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Original article

Effects of novel beta-lactam, MC-100093, and ceftriaxone on astrocytic glutamate transporters and neuroinflammatory factors in nucleus accumbens of C57BL/6 mice exposed to escalated doses of morphine

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ABSTRACT

Chronic exposure to opioids can lead to downregulation of astrocytic glutamate transporter 1 (GLT-1), which regulates the majority of glutamate uptake. Studies from our lab revealed that beta-lactam antibiotic, ceftriaxone, attenuated hydrocodone-induced downregulation of GLT-1 as well as cystine/glutamate antiporter (xCT) expression in central reward brain regions. In this study, we investigated the effects of escalating doses of morphine and tested the efficacy of novel synthetic non-antibiotic drug, MC-100093, and ceftriaxone in attenuating the effects of morphine exposure in the expression of GLT-1, xCT, and neuroinflammatory factors (IL-6 and TGF-β) in the nucleus accumbens (NAc). This study also investigated the effects of morphine and beta-lactams in locomotor activity, spontaneous alternation percentage (SAP) and number of entries in Y maze since opioids have effects in locomotor sensitization. Mice were exposed to moderate dose of morphine (20 mg/kg, i.p.) on days 1, 3, 5, 7, and a higher dose of morphine (150 mg/kg, i.p.) on day 9, and these mice were then behaviorally tested and euthanized on Day 10. Western blot analysis showed that exposure to morphine downregulated GLT-1 and xCT expression in the NAc, and both MC-100093 and ceftriaxone attenuated these effects. In addition, morphine exposure increased IL-6 mRNA and TGF-β mRNA expression, and MC-100093 and ceftriaxone attenuated only the effect on IL-6 mRNA expression in the NAc. Furthermore, morphine exposure induced an increase in distance travelled, and MC-100093 and ceftriaxone attenuated this effect. In addition, morphine exposure decreased the SAP and increased the number of arm entries in Y maze, however, neither MC-100093 nor ceftriaxone showed any attenuating effect. Our findings demonstrated for the first time that MC-100093 and ceftriaxone attenuated morphine-induced downregulation of GLT-1 and xCT expression, and morphine-induced increase in neuroinflammatory factor, IL-6, as well as hyperactivity. These findings revealed the beneficial therapeutic effects of MC-100093 and ceftriaxone against the effects of exposure to escalated doses of morphine.

1. Introduction

Opioid use has been a major health issue, and overdose leading to death has increased over the years (Rudd et al., 2016). There are several abused opioid drugs, including morphine, hydrocodone, oxycodone, fentanyl, and other synthetic opioids as well as the illegal morphine

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derivative such as heroin (Schaefer et al., 2017). Morphine is considered more potent than hydrocodone and oxycodone, however, fentanyl is more potent than morphine (Schaefer, et al., 2017). All opioids share the same pharmacological mechanism of action for the development of drug dependence and other peripheral effects, which are dependent on the potency and duration of exposure of a selected opioid. Opioids dependence and tolerance are neurobiological and pharmacological effects that are associated with changes in the brain neurochemistry (Koob, 2020;Kosten and George, 2002). Alternatively, opioids exposure increased locomotor activity and sensitization as well as opioid seeking behaviors (Alshehri et al., 2018;Berríos-Cárcamo et al., 2022;Emery et al., 2015;Gaulden et al., 2021;Nazarian et al., 2011). Opioids act through their receptors to regulate several neurotransmitters. Among them glutamate has been considered as a major neurotransmitter affected by exposure to opioids (Reeves et al., 2022). Recent study from our laboratory revealed that exposure to hydrocodone overdose increased locomotor activity and sensitization, and this effect might be associated with alteration in the glutamatergic signaling (Wong and Sari, 2024).

It is well known that glutamate uptake is regulated mainly by astrocytic glutamate transporter 1 (GLT-1), which modulates the majority of extracellular glutamate concentrations at the synaptic cleft (Danbolt, 2001). In addition, cystine/glutamate antiporter (xCT) is co-localized with GLT-1 in astrocytes to modulate glutamate homeostasis. Studies showed that chronic exposure to morphine reduced glutamate uptake, and this effect was associated with downregulation of glutamate transporters, mainly GLT-1 (Dunbar and Pulai, 1998;Mao et al., 2002; Marek et al., 1991;Ozawa et al., 2001;Wang et al., 2016). Furthermore, recent study from our lab showed that chronic exposure to hydrocodone reduced the expression of GLT-1 and xCT in the nucleus accumbens (NAc) (Wong and Sari, 2023). Glutamate spillover has been associated with relapse to heroin, which might be caused by reduced glutamate uptake (Shen et al., 2014). It is evident that chronic exposure to opioids has been shown to be associated with downregulation of GLT-1 expression, which is a neuropathological state observed with several drugs of abuse (Alasmari et al., 2018;Alhaddad et al., 2022;Alshehri, et al., 2018;Das et al., 2022;Das et al., 2015;Gregg et al., 2016;Knackstedt et al., 2010;Sari et al., 2009;Sondheimer and Knackstedt, 2011). These latter studies from our lab and others showed clearly that the beta-lactam antibiotic, ceftriaxone, attenuated drugs of abuse-induced downregulation of GLT-1 and xCT in mesocorticolimbic brain regions, including the NAc. Importantly, we recently revealed that a novel synthetic beta-lactam non-antibiotic, MC-100093, reduced ethanol intake and attenuated ethanol-induced downregulation of GLT-1 and xCT expression in the NAc of alcohol-preferring rats (Alhaddad, et al., 2022). Although, several studies showed the effects of chronic exposure to drugs of abuse, including opioids, less is known about the effects of escalated doses of opioids, with regard to particularly the overdose, on the expression of GLT-1 and xCT, as well as the attenuating effects with the novel synthetic beta-lactam, MC-100093. In this study, we tested the effects of repeated exposure to moderate dose every other day for one week and then challenged the mice with a single higher dose of morphine in the expression of GLT-1 and xCT as well as target neuroinflammatory factors, interleukin-6 (IL-6) and transforming growth factor-β (TGF-β) in the NAc. We targeted the NAc as a brain reward region involved in the regulation of glutamate signaling and glutamate homeostasis, and it has a crucial role in the development of dependence to drugs of abuse, including opioids (Hearing et al., 2018;Scofield et al., 2016). This study also investigated the effects of escalated doses of morphine and beta-lactams in behavioral paradigms such as locomotor activity, spontaneous alternate percentage and short memory Y maze. It is important to note that the main goal of this study was to determine the preclinical therapeutic effects of our novel synthetic beta-lactam, MC-100093, in mice exposed to escalated doses of morphine with higher dose (150 mg/kg, i.p.) that is considered as an overdose in targets astrocytic glutamate transporters (GLT-1 and xCT) and neuroinflammatory signaling (IL-6 and TGFβ) as well as locomotor activity behaviors.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice were tested at the age of eight weeks old. After habituation to the vivarium, mice were single-housed in a temperature-controlled (21 °C) and humidity-controlled (30 %) vivarium with a 12/12 h light–dark cycle. Mice had access to food and water throughout the experiments. The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the Research Ethics Committee at King Saud University (ethics reference# KSU-SE-22–48).

2.2. Drug treatment procedures

Mice were divided in four groups (n = 8–9 per group). The control and treatment groups were as follows: **1)** vehicle group received saline (i.p.) on Days 1, 3, 5, 7, and 9; **2)** morphine group received 20 mg/kg morphine (i.p.) on Days 1, 3, 5, 7 and mice were challenged with higher dose of morphine (150 mg/kg i.p.) on day 9; and **3)** morphine-ceftriaxone group received 20 mg/kg morphine (i.p.) on Days 1, 3, 5, 7 and then challenged with higher dose of morphine (150 mg/kg, i.p.) on day 9, and ceftriaxone (200 mg/kg, i.p.) was administered from Day 4-Day 8; and **4)** morphine-MC-100093 group received 20 mg/kg morphine (i.p.) on Days 1, 3, 5, 7 and then challenged with higher dose of morphine (150 mg/kg, i.p.) on day 9, and MC-100093 (50 mg/kg, i.p.) was administered from Day 4-Day 8 (Fig. 1). Mice were tested for locomotion activity and memory Y maze behaviors after administering higher dose of morphine on Day 9. Mice were then sacrificed by CO_2 inhalation, followed by cardiac puncture for blood collection and then decapitated on Day 10 (Fig. 1). The brains were dissected and frozen at −80 °C. The NAc (shell and core) were micropunched using a cryostat apparatus and tissue was stored at −80 °C for Western blot and a real-time reverse transcription–polymerase chain reaction (RT-PCR) assays.

2.3. Behavioral testing

2.3.1. Locomotor activity

Locomotion activity test was conducted on day 9 after administering higher dose of morphine using activity monitoring system as performed in a previous study (York et al., 2013). Supermex apparatus ((Muromachi Kikai, Tokyo, Japan) and a monitoring sensor were connected to chambers (25 × 45 × 20 cm^3 for each chamber). Habituation was performed for each mouse before conducting the test. After administering higher dose of morphine, each mouse was placed in the chamber and tracked by activity monitoring software for 10 min. The total distance travelled for each mouse was measured and analyzed using a computerized and automated system (Comp ACT AMS, Muromachi Kikai).

2.3.2. Short memory Y-maze test

To investigate the effects of escalated doses of morphine and β-lactam treatments on short memory recognition, Y-maze test was conducted as performed previously (Alzarea and Rahman, 2019). In this test, the cognition of mice to explore new arms that were not explored in the previous two visits was evaluated. The apparatus has three arms (A, B, and C) and each mouse was placed in the center of the apparatus to explore the arms for 5 min. Three different subsequent responses of alternation (e.g. B, C, A) of arm visits were determined to calculate the spontaneous alternation percentage (SAP) as described in equation below. In addition, the number of responses of alternation for each mouse was detected. These parameters were determined on day 9 following exposure to higher dose of morphine.

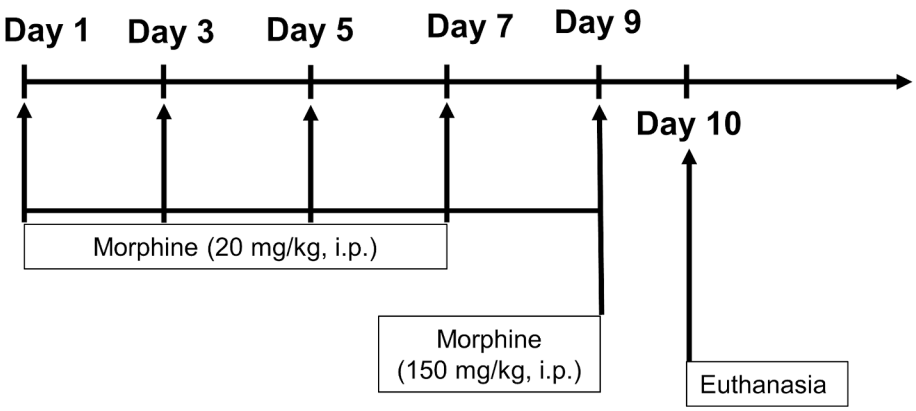

Fig. 1. Experimental design and timeline of the study.

$$\text{Spontaneous alternation percentage} = \left[\frac{\text{Number of responses of alternation}}{\text{Total arm entries} - 2}\right] \times 100 \text{ (equation)}$$

2.4. Brain tissue harvesting

Mice were sacrificed by CO_2 inhalation, followed by cardiac puncture for blood collection and then decapitated on Day 10. Brains were dissected and frozen at −80 °C. Brain reward region, nucleus accumbens (NAc), was micropunched using a cryostat apparatus.

2.5. Western blot for determination of GLT-1 and xCT expression in the nucleus accumbens

The expression of GLT-1 and xCT in the NAc was determined using Western blot assay as performed and described in previous studies (Alhaddad et al., 2022; Alshehri et al., 2017; Wong and Sari, 2024). RIPA lysis buffer containing protease and phosphatase inhibitors was used for the NAc tissues homogenization. The BCA protein assay was used to determine the amount of proteins in the NAc homogenate for each sample. Mixtures of equal amount of protein from each tissue sample and laemmli dye were prepared for loading on polyacrylamide gels. After gel running, proteins on the gels were transferred onto polyvinylidene difluoride (PVDF) membrane using dry transfer apparatus. Membranes were then incubated with 3 % non-fat milk for one hour in Tris-buffered saline with Tween-20 (TBST). Primary antibodies: rabbit anti-GLT-1 (ABclonal, Wuhan, China), rabbit anti-xCT (ABclonal, Wuhan, China), and rabbit anti-β-actin (ABclonal, Wuhan, China) were incubated with the membranes for 24 h at 4 °C degree. On the following day, 5-time wash for 5 min for each wash was performed on the membranes. Membranes were then blocked with 3 % non-fat milk in TBST for 30 min. Membranes were then incubated with the corresponding secondary antibodies for 90 min. Subsequently, membranes were washed with TBST for five times. The membranes were then carefully dried and exposed to chemiluminescent reagents for detection of proteins. ImageJ software (Version 1.53 t 24, NIH) was used to quantify the detected blots. Our data were normalized 100 % (relative to control group) to compare between control and morphine groups or 100 % (relative to morphine group) to compare between morphine and morphine treated with β-lactams groups as performed in previous studies (Alhaddad et al., 2022; Li et al., 2003; Wong and Sari, 2024).

2.6. A real-time reverse transcription–polymerase chain reaction (RT-PCR) protocol for determination of IL-6 mRNA and TGF-β mRNA expression in the nucleus accumbens

Gene expression of selected neuroinflammatory markers, Il-6 and TGF-β, were determined in the NAc of the control and treatment groups as performed in a previous study (Humphreys et al., 2014). Following the manufacturer's instructions, the TRIzol™ reagent (Thermo Scientific, Waltham, MA, USA) was used to extract the total RNA from the NAc. Using a NanoDropTM 8000 Spectrophotometer (Thermo Scientific, USA), the purity and amount of extracted RNA samples were assessed. After the extracted RNA was reverse transcribed into cDNA using cDNA Synthesis SuperMix (Bimake, Houston, TX, USA), gene expression was measured using SYBR Green Master Mix (Bimake, Houston, TX, USA) via the Applied Biosystems 7500 Fast Real-Time PCR System. The relative expression of IL-6 and TGF-β genes were then assessed between the groups using the ΔΔCt methodology. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used in the current study as the housekeeping gene. Briefly, GAPDH *CT* values were subtracted from the mean *CT* values of the gene of interest in order to obtain Δ*CT*. The average Δ*CT* values for the control group were further subtracted from the Δ*CT* values for the experimental groups to obtain ΔΔ*CT*. Next, the relative fold change from control for each sample was represented by computing $2^{-\Delta\Delta CT}$ and was reported as mean ± SEM. The primer sequences used in this study are listed in Table 1 (IDT, Leuven, Belgium).

2.7. Gas Chromatography–Mass spectrometry (GC–MS)

GC–MS was used to quantify serum morphine concentrations 1 h and 24 h after exposure to higher dose of morphine as previously described (Saad et al., 2019). To make the Internal Standard (IS) stock solution (1 mg/mL), 10 mg of morphine-D3 powder was dissolved in 10 mL of methanol and then added to a 10 mL volumetric flask. A 10 mL volumetric flask containing 10 mg of powdered morphine was filled with 10 mL of methanol to create a morphine standard stock solution (1 mg/mL). In a 7-mL polypropylene tube, 100 µL of serum was spiked with 50 µL of IS (1.0 µg/mL), and 900 µL of 0.01 M KH2PO4 was further added to the solution. The mixture was vortexed for one minute, and then the tube was centrifuged for five minutes at 15,000 x g. Solid phase extraction was applied straight to the clear supernatant after it had been decanted into a fresh tube. Appropriate volumes of the working morphine standard solutions were added to blank serum to obtain five-point calibration curves with a range of 50 ng/mL to 3000 ng/mL (50, 100, 200, 400, and 3000 ng/mL). A nitrogen stream was used to evaporate the eluent solutions until they were completely dry at 50 °C. In order to derivatize the residue, it was reconstituted in 50 µL of BSTFA +

Table 1
The primer sequences used in this work.

Genes	Forward- Primer Sequences (5′→ 3′)	Reverse- Primer Sequences (5′→ 3′)
Il-6	TACCACTTCACAAGTCGGAGGC	CTGCAAGTGCATCATCGTTGTTC
TGF-β	TGATACGCCTGAGTGGCTGTCT	CACAAGAGCAGTGAGCGCTGAA
Gapdh	CATGGCCTTCCGTGTTCCTA	CCTGCTTCACCACCTTCTTGAT

1 % TMCS and 50 µL of ethyl acetate. It was then sealed, vortexed, and incubated for 30 min at 70 °C. The mixture was added into insert vials in the autosampler of the GC–MS for analysis after cooling in room temperature. Inter and Intra-assay accuracy and precision were calculated using %bias and percent of relative standard deviation (%RSD) approaches, respectively, to validate the methodology. Limit of detection (LOD), curve limit of quantification (LOQ), linearity and recovery were also obtained to further validate the methodology.

2.8. Statistical analyses

Data obtained from the current study were analyzed using GraphPad prism software. Two Way Analysis of variance (ANOVA) was used to compare body weight between control, morphine, morphine/MC-100093, and morphine/ceftriaxone groups. One-way ANOVA was used to evaluate the significant changes of behavioral parameters among groups. Independent *t* test was used to measure the significant differences between morphine and control, morphine/MC-100093, or morphine/ceftriaxone groups on total distance travelled and short memory recognition. One-way ANOVA followed by Fisher's least significant difference (LSD) for multiple comparisons test were used to evaluate the significant effects of morphine, MC-100093 or ceftriaxone treatment on protein expression of GLT-1 and xCT, and gene expression of IL-6 and TGF-β. The obtained data were shown as mean ± SEM, and the significance was considered when $p \leq 0.05$.

3. Results

3.1. Effects of exposure to escalated doses of morphine, and treatment with MC-100093 and ceftriaxone in body weight

The effects of morphine and beta-lactams (MC-100093 and ceftriaxone) in body weight were evaluated throughout the days of i.p. injections of these drugs. Statistical analysis revealed main effect of treatment [F (3,187) = 4.18; $p < 0.01$]. However, the analysis did not reveal main effect of time (F (5, 187) = 0.1679861, $p > 0.05$) or interaction (F (15, 187) = 0.04776768, $p > 0.05$). Statistical analysis did not show any significant difference in body weight between control, morphine and beta-lactams treated groups ($p > 0.05$) (Fig. 2).

3.2. Effects of MC-100093 and ceftriaxone in locomotor activity, percentage of spontaneous alternation behavior and Y maze behavior in mice exposed to repeated moderate dose of morphine and single higher dose of morphine

Statistical analysis revealed significant changes in the total distance travelled among groups (F (3, 21) = 15.44, $p < 0.0001$). In addition, the analysis showed significant increase in total distance travelled in morphine group as compared to control group (t = 5.364, df = 11, $p < 0.001$), morphine group as compared to morphine-ceftriaxone group (t = 4.651, df = 11, $p < 0.001$), and morphine group as compared to morphine-MC100093 group (t = 2.386, df = 11, $p < 0.05$) (Fig. 3**A**). Statistical analysis did not show significant changes in the spontaneous alternation percentage (SAP) among groups (F (3, 21) = 1.281, $p > 0.05$). However, there was a decrease in SAP in morphine treated group as compared to control saline group (t = 2.268, df = 11, $p < 0.05$) (Fig. 3B). There were no significant differences between morphine and morphine-MC100093 (t = 0.3558, df = 11, $p > 0.05$) or morphine and morphine-ceftriaxone groups (t = 1.199, df = 11, $p > 0.05$) in the SAP (Fig. 3B). Additionally, statistical analysis showed significant changes in the number of arm entries in Y maze among the groups (F (3, 21) = 3.263, $p < 0.05$). The analysis revealed that there was an increase in the number of arm entries in Y maze in morphine-treated group as compared to control saline group (t = 2.510, df = 11, $p < 0.05$) (Fig. 3C). However, there were no significant difference in the number of arm entries in Y maze between morphine and morphine-ceftriaxone (t = 0.3625, df = 11, $p > 0.05$) or morphine and morphine-MC100093 (t = 0.4805, df = 11, $p > 0.05$) groups (Fig. 3C).

3.3. Effects of MC-100093 and ceftriaxone in GLT-1 and xCT protein expression as well as IL-6 and TGF-β mRNA expression in the nucleus accumbens in mice exposed to escalated doses of morphine

We further investigated whether ceftriaxone and MC-100093 would

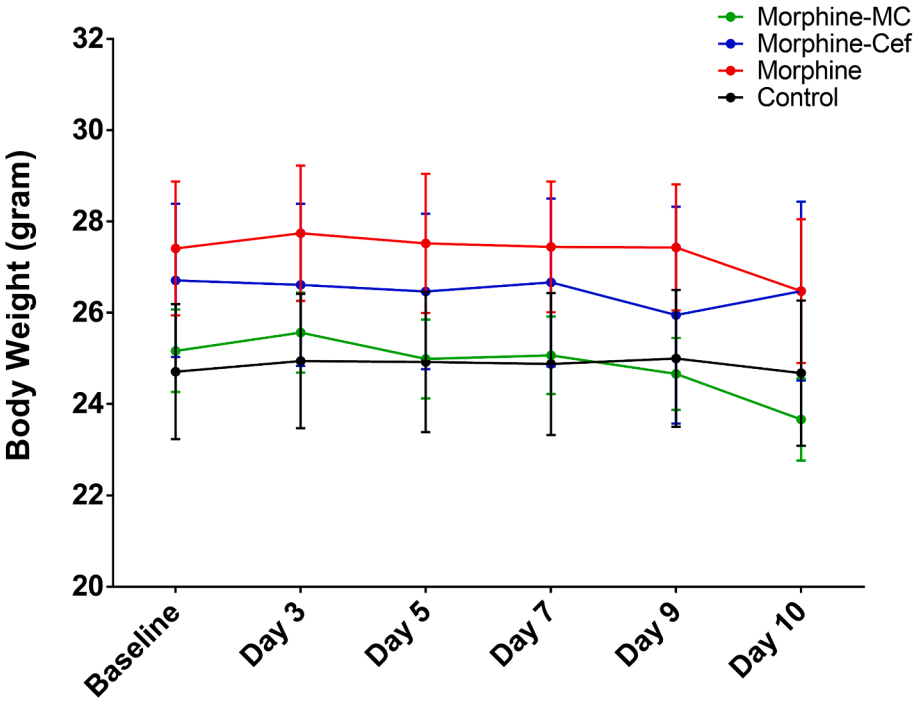

Fig. 2. Effects of repeated exposures to moderate dose and single higher dose of morphine and treatment with ceftriaxone and MC-100093 in body weight of male C57BL/6 mice. Statistical analyses didn't show any significance difference between control and treatment groups in body weight. N = 8–9/group.

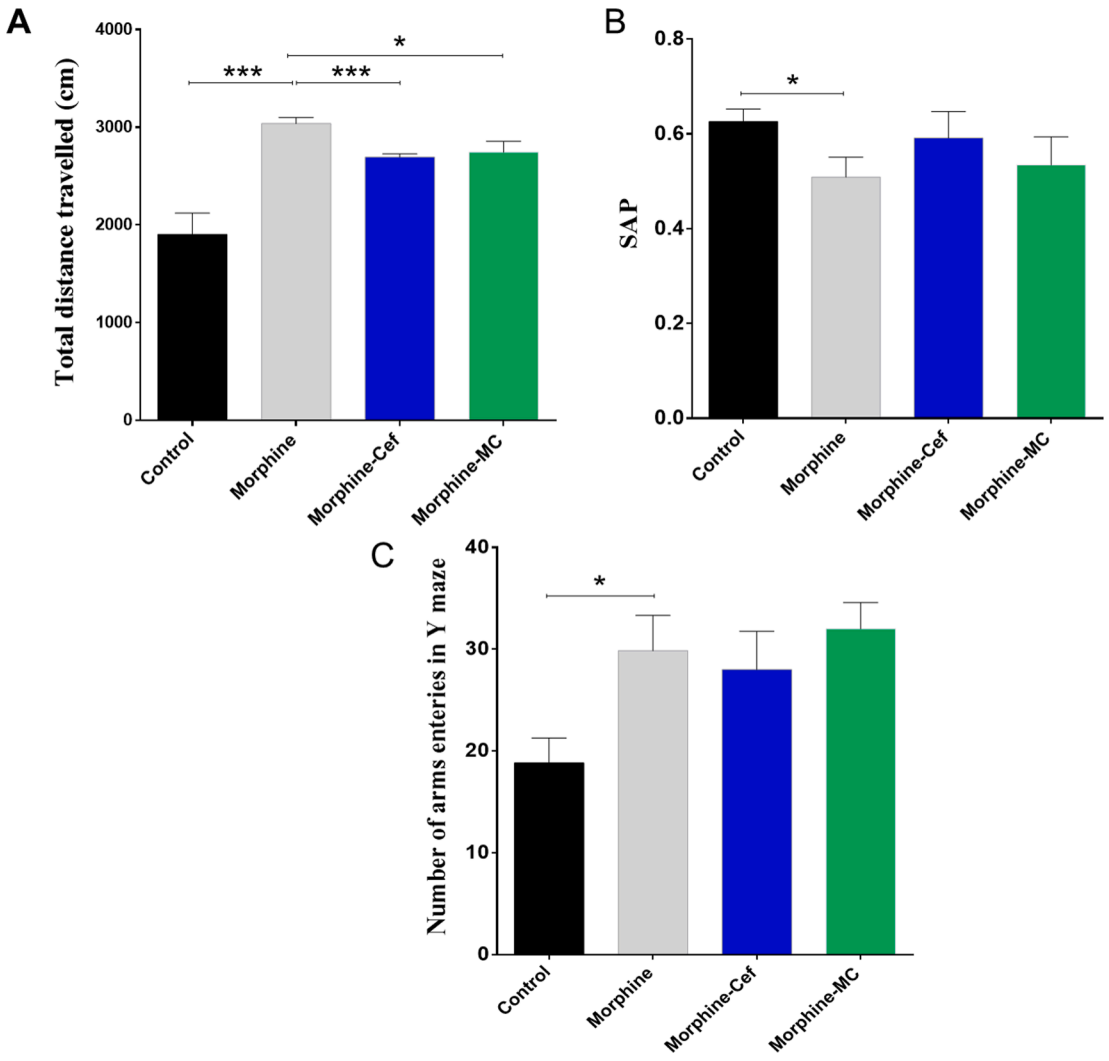

Fig. 3. Effects of ceftriaxone and MC-100093 in locomotor activity and short memory Y maze in mice exposed to repeated moderate dose and single higher dose of morphine. (A) Locomotor behavioral analysis showed that ceftriaxone and MC-100093 attenuated morphine-induced increases in total distance travelled. B) Short memory Y maze analysis did not show any significance difference in the spontaneous alternation percentage (SAP) between morphine, and ceftriaxone-morphine- and MC-100093-morphine-treated groups. C) Statistical analysis did not show any significance difference in the number of arms entries in Y Maze between morphine and ceftriaxone-morphine and MC-100093-moprhine treated groups. *$p < 0.05$; ***$p < 0.001$; N = 6–7 per group.

attenuate the effects of escalated doses of morphine in the expression of GLT-1 and xCT proteins, and IL-6 mRNA and TGF-β mRNA in the NAc. One-way ANOVA did not show significant changes in GLT-1 protein expression in the NAc among the groups ($F(3, 16) = 3.139$, $p = 0.0545$). However, the statistical analysis, using Fisher's LSD for multiple comparisons test, revealed a significant decrease in GLT-1 expression in morphine group as compared to control group ($p < 0.05$), and MC-100093 and ceftriaxone treatments attenuated morphine-induced downregulation of GLT-1 expression in the NAc ($p < 0.05$) (Fig. 4). Moreover, one-way ANOVA revealed significant changes in xCT protein expression in the NAc among the groups ($F(3, 16) = 3.706$, $p < 0.05$). The statistical analysis revealed downregulation of xCT expression in the NAc in morphine group as compared to control group ($p < 0.01$), and MC-100093 and ceftriaxone attenuated morphine-induced downregulation of xCT expression in the NAc ($p < 0.05$) (Fig. 4). Furthermore, statistical analysis showed significant changes in IL-6 mRNA expression in the NAc among the groups ($F(3, 16) = 5.427$, $p < 0.01$). The analysis revealed an increase of IL-6 mRNA expression in the NAc in morphine group as compared to control group ($p < 0.05$), MC-100093-morphine group ($p < 0.01$) and ceftriaxone-morphine group ($p < 0.01$) (Fig. 5). Finally, one-way ANOVA did not show significant changes in TGF-β mRNA expression in the NAc among the groups ($F(3, 16) = 3.166$, $p = 0.0532$). However, the statistical analysis revealed an increase of TGF-β mRNA expression in the NAc in morphine group as compared to control saline group ($p < 0.05$), and this also was observed in morphine-MC100093 group compared to control group ($p < 0.05$) (Fig. 5).

3.4. Detection of serum morphine concentrations of morphine after exposure to higher dose of morphine using validated GC–MS technique

We determined the serum concentrations of morphine at 1 h and 24 h after exposure to higher dose of morphine in C57BL/6 mice. Firstly, we validated the detection methodology after determining LOQ, LOD, recoveries, precision, accuracy, linearity, and the coefficient of determination (R^2) (Table 2). After validating the methodology of morphine detection in the serum, we determined the concentrations of morphine in the serum at 1 h and 24 h after exposure to higher dose of morphine. Our serum samples showed that the concentrations of morphine in the serum were higher after 1 h of i.p. injections and decreased by ~ 40 % after 24 hrs (Table 3, N = 5/ for each group).

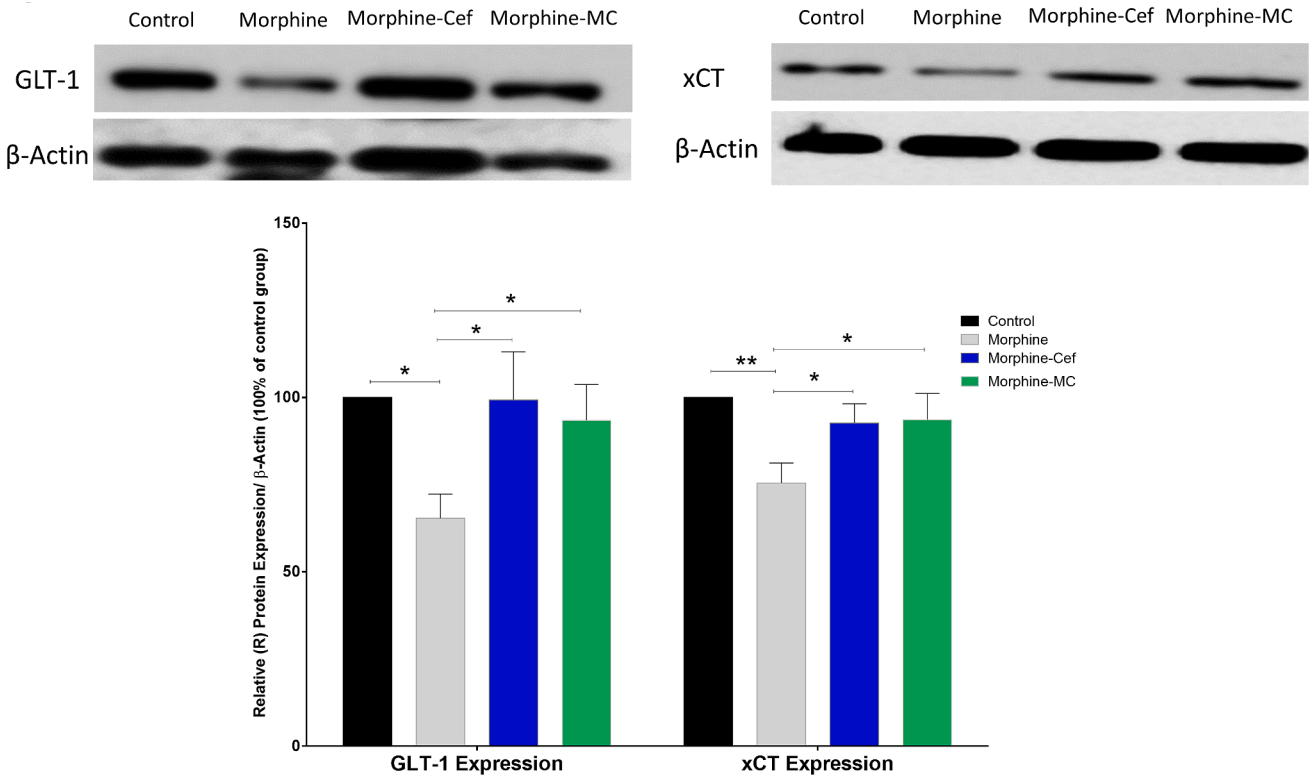

Fig. 4. Effects of ceftriaxone and MC-100093 in protein expression of GLT-1 and xCT in the NAc of mice exposed to repeated moderate dose and single higher dose of morphine. Quantitative analysis of Western blots showed that morphine exposure downregulated GLT-1 protein expression in the NAc as compared to control group, and ceftriaxone and MC-100093 increased GLT-1 protein expression as compared to morphine treated group. Similarly, morphine exposure downregulated xCT protein expression in the NAc, and ceftriaxone and MC-100093 increased xCT protein expression as compared to morphine treated group. Control group data are represented as 100 %, and data are represented as mean ± S.E.M. $*p < 0.05$; $**p < 0.01$. N = 5 per group.

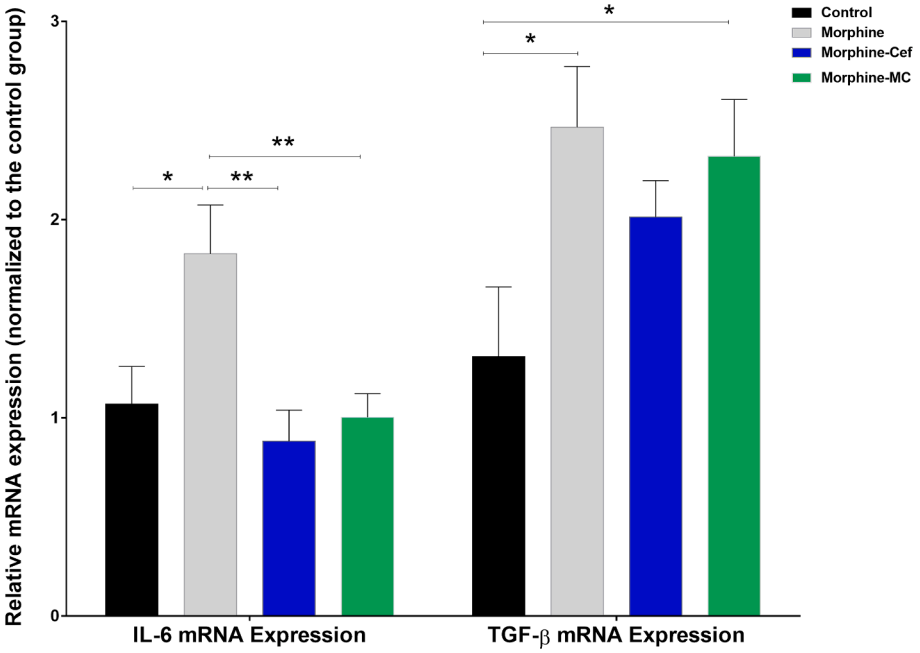

Fig. 5. Effects of ceftriaxone and MC-100093 in IL-6 mRNA (C) and TGF mRNA (D) in the NAc of mice exposed to repeated moderate dose and single higher dose of morphine. RT_PCR quantitative analysis showed that morphine exposure increased IL-6 mRNA expression in the NAc as compared to control group. Importantly, ceftriaxone and MC-100093 attenuated morphine-induced upregulation of IL-6 mRNA expression in the NAc. Furthermore, morphine exposure increased TGF-β mRNA expression in the NAc as compared to control group. There was a significant difference between control and morphine-MC100093 groups. However, there were no significance differences in the expression of TGF-β mRNA between morphine, morphine-MC100093 and morphine-ceftriaxone in the NAc. Control group data are represented as 100 %, and data are represented as mean ± S.E.M. $*p < 0.05$; $**p < 0.01$; N = 5 per group.

Table 2
The values of the validated parameters.

Validation Parameter	Values	
Linearity range	50–3000 ng/mL	
the coefficient of determination (R^2)	0.9945	
Limit of detection	10.91 ng/mL	
Curve limit of quantification	50 ng/mL	
Recovery	90.87 %- 112.96 %	
Accuracy (% bias)	Inter-assay	Intra-assay
	Within ± 11.8 %	Within ± 11.5 %
Precision (% RSD)	Inter-assay	Intra-assay
	Within ± 11.4 %	Within ± 15.5 %

Table 3
Serum concentrations of morphine in C57BL/6 mice 1 h and 24 h after overdose exposure.

Time after overdose exposure	Analyte	Internal Standard	Number of samples	Mean	Standard deviation
1 h	Morphine	Morphine D3	5	2131.11 ng/mL	407.34
24 h	Morphine	Morphine D3	5	1307.78 ng/mL	315.01

4. Discussion

This study tested moderate dose of morphine 20 mg/kg (i.p.) as repeated dosing and then acute higher dose of morphine 150 mg/kg (i.p.), which is considered as an overdose but not lethal since study reported that animals exposed to morphine at higher dose (~223 mg/kg, i.p.) may lead to 60–80 % mortality (Strandberg et al., 2006). Based on the finding from this latter study, we have chosen 20 mg/kg (i.p.) morphine as moderate dose and 150 mg/kg (i.p.) as higher dose, which is considered sublethal. Thus, this study reports that exposure to escalated doses of morphine reduced the expression of two key astrocytic glutamate transporters, GLT-1 and xCT, and increased IL-6 and TGF-β expression in the NAc. In addition, exposure to escalated doses of morphine increased locomotor activity and the number of arm entries in Y maze, however, there was a decrease in the SAP. The effect of morphine on locomotor activity is in accordance with several studies tested morphine and other opioids (Brady and Holtzman, 1981;Niikura et al., 2013;Santos et al., 2023;Zhang and Kong, 2017). In addition, our study is in accordance with previous study demonstrating exposure to morphine reduced spontaneous alternation in mice (Kitanaka et al., 2015;Mohammadkhani et al., 2024). Other study showed reduction in SAP with exposure to morphine in rats (Alipour et al., 2023). Furthermore, another study showed that exposure to morphine during adolescent age alters short-term memory in adult rats (Azadi et al., 2021). Glutamate plays a critical role in memory and learning (McEntee and Crook, 1993). Indeed, study demonstrated that intracerebral injection of dihydrokainic acid, a GLT-1 blocker, impairs memory performance (Tian et al., 2019). This suggests the importance of modulating GLT-1 expression in memory performance associated with exposure to opioids.

Our present study focused on investigating GLT-1 as a major glutamate transporter that regulates most of the extracellular glutamate concentrations at the synaptic cleft (Danbolt, 2001), and xCT is a cystine/glutamate antiporter that regulates extracellular glutamate concentrations (Baker et al., 2002;Bannai, 1984). Both GLT-1 and xCT are colocalized in astrocytes to regulate excess of glutamate at the synaptic cleft. Studies from our group and others clearly showed that chronic exposure to drugs of abuse (e.g. ethanol, cocaine, methamphetamine, and opioids) can lead to downregulation of GLT-1 and xCT expression in the mesocorticolimbic brain regions; and this effect was associated with reduction in glutamate uptake and concomitant increase in extracellular glutamate concentrations at the synaptic cleft (Abulseoud et al., 2012;Aghajanian et al., 1994;Alasmari, et al., 2018; Alhaddad, et al., 2022;Alshehri et al., 2017;Das, et al., 2022;Das, et al., 2015;Han et al., 2008;Knackstedt, et al., 2010;Wong and Sari, 2023). It is important to note that downregulation of xCT may reduce extracellular glutamate concentration, which can lead to loss of glutamatergic tone mediated through presynaptic mGluR2/3, and consequently causing increased in synaptic glutamate release (Javitt et al., 2011; Moran et al., 2005). This suggests that the interaction between GLT-1, xCT and mGluR2/3 is one of the signaling mechanisms for regulating glutamate homeostasis. Downregulation of GLT-1 is key factor in the development of dependence and tolerance to drugs of abuse, including opioids (Abulseoud et al., 2022;Smaga et al., 2020). In accordance with our present finding, studies have shown that chronic exposure to morphine downregulated GLT-1 expression in the brain, an effect that was associated with an increase in the extracellular glutamate concentrations (Ozawa, et al., 2001;Wang, et al., 2016). In this study, xCT was also downregulated in the NAc with repeated exposures to moderate dose of morphine exposure and single higher dose of morphine, and this effect was also observed in a previous study from our laboratory that tested hydrocodone at lower dose with shorter duration of exposure (Alshehri, et al., 2018). In accordance, a recent study from our laboratory showed that exposure to moderate dose of hydrocodone for 14 days downregulated xCT as well as GLT-1 in the NAc, amygdala and dorsomedial prefrontal cortex (Wong and Sari, 2023). Together, studies from our laboratory and others suggest clearly that exposure to opioids can lead to downregulation of GLT-1 and xCT expression in the mesocorticolimbic brain regions, and this effect might be associated with hyperglutamatergic state, which is critical in the development of drug dependence and neurotoxicity. Several studies suggest that there is interaction between several glutamate transporters, including GLT-1, and opioid receptors and this might be critical for the pharmacological mechanism involving opioid dependence (Alasmari et al., 2022; Liang et al., 2014;Meyer et al., 2017;Reeves et al., 2021;Xia et al., 2006).

Furthermore, studies from our laboratory have been focused on finding drugs that upregulate GLT-1 expression to attenuate the effects of several drugs of abuse, including opioids, ethanol, cocaine, methamphetamine, and nicotine. Thus, normalizing GLT-1 expression in central reward brain regions using beta-lactams (e.g. ceftriaxone and ampicillin/sulbactam) has been critical in reducing ethanol intake, cocaine, methamphetamine, and nicotine seeking behaviors, and attenuating the effect of chronic exposure to opioids (e.g. hydrocodone) (Abulseoud et al., 2012; Alajaji et al., 2013; Knackstedt et al., 2010; Philogene-Khalid et al., 2022; Sari et al., 2009; Stennett et al., 2017; Wong and Sari, 2023, 2024). In fact, ceftriaxone, known to upregulate GLT-1, has been effective in animal models of neurological diseases and drugs of abuse involving a hyperglutamatergic state (Gao et al., 2020; Rothstein et al., 1992;Rothstein et al., 2005). In this study, we tested ceftriaxone, a beta-lactam antibiotic known to upregulate GLT-1 (Fan et al., 2018;Rothstein, et al., 2005;Sari, et al., 2009), and more importantly we tested our novel synthetic beta-lactam, MC-100093, which doesn't have any antibiotic action. MC-100093 has been found to upregulate GLT-1 in animal models of ethanol intake and cocaine seeking behavior (Alhaddad et al., 2022; Knackstedt et al., 2021; Leon et al., 2023). The present study revealed that this novel beta-lactam drug attenuated the effect of repeated exposures to moderate dose of morphine and single higher dose of morphine in GLT-1 and xCT expression in the NAc, and its effect was similar to ceftriaxone. This is the first study revealing the efficacy of MC-100093 to attenuate the downregulation of GLT-1 and xCT in the NAc of mice exposed to escalated doses of morphine. Modulating the expression of GLT-1 and xCT in the brain with MC-100093 as well as ceftriaxone in this animal model has been associated with a decrease in locomotor activity. This decrease in locomotor activity might be associated with modulation of glutamate over-excitation. In fact, previous study showed that blockade of glutamate uptake in the NAc through bilateral injection of l-*trans*-pyrrolidine-2,4-dicarboxylic acid increased locomotor activity (Kim and Vezina,

1999). In this present study, we found that MC-100093 as well as ceftriaxone reversed the effect of morphine-induced reduction in GLT-1 expression, and consequently may have an effect in reducing excess of glutamate at the synaptic cleft through the glutamate uptake mechanism involving astrocytes where GLT-1 and xCT are co-localized. The normalizing effect of glutamate signaling with these beta-lactams may have an effect in modulating locomotor activity affected by escalated doses of morphine.

In this study, we further explored the effects of morphine and beta-lactams on neuroinflammatory cytokine IL-6, and growth factor TGF-β in the NAc of mice exposed to escalated doses of morphine. It has been suggested that there is influence of inflammatory mediators on the expression of astrocytic glutamate transporters, including GLT-1 (Tilleux and Hermans, 2007). In addition, glutamate release and uptake from astrocytes, which expresses GLT-1 and xCT, are controlled by signaling pathways associated with inflammatory factors, including TNF-α (Vesce et al., 2007). In vitro study demonstrated that increased in the concentration of glutamate is associated with increase in IL-1β and TNF-α in neuronal culture (Ye et al., 2013). Thus, changes in glutamate homeostasis with exposure to opioids might have direct or indirect mechanism of action to produce cytokines, including IL-6. Our data related to the effects of exposure to escalated doses of morphine in the expression of IL-6 in the NAc are in accordance with study that showed tramadol exposure for eight weeks increased several pro-inflammatory cytokines, including IL-6, in the cerebrum of rats (Mohamed and Mahmoud, 2019). In addition, exposure of hippocampal cultures to morphine induced increases in neuroinflammatory cytokines, including IL-6 (Osmanlioglu et al., 2020). Alternatively, a recent study from our group revealed that exposure to repeated exposures to fentanyl moderate dose followed by higher dose of fentanyl induced increases in IL-6 in liver, and this effect was attenuated by ceftriaxone treatment (Alasmari et al., 2023). In the present study, both MC-100093 and ceftriaxone attenuated morphine-induced increase in IL-6 mRNA expression in the NAc. To the best of our knowledge, this study is one of the first to show the efficacy of MC-100093 and ceftriaxone in reducing the production of IL-6 as result of repeated exposures to moderate dose of morphine and single higher dose of morphine.

Although, morphine exposure induced increased in TGF-β mRNA expression, neither MC-100093 or ceftriaxone had any modulatory effect. This suggests that the modulatory effect of IL-6 might be mediated through changes in glutamate homeostasis involving GLT-1 and xCT, and TGF-β might be regulated through a different mechanism. It is important to note that morphine increased the expression of TNF-α, and this effect is suggested to be associated with increased in glutamate transmission (Shen et al., 2012). We believe that reduction in the expression of GLT-1 and xCT with morphine exposure may increase glutamate transmission or increase extracellular glutamate concentration in the synaptic cleft leading to oxidative stress and increased in cytokines as part of the inflammatory mechanism. MC-100093 and/or ceftriaxone attenuated morphine-induced downregulation of GLT-1 and xCT, and consequently attenuated morphine-induced increase in IL-6 expression. Additional studies are warranted to determine the mechanism of upregulating the expression of GLT-1 and xCT with MC-100093 and ceftriaxone in association with reduction of neuroinflammatory cytokines, including IL-6.

In conclusion, this study was the first to determine the efficacy of novel synthetic beta-lactam, MC-100093, in reversing the effects of repeated exposures to moderate dose and single higher dose of morphine in GLT-1 and xCT expression in the brain. MC-100093 and ceftriaxone attenuated morphine-induced increase in neuroinflammatory cytokine, IL-6. Finally, MC-100093 and ceftriaxone treatments attenuated the effect of morphine-induced increase in locomotor activity. The beneficial therapeutic uses of beta-lactams for the treatments of drug dependence have been extensively studied in preclinical animal models of drugs of abuse, including alcohol, opioids, cocaine, methamphetamine and other drugs of abuse (Abulseoud, et al., 2022;Esmaili-Shahzade-Ali-Akbari et al., 2023). Although, ceftriaxone has been one of the beta-lactams antibiotics studied to attenuate drug seeking behaviors involving through upregulation of GLT-1 and xCT, the novel synthetic beta-lactam, MC-100093, which doesn't have any antibiotic action, is now considered as a potential therapeutic drug to attenuate drug-induced downregulation of GLT-1, and consequently reducing drugs of abuse seeking behaviors as demonstrated by this present study and others (Alhaddad, et al., 2022;Knackstedt, et al., 2021;Leon, et al., 2023;Wong and Sari, 2023). Our future studies are focusing now on synthetizing new derivatives for increasing the efficacy and bioavailability of MC-100093, and expanding the preclinical studies for the identification of lead compound that may have potential clinical therapeutic effects for treating drug addiction as well as any other psychiatric disorders and neurological diseases involving hyperglutamatergic state.

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CRediT authorship contribution statement

Youssef Sari: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Ghadeer M.S. Swiss:** Data curation, Formal analysis, Validation. **Fatin A. Alrashedi:** Data curation, Formal analysis, Validation. **Kholoud A. Baeshen:** Data curation, Formal analysis, Validation. **Sultan A. Alshammari:** Data curation, Formal analysis, Visualization. **Shakir D. Alsharari:** Conceptualization, Methodology. **Nemat Ali:** Investigation, Methodology, Visualization. **Abdullah F. Alasmari:** Investigation, Resources, Software. **Ali Alhoshani:** Formal analysis, Resources, Validation, Visualization. **Alaa A. Alameen:** Data curation, Formal analysis, Validation. **Wayne E. Childers:** Methodology, Resources. **Magid Abou-Gharbia:** Conceptualization, Funding acquisition, Resources. **Fawaz Alasmari:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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