MC-100093, a novel beta-lactam GLT-1 enhancer devoid of antimicrobial properties attenuates cocaine relapse in rats

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List of non-standard abbreviations
BCA: bicinchoninic acid
Ca: calcium
Cl: chloride
FDA: Food and Drug Administration
FR-1: fixed ratio 1
GLT-1: glutamate transporter-1
H: hydrogen
HRP: horse radish peroxidase
Hz: hertz
ID: inner diameter
IV: intravenous
IP: intraperitoneal
K: potassium
LC/MS/MS – liquid chromatography/tandem mass spectrometry
LLOQ – lowest level of quantitation
MC-100093: Moulder Center-100093
Mg: magnesium
NA: nucleus accumbens
Na: sodium
NADPH: Nicotinamide adenine dinucleotide phosphate
O: oxygen
OD: outer diameter
PO₄: phosphate
PVDF: Polyvinylidene difluoride
RIPA: Radioimmunoprecipitation assay buffer
SDS: sodium dodecyl sulfate
U: units
UDP: uridine diphosphate
Abstract

Cocaine use disorder (CUD) currently lacks FDA-approved treatments. In rodents, the glutamate transporter-1 (GLT-1) is downregulated in the nucleus accumbens following cocaine self-administration and increasing the expression and function of GLT-1 reduces the reinstatement of cocaine-seeking. The beta-lactam antibiotic ceftriaxone upregulates GLT-1 and attenuates cue- and cocaine-induced cocaine seeking without affecting motivation for natural rewards. While ceftriaxone shows promise for treating CUD, it possesses characteristics that limit successful translation from bench to bedside, including poor brain penetration, a lack of oral bioavailability and a risk of bacterial resistance when used chronically. Thus, we aimed to develop novel molecules that retained the GLT-1 enhancing effects of ceftriaxone but displayed superior drug-like properties. Here we describe a new monocyclic beta-lactam, MC-100093, as a potent up-regulator of GLT-1 that is orally bioavailable and devoid of antimicrobial properties. MC-100093 was synthesized and tested in vitro and in vivo to determine physiochemical, pharmacokinetic and pharmacodynamic properties. Next, adult male rats underwent cocaine self-administration and extinction training. During extinction training, rats received one of four doses of MC-100093 for 6-8 days prior to a single cue-primed reinstatement test. Separate cohorts of rats were used to assess nucleus accumbens GLT-1 expression and MC-100093 effects on sucrose self-administration. We found that 50 mg/kg MC-100093 attenuated cue-primed reinstatement of cocaine-seeking while upregulating GLT-1 expression in the nucleus accumbens core. This dose did not produce sedation, nor did it decrease sucrose consumption or body weight. Thus, MC-100093 represents a potential treatment to reduce cocaine relapse.
Significance Statement

Increasing GLT-1 activity reliably reduces drug-seeking across classes of drugs, however, existing GLT1-enhancers have side effects and lack oral bioavailability. To address this issue, novel GLT-1 enhancers were synthesized and the compound with the most favorable pharmacokinetic and pharmacodynamic properties, MC-100093, was selected for further testing. MC-100093 attenuated cued cocaine-seeking without reducing food-seeking or locomotion and upregulated GLT-1 expression in the nucleus accumbens.
Introduction

Approximately 1 million Americans meet the criteria for cocaine use disorder (CUD) (Center for Behavioral Health Statistics and Quality, 2015). While many cocaine users are able to attain long periods of drug abstinence, approximately 85% of users relapse, typically when exposed to cocaine-paired cues or stress or after taking a small amount of the drug again (O’Brien, 2003; Bossert et al., 2005; Epstein et al., 2006). There are currently no FDA-approved medications to reduce the risk of relapse. In order to develop such medications, animal models of cocaine relapse are used to understand the neurobiology underlying this behavior. The extinction-reinstatement model of relapse allows animals to self-administer intravenous cocaine in an operant chamber for a number of weeks, followed by extinction training. During extinction training, the operation (e.g. lever press) made to previously obtain drug is no longer reinforced. Drug-seeking typically declines over the course of 2-3 weeks, at which time reinstatement testing is conducted. The same stimuli which prompt relapse in humans (e.g. drug-associated cues, stress, a drug “prime”) also prompt the reinstatement of the drug-seeking response in animals. This model has been used extensively to determine the neurobiology mediating relapse and identify compounds that attenuate relapse.

Following 2-3 weeks of extinction training, at the time in which rats would be placed back into the operant chamber for a reinstatement test, the expression and function of the major glutamate transporter GLT-1 is decreased in a brain region critical for reinstatement, the nucleus accumbens (NA) core (Knackstedt et al., 2010; Trantham-Davidson et al., 2012; Fischer et al., 2013; LaCrosse et al., 2017; Kim et al., 2018). Some, but not all, members of the beta-lactam family of antibiotics increase GLT-1 expression and function in a screen of more than one thousand FDA-approved compounds (Rothstein et al., 2005). Ceftriaxone is the most potent in this class of antibiotics and has since been demonstrated to increase GLT-1 function in animal models of many neurological diseases, including ischemia, Alzheimer’s Disease,
Parkinson’s Disease, amyotrophic lateral sclerosis, and addiction [for review see (Soni et al., 2014; Tai et al., 2019; Smaga et al., 2020)].

Following cocaine self-administration, sub-chronic (5-7 days) ceftriaxone rescues GLT-1 expression in the NA core and attenuates the reinstatement of cocaine seeking that is primed by cues and cocaine (Sari et al., 2009; Knackstedt et al., 2010; Fischer et al., 2013; LaCrosse et al., 2017; Bechard et al., 2018, 2020). These effects persist weeks after the cessation of ceftriaxone administration, indicating that short-term administration of ceftriaxone restores glutamate homeostasis in the long term (Sondheimer and Knackstedt, 2011). Ceftriaxone does not produce sedation or affect the consumption of regular chow, sucrose or sweetened condensed milk in rats or mice (Knackstedt et al., 2010; Ward et al., 2011; Weiland et al., 2015), indicating a specific effect on the motivation to seek cocaine after a period of abstinence. Notably, both pharmacological and genetic inhibition of GLT-1 in the nucleus accumbens prevent ceftriaxone from attenuating relapse, indicating a necessary role for this protein in mediating the effects of ceftriaxone (Fischer et al., 2013; LaCrosse et al., 2017). In support of a crucial role for GLT-1 function in mediating relapse to cocaine, other GLT-1 enhancers attenuate cocaine-seeking. Like ceftriaxone, chronic administration of propentofylline attenuates both cue- and cocaine-primed reinstatement of cocaine seeking (Reissner et al., 2014). Propentofylline also restores GLT-1 expression in the nucleus accumbens and the attenuation of reinstatement is dependent on such upregulation. Riluzole increases GLT-1 and attenuates cue- and cocaine-primed reinstatement (Sepulveda-Orengo et al., 2018).

While ceftriaxone has reliably been demonstrated to attenuate cocaine-seeking, it possesses qualities that limit its translational potential. Chronic administration of antimicrobial agents such as ceftriaxone can induce antibiotic-resistant strains of bacteria and undesirable side effects common to many antibiotics (e.g. gastrointestinal distress). Furthermore, ceftriaxone must be administered intraperitoneally or intravenously and possesses low brain
bioavailability. Interestingly, in our hands, other beta-lactam-containing compounds such as cefazolin, clavulanic acid, amoxicillin, and Augmentin (the combination of clavulanic acid and amoxicillin) do not attenuate the reinstatement of cocaine-seeking (Weiland et al., 2015; Bechard et al., 2019). This is unfortunate as these compounds are orally bioavailable and would thus be a more attractive potential treatment for cocaine use disorder. In order to address this critical need, we developed additional drug-like beta-lactam derivatives with the goal of generating compounds devoid of antimicrobial properties that enhance GLT-1 function upon oral administration. Here we report that one such compound, MC-100093, fits these characteristics and reduces the reinstatement of cocaine-seeking.

**Experimental Procedures**

**Animals**

Adult male Sprague-Dawley rats were purchased from Jackson labs (Pharmacokinetic studies) and Charles River (cocaine and sucrose studies). Rats were housed in a temperature-controlled vivarium at Temple University (Pharmacokinetic studies) or the University of Florida (cocaine and sucrose studies). Rats used for the cocaine/sucrose studies were single-housed on a 12-h reverse light cycle with lights off at 7 am. Animals received 20 g/day chow and water ad libitum. All rats were acclimated for a minimum of 2 days before being placed on a pharmacokinetic study and 5 days for cocaine/sucrose experiments. All animal procedures were approved by the Institutional Animal Care and Usage Committees of Temple University and the University of Florida, and were in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Drugs**

MC-100093 (Figure 1) was prepared at Temple University School of Pharmacy’s Moulder Center for Drug Discovery Research in two steps using the synthesis described in US
patent 9,975,879 and obtained as a white powder (Abou-Gharbia, 2019). MC-100093 was dissolved in sterile 0.9% saline for in vivo administration. Cocaine was generously provided by the National Institutes on Drug Abuse Drug Supply Program (Research Triangle Institute, Research Triangle Park, NC) and was dissolved in sterile 0.9% saline.

*In Vitro Physicochemical/ADME Assays of MC-100093*

Aqueous solubility of MC-100093 at pH 7.4 was assessed using the commercially available Millipore MultiScreen™ Solubility filter system (Millipore, Billerica, MA). Analysis was performed by LC/MS/MS on a Waters Xevo TQ using positive electrospray ionization. Assay acceptance criteria were 20% for all standards and 25% for the LLOQ.

Microsomal stability was assessed by incubating MC-100093 at 37°C in the presence of human or rat liver microsomes and an NADPH regenerating system according to standard procedures (Yang et al., 2007). Phase II conjugative metabolism was assessed in allomethicin-activated microsomes in the presence of UDP glucuronosyltransferase cofactors according to standard procedures (Kilford et al., 2009). Microsomal protein content was adjusted to give accurate rates of substrate consumption. Analysis was performed by LC/MS/MS as described above.

Cytochrome P450 inhibition was assessed using the three major human cytochrome P450 enzymes, 3A4, 2D6 and 2C9. Expressed enzymes were used to minimize non-specific binding and membrane partitioning issues (McMasters et al., 2007). The 3A4 assay employed testosterone as a substrate and was analyzed using LC/MS/MS technology as described above. The 2D6 and 2C9 assays use fluorescent substrates and were analyzed on an Envision plate reader (PerkinElmer, Waltham, MA).

Plasma protein binding and microsomal partitioning were measured by equilibrium dialysis (Harvard Apparatus, Holliston, MA) under CO₂ as previously described (Kochansky et
al., 2008) and analyzed by LC/MS/MS as described above. Permeability was assessed with a bi-directional MDCK-MR1 assay using standard procedures (Pastan et al., 1988; Wang et al., 2005), commercially available cells and 1 uM substrate concentrations to minimize transporter saturation. The MDCK-MR1 cell line is also used to monitor p-glycoprotein (P-gp) efflux liability. Results were analyzed by LC/MS/MS as described above.

In Vivo Pharmacokinetic Assessment of MC-100093

A total of 9 rats were used; three rats were used for each dosing regimen. For IV and oral dosing, test compound was formulated in sterile saline and dosed at 1mg/kg IV and 10 mg/kg PO. A pre-dose plasma sample was collected prior to administration of MC-100093. Blood samples (typically 100 uL) were collected at 0 (pre-dose), 5, 15, and 30 minutes, 1, 2, 4, 8, and 24 hours, placed in tubes containing EDTA, centrifuged, and the plasma frozen until LC/MS/MS analysis. For measuring brain concentrations, a separate set of rats (3 rats per time point) were dosed IV (1 mg/kg), blood samples were collected at the tmax (0.1 hr), the animals were euthanized with CO₂, and brain samples were collected for analysis. Results were analyzed by LC/MS/MS as described above. Pharmacokinetic parameters were determined by non-compartmental or multi-compartmental equations as appropriate using the WinNonlin program. Models were compared using the Akaike Information Criteria (Akaike, 1974). Pharmacokinetic parameters were compared for statistical significance using paired t-tests. Studies in adult male C57Bl6 mice were conducted in the same manner, but three mice used for each time point and sacrificed after each blood draw. A total of 27 mice were used.

In vitro Selectivity Assessment of MC-100093

MC-100093 was screened for its affinity at 43 GPCRs (G-protein coupled receptors) and transporters found in the brain as well as the cardiovascular toxicity target Kv11.1 (human ether-
a-go-go related potassium channel, hERG) at a concentration of 10 µM through the NIMH Psychoactive Drug Screening Program (Besnard et al., 2012).

**Antimicrobial Susceptibility Testing**

MIC (minimum inhibitory concentration) testing was performed according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2013) as described elsewhere (Eidem et al., 2015). Briefly, individuals wells of a 96-well microtiter plate were inoculated with ~ 10^5 CFU (colony-forming units) of *S. aureus* USA300, *S. aureus* RN8175 or *E. coli* C600N, containing 2-fold increasing concentrations of MC-100093 (0 to 512 µg/ml) and incubated for 18 hours in Mueller-Hinton broth. The assays were also performed in the presence of 2.5% bovine serum albumin. The MIC was defined as the lowest concentration of test compound at which there was no visible bacterial growth in the wells.

**Glutamate uptake assay**

Mouse astrocyte-neuron co-cultures were treated with MC-100093 (10 µM) for 72 hr prior to harvesting for a tritiated glutamate uptake assay. Samples were incubated at 37°C for 10 min in Na^+ buffer (5mM Tris-HCl, pH 7.2, 10mM HEPES, 140mM NaCl, 2.5mM KCl, 1.2mM CaCl_2, 1.2mM MgCl_2, 1.2mM K_2HPO_4, and 10mM D-glucose). Glutamate uptake reaction was started by incubating cells for 5 minutes at 37°C in Na^+ buffer containing 0.5 µM L-glutamate and 0.3 µCi L-[3H]glutamate (Perkin-Elmer) per sample, followed by rapid washing twice with ice-cold Na^+ -free assay buffer (5mM Tris-HCl, pH 7.2, 10mM HEPES, 140mM Choline-Cl, 2.5mM KCl, 1.2mM CaCl_2, 1.2mM MgCl_2, 1.2mM K_2HPO_4, and 10mM D-glucose). Cells were lysed with 0.1N NaOH solution and radioactivity will be measured using a scintillation counter.

**Cocaine self-administration, extinction and reinstatement**

**Surgical Procedures**

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Rats were anesthetized using a mixture of ketamine (87.5 mg/kg, IP) and xylazine (5 mg/kg, IP) and surgically implanted with catheters in the jugular vein. Catheters (SILASTIC silicon tubing, ID 0.51 mm, OD 0.94 mm, Dow Corning, Midland, MI) were implanted in the jugular vein, secured with 4-0 silk sutures, and threaded subcutaneously between the shoulder blades to exit via an incision in the back. Catheter tubing was connected to a stainless-steel cannula (Plastics One, Roanoke, VA, USA) embedded in a rubber harness (Instech, Plymouth Meeting, PA, USA). The harness was worn for the duration of cocaine self-administration. Ketorolac (2 mg/kg, IP) was administered post-operatively and 4 days following surgery for analgesia. The antibiotic Timentin (10 mg/kg) was administered IV (0.1 mL) for 4 days post-surgery. Catheters were flushed with 0.1 mL of heparinized saline (100 U/mL) before and after each self-administration to ensure prolonged catheter patency. Catheter patency was tested periodically with methohexital sodium (10 mg/mL; Eli Lilly, Indianapolis, IN, USA), which results in a temporary loss of muscle tone.

**Cocaine Self-Administration, Extinction and Reinstatement Procedures**

Rats were trained to self-administer cocaine (or receive yoked-saline infusions) in a standard two-lever operant chamber (Med Associates, St. Albans, VT), whereby presses on the active lever resulted in the intravenous (IV) delivery of 0.35 mg cocaine/infusion. An FR-1 schedule of reinforcement was employed and each drug infusion was paired with auditory (a 2900 Hz tone) and visual cues (the illumination of a light over the active lever). Throughout the 2-hour self-administration session, presses on the inactive lever were recorded but had no programmed consequence. Each infusion of cocaine was followed by a 20 second time-out period during which time no drug was delivered. A yoked-saline control group (n=7) received IV saline (0.9% physiological saline) when their yoked counterpart received cocaine. Rats continued in the self-administration phase until meeting a criterion of 10 or more cocaine infusions per session for 12 sessions. This was done to ensure consistency with other studies in
our lab assessing the same dependent variables. Two rats were excluded from future testing due to catheter failure during self-administration. Upon completion of the operant self-administration phase, rats began daily 2-hour extinction training sessions, during which presses on the previously active lever no longer produced infusions or cue presentation. Once animals experienced a minimum of 10-12 extinction sessions and active lever pressing had reached criteria (25% of self-administration levels), a subset of rats underwent reinstatement testing (n=30), while the remaining cocaine (n=14) and yoked-saline rats (n=7) were sacrificed for western blotting without reinstatement testing. For 6-8 days prior to these endpoints, rats to be tested for reinstatement were administered 0, 25, 50, or 100 mg/kg MC-100093 (in 1 mL/kg sterile saline) after the daily extinction session, in accordance with the timing of ceftriaxone injections in our previous work [e.g. (Knackstedt et al., 2010; Bechard et al., 2018; Schwendt et al., 2018)]. The rats to be used for western blotting received either 0 (Vehicle) or 50 mg/kg MC-100083. The number of rats receiving each dose of MC-100093 are depicted in Figures 2 and 3. Rats that underwent a cue-primed reinstatement test were placed into the self-administration chambers and presses on the active lever resulted in presentation of the cues previously associated with cocaine infusions (light + tone) but no cocaine. MC-100093 was not administered prior to the reinstatement test.

**Locomotor testing**

To ensure that MC-100093 was not inducing sedation and thereby inhibiting the reinstatement of cocaine-seeking, rats in the 0 and 50 mg/kg MC-10093 groups underwent locomotor testing 48 hours after reinstatement testing. MC-100093 injections continued in the days between reinstatement and locomotor testing but were not given the day of the locomotor test. Behavioral activity was measured in a photocell apparatus (San Diego Instruments, San Diego, CA). Subjects were placed into the apparatus for a 60-min baseline period during which time activity was monitored. Rats then received a saline injection (1 mL/kg IP) followed 60 min
later by cocaine (10 mg/kg IP). Locomotion was recorded in 10-min increments throughout the 240 minutes. Motor activity was quantified as total distance traveled.

**Western blotting**

Following 10-12 days of extinction training (and MC-100093 or vehicle treatment), rats were rapidly decapitated without anesthesia. The NA core was dissected on ice and homogenized in sucrose buffer containing protease inhibitors. After spinning at 1000g, the supernatant was retrieved and spun at 10000 g. The resulting pellet was re-suspended in sucrose buffer and protease inhibitors, and spun at 10000 g. The supernatant was discarded and the resulting pellet contained the membrane (P2) fraction and was suspended in 1% SDS in RIPA. Protein content was measured using the BCA assay. For western blotting, proteins were separated using 10% SDS-PAGE and transferred to PVDF membrane. Membranes were blocked in 5% milk and probed with primary antibody against GLT-1 (EMD Millipore guinea pig anti-GLT-1, 1:8000). Membranes were incubated with HRP-conjugated secondary antiserum (Jackson ImmunoResearch Lab) at room temperature (anti-guinea pig 1:5,000). Rabbit anti-calnexin (1:40,000) was utilized as a loading control. Bands were visualized using ECL Plus (GE Healthcare). Band density was quantified with NIH Image J software.

**Sucrose self-administration**

Rats (n=6) were trained to self-administer sucrose pellets (45 mg, BioServ) in a standard two-lever operant chamber during daily 2 hr sessions. One press on the active lever delivered one sucrose pellet. Rats were administered vehicle (0.9% physiological saline, 0.3 mL IP) for the first 5 days of self-administration and until lever presses were stable. After the fifth sucrose self-administration session, rats received MC-100093 (50 mg/kg IP) once daily for 5 days. Injections occurred following the self-administration sessions, as in the cocaine experiments.

**Statistical analyses**
Graph Pad Prism (version 7.05) was used to analyze the behavioral and western blotting data. The alpha value was set at $p \leq 0.05$. Cocaine self-administration and extinction data (infusions, active and inactive lever presses), weight, and locomotor behavior were compared between groups using 2-way Repeated Measures (RM) Analyses of Variance (ANOVAs) with Dose as the between-subjects factor and Time as the within-subjects factor. Active and inactive lever pressing during the reinstatement test were compared to pressing during the average of the last three days of extinction using a 2-way RM ANOVA with Dose as a between-subjects factor and Test as the within-subjects factor. For western blotting data, the integrated optical density of individual GLT-1 bands was first normalized to calnexin optical density. These values were then normalized to the Saline-0 mg/kg control group by computing the average density for the Saline-0 mg/kg group on each gel and dividing all samples on this gel by this average. This yielded a "percent control value" and these values were compared between groups with a one-way ANOVA. Sucrose self-administration was analyzed with a RM one-way ANOVA on the daily sucrose self-administration and a paired-samples t-test was also conducted to compare the total number of sucrose pellets consumed prior to and during MC-100093 administration. Significant interactions or main effects from the one-way ANOVA were followed up with Sidak's multiple comparisons test.

Results

Physiochemistry and Pharmacokinetics

MC-100093 increased glutamate uptake in a neuron/astrocyte co-culture model ($t_{(4)} = 3.23; p = 0.0319$). MC-100093 is devoid of antibacterial activity, in representative gram positive (S. aureus USA300, S. aureus RN8175) and gram negative (E. coli C600N) strains. In a preliminary selectivity study, MC-100093 showed no activity in a panel of 43 GPCRs found in the brain at concentrations up to and including 10 uM (NIMH PDSP). This includes many of the pathways thought to play a role in cocaine dependence and reinstatement (Somaini et al., 2011).
as well as the potential cardiovascular toxicity targets human ether-a-go-go related ion channel (hERG) and serotonin 5HT2b. Table 1 provides a list of the targets that MC-100093 was screened against.

_In vitro_ ADME (Absorption, Distribution, Metabolism, Excretion) screening demonstrated that the physicochemical properties of MC-100093 are consistent with marketed, orally bioavailable drugs. MC-100093 is soluble in aqueous media, virtually inactive at inhibiting the three major metabolic cytochrome P450 enzymes responsible for drug-drug interaction (Cyp3A4, 2D6, and 2C9) and metabolically stable in rat, mouse and human liver microsomes (see Table 2). The lack of in vitro metabolism suggests that the pharmacokinetics of MC-100093 should be similar in the rat and mouse. Protein binding is relatively low (35%) and the compound is not retained in membrane lipids to a great extent (free, unbound fraction = 83%). Taken together, these properties are suggestive of acceptable pharmacokinetic properties, and an _in vivo_ PK assessment in rats (IV and PO) has shown that this is indeed the case.

As shown in Tables 3 and 4, the compound is orally bioavailable (F% = 28%) in the rat, has excellent exposure when dosed at 1 mg/kg IV and 10 mg/kg PO, a moderate half-life that is likely elimination-driven rather than metabolism-driven, and exposure to the brain is substantially higher than for ceftriaxone when administered IP in the mouse (14% brain penetration (Table 4) for MC-100093 vs. 1% for ceftriaxone) (Granero _et al._, 1995). Additional PK studies in mice (25 mg/kg, IP, Table 3) demonstrated that the compound can be successfully administered in animal studies. Brain exposure in this study was also significantly higher than that observed for ceftriaxone (14x), and the metabolic half-life is sufficient to support _in vivo_ studies.

To rule out the possibility that the effect of MC-100093 on cocaine reinstatement was not the result of off-target activity known to be involved in animal models of cocaine reinstatement, we screened our compound for affinity at 43 GPCRs and transporters (Table 1) through the
NIMH Psychoactive Drug Screening Program (Besnard et al., 2012). MC-100093 showed no appreciable affinity for these targets as well as the cardiovascular toxicity target hERG (human ether-a-go-go related potassium channel, Kc11.1) at a concentration of 10 μM.

Cocaine self-administration, extinction and reinstatement

We assigned rats to receive one of four doses of MC-100093 during extinction training such that the groups had similar prior cocaine intake and lever pressing histories. Two-way RM ANOVAs revealed no main effects of Dose or Dose x Time interactions for infusions (Fig. 2A), active (Fig. 2B) or inactive (Fig. 2D) lever presses during cocaine self-administration. There was a main effect of Time for the number of infusions attained \[F_{(11, 275)} = 20.33, p<0.0001\] during self-administration. There were also main effects of Time for active lever presses \[F_{(11, 275)} = 51.83, p<0.0001\] and inactive lever presses \[F_{(23, 598)} = 7.97, p<0.001\] during self-administration and extinction. We compared body weight for the 6 days prior to initiating MC-100093 injections and the 7 days of injections (Fig. 2C), finding no effect of Dose and no Dose x Time interaction. There was a main effect of Time on weight \[F_{(12, 300)} = 75.02, p<0.0001\], as weight increased over the 13 days for all groups, indicating that MC-100093 did not affect motivation to consume regular chow. Reinstatement of cocaine seeking is defined as the resumption of an extinguished drug-seeking response. Thus, we compared lever pressing during extinction (average of the last three days) to that during the cue-primed test. For active lever presses (Fig. 2E), we found a Dose x Time interaction \[F_{(3, 26)} = 3.754, p<0.05\] and a main effect of Time \[F_{(1, 26)} = 113.90, p<0.001\]. Post-hoc tests showed that while all groups reinstated (p<0.05 comparing extinction to reinstatement lever pressing), the 50 mg/kg dose attenuated active lever pressing relative to the 0 and 25 mg/kg treatment groups. There was no Dose x Time interaction for inactive lever presses, as inactive lever pressing remained low throughout extinction and test (Fig. 2F).

Western Blotting
Upon detecting that the 50 mg/kg dose of MC-100093 attenuated reinstatement, we ran a separate cohort of rats through self-administration and extinction for the purposes of assessing the effects of this dose on GLT-1 expression in the NA core at the time in which rats would be placed into the chamber for a reinstatement test. This was done because we have previously assessed GLT-1 expression after extinction training but in the absence of a reinstatement test (Knackstedt et al., 2010). In this cohort of rats, there was an effect of Time on infusions \( F(11, 143) = 2.406, p<0.01; \) Fig. 3A]. While there was no effect of Dose and no Dose x Time interaction on infusions, we verified no group differences in cocaine intake by comparing the total number of infusions earned with an unpaired t-test, finding no differences (Fig. 3C). There was a main effect of Time on both active \( F(21, 273) = 5.232, p<0.001, \) Fig. 3B] and inactive \( F(21, 273) = 2.681, p<0.0001; \) Fig. 3D] lever presses during self-administration and extinction. However, there was not a main effect of Dose nor a Dose x Time interaction for these variables. Nucleus accumbens membrane GLT-1 expression differed by Group \( F(2, 17) = 3.482, p=0.05; \) Fig. 3E), with post-hoc tests showing that the cocaine-vehicle (0 mg/kg) group displayed reduced expression relative to the yoked-saline control group (p<0.05). The cocaine group that received 50 mg/kg MC-100093 did not display a reduction in GLT-1 expression, indicative of a restoration of expression.

**Locomotor and Sucrose Self-administration Controls**

Following subchronic administration of vehicle (0 mg/kg) or 50 mg/kg MC-100093, rats were tested for spontaneous and cocaine-induced locomotion. A main effect of Time \( F(23, 276) = 15.08, p<0.0001; \) Fig. 4A] was observed, but no effect of Dose and no Dose x Time interaction were detected. Thus, both groups exhibited similar spontaneous and cocaine-induced locomotion. To determine whether MC-100093 decreases the motivation to seek a natural reward, a within-subjects assessment of sucrose self-administration during vehicle and 50 mg/kg MC-100093 was conducted. A one-way ANOVA with RM on time found a significant main
effect \[F(2.666, 13.33) = 5.243, p<0.05\]. Post-hoc tests found that several days, both prior to and during MC-100093 treatment, were greater than Day 5 intake (Fig. 4B). The first MC-100093 injection was administered after Day 5 sucrose self-administration session, and thus the decrease in intake on Day 5 was not due to this drug. A paired-samples t-test conducted on the total number of sucrose pellets consumed found a significant increase in the number of pellets consumed during MC-100093 treatment relative to the 5 days of vehicle treatment \[t(5) = 3.227, p<0.05; \text{Fig 4B inset}\].

Discussion

We entered into a drug discovery project with the goal of identifying more potent, more selective drugs than ceftriaxone that also possess reduced antibiotic activity. Our efforts led to the design and synthesis of MC-100093, a novel beta-lactam derivative that does not possess antimicrobial properties. The molecule’s stereoselective synthesis is short, straightforward and high yielding. MC-100093 is structurally distinct from the beta-lactam antibiotics reported to enhance GLT-1 uptake (Rothstein et al., 2005). It is a monocyclic monobactam whereas the antibiotics found to possess GLT-1 enhancing effects possess the bicyclic penicillin or cephalosporin scaffolds. MC-100093 displays many physicochemical and in vitro ADME properties expected of a good drug candidate (Table 1). The molecule is highly soluble in aqueous media (> 10,000 \(\mu\text{M}\)). It is stable to cytochrome P450-mediated oxidative metabolism in both mouse and human liver microsomes. It does not show any appreciable inhibition of the three major metabolizing liver P450 enzymes CYP3A4, CYP2D6 and CYP2C9, which suggests that there is a low potential for causing drug-drug interactions. Importantly, MC-100093 shows no anti-microbial activity against representative gram-positive and gram-negative strains of bacteria at concentrations up to 256 \(\mu\text{g/mL}\). Use of the compound would not lead to the development of bacterial resistance, which is one of the issues with ceftriaxone that precludes its long-term use as an anti-addictive agent. MC-100093 showed no affinity for 43 GPCRs and
transporters at a concentration of 10 μM, including a several targets (e.g., dopamine, acetylcholine, AMPA, kappa opioid) known to have effects in models of cocaine dependence and reinstatement (Somaini et al., 2011). While the list is not exhaustive, that data, coupled with the fact that MC-100093 does not meet the pharmacophoric requirements for other known mechanisms that show activity in cocaine reinstatement models such as orexin antagonism (Roecker et al., 2016), suggest that MC-100093 is not acting through these mechanisms to prevent cue- and cocaine-induced reinstatement.

MC-100093 also displays superior in vivo pharmacokinetic parameters in rats compared to ceftriaxone [Table 2, (Granero et al., 1995)]. While MC-100093 is more hydrophilic (logP = -1.35) than compounds normally expected to display good intestinal permeability (logP 1-3, (Pajouhesh and Lenz, 2005), the compound does display reasonable oral bioavailability in rats (28%, Table 2). In contrast, ceftriaxone is not orally bioavailable and must be administered intramuscularly or intravenously to humans. MC-100093 penetrates into the CNS better than ceftriaxone, displaying a brain/plasma ratio of 14% in mice (Table 4) compared to 1% for ceftriaxone (Nau et al., 2010). Thus, MC-100093 represents an orally bioavailable GLT-1 enhancer that penetrates the CNS better than ceftriaxone.

Treatment with MC-100093 (10 μM) enhanced uptake of glutamate into astrocytes in a mouse neuron/astrocyte co-culture model to a maximum of 123.5% over control values, with an IC₅₀ value = 0.1 μM. Treatment with ceftriaxone showed a similar maximal effect on enhancement of glutamate uptake (130.2%), but was 10-fold less potent than MC-100093, giving an IC₅₀ = 1.4 μM. When administered to rats with a prior history of cocaine self-administration, 50 mg/kg MC-100093 restored the expression of NA core GLT-1. While ceftriaxone has been demonstrated to increase GLT-1 expression and glutamate uptake in cell culture, a number of studies have reported that when ceftriaxone is administered to healthy rodents, it is unable to upregulate GLT-1 in the nucleus accumbens [for review see (Smaga et
Thus, we did not screen MC-100093 for its ability to increase GLT-1 protein expression in cocaine-naïve rats. This will be addressed in future work.

MC-100093 displayed an inverted u-shaped dose response function for the ability to attenuate cue-primed reinstatement of cocaine seeking, with the 50 mg/kg dose showing efficacy and the 25 and 100 mg/kg doses not affecting reinstatement. The effective dose of MC-100093 in these models was significantly lower than that seen with ceftriaxone in rats (100-200 mg/kg IP; (Sari et al., 2009; Knackstedt et al., 2010)). The inverted u-shaped dose response is not uncommon in operant models as well as other behavioral and learning/memory models. The exact causes for this phenomenon are not completely understood, but one hypothesis involves system plasticity and the attempt of the system to restrict the magnitude of the response within pre-established control values (Calabrese, 2008). For example, here, 100 mg/kg MC-100093 may increase GLT-1 to supra-physiological levels, which would decrease basal (pre-reinstatement) glutamate levels further, producing additional post-synaptic glutamate receptor adaptations (LaCrosse et al., 2017) and thus greater reinstatement of cocaine seeking. To our knowledge, ceftriaxone doses higher than 200 mg/kg have been not been tested in the cocaine reinstatement model in rats, and thus it is not known if the same inverted u-shaped curve would be observed for ceftriaxone.

Thus, like ceftriaxone, MC-100093 attenuates cue-primed relapse to cocaine-seeking without reducing motivation to consume regular chow or sucrose pellets or affecting body weight (Weiland et al., 2015). We found no effect of MC-100093 on spontaneous or cocaine-induced locomotion. We have previously reported that in both cocaine-experienced and cocaine-naïve rats, subchronic ceftriaxone (200 mg/kg IP) increases spontaneous locomotion relative to vehicle-treated rats (Knackstedt et al., 2010; Schwindt et al., 2018). In both of these reports, locomotor testing occurred 24 hours after the final ceftriaxone injection, indicating a long-term effect on locomotion. When locomotion was assessed in the home cage, 8 days of ceftriaxone...
(200 mg/kg IP) similarly increased spontaneous locomotion (Bellesi et al., 2012). Here, the same dose of MC-100093 that attenuated reinstatement (50 mg/kg) did not alter spontaneous or cocaine-induced locomotion. While this indicates that sedation was not the cause of the attenuation in cocaine-seeking during the cue test, it also indicates that the neurochemical effects of MC-100093 are not identical to those of ceftriaxone.

The data presented here indicate that MC-100093 is a strong candidate for a medication to reduce cocaine relapse, with advantages over the antibiotic ceftriaxone. Future work will examine whether MC-100093 exerts the same effects on neurobiology underlying cocaine relapse as does ceftriaxone, such as normalization of NA core physiology, surface glutamate receptor expression, and glutamate release and reuptake (Trantham-Davidson et al., 2012; LaCrosse et al., 2017).

Acknowledgements

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*Performed data analysis*: Lori Knackstedt, Svetlana Vidensky, John Gordon, Paul Dunman, Ben Blass and Wayne Childers
Wrote or contributed to the writing of the manuscript: Lori Knackstedt, Wayne Childers, Magid Abou-Gharbia
Bibliography


stress susceptibility and enhanced cocaine seeking with a role for mGlu5 receptors. *Transl Psychiatry* 8:209.


Figure Legends

Figure 1. Structures of ceftriaxone and MC-100093. The beta-lactam moiety is shown in red.

Figure 2. MC-100093 inhibits relapse to cocaine-seeking in a dose-dependent manner. Rats were treated with MC-100093 (0, 25, 50, 100 mg/kg IP) for 6-8 days during extinction prior to a cue-primed reinstatement test. A. Rats later receiving 0, 25, 50 or 100 mg/kg MC-100093 did not differ in the mean number of infusions attained during self-administration. B, D. Rats later assigned to different doses of MC-100093 did not differ in the number of active or inactive lever presses during self-administration or extinction training. C. Weight continued to increase in all groups following the initiation of injections, indicating no effects on motivation to consume chow. E. All groups reinstated cocaine-seeking, evidenced by an increase in active lever pressing during the Cue-primed test relative to extinction (average of last three days). Only the 50 mg/kg dose of MC-100093 attenuated active lever presses during the cue-primed test. F. No group differences in inactive lever pressing were observed. * = p<0.05 relative to extinction. # = p<0.05 relative to 50 mg/kg.

Fig. 3. Following cocaine self-administration and extinction training, MC-100093 restores GLT-1 expression in the nucleus accumbens core. A,C. There were no differences in number of cocaine infusions earned in rats later treated with 0 or 50 mg/kg MC-100093. There were no differences in active (B) or inactive lever pressing (D) in rats later treated with 0 or 50 mg/kg MC-100093. E. Western blotting of nucleus accumbens tissue revealed a decrease in GLT-1 membrane expression in cocaine-extinguished rats that were treated with 0, but not 50 mg/kg MC-100093. F. Sample western blots. * = p<0.05 compared to Saline-0 mg/kg.

Fig. 4. Subchronic MC-100093 does not produce sedation or decrease sucrose self-administration. A. MC-100093 does not alter spontaneous (0-60 min) or cocaine-induced locomotion (120-240 min). B. MC-100093 treatment began after the 2 hr sucrose self-administration session on Day 5. Rats increased sucrose consumption during treatment with
MC-100093, indicating that its effects on cocaine-seeking are not due to motivational deficits. *

= p<0.05 relative to Day 5. ^= p<0.05 paired samples t-test.
Table 1. MC-100093: Receptor Binding Results from Psychoactive Drug Screening Program. All tests run at 10 μM. All results < 10% inhibition of standard ligand binding @ 10 μM.

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<th>Adrenergic</th>
<th>Benzodiazepine</th>
<th>Dopamine</th>
<th>Gamma Amino Butyric Acid</th>
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<tr>
<td>Alpha-1A</td>
<td>Rat brain Bz</td>
<td>D1</td>
<td>GABA-A</td>
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<td>Alpha-1B</td>
<td>Peripheral Bz</td>
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Table 2: Properties, in vitro screening and in vitro ADME results for MC-100093

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<tr>
<th>Structure</th>
<th>MW</th>
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<th>E Coli C600N</th>
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<td>IC₅₀ % Ctrl</td>
<td>MIC (µg/mL)</td>
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<td>72.88</td>
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Table 3: Rat PK for MC-100093.

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<th>Route</th>
<th>Dose (mg/kg)</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (mg/ml)</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; (h)</th>
<th>AUC&lt;sub&gt;tot&lt;/sub&gt; (mg·hr/l)</th>
<th>T&lt;sub&gt;1/2&lt;/sub&gt; (h)</th>
<th>CL (L/h)</th>
<th>VZ (L)</th>
<th>Vss (L)</th>
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<td>1.5</td>
<td>5.26</td>
<td>1.8</td>
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<td>3.2</td>
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Table 4: Mouse PK for MC-100093 after IP dosing.

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<th>Dose (mg/kg)</th>
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<th>T&lt;sub&gt;max&lt;/sub&gt; (h)</th>
<th>AUC&lt;sub&gt;tot&lt;/sub&gt; (mg*hr/m)</th>
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<th>CL (L/h)</th>
<th>VZ (L)</th>
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<td>11.5</td>
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Footnote

Role of Funding Source: This work was funded by the National Institutes of Health National Institute on Drug Abuse grant [DA037270] awarded to Drs. Abou-Gharbia and Rothstein. The NIH had no further role in study design, data analysis or the writing of the manuscript.
Figure 2
Figure 3
Figure 4

(A) Graph showing the distance travelled (cm²) over time (min) for different groups: Vehicle (n=7), 50 mg/kg saline (n=8), and MC-100093.

(B) Graph showing the mean sucrose pellets/2 hr per day from Day 1 to Day 10. The inset shows the total sucrose pellets for Vehicle and MC-100093.