Mass Spectrometric Imaging of the Brain Demonstrates the Regional Displacement of 6-Monoacetylmorphine by Naloxone

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ABSTRACT: Overdose is the main cause of mortality among heroin users. Many of these overdose-induced deaths can be prevented through the timely administration of naloxone (NLX), a nonselective mu (μ)-, kappa (κ)-, and delta (δ)-opioid receptor antagonist. NLX competitively inhibits opioid-overdose-induced respiratory depression without eliciting any narcotic effect itself. The aim of this study was to investigate the antagonistic action of NLX by comparing its distribution to that of 6-monacetylmorphine (6-MAM), heroin’s major metabolite, in a rodent model using mass spectrometric imaging (MSI) in combination with liquid chromatography−tandem mass spectrometry (LC−MS/MS).

Male Sprague−Dawley rats (n = 5) received heroin (10 mg kg$^{-1}$) intraperitoneally, NLX (10 mg kg$^{-1}$) intranasally, and NLX injected intranasally 5 min after heroin administration. The animals were sacrificed 15 min after dose and brain tissues were harvested. The MSI image analysis showed a region-specific distribution of 6-MAM in the brain regions including the corpus callosum, hippocampal formation, cerebral cortex, corticospinal tracts, caudate putamen, thalamus, globus pallidus, hypothalamus, and basal forebrain regions of the brain. The antagonist had a similar biodistribution throughout the brain in both groups of animals that received NLX or NLX after heroin administration. The MSI analysis demonstrated that the intensity of 6-MAM in these brain regions was reduced following NLX treatment. The decrease in 6-MAM intensity was caused by its displacement by the antagonist and its binding to these receptors in these specific brain regions, consequently enhancing the opioid elimination. These findings will contribute to the evaluation of other narcotic antagonists that might be considered for use in the treatment of drug overdose via MSI.

1. INTRODUCTION

Worldwide, approximately 69 000 people die from an opioid overdose every year.1 According to the Centres for Disease Control and Prevention (CDC), in 2015, over 33 091 people died from an opioid overdose (including heroin and prescription drugs) in the United States.2 What is even more staggering is the dramatic increase in the number of drug overdose and opioid-related deaths each year. In fact, according to the CDC, heroin overdose mortality rates increased by 20.6% from 2014 to 2015.3 Heroin-induced deaths are primarily a result of respiratory depression.4

Opioid-induced respiratory depression primarily involves the medullary respiratory center.5 The chemoreceptors detect the levels of carbon dioxide in the blood by monitoring the pH changes in the blood. The medulla oblongata, which controls the ventilation, sends signals to the respiratory muscles.4,5 Heroin inhibits these chemoreceptors via mu (μ)-opioid receptors and through the μ- and delta (δ)-opioid receptors in the medulla.5,6 This results in a decreased sensitivity of the chemoreceptors to the increased level of carbon dioxide in the system, leading to respiratory depression.5,7 The reported lethal dose of heroin in humans ranges approximately from 12 to 180 mg.8,9 The reported LD$_{50}$ of heroin is 22 mg kg$^{-1}$ intravenously (iv) and 23 mg kg$^{-1}$ (iv) in mice and rats, respectively.10 Most deaths occur approximately 1−3 h after the injection of the drug, thus providing a sufficient window for pharmaceutical intervention.11

Many of the deaths caused by heroin overdose can be prevented through the administration of naloxone (NLX) (1-N-allyl-14-hydroxynordihydromorphinone), a nonselective μ-, kappa (κ)-, and δ-opioid receptor antagonists, with a high affinity for the μ-opioid receptor.12,13 NLX is a safe and effective antagonist used in emergency situations to com-
petitively inhibit the pharmacologic effects of narcotics, including respiratory depression, following opioid administration.13–16 These antagonists bind to the opioid receptor sites, thereby preventing the binding of opiates without enforcing any narcotic effect itself.17,18 NLX has high lipophilicity and is able to rapidly cross the blood–brain barrier (BBB).19–20 Consequently, the antagonist exerts an immediate onset of action with clinical effects being observed as early as 3 min after intravenous (iv) and intranasal (in) administration.21–25 NLX is commonly administered via iv injection; however, there is a growing interest in the route as recent studies have shown it to be clinically effective in rapidly reversing opioid-induced pharmacologic effects in an emergency situation.23–25 This is especially important since the nasal cavity provides direct access to the central nervous system (CNS) via the olfactory pathways.22,26 To effectively reverse the opiate overdose, the recommended clinical dose of NLX ranges from 0.4 to 1.2 mg, repeated up to 10 mg kg⁻¹ if necessary. One study has demonstrated that a NLX dose of 1.04 mg produces 50% receptor occupancy (enough to counteract opioid overdose) via a nontomographic positron detection system.21 Lewanowitsch et al. reported that a NLX dose of 3 mg kg⁻¹ counteracts the respiratory and analgesic effects of heroin (7 mg kg⁻¹) after intraperitoneal (ip) administration.27 There is increasing evidence highlighting the importance of NLX in the treatment of opioid toxicity, consequently decreasing the mortality and morbidity.28

Heroin is rapidly transformed into its active metabolites, 6-MAM and morphine, with studies showing that the narcotic effects of heroin occur primarily via its major metabolite, 6-MAM.29,30 In addition, the maximal brain concentrations (T_max) of 6-MAM were achieved at 15 min after heroin administration,28,31 similar to the reported T_max of NLX.32,33 Thus, it was pivotal to investigate the antagonistic activity of NLX against the active heroin metabolite, 6-MAM and after 15 min of the drugs’ administration. Furthermore, we have previously demonstrated that the localization of 6-MAM in the rat brain correlates well with the distribution of μ-opioid receptors, using mass spectrometric imaging (MSI).31 Recently, our group reported the brain distribution of methadone and naltrexone in brain regions that are densely packed with μ-opioid receptors, the primary site of action of heroin, via MSI in the rodent model.35 Therefore, the MSI technique can be used to investigate the antagonistic properties of drugs being used in the treatment of heroin overdose. The aim of this study was to understand the effects of NLX, an opioid antagonist used in life-threatening emergency settings, to reverse an opiate overdose by comparing its distribution to that of 6-MAM via MSI technology.

2. RESULTS

NLX and 6-MAM were simultaneously quantified using the developed LC−MS/MS method resulting in retention times (RT) of 5.8 and 6.6 min, respectively. The transitions monitored were as follows: NLX (328 → 310 m/z), 6-MAM (328 → 268 m/z), and internal standard (342 → 324 m/z). The limit of detection (LOD) was determined to be 0.1 and 4 ng mL⁻¹ for NLX and 6-MAM in both brain and plasma, respectively. The lower limit of quantification (LLOQ) determined was 1 ng mL⁻¹ for NLX and 20 ng mL⁻¹ for 6-MAM in both matrices. NLX and 6-MAM had mean recoveries of >85% in both matrices, at three QC levels (LQC, MQC, and HQC) with a relative standard deviation (RSD) values of less than 15%, which is within the limits set by the European Medicine’s Agency (EMA) (Supporting Information Table S1). Three QC levels were evaluated for intraday and interday precision and accuracy for both analytes, with the %RSD of below 15%, in compliance with EMA guidance (see Supporting Information Tables S2 and S3).36

The mean brain concentrations of NLX and 6-MAM following the administration of 10 mg kg⁻¹ of each of the drugs to healthy Sprague–Dawley (SD) rats are presented in Figure 1. The mean plasma concentrations of both analytes are provided in the Supporting Information in Figure 1. The concentrations of 6-MAM in the plasma and brain were 201.47 ± 8.95 and 1198.80 ± 7.14 ng mL⁻¹, respectively. Following the administration of NLX 5 min after heroin, the concentrations of 6-MAM were 186.11 ± 7.95 and 545.14 ± 5.33 ng mL⁻¹ in the plasma and brain, respectively. The plasma NLX concentrations were 18.62 ± 3.73 and 1245.71 ± 10.20 ng mL⁻¹ in the brain. In animals pretreated with heroin, the concentrations of NLX were 15.90 ± 3.87 and 1193.66 ± 8.40 ng mL⁻¹ in the plasma and brain, respectively.

The MSI analysis of coronal brain tissue sections of NLX and heroin-treated animals, conducted using LIFT mode with a mass window of 310 + 0.25% m/z (NLX) and 268 ± 0.25% m/z (6-MAM), after 15 min of the drugs’ administration, with their corresponding hematoxylin and eosin stain (H&E) images are shown in Figure 2. The MSI analysis revealed a region-specific distribution of 6-MAM in the brain, localizing in the corpus callosum, hippocampal formation, cerebral cortex, corticospinal tracts, caudate putamen, thalamus, globus pallidus, hypothalamus, and basal forebrain regions (Figure 2A). The intensity of 6-MAM in these specific regions was reduced and replaced by NLX after treatment (Figure 2B). The antagonist was widely distributed throughout the brain but with highest intensities in the corpus callosum, caudate putamen, corticospinal tracts, and basal forebrain regions of the brain in both groups that received it via in administration (Figure 2D) and ip administration (Figure 2E).

3. DISCUSSION

Heroin is considered a prodrug, as the pharmacologic effects of heroin occurs primarily via its metabolite, 6-MAM.34 Following
demonstrating its short duration of action. Recent studies showing the rapid metabolism and elimination of NLX, were generated from brain tissues sectioned at Hematoxylin and eosin (H&E) stained brain section. All of the ion images were generated from 0 to 70% of the maximum intensity. All images administration (10 mg kg\(^{-1}\)) resulted in the high concentrations of 6-MAM (1193.66 \(\pm\) 119.31 ng mL\(^{-1}\)) after 30 min, after 10 mg kg\(^{-1}\) administered with saline, analyzed with the same mass filters as NLX) using LIFT mode with a mass window of 310 \(\pm\) 0.25% m/z (NLX), Hematoxylin and eosin (H&E) stained brain section. All of the ion images were generated from 0 to 70% of the maximum intensity. All images were generated from brain tissues sectioned at \(-0.82\) mm anterior bregma. Scale bar = 5 mm.

Figure 2. Coronal brain images of (A) 6-MAM after ip administration of a 10 mg kg\(^{-1}\) dose of heroin, followed 5 min later by in administration of NLX (10 mg kg\(^{-1}\)) to SD rats, and (C) control brain sections (animals administered with saline, analyzed with the same mass filters as 6-MAM) using the LIFT mode with a mass window of 268 \(\pm\) 0.25% m/z, total-ion-current (TIC) normalization. (D) NLX image after in administration of a 10 mg kg\(^{-1}\) dose of NLX. (E) NLX image after ip administration of heroin (10 mg kg\(^{-1}\)), followed 5 min later by in administration of NLX (10 mg kg\(^{-1}\)) to SD rats and (F) control brain sections (animals administered with saline, analyzed with the same mass filters as NLX) using LIFT mode with a mass window of 310 \(\pm\) 0.25% m/z (NLX). (G) Hematoxylin and eosin (H&E) stained brain section. All of the ion images were generated from 0 to 70% of the maximum intensity. All images were generated from brain tissues sectioned at \(-0.82\) mm anterior bregma. Scale bar = 5 mm.

the administration of NLX 5 min after heroin injection, the concentrations of 6-MAM in the brain is decreased. An explanation for the reduction in 6-MAM brain concentrations after the treatment of the antagonist is that the administration of NLX resulted in the replacement of 6-MAM and a more rapid clearance of the opiate from the brain. Similarly, Saccone et al. (2016) employed positron emission tomography (PET) imaging to demonstrate a more rapid clearance of the potent \(\mu\)-opioid receptor agonist, carfentanil from the brain in the presence of NLX in Rhesus monkeys. Even with this efficacy, NLX (\(T_{1/2} 30–40\) min\)) has a longer duration of action than 6-MAM (\(T_{1/2} 9–18\) min). NLX entered the brain rapidly, resulting in the high concentrations of 1245.71 \(\pm\) 10.20 ng mL\(^{-1}\) after 15 min of drug administration (10 mg kg\(^{-1}\) inh) and 1193.66 \(\pm\) 8.40 ng mL\(^{-1}\) after heroin injection (10 mg kg\(^{-1}\) ip), followed 5 min later by NLX (10 mg kg\(^{-1}\) in). Plasma NLX concentrations were low at 18 ng mL\(^{-1}\) after NLX administration (10 mg kg\(^{-1}\) inh) and 15 ng mL\(^{-1}\) in animals pretreated with heroin (10 mg kg\(^{-1}\) ip) and NLX (10 mg kg\(^{-1}\) inh). The rapid tissue penetration and high concentrations of NLX in the brain is responsible for its immediate onset of action and potency as an effective opioid antagonist. Similarly, Ngai et al. (1976) demonstrated rapid entry of NLX into the brain by showing high brain-to-serum concentrations ratio (2.7–4.6), following the iv administration of a 5 mg kg\(^{-1}\) dose. In a study of [\(^{14}\)C] labeled NLX, peak radioactivity was detected at 15 min, with 82% of the radioactivity reduced after 30 min, after 10 mg kg\(^{-1}\) sc dose, showing the rapid metabolism and elimination of NLX, demonstrating its short duration of action. Recent studies have shown that NLX is clinically effective for the reversal of opioid intoxication in an emergency setting and can be safely administered in prehospital and hospital environments. Intranasal route of administration has the advantage of rapid onset of action, bypassing the first-pass metabolism through the direct transport to the central nervous system via nasal mucosa.  

MSI was used to determine the distribution of 6-MAM in the brain tissue after the ip injection of heroin (10 mg kg\(^{-1}\)), followed 5 min later by the in administration of NLX (10 mg kg\(^{-1}\)). Ion distribution images of 6-MAM in brain section using matrix-assisted laser desorption ionization (MALDI)-imaging data set. Regions of interest (ROIs) were used to estimate the drug ion abundance in each brain compartment compared to the whole brain tissue. 6-MAM was localized in the corpus callosum, hippocampal formation, cerebral cortex, corticospinal tracts, caudate putamen, thalamus, globus pallidus, hypothalamus, and basal forebrain regions of the brain, in correlation with our previous study. The antagonist was widely distributed throughout the brain but with the highest intensities in dentate gyrus, hippocampus, within the limbic system, striatum, nucleus accumbens, fornix, cortex, and ventral pallidum, and thalamus and hypothalamic regions of the brain in both groups of animals that received NLX or heroin followed by NLX. These representative images demonstrate that the intensity of 6-MAM in these brain regions was markedly reduced following NLX treatment. The decrease in 6-MAM intensity is hypothesized to be caused by the displacement of bound 6-MAM by the antagonist and the binding of NLX to these receptors in these specific brain regions, consequently enhancing the opioid elimination.

The patterns of localization of NLX and 6-MAM are consistent with the distribution of opiate receptors in the brain, as reported by autoradiography studies. Trusk et al. detected elevated regional cerebral blood flow (RCBF) in specific brain regions including the cortex, basal ganglia, limbic system, midbrain tegmentum, superior colliculus, periaqueductal gray, internal capsule, and fornix following heroin.
administration. Geary et al. reported the localization of NLX in brain areas known to have high concentrations of opioid receptors including the striatum, nucleus accumbens, and cingulate cortex, using autoradiography.

These findings regarding the antagonistic activity of NLX are in correlation with previous reports. Saccone et al. employed PET imaging to demonstrate high radiotracer activity in the dense brain regions in opiate receptors, including the thalamus, striatum, and pons, after the injection of [11C]carfentanil (CFN) in male rhesus monkeys. NLX decreased the receptors available for binding, by 55–66%, with a high degree of [11C] CFN blockage observed in the parietal and frontal cortices of the brain. Similarly, Frost et al. reported high levels of carfentanil-induced activity in the brain regions dense in opiate receptors using PET. These activities were then reduced following NLX treatment, thereby demonstrating specific opiate receptor blockade in the brain. In addition, Xi et al., using fMRI demonstrated the ability of NLX to effectively reverse the heroin overdose, suggesting receptor-specific heroin-induced neuronal activation and inhibition.

Opioid receptors are highly concentrated in the respiratory control centres of the brain including the brainstem, insula, thalamus, and anterior cingulate cortex. The μ-opioid receptors are responsible for the opioid-induced analgesic effects and respiratory depression. Thus, since the physiological effects of heroin are mediated mainly by the activation of μ-opioid receptors, treatment with NLX selectively blocks opioid μ-receptor activity. Our findings show that action sites of heroin are region specific and consistent with the distribution of opioid μ-receptors in the brain. NLX was widely distributed throughout the brain, with high intensity in brain regions known to have high concentration μ-opioid receptors, indicating the nonselectivity property of the antagonist and its high affinity for opioid μ-receptors. These brain structures are involved in learning and memory (dentate gyrus structure of the hippocampus); movements (caudate putamen); memory reinforcement (the fornix part of the limbic system); endocrine-regulation (thalamus and hypothalamus); and smell (Piriform cortex part of the cerebrum).

4. CONCLUSIONS

The ability of the narcotic antagonist NLX to displace 6-MAM at specific sites in the brain was investigated using MSI. These results confirm that treatment with NLX after heroin administration resulted in the rapid removal of 6-MAM bound to opioid receptors in SD rat brain tissue. To the best of our knowledge, this is the first study to investigate the distribution of NLX in the brain and its antagonistic effects against 6-MAM, using MSI. We have demonstrated that MSI can be applied to study the distribution and antagonistic property of NLX in rat model, further demonstrating the antagonistic property of the drug. These findings will contribute to the evaluation of other narcotic antagonist properties of drugs that might be considered for use in the treatment of drug overdose via MSI.

5. MATERIALS AND METHOD

5.1. Chemicals and Reagents. NLX was purchased from Sigma-Aldrich (Munich, Germany), and naltrexone hydrochloride (internal standard) was bought from DLD Scientific (Gauteng, South Africa). Morphine purchased from Fresenius Kabi (Gauteng, South Africa) was subjected to acetylation to produce heroin as described previously by Tekleze et al. (2017); research protocol was approved by the University of KwaZulu-Natal Animal Research Ethics Committee (AREC/067/015D). Ammonium formate, acetic anhydride, trimethylamine, sodium fluoride, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (Munich, Germany). Liquid chromatography mass spectrometry (LC–MS) grade methanol, acetonitrile, and dichloromethane were obtained from Sigma-Aldrich (Munich, Germany). Formic acid (99%) was acquired from Merck Ltd (Durban, South Africa) and α-cyano-4-hydroxycinnamic acid (CHCA) was purchased from Bruker Daltonics (Bremen, Germany). IsoFor (isoflurane) was purchased from Safeline pharmaceuticals (Gauteng, South Africa). Water was purified using a Milli-Q purification ultrapure water system from Millipore Corporation (Bedford, MA).

5.2. Animal Experiments and Sample Collection. Male Sprague–Dawley rats (weight 120 ± 10 g) were purchased from the Biomedical Resource Unit (BRU), University of KwaZulu-Natal (Durban, South Africa). The animals were housed in polycarbonate cages in standard housing conditions (humidity 50–70%; temperature 21–24 °C; a 12 h light/dark cycle). The animals were provided with food and water ad libitum. The research protocol was approved by the University of KwaZulu-Natal Animal Research Ethics Committee (AREC/092/015D).

Animals were divided into four groups (n = 5) and received one of the following regimens; group 1 (control): saline only; group 2: heroin (10 mg kg⁻¹ ip) freshly prepared in a saline solution; group 3: NLX (10 mg kg⁻¹) freshly prepared in saline solution; and group 4: heroin (10 mg kg⁻¹ ip), followed 5 min later by NLX (10 mg kg⁻¹ in). For the in administration, the rats were first anesthetized with a cocktail of ketamine/xylazine at a dose of 80 and 20 mg kg⁻¹, respectively. The animals were placed in a supine position with their nose at an upright 90° angle. A micropipette (P10) was used to administer NLX drops at the opening of the nostrils, allowing the animal to snort the drop into the nasal cavity. Animals were then terminated 15 min after the administration of heroin (group 2) and NLX (groups 3 and 4) with an overdose of IsoFor. After euthanasia, blood was drawn by cardiac puncture into heparinized tubes containing sodium fluoride at a ratio of (1:4) to the final concentrations of 4 mg mL⁻¹ sodium fluoride to prevent the degradation of heroin and its metabolites.

Consequently, collected blood samples were centrifuged at 4 °C for 5 min at 3500 rpm. Aliquots of plasma (1.5 mL) were transferred and diluted 1:1 with ice-cold 10 mM formate buffer (pH 3.0, with 4 mg mL⁻¹ sodium fluoride) and immediately stored at −80 °C the before analysis. Subsequently, the brain was surgically removed, frozen in liquid nitrogen vapor, and immediately stored at −80 °C. Each frozen brain was divided into two hemispheres where one-half was allocated for MALDI-MSI–MS/MS analysis. The brain hemisphere was weighed and homogenized in three volumes of Milli-Q water (3 mL g⁻¹ tissue), and the homogenates were subjected to solid-phase extraction (SPE).

5.3. LC–MS/MS Conditions and Analysis. LC–MS/MS quantification has become the gold standard tool for assessing the concentration profiles of a wide range of therapeutics in different biological matrices. The high-performance liquid chromatography (HPLC) system was coupled with an amaZon speed ion trap (IT) fitted with an electrospray...
ionization (ESI) ion source from Bruker Daltonics (Bremen, Germany). The HPLC system included a Thermo Scientific Dionex UltiMate 3000 binary pump and an autosampler ( Dionex Softron GmbH, Germany). Chromatographic separation was conducted using a YMC C18 Triart (150 × 3.0 mm² and 3 μm particle size) analytical column (YMC Europe, Gmbh, Germany). A LC–MS/MS method was developed for the quantification of NLX and 6-MAM as per the European Medicine’s Agency guidelines (see Supporting Information for details).  

5.4. MSI Analysis. 5.4.1. Tissue Processing for MSI. NLX-or heroin-treated frozen brain tissues were cut into 10 μm thick sections and thaw-mounted onto indium titanium oxide (ITO)-coated glass slides (Bruker Daltonics, Germany) using a Leica CM 1100 cryostat (Leica, Germany). All brain tissues were sectioned at −0.82 mm anterior bregma. Prior to MSI analysis, the glass slides were desiccated overnight. MALDI matrix solution, α-cyano-4-hydroxycinnamic acid (CHCA, 7 mg mL⁻¹, in 50% ACN: 0.2% TFA), was applied onto the slides using an automatic matrix sprayer (ImagePrep, Bruker Daltonics, Germany). Calibration standards (NLX: 0.1–50 ng mL⁻¹ and 6-MAM: 20–1250 ng mL⁻¹) were spotted (1 μL) on an untreated brain section. The limit of detection (LOD) for each analyte was determined from the standards spotted on untreated brain tissue.

5.4.2. MALDI–TOF Mass Spectrometry Imaging. MSI experiments were conducted using an Autoflex III Smartbeam MALDI–TOF (Bruker Daltonics, Bremen, Germany) instrument with a FlexControl 3.4 (Bruker Daltonics, Germany) acquisition software. A LIFT method, in the positive-ion mode was employed to obtain the mass spectra with a detection m/z range of 260–394 and 200–380 for NLX and 6-MAM, respectively. The raster width for imaging was set at 100 μm. The images were normalized using the root mean square (RMS) method. The presence of the parent drug and unique fragmentation patterns were observed in each sample. The fragment mass of NLX (310 m/z and 6-MAM (268 m/z) were monitored and used for the visualization of the drug in the rat brain section. MS imaging data were processed using Flex Analysis 3.4 and FlexImaging 4.0 (Bruker Daltonics, Germany).

5.5. Statistical Analysis. Statistical analysis was conducted by GraphPad Prism V6.0 (GraphPad Software Inc., La Jolla) using an unpaired t-test and the Welch’s correction post-hoc test. Data is represented as a mean ± SD, a p < 0.05 was considered statistically significant.

■ ASSOCIATED CONTENT

4 Supporting Information  
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b03570.  
Method development and optimization; LC–MS/MS method validation data; and pharmacokinetics and brain distribution data (PDF)

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B.G.T., A.P., S.N., T.N., S.B., and S.M. designed the study. B.G.T., A.P., S.N., and S.M. performed all experiments. B.G.T., A.P., S.N., S.M., and S.B. wrote the manuscript. N.D.G., T.N., H.K., T.G., and S.B. supervised and funded the acquisition for this work. All authors have read and reviewed the manuscript.

Notes  
The authors declare no competing financial interest.

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