes, and lethality in rodents when administered before or after cocaine (Cooper et al., 2006; Jutkiewicz et al., 2009; Ko et al., 2007; Wood et al., In Press).

The wild-type (wt) CocE cannot be used as a pharmacotherapy for cocaine abuse due to its 13.7 min half-life at 37°C (Cooper et al., 2006; Gao et al., 2009; Ko et al., 2007). We have previously identified a mutant of CocE (T172R and G173Q) that extends the half-life of CocE to ~4.5 hours, as assessed by *in vitro* kinetic assays (at 37°C) or *in vivo* by protection against cocaine-induced lethality in mice (Gao et al., 2009). These mutations are thought to improve stability by burying additional surface area and/or through the formation of additional hydrogen bonds between domains I and II of CocE (Gao et al., 2009; Narasimhan and Sunahara, personal communication).

The increased stability of CocE-T172R/G173Q allowed the enzyme to block the reinforcing effects of cocaine during one-hour cocaine self-administration sessions following pretreatment with CocE-T172R/G173Q (Collins et al., 2009). This behavioral effect of CocE was specific to cocaine-reinforced behavior, and did not block responding for food or WIN-35065-2, a cocaine analog not hydrolyzed by CocE. These data support the hypothesis that CocE hydrolyzes cocaine rapidly enough to prevent its reinforcing effects. Although CocE-T172R/G173Q is capable of blocking the reinforcing effects of cocaine after an immediate pre-treatment and can protect against cocaine toxicity for up to 4.5 hours, its actions are still too short in duration to make the enzyme a feasible candidate to treat cocaine abuse (although this duration would be sufficient to cocaine toxicity indication).

Molecular Pharmacology Fast Forward. Published on January 19, 2010 as DOI: 10.1124/mol.109.060806
This article has not been copyedited and formatted. The final version may differ from this version.

Given that rapid cocaine hydrolysis is sufficient to block the reinforcing properties of cocaine (Collins et. al., 2009) and the reinstatement of cocaine-reinforced responding (Brimijoin et al., 2008), we set out to derive a mutant of CocE that would be more appropriate for cocaine abuse therapy. Here we describe CocE-L169K/G173Q, the most thermostable CocE variant characterized to date ($t_{1/2}$ at 37°C=2.9 days). CocE-L169K/G173Q was characterized in both *in vitro* stability assays and *in vivo* duration of action studies. We also provide structural evidence as to why this enzyme is the most thermostable variant to date. Initial pharmacokinetic data from this long-acting mutant compared to wt CocE is also presented.

Methods:

Site directed mutagenesis: Point mutations were introduced into the CocE sequence present in the bacterial expression vector pET-22b (+) using a modified QuickChange (Stratagene) mutagenesis protocol and confirmed by sequencing in both directions over the entire coding region. Wt and CocE mutants were expressed and purified as described previously (Gao et al., 2009; Narasimhan and Sunahara, personal communication).

Spectrophotometric cocaine assay: Cocaine hydrolysis was measured spectrophotometrically using a protocol adapted from (Turner et al., 2002). Varying concentrations of cocaine (0.5, 2.5, 5, 12.5, 25, 50, 100, and 150 µM) in PBS Buffer (50 mM Tris pH 8.0, 150 mM NaCl) were added to a UV-permeable 96 well plate (100 µl). CocE was added to the 96-well plate to give a final concentration of 10 ng/mL and a final volume of 200 µL. . Cocaine hydrolysis was followed by the change in absorbance at 240 nm at 10 second time points for 20 min using a SpectraMax Plus 384 UV plate reader (Molecular Devices, Sunnyvale, CA) using SOFTmax Pro software (Version 3.1.2). The V_{max} and K_m of the enzyme were determined using Prism (GraphPad, San Diego).

Molecular Pharmacology Fast Forward. Published on January 19, 2010 as DOI: 10.1124/mol.109.060806
This article has not been copyedited and formatted. The final version may differ from this version.

In vitro measurements of thermostability: Mutants were incubated at 37 ˚C in human plasma (obtained from the University of Michigan Hospital blood bank) at a concentration of 60 μg/mL. All samples were prepared immediately before incubation for the indicated times, and cocaine hydrolyzing activity was assayed as described above.

Crystallization and structure determination: CocE L169K/G173Q was purified as previously described (Gao et al., 2009). CocE crystals were grown by hanging drop vapor diffusion in VDX plates on siliconized glass cover-slips (Hampton Research). One μL of CocE at 5 mg/mL was combined with one μL well solution (1.7 M Ammonium sulfate, 10 mM Tris-HCl, pH 7.3, 25 mM NaCl) and incubated at 293 K over 1 mL of well solution. Crystals reached their maximum size within two days and were harvested within one week of tray setup. Five μL of cryo-protectant (1.5 M Ammonium sulfate, 5 mM Tris-HCl, pH 7.3, 10 mM HEPES pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA, 825 mM NaCl, 25% glycerol) were added to the drop during harvest to prevent ammonium sulfate crystal formation. CocE crystals were transferred to a 20 μL drop of cryo-protectant for one to five minutes then flash-frozen in liquid nitrogen. X-ray diffraction data were collected at the Advanced Photon Source, LS-CAT, beamline 21-ID-D using one second exposures at a wavelength of 1.02 Å. Data were integrated and scaled using HKL2000 (Otwinowski and Minor, 1997) and refined against the structure of wt CocE (Larsen et al., 2002). Modeling was performed by alternating rounds of refinement using refmac5 (Collaborative Computational Project, 1994) and manual density fitting using Coot (Emsley and Cowtan, Dec 2004). Coordinates were validated by MolProbity (Lovell et al., 2003) and deposited into the Protein Data Bank under accession code 3IDA.

Intravenous enzyme administration: Mice were placed in small restraint chambers (outer tube diameter: 30 mm, inner tube diameter: 24 mm, model no. BS4-34-0012; Harvard

Apparatus Inc., Holliston, MA) that left the tail exposed. The tail was then placed under a heat lamp for 7 seconds to increase blood flow and bring the tail vein to the surface. The tail was wiped with an alcohol pad and a 30.5 gauge precision glide needle was inserted into one of the lateral veins of the tail. After injection of 0.2 mL, the needle was removed and the bleeding was stanched using sterile gauze and pressure on the injection site.

Behavioral toxicity: Cocaine-induced toxicity was evaluated by observing the occurrence of convulsions and/or lethality in male NIH Swiss mice (25-30 g) (Harlan Inc. Indianapolis, IN). Mice were housed in groups of nine mice per cage (16x28x20 cm) and allowed *ad libitum* access to food and water. Animals were maintained on a 12 hour light-dark cycle with lights on at 7:00 AM. Experiments were performed according to guidelines established by the National Institutes of Health in the Guide of the Care and Use of Laboratory Animals. Experimental protocols were approved by the University Committee on the Use and Care of Animals (UCUCA) at the University of Michigan.

Mice were given intravenous tail vein injections of CocE (1 mg, 0.3 mg, 0.1 mg, 0.032 mg, and 0.01 mg) or saline 1 min prior to an intraperitoneal challenge dose of cocaine (100, 180, 320, 560, or 1000 mg/kg). The mice were observed for behavior in a Plexiglas container (16x28x20cm) for 45 minutes or until death. Assessment of the *in vivo* duration of action used the above protocol except that CocE (1 mg) was given at longer times pre-treatment to a challenge dose of 180 mg/kg cocaine. Convulsions were defined as loss of righting for more than 5 seconds. Lethality was defined as a loss of respiration and heart beat.

Serum collection: Blood was collected via (cheek pouch blood sampling) using a mouse bleeding lancet (GoldenRod 4.0 mm animal lancet; MEDIpoint Inc., Mineola, NY). Blood was collected in tubes (BD Microtainer) and placed on ice. Bleeding was stopped using a sterile gauze pad applied with direct pressure. The animal was then returned to its home cage and

Molecular Pharmacology Fast Forward. Published on January 19, 2010 as DOI: 10.1124/mol.109.060806
This article has not been copyedited and formatted. The final version may differ from this version.

allowed to recover. Samples were spun at 4000 rpm for 5 minutes to separate the serum fraction. Each mouse gave approximately 50 µL serum.

Western analysis: Plasma samples (20 µg total plasma protein) were resolved by SDS-PAGE on 10% polyacrylamide gels. Protein was transferred to a nitrocellulose membrane and probed with a rabbit anti-CocE polyclonal primary antibody (a kind gift from Dr. Donald Landry at Columbia University) and HRP-linked anti-rabbit secondary antibody (BioRad). The membranes were stripped and re-probed with rabbit anti-mouse apolipoprotein antibody (Affinity BioReagents PA1-23059) and the same secondary antibody for loading control. Western blots for CocE using these conditions can detect CocE to about 10 ng. Analysis of band densities from scanned films was performed with ImageJ Software (NIH). Area under curve analysis was done to determine densities of both CocE and ApoA1 bands. To normalize CocE densities, the fraction of the mean ApoA1 density was calculated for each ApoA1 band and divided from each CocE density.

Cocaine self-administration: Male Sprague Dawley rats (300-350 g) (Harlan Inc., Indianapolis, IN) were housed in groups of 3 animals per cage with *ad libitum* access to food and water on a 12 hour light-dark cycle (lights on at 7:00 AM). After catheter implantation, rats were singly housed. All experimental protocols were approved by UCUCA at the University of Michigan.

Rats were implanted with an indwelling femoral vein catheter under ketamine (90 mg/kg) and xylazine (10 mg/kg) anesthesia. The catheter is run under the skin and fixed to a metal plate that is sewn into the muscle of the back under the skin. This plate can then be attached to an IV catheter line present in the self administration box. After a 5 day recovery period post-surgery, rats were initiated into the self-administration procedure. Training and testing occurred at the same time each day at the middle of the light cycle. Rats were placed for one hour

Molecular Pharmacology Fast Forward. Published on January 19, 2010 as DOI: 10.1124/mol.109.060806
This article has not been copyedited and formatted. The final version may differ from this version.

sessions into a standard operant chamber equipped with a "nose-poke" apparatus, in which an entry into a hole was recorded with a photo-beam break and counted as one response. Rats were required to respond with a nose poke for an injection of 0.56 mg/kg cocaine (0.1 ml/kg injection volume) on a fixed ratio (FR) 1 schedule (one nose poke delivers one cocaine injection). Once responding stabilized in this phase of training, rats were gradually moved up to an FR 5 and then switched to a dose of 0.1 mg/kg/injection cocaine in order to maintain the most reliable behavior throughout the session. Once animals responded consistently (≤ 20% variation in responding and no upward or downward trend) for three days, they were given either 1 mg CocE-L169K/G173Q or saline at the indicated times prior to the session. After the test day, rats continued the training schedule until responding recovered to baseline. Animals were also tested by substituting saline for cocaine. Immediate CocE pretreatment and saline substitution conditions were compared to cocaine-reinforced responding behavior maintained by 0.1 mg/kg/injection by one-way ANOVA with Bonferroni post-tests. CocE pretreatments at 1 and 2 hours before the session were compared to baseline responding by students t-test.

Drugs: Cocaine hydrochloride was obtained from the National Institute on Drug Abuse (Bethesda, MA).

Results

Thermostability of mutants: Previous studies showed that CocE-L169K alone has a 33-fold longer half-life at 37 °C than wt CocE (measured by time to 50% loss of V_{max}) (Narasimhan and Sunahara, personal communication). However, CocE-L169K had an increased K_m that decreased the catalytic efficiency of the enzyme 12-fold. To compensate for this deficiency, L169K was paired with single mutations to test if the elevated K_m could be corrected, or, if the half-life could be extended in a synergistic manner. Mutants were assessed for initial activity using the spectrophotometric cocaine hydrolysis assay, and (if initial kinetics were better than or

Molecular Pharmacology Fast Forward. Published on January 19, 2010 as DOI: 10.1124/mol.109.060806
This article has not been copyedited and formatted. The final version may differ from this version.

comparable to wt CocE) were subjected to incubation at 37°C for 24 hrs to test for thermostability (Table 1). The L169K mutant retained 55% of its catalytic efficiency after a 24 hr incubation period at 37°C. The combination of L169K and T172R ($t_{1/2}$@ 37°C CocE-T172R= 46.8 min (Narasimhan and Sunahara, personal communication)) produced an enzyme with a lower V_{max} and a higher K_m than wt (5.6-fold loss in catalytic efficiency). Combination of L169K and N197K (predicted to create novel contacts with the dimer partner of CocE) had similar kinetics to wt CocE, but did not retain any measurable activity after 24 hours. The addition of other point mutations that we predicted to either increase the stabilization of domain 2 by creating additional buried surface area between domain 1 and domain 2 (N42V), or create a new hydrogen bond between the subunits of the CocE dimer (A193D), failed to increase thermostability, increase V_{max}, or further reduce the $K_{m.}$

However, combining L169K and G173Q conferred a prolonged half-life at 37°C, and retained nearly 75% activity after 24 hours. Although the K_m was 4-fold elevated above that of the wt K_m (30 μM compared to 7 μM) it was half that of CocE-L169K. Importantly, CocE-L169K/G173Q exhibited a higher V_{max} than any CocE variant characterized to date. The higher V_{max} of CocE-L169K/G173Q compensates for its slightly increased K_m, such that CocE-L169K/G173Q had a catalytic efficiency of 219.7 $min^{-1}\mu M^{-1}$, similar to that of wt (349.7 $min^{-1}\mu M^{-1}$), which represented a 7-fold improvement over CocE-L169K alone (29.6 $min^{-1}\mu M^{-1}$). Impressively, the half-life of CocE-L169K/G173Q at 37°C (Figure 1a) was 340-fold longer than wt, corresponding to 2.9 days. After a 10 day incubation at 37°C, CocE-L169K/G173Q still retained approximately 15% activity.

Temperature of inactivation experiments were conducted by incubating CocE-L169K/G173Q for 10 minutes at a variety of temperatures (Figure 1b). The V_{max} of CocE-L169K/G173Q decreased as the temperature of incubation increased above 42 °C, with the temperature of inactivation between 42°C and 48°C. This is a significant improvement over wt, which has a temperature of inactivation of 37° (Narasimhan and Sunahara, personal

communication), and closer to that of BchE, which inactivates between 54-57°C (Edwards and Brimijoin, 1983).

Crystal structure of CocE-L169K/G173Q: CocE consists of three domains; Domain I has an α/β hydrolase fold and contains the catalytic residues, Domain II consists primarily of a pair of large helices, and Domain III has a jelly roll-like topology. All three domains contribute to the active site. The CocE-L169K/G173Q structure was determined to a resolution of 1.6 Å (Table 2), and can be superimposed onto the four previously determined structures of wt CocE with an RMSD of < 0.58 Å for all Cα atoms (PDB ID: 1JU3, 1JU4, 1L7R, 1L7Q). The crystal packing of CocE-L169K/G173Q is the same as in previous structures, and is consistent with CocE existing as a homodimer.

The L169K and G173Q mutations are both located in helix 2 of domain II. The mutated side chains are easily distinguished in the electron density maps (Figure 2b) and each creates new interactions between domain I and II. The Gln173 creates a hydrogen bond to the backbone oxygen of Pro43 in domain I. The Lys169 reaches over a portion of the entrance to the active site to create Van der Waals interactions with Tyr44 of domain I. Unlike for a leucine side chain, the ζ-nitrogen of Lys169, is able to favorably interact with solvent, and in the structure stabilizes a glycerol molecule that was used as a cryoprotectant. Notably, the loop between helices 2 and 3 of domain II, which exhibit multiple conformations in other CocE structures determined, adopt a single conformation in the CocE-L169K/G173Q structure, suggesting greater stability in this region of the protein. As reported previously, DTT and CO_2 present during the protein purification was observed to form a tetrahedral 1,4-dithio-2,3-butylene carbonate (DBC) adduct with Ser117 in the active site (Narasimhan and Sunahara, personal communication).

Molecular Pharmacology Fast Forward. Published on January 19, 2010 as DOI: 10.1124/mol.109.060806
This article has not been copyedited and formatted. The final version may differ from this version.

Duration of action against cocaine-induced lethality. Cocaine administration at a dose of 180 mg/kg into the intraperitoneal cavity of NIH Swiss mice produced lethality in 100% of animals. Pretreatment with CocE-L169K/G173Q produced dose-dependent, rightward shifts in the cocaine dose response curve (Figure 3a). CocE-L169K/G173Q exhibited a subtle degree of protection even with a very low dose of 0.01 mg, and increased the LD_{100} of cocaine to 1000 mg/kg at a dose of 0.1 mg CocE-L169K/G173Q, one quarter log unit lower than what is needed for the same effect with wt CocE (0.32 mg). The time-to-death after cocaine injection was also increased by CocE-L169K/G173Q. With saline pretreatment, animals died within 2-3 minutes after administration of 180 mg/kg cocaine. However, with a pretreatment of 0.032 mg CocE-L169K/G173Q, it took a dose of 560 mg/kg cocaine to produce near 100% lethality and produced a slight increase in the time to death of some animals (animals died within 2-7 minutes after 560 mg/kg cocaine, data not shown).

CocE-L169K/G173Q pretreatments of longer duration were able to protect animals from cocaine induced lethality (Figure 3b). We have previously demonstrated that wt CocE is rendered ineffective at preventing lethality from a challenge dose of cocaine after a 30 minute pretreatment (Ko et al., 2007). CocE-T172R/G173Q (1 mg) protected 50% of animals from a LD_{100} dose (180 mg/kg) of cocaine up to 4 hours after CocE administration (Gao et al., 2009). CocE-L169K/G173Q (1 mg), given 7-8 hours before administration of 180 mg/kg cocaine, protected 50% of the animals, representing a nearly 16-fold improvement over the wt enzyme.

***In vivo* protection against the reinforcing effects of cocaine.** Sprague Dawley rats were trained to respond for cocaine (0.1 mg/kg/injection) on a fixed ratio (FR) 5 schedule. After saline pretreatments, animals responded for 75-100 injections of 0.1 mg/kg cocaine per one-hour session. When saline was substituted as the reinforcer, total responses were reduced and clustered at the beginning of the sessions (data not shown). Immediate pretreatment with CocE-L169K/G173Q produced a reduction in the number of reinforcers earned to a level not

Molecular Pharmacology Fast Forward. Published on January 19, 2010 as DOI: 10.1124/mol.109.060806
This article has not been copyedited and formatted. The final version may differ from this version.

significantly different than saline-reinforced responding (Figure 4). Both saline substitution and immediate pretreatment with CocE-L169K/G173Q only produced about 25% of the responding of baseline sessions, similar to the behavior seen in the initial session of responding extinction. One hour pretreatment times also resulted in a reduction in responding, the degree to which was smaller than that with the immediate pretreatment and not statistically significant (three of five animals reduced responding to under 50% of control levels). Two hour pretreatments with CocE-L169K/G173Q resulted in a cumulative average of responses that was not significantly different than saline pretreatment.

***In vivo* serum half-life:** The presence of wt and CocE-L169K/G173Q remaining in the serum of NIH Swiss mice was analyzed using western blot analysis on serum samples taken at increasing times after CocE administration (Figure 5a, b). Wt and CocE-L169K/G173Q appear to be eliminated from serum following a similar time course. The elimination time course yields a serum half-life of 2.3 hours for CocE-L169K/G173Q and 2.2 hours for wt CocE (one-phase decay model, r^2=0.8 for both enzymes tested). Although the *in vitro* half lives of these enzymes at 37°C is 340-fold different, the *in vivo* serum half-life is virtually identical and follows the same pattern of elimination.

Discussion

This study demonstrates the superior stability of CocE-L169K/G173Q *in vitro,* its extended duration of action *in vivo*, and provides the first description of CocE pharmacokinetics in rodents. Wt CocE prevents the acute toxic effects of cocaine (Cooper et al., 2006; Jutkiewicz et al., 2009; Ko et al., 2007; Wood et al., In Press), but its instability at 37°C, and its short duration of action *in vivo* limit its therapeutic potential. However, CocE-L169K/G173Q appears to be the first attractive CocE candidate for treatment of cocaine abuse (should its half-time in

Molecular Pharmacology Fast Forward. Published on January 19, 2010 as DOI: 10.1124/mol.109.060806
This article has not been copyedited and formatted. The final version may differ from this version.

serum be extended), due to its long duration of action and ability to block the reinforcing properties of cocaine.

CocE-L169K/G173Q displays a significantly higher V_{max} than the wt or T172R/G173Q mutant (Gao et al 2009). The increase in catalytic turnover is accompanied by a slight increase in the K_m of the enzyme. Crystallographic evidence suggests that the elevation in K_m is most likely due to the extended side chain of Lys169, which may partially occlude access to the active site pocket. This increase in K_m, however, does not affect the enzyme's function at the very high cocaine concentrations which cause toxicity *in vivo*. Our data also suggests that this defect does not seem to hinder CocE-L169K/G173Q's efficacy at rapidly clearing low concentrations of cocaine as seen in rats in self-administration models. The increased K_m does not hinder CocE-L169K/G173Qs ability to hydrolyze cocaine at a faster rate than CocE-T172R/G173Q even at the very low cocaine concentrations seen in these studies (0.1 mg/kg/injection or approximately 0.49 μM when dispersed in total body water of the rat), due to CocE-L169K/G173Q's much higher V_{max} (predicted by Michaelis-Menten rate equation).

The L169K/G173Q mutations dramatically increased the thermostability of the enzyme from 37°C for wt to 48-55°C for CocE-L169K/G173Q. Butyrylcholinesterase has been reported to have a temperature of inactivation of 54-57°C (Brimijoin et al., 2008). Furthermore, these two mutations slow aggregation of CocE, as seen by size exclusion chromatography (data not shown). After incubation at 37°C, wt CocE is 100% aggregated within one hour (Narasimhan and Sunahara, personal communication) while CocE-L169K/G173Q is only 30-40% aggregated after 24 hours.

Analysis of the crystal structure of CocE-L169K/G173Q reveals a complex molecular mechanism for its improved thermal stability. Lys169 and Gln173 provide additional anchor points between domain I and the conformationally flexible region of domain II. The newly introduced hydrogen bond between Gln173 (domain II) and Pro43 (domain I), the novel Van der Waals interaction between Lys169 (domain II) and Tyr44 (domain I), and more favorable

Molecular Pharmacology Fast Forward. Published on January 19, 2010 as DOI: 10.1124/mol.109.060806
This article has not been copyedited and formatted. The final version may differ from this version.

interactions with solvent by the Lys169 side chain also likely contribute to CocE-L169K/G173Q's thermostability.

In vivo studies in mice confirm that CocE-L169K/G173Q displays an increased potency and duration of action over wt. The higher potency of CocE-L169K/G173Q is most likely due to the combination of its higher V_{max} and increased stability at 37°C, allowing it to quickly degrade high concentrations of cocaine and remain active over a longer period of time. It is reasonable to expect that at concentrations of cocaine resembling a human overdose situation, cocaine would be hydrolyzed more rapidly by CocE-L169K/G173Q than by wt CocE, although less of a difference in turnover rate would be seen at lower cocaine concentration, due to the elevated K_m of CocE-L169K/G173Q.

The rat self-administration study presented here that examines the effects CocE-L169K/G173Q expands on the work previously conducted by Collins et al (2009). They demonstrated that CocE-T172R/G173Q did not suppress food- or WIN-35065-2 (a non-hydrolyzable cocaine analog)-reinforced operant responding at doses that suppressed cocaine-reinforced responding. These results demonstrate that CocE specifically eliminates the reinforcing properties of cocaine, and does not simply suppress operant responding. The Collins study also highlighted a dose-response relationship between CocE-T172R/G173Q and cocaine-reinforced responding. No change in responding was observed with the lowest dose of 0.032 mg CocE-T172R/G173Q given to rats responding for 0.1 mg/kg/injection cocaine. A dose of 0.1 mg CocE-T172R/G173Q increased the rate of responding for 0.1 mg/kg/injection (as would be expected to surmount a low dose of an "antagonist"). At the higher doses of CocE-T172R/G173Q, 0.32 and 1.0 mg, responding for cocaine decreased by one-half, and to saline levels, respectively. The time course data presented here on CocE-L169K/G173Q (Figure 5) demonstrates that after a 2-hour pretreatment, there is still enough circulating CocE to reduce operant responding slightly. It is likely that with longer pre-treatment times, operant responding for cocaine would increase due to very low levels of CocE remaining in the serum.

Molecular Pharmacology Fast Forward. Published on January 19, 2010 as DOI: 10.1124/mol.109.060806
This article has not been copyedited and formatted. The final version may differ from this version.

The duration of action of CocE-L169K/G173Q *in vitro* greatly surpasses wt and the previously reported thermostable mutant CocE-T172R/G173Q. However, its *in vivo* duration of action (7-8 hrs) is significantly shorter than the *in vitro* half-life at 37°C (2.9 days). In measures of protection against cocaine-induced lethality, wt, CocE-L169K and CocE-T172R/G173Q all lose their protective effects over a time course similar to their respective *in vitro* half-lives at 37°C (Gao et al., 2009; Narasimhan and Sunahara, personal communication). This discrepancy between the CocE-L169K/G173Q $t_{1/2}$ *in vitro* and *in vivo* is also observed in the protection against the reinforcing properties of cocaine measured in the rat self-administration model. Some reduction in responding can be seen after a 1-hour pretreatment of CocE-L169K/G173Q; however this protection is almost eliminated after 2 hours. CocE dose-response analyses by Collins *et al*. (2009) illustrate that plasma levels of CocE-T172R/G173Q need to be sufficiently high or else the effect of CocE is surmountable. This leads us to hypothesize that the discrepancy between the *in vitro* stability and *in vivo* protection against lethality and self-administration data of CocE-L169K/G173Q, may be due to a reduction in plasma levels of the enzyme.

Indeed, western blot analysis on serum from mice injected with CocE-L169K/G173Q shows that the enzyme is eliminated from the serum with a $t_{1/2}$ of 2.3 hours. Processes unrelated to thermostability most likely explain the relatively short pharmacokinetic properties of the enzyme. One possibility is protease degradation, which may remove or destroy the regions of CocE recognized by the polyclonal anti-body and yield results that mimic the disappearance of CocE. Glomerular filtration is unlikely, as CocE is about 134 kDa as a dimer (Narasimhan and Sunahara, personal communication). However, it is possible that the dimer interface may be disrupted *in vivo* and the monomer or smaller proteolysed products are filtered from the blood before forming aggregates (CocE aggregates can be observed in mouse serum with size exclusion chromatography, data not shown).

Molecular Pharmacology Fast Forward. Published on January 19, 2010 as DOI: 10.1124/mol.109.060806
This article has not been copyedited and formatted. The final version may differ from this version.

It is of great interest that both the thermally stable CocE-L169K/G173Q and the wt CocE disappear from the plasma with the same kinetics. Active CocE is a dimer, whereas a monomeric form has never been isolated *in vitro*. As CocE is heat-inactivated, the protein forms aggregates both *in vitro* and *in vivo* (determined by size exclusion chromatography, Narasimhan and Sunahara, personal communication). Wt CocE aggregates *in vitro* within one hour at 37°C (Narasimhan and Sunahara, personal communication), consistent with its thermal inactivation, however CocE-L169K/G173Q maintains as a dimer form for at least 120 hours (data not shown). The observation that both dimers and aggregates are eliminated with the same half-time illustrates that the state in which the esterase exists does not affect the CocE elimination process *in vivo,* despite corresponding to the activity of the enzyme.

The long *in vitro* half-life of CocE-L169K/G173Q at 37°C greatly surpasses any combination of mutations to date. The three-day half-life *in vitro* is a promising step towards development of a therapy against cocaine addiction. After ten days at 37°C, this enzyme still retains 15% of its original activity and displays intact Michaelis-Menten kinetics. The concentrations of cocaine typically seen in cocaine abusers (0-1 mg/L or 0.6-3 μM, (Couper and Logan, 2004)) should be cleared in approximately the same time by both the wt and L169K/G173Q enzyme. The extended half-life and improved Michaelis-Menten parameters of CocE-L169K/G173Q suggest that this CocE enzyme represents a strong candidate for a cocaine abuse therapy indication.

Creation of the thermally stable CocE-L169K/G173Q was a necessary advancement toward treatment of cocaine addiction by CocE. This enzyme demonstrates that the instability of wt CocE at 37ºC can be surmounted. CocE-L169K/G173Q has also demonstrated that the thermostability of the CocE enzyme does not affect the *in vivo* rate of elimination from the serum. The short plasma half-life of CocE-L169K/G173Q presents an obstacle that must be overcome if CocE is to be used as a treatment for cocaine abuse. Strategies that have shown

Molecular Pharmacology Fast Forward. Published on January 19, 2010 as DOI: 10.1124/mol.109.060806
This article has not been copyedited and formatted. The final version may differ from this version.

success at increasing circulating protein half-life *in vivo*, including PEGylation, encapsulation in red blood cells and sugar modification, are currently under investigation.

Acknowledgements

We thank Davina Barron and Adam Kynaston, for their excellent technical assistance. Thanks also to Dr. R. D. Davenport for providing us with the human plasma samples.

References

Benowitz NL (1993) Clinical pharmacology and toxicology of cocaine. *Pharmacol Toxicol.* **72**(1):3-12.

Billman GE (1990) Mechanisms responsible for the cardiotoxic effects of cocaine. *FASEB J.* **4**(8):2469-2475.

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**(3):904-908.

Brimijoin S, Gao Y, Anker JJ, Gliddon LA, Lafleur D, Shah R, Zhao Q, Singh M and Carroll ME (2008) A cocaine hydrolase engineered from human butyrylcholinesterase selectively blocks cocaine toxicity and reinstatement of drug seeking in rats. *Neuropsychopharmacology*. **33**(11):2715-25.

Browne SP, Slaughter EA, Couch RA, Rudnic EM and McLean AM (1998) The influence of plasma butyrylcholinesterase concentration on the in vitro hydrolysis of cocaine in human plasma. *Biopharm Drug Dispos.* **19**(5):309-314.

Carroll FI, Howard JL, Howell LL, Fox BS and Kuhar MJ (2006) Development of the dopamine transporter selective RTI-336 as a pharmacotherapy for cocaine abuse. *AAPS J.* **8**(1):E196-203.

Collaborative Computational Project N (1994) The CCP4 Suite: Programs for Protein Crystallography. *Acta Cryst.* **D50**:760-763.

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**(2):445-55.

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**(6):1885-1891.

Couper FJ and Logan BK (2004) Drug and Human Performance Fact Sheets, National Highway Traffic Safety Administration, Washington D.C.

Crumb WJ, Jr. and Clarkson CW (1990) Characterization of cocaine-induced block of cardiac sodium channels. *Biophys J.* **57**(3):589-599.

Daniels A, Ayala E, Chen W, Coop A and Matsumoto RR (2006) N-[2-(m-methoxyphenyl)ethyl]-N-ethyl-2-(1-pyrrolidinyl)ethylamine (UMB 116) is a novel antagonist for cocaine-induced effects. *Eur J Pharmacol.* **542**(1-3):61-68.

Duysen EG, Bartels CF and Lockridge O (2002) Wild-type and A328W mutant human butyrylcholinesterase tetramers expressed in Chinese hamster ovary cells have a 16-hour half-life in the circulation and protect mice from cocaine toxicity. *J Pharmacol Exp Ther.* **302**(2):751-758.

Edwards JA and Brimijoin S (1983) Thermal inactivation of the molecular forms of acetylcholinesterase and butyrylcholinesterase. *Biochimica et Biophysica Acta.* **742**:509-516.

Emsley P and Cowtan K (2004) Coot: model-building tools for molecular graphics. ACTA *Crystallographica Section D- Biological Crystallography.* **60**(Sp. Iss. 1):2126-2132.

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**(2):318-323.

Gao Y and Brimijoin S (2004) An engineered cocaine hydrolase blunts and reverses cardiovascular responses to cocaine in rats. *J Pharmacol Exp Ther.* **310**(3):1046-1052.

Gao Y, LaFleur D, Shah R, Zhao Q, Singh M and Brimijoin S (2008) An albumin-butyrylcholinesterase for cocaine toxicity and addiction: catalytic and pharmacokinetic properties. *Chem Biol Interact.* **175**(1-3):83-87.

Gorelick DA (1997) Enhancing cocaine metabolism with butyrylcholinesterase as a treatment strategy. *Drug Alcohol Depend.* **48**(3):159-165.

Hoffman RS, Morasco R and Goldfrank LR (1996) Administration of purified human plasma cholinesterase protects against cocaine toxicity in mice. *J Toxicol Clin Toxicol.* **34**(3):259-266.

Johanson CE and Fischman MW (1989) The pharmacology of cocaine related to its abuse. *Pharmacol Rev.* **41**(1):3-52.

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**(3):409-20.

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**(2):926-933.

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**(1):17-21.

Lovell SC, Davis IW, Arendall III WB, de Bakker PIW, Word JM, Prisant MG, Richardson JS and Richardson DC (2003) Structure validation by C-alpha geometry: phi, psi, and C-beta deviation. *Protein Struct Funct Genet.* **50**:437-450.

Lynch TJ, Mattes CE, Singh A, Bradley RM, Brady RO and Dretchen KL (1997) Cocaine detoxification by human plasma butyrylcholinesterase. *Toxicol Appl Pharmacol.* **145**(2):363-371.

Mattes CE, Lynch TJ, Singh A, Bradley RM, Kellaris PA, Brady RO and Dretchen KL (1997) Therapeutic use of butyrylcholinesterase for cocaine intoxication. *Toxicol Appl Pharmacol.* **145**(2):372-380.

Negus SS, Mello NK, Kimmel HL, Howell LL and Carroll FI (2009) Effects of the monoamine uptake inhibitors RTI-112 and RTI-113 on cocaine- and food-maintained responding in rhesus monkeys. *Pharmacol Biochem Behav.* **91**(3):333-338.

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

Otwinowski Z and Minor W (1997) Processing of X-ray Diffraction Data Collected in Oscillation Mode in *Methods in Enzymology, Volume 276: Macromolecular Crystallography* (Carter Jr. CW and Sweet RW eds), Academic Press, New York.

Rothman RB, Blough BE and Baumann MH (2007) Dual dopamine/serotonin releasers as potential medications for stimulant and alcohol addictions. *AAPS J.* **9**(1):E1-10.

Substance Abuse and Mental Health Services Administration (2007) Results from the 2006 National Survey on Drug Use and Health: National Findings, Rockville, MD.

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**(41):12297-12307.

Uhl GR, Hall FS and Sora I (2002) Cocaine, reward, movement and monoamine transporters. *Mol Psychiatry.* **7**(1):21-26.

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

Xie W, Altamirano CV, Bartels CF, Speirs RJ, Cashman JR and Lockridge O (1999) An improved cocaine hydrolase: the A328Y mutant of human butyrylcholinesterase is 4-fold more efficient. *Mol Pharmacol.* **55**(1):83-91.

Molecular Pharmacology Fast Forward. Published on January 19, 2010 as DOI: 10.1124/mol.109.060806
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

a) This work was supported by the National Institutes of Health [Grants DA021416, GM007767, DA025100, DA013930]. JJGT is supported by National Institutes of Health [Grants DA025100, HL086865, and HL071818]. The project described was also supported by grant number GM007767 from NIGMS and its contents are solely the responsibility of the authors and do not necessarily represent the official views of NIGMS.

b) Brim, R.L., Nance, M., Tesmer, J., Sunahara, R. K., Woods, J. H. “Mutant Bacterial Cocaine Esterase with a Three-day Half-life Possess Characteristics of a Cocaine Abuse Therapy”, American Society of Pharmacology and Experimental Therapeutics Annual Meeting. New Orleans LO, 2009

c) Please send reprint requests to:
James H. Woods
Department of Pharmacology
University of Michigan Medical School
1301 Medical Sciences Research Building III
1150 W Medical Center Drive
Ann Arbor, MI 48109-0600
jhwoods@umich.edu

d) Department of Pharmacology
University of Michigan Medical School
1301 Medical Sciences Research Building III
1150 W Medical Center Drive
Ann Arbor, MI 48109-0600
(RLB, DN, JJGT, RKS, JHW)

Life Sciences Institute
University of Michigan
210 Washtenaw Ave
Ann Arbor, MI 48109-2216
University of Michigan
Ann Arbor, MI
(MRN, JJGT)

Department of Biophysics
University of Michigan
4028 Chemistry Building
930 North University Avenue
Ann Arbor, MI 48109-1055
(DWY)

Department of Pharmaceutical Sciences
College of Pharmacy
University of Kentucky
725 Rose Street
Lexington, KT 40536
(CGZ)

Molecular Pharmacology Fast Forward. Published on January 19, 2010 as DOI: 10.1124/mol.109.060806
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 1. *In vitro* stability of CocE-L169K/G173Q **A** Ten day time course of CocE activity at 37 ˚C. CocE was incubated at 60 µg/mL for the time indicated. Activity was assessed using the spectrophotometric cocaine hydrolysis assay. V_{max} and k_{cat} were determined and the catalytic efficiency was plotted as a percentage of the catalytic efficiency of non-incubated enzyme. A single phase exponential decay model shows that 50% activity is retained until approximately 2.9 days. **B** Heat inactivation of CocE-L169K/G173Q. CocE was incubated at 60 µg/mL at the temperatures indicated for 10 min. Incubations were stopped on ice, and V_{max} was determined by the spectrophotometric assay. V_{max} activity was plotted as a percentage of the un-incubated CocE V_{max} kept at 4˚C. CocE-L169K/G173Q retains over 50% activity up to 42˚C. At higher temperatures, the activity rapidly drops, but residual activity is observed up to 65˚C.

Figure 2. Crystal Structure of CocE-L169K/G173Q. A Domain structure of CocE. Domain I (grey) contains the catalytic residues, domain II (green) is helical with two pronounced anti-parallel helices (H2 and H3). Domain III is shown in purple. The active site is formed at their intersection. Domains I and III are shown as their solvent-excluded surfaces. The L169K and G173Q mutations (ball and stick side chains) are found in H2 of domain II, close to the entrance to the active site, partially occupied here by a molecule of glycerol. **B** Refined model of CocE-L169K/G173Q. 2|Fo|-|Fc| electron density is shown as a blue wire cage contoured at the 1 σ level, with |Fo|-|Fc| density show in green contoured at 3 σ and red at -3 σ. Lys169 is observed in two distinct conformations that interact with a bound molecule of glycerol from the harvesting solution. Carbon atoms are shown as their respective domain colors (see panel A), oxygens are red, and nitrogens blue.

Figure 3. *In vivo* potency and duration of action of CocE-L169K/G173Q **A** Potency of CocE-L169K/G173Q against cocaine induced lethality. CocE-L169K/G173Q was administered to NIH Swiss mice intravenously one minute prior to a cocaine challenge given intraperitineally. Increasing doses of CocE-L169K/G173Q cause significant rightward shifts in the cocaine dose-response curve. **B** CocE-L169K/G173Q pretreatment protects against cocaine lethality more than wt CocE. CocE is administered intravenously at time of pretreatment. A lethal dose of 180 mg/kg is used as a challenge dose at time 0. The percentage of mice experiencing lethality after a one hour post-cocaine period is plotted.

Figure 4. CocE-L169K/G173Q protection against cocaine-reinforced operant responding in Sprague Dawley rats CocE-L169K/G173Q (1 mg) was given as a pretreatment to cocaine self-administration sessions at the times indicated. Rats in the saline substitution condition received no cocaine from nose-pokes during the session.

*** One-way ANOVA $F(2, 12)=27.4$, Bonferroni post test $p<0.001$
* Students t-test $p<0.05$

Figure 5. *In vivo* CocE plasma half-life **A** Representative western blots of CocE from mouse serum over time. CocE (L169K/G173Q or wt) was administered to mice intravenously via the lateral tail vein. Blood samples were taken at the times indicated by submandibular sampling. Serum was collected and 20 µg total serum protein was run on a 10% SDS PAGE gel. Blotting was performed with rabbit anti-CocE antibody and rabbit anti-Apolipoprotein A1 antibody. Wt and CocE-L169K/G173Q were both tested in 3 independent groups of animals followed by serum analysis. **B** Quantification of wt and CocE-L169K/G173Q Western blot densities analyzed with Image J software (NIH). All time points are adjusted as a fraction of the apoA1 loading control. Fit to a one phase exponential decay model, the half-life of CocE-L169K/G173Q was determined to be 2.3 hours post administration (wt=2.2 hours). 2-way ANOVA $p=0.92$

Molecular Pharmacology Fast Forward. Published on January 19, 2010 as DOI: 10.1124/mol.109.060806
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1. **Cocaine Hydrolysis by CocE Variants** V_{max} and K_m, were measured spectrophotometrically immediately after thaw from -80°C and after 24 hr incubation at 37°C. Catalytic efficiency was calculated from the V_{max} and K_m values and is expressed as min^{-1} μM^{-1}. CocE variants that lost all activity, or had negligible activity, are reported here as no Michaelis-Menten Kinetics remaining.

CocE Variant	V_{max} (min^{-1})	K_m (μM)	Catalytic Efficiency (min^{-1} μM^{-1})	V_{max} 24hrs, 37°C (min^{-1})	K_m 24hrs, 37°C (μM)	Catalytic Efficiency 24hrs, 37°C	Catalytic Efficiency remaining at 24hrs
Wild-type	2510	7.2	348.6	270	7.3	37.0	10.8%
L169K[a]	3100[a]	105.0[a]	29.5[a]	950[b]	59.8[b]	15.9	55%
L169K/T172R	1510	24.2	62.4			N/D[c]	
L169K/N197K	3120	58.3	53.5			N/M[d]	
L169K/G173Q	6670	30.3	220.1	3890	22.9	170.0	77.5%
L169K/G173Q/N42V	3180	56.4	56.4			N/M[d]	
L169K/G173Q/A193D	2350	21.4	110.0	340	30.6	11.1	10.0%

[a] Indicates data cited from (Narasimhan et al., submitted)
[b] Personal Communication (D. Narasimhan)
[c] Not Determined
[d] No Michaels-Menten Kinetics remaining at 24 hours

Molecular Pharmacology Fast Forward. Published on January 19, 2010 as DOI: 10.1124/mol.109.060806
This article has not been copyedited and formatted. The final version may differ from this version.

Table 2. Crystallographic data and refinement statistics

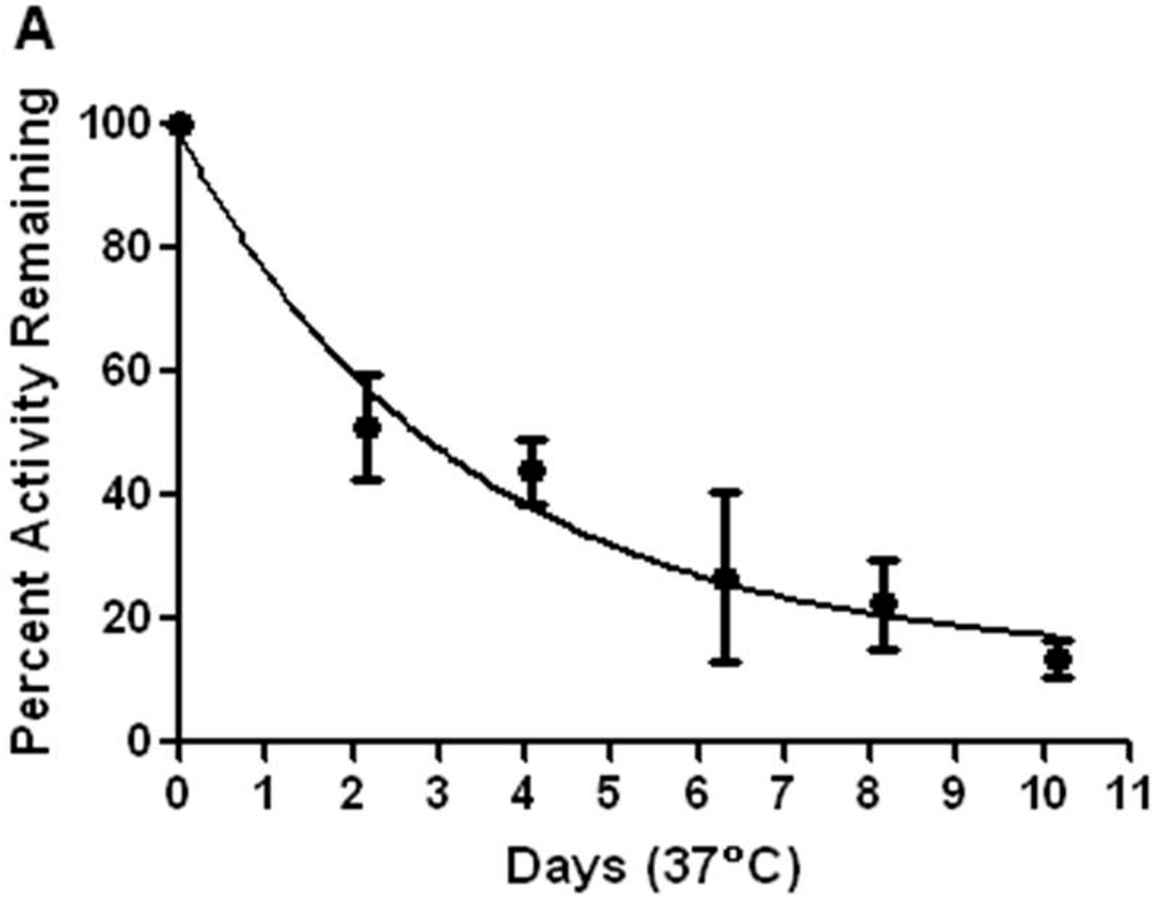
X-ray Source:	APS LS-CAT 21-ID-D
Wavelength (Å)	1.02
Resolution (Å)	25.0 - 1.60 Å
Space group	$P6_522$
Cell constants (Å, °)	*a=b=108.3, c=227.2,* $\alpha=\beta=90.0^{\circ}$, $\gamma=120.0^{\circ}$
Unique reflections	104,076
Average redundancy	9.5 (8.3)
R_{sym} (%)[a]	9.4 (61.3)
Completeness (%)	99.1 (98.3)
$/<\sigma_I>$	*22.4 (3.1)*
Refinement resolution (Å)	*24.6-1.60 Å*
Total reflections used	*97,839*
Protein atoms	*4,799*
Non-protein atoms	*761*
RMSD bond lengths (Å)	*0.012*
RMSD bond angles (°)	*1.19*
Estimated coordinate error (Å)	*0.08*
Ramachandran plot statistics:	
Most favored, disallowed (%)	*96.7, 0.2*
R_{work}[b]	*17.7 (22.5)*

R_{free}[c]	19.4 (25.5)

[a] $R_{sym} = \Sigma_{hkl}\Sigma_i |I(hkl)_i - I(hkl)|/\Sigma_{hkl} I(hkl)_i$, where I(hkl) is the mean intensity of i reflections after rejections.
[b] $R_{work} = S_{hkl}||F_{obs}(hkl)| - |F_{calc}(hkl)||/S_{hkl} |F_{obs}(hkl)|$; no I/s cutoff was used during refinement.
[c] 5% of the truncated data set was excluded from refinement

Figure 1

Molecular Pharmacology Fast Forward. Published on January 19, 2010 as DOI: 10.1124/mol.109.060806
This article has not been copyedited and formatted. The final version may differ from this version.



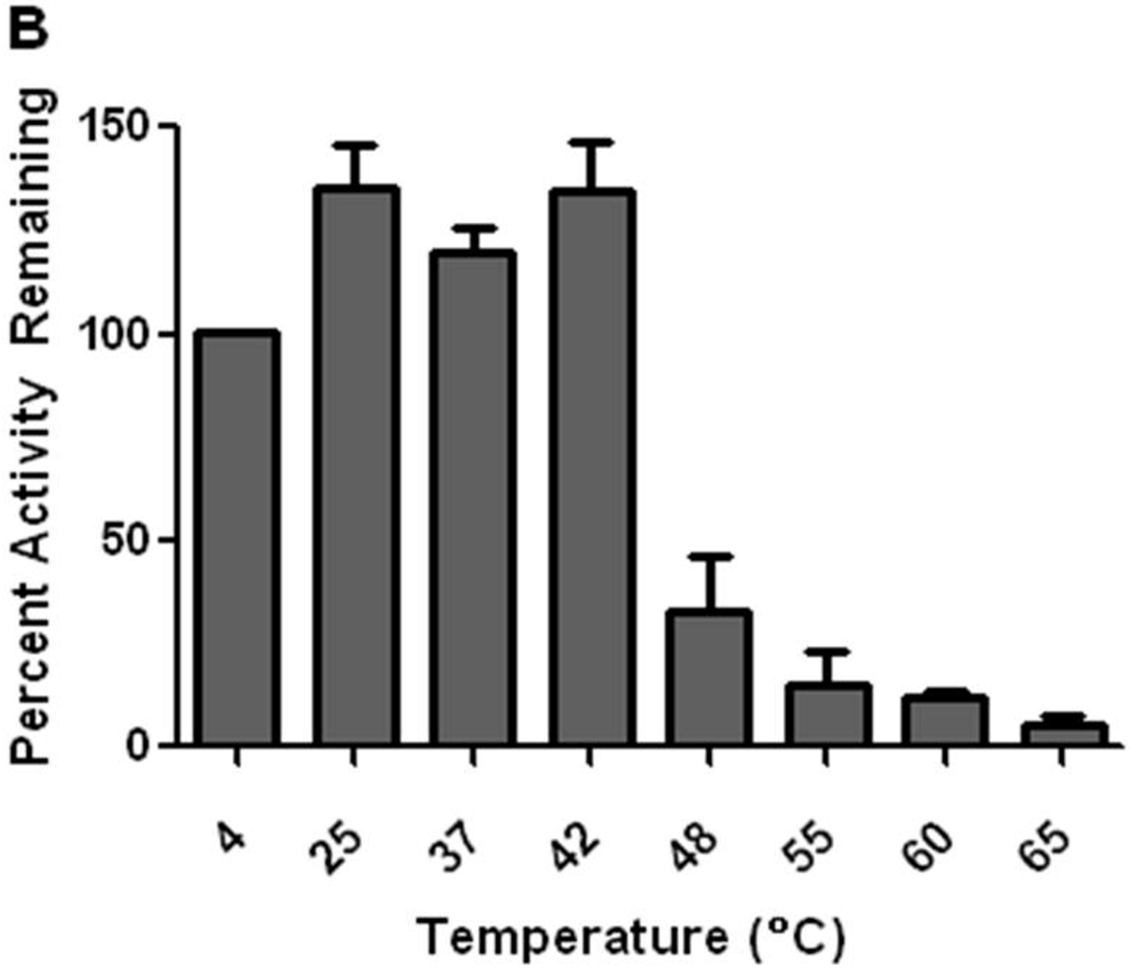
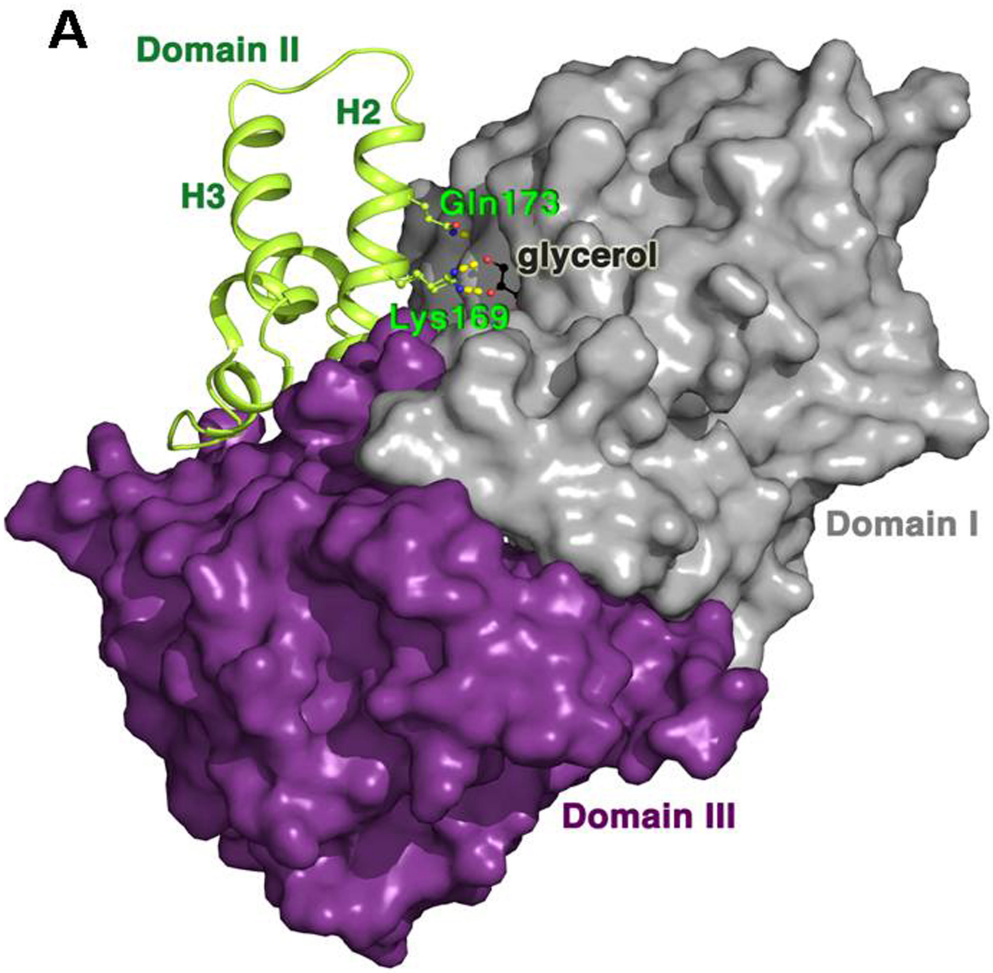


Figure 2

Molecular Pharmacology Fast Forward. Published on January 19, 2010 as DOI: 10.1124/mol.109.060806
This article has not been copyedited and formatted. The final version may differ from this version.



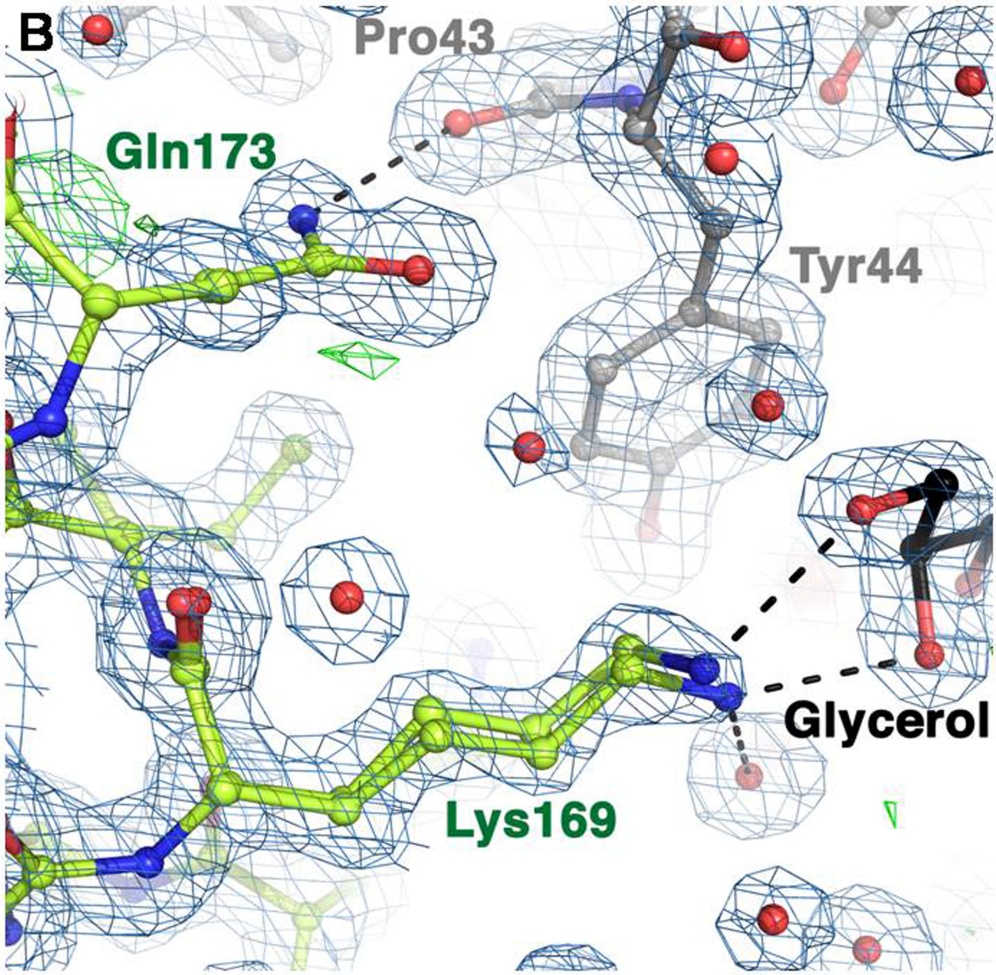
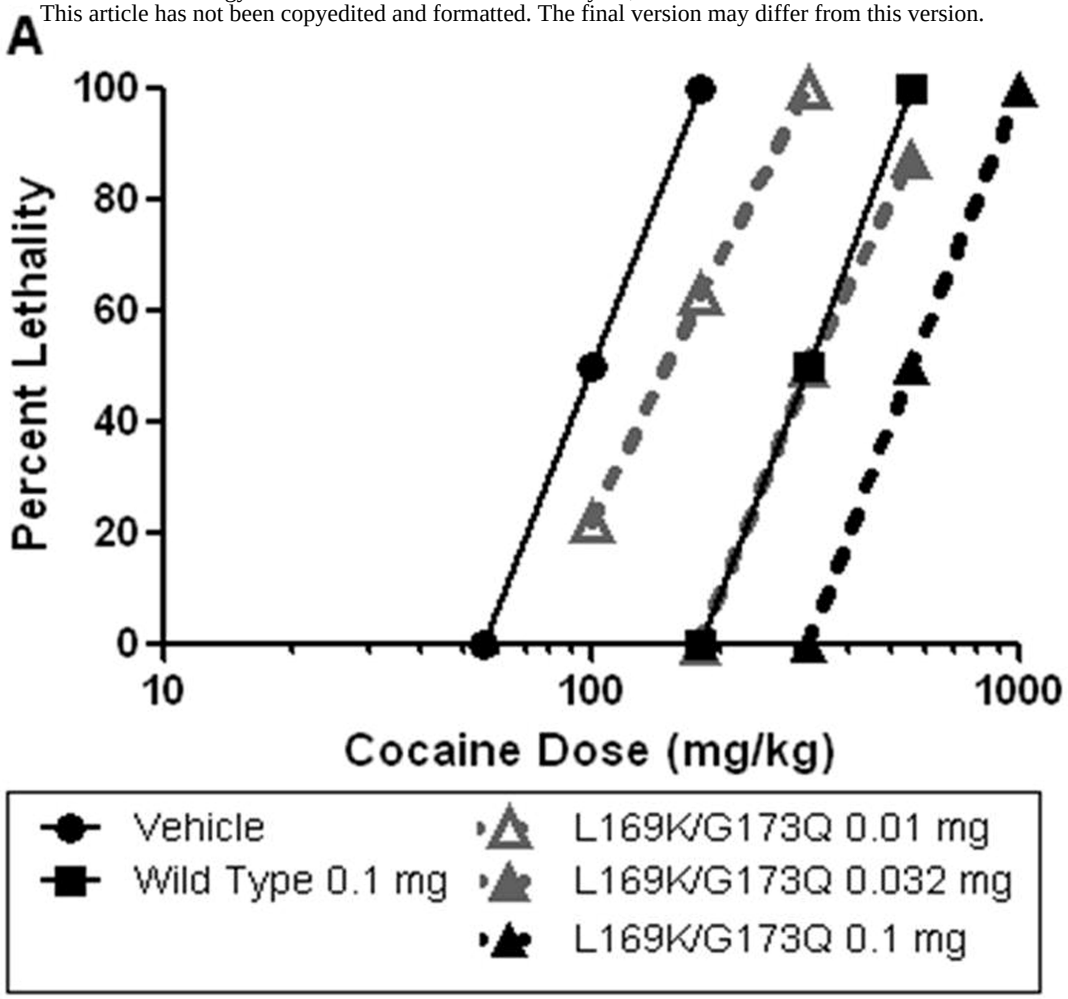


Figure 3

Molecular Pharmacology Fast Forward. Published on January 19, 2010 as DOI: 10.1124/mol.109.060806



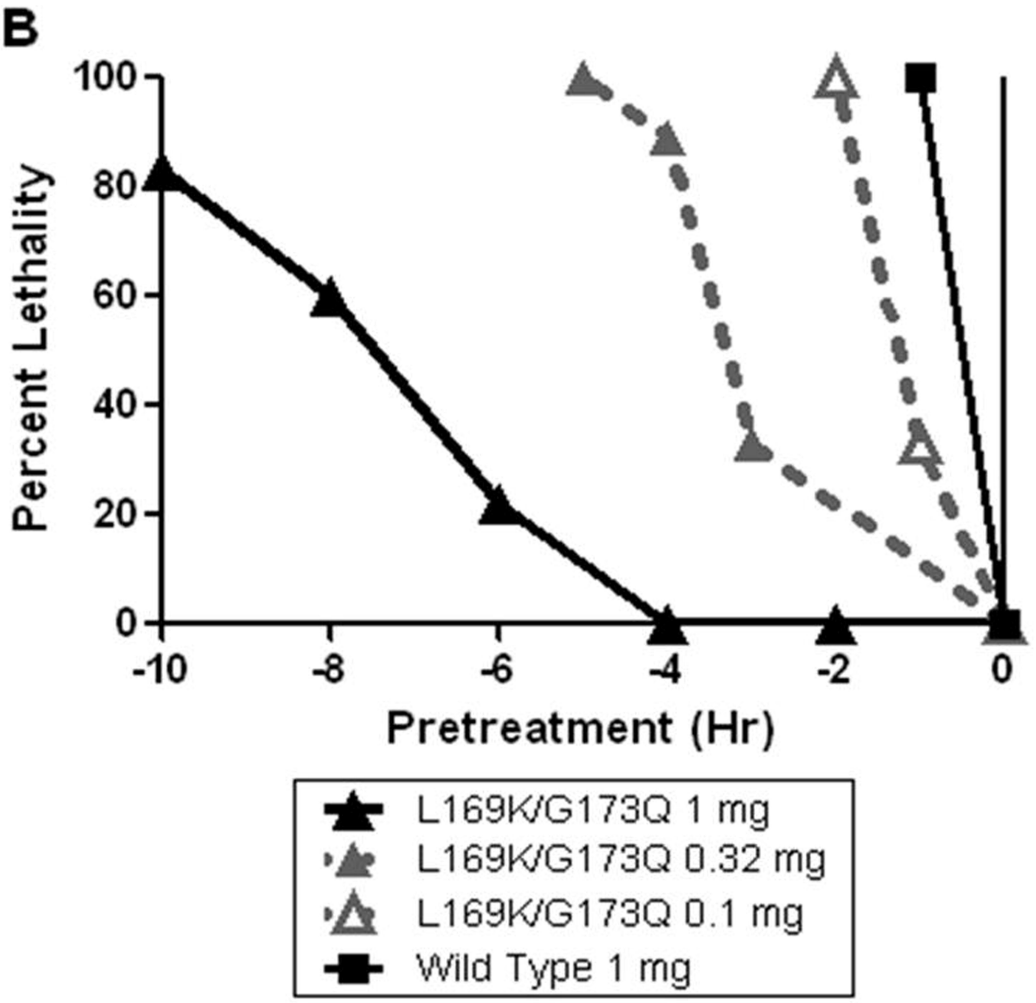


Figure 4

Molecular Pharmacology Fast Forward. Published on January 19, 2010 as DOI: 10.1124/mol.109.060806
This article has not been copyedited and formatted. The final version may differ from this version.

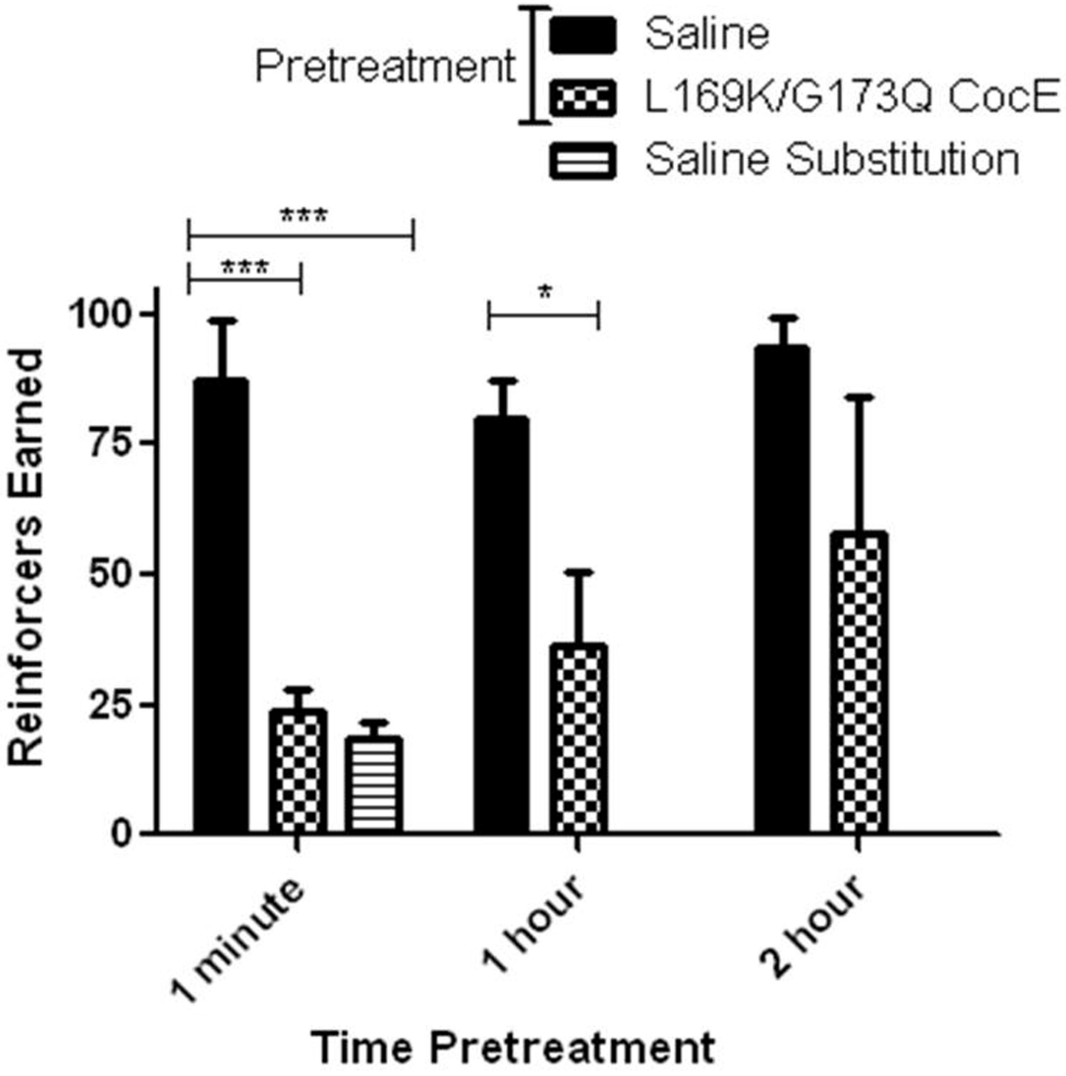


Figure 5

Molecular Pharmacology Fast Forward. Published on January 19, 2010 as DOI: 10.1124/mol.109.060806
This article has not been copyedited and formatted. The final version may differ from this version.