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SHORT COMMUNICATION

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Investigations on the in vitro and in vivo metabolic fate of the new synthetic opioid desmethylmoramide using HPLC–HRMS/MS for toxicological screening purposes

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Abstract

New synthetic opioids are an increasing challenge for clinical and forensic toxicologists that developed over the recent years. Desmethylmoramide (DMM), a structural analogue of methadone, is one of the most recent appearances on the drug market. This study investigated its metabolic fate in rat and pooled human liver S9 fraction (pHLS9) to allow the identification of suitable urinary screening targets beyond the parent compound. The analysis of rat urine after the administration of DMM revealed five metabolites, which were the result of pyrrolidine ring or morpholine ring hydroxylation and combinations of them. Additionally, an *N*′,*N*-bisdesalkyl metabolite was formed. Incubations of DMM using pHLS9 revealed a pyrrolidine hydroxy metabolite, as well as an *N*-oxide. No Phase II metabolites were detected in either rat urine or incubations using pHLS9. The metabolism of DMM did in part comply with that of its archetype dextromoramide (DXM). Although morpholine ring hydroxylation and *N*-oxidation were described for DXM and detected for DMM, phenyl ring hydroxylation was not found for DMM but described for DXM. An analysis of 24 h pooled rat urine samples after DMM administration identified the hydroxy and dihydroxy metabolite as the most abundant excretion products, and they may, thus, serve as screening targets, as the parent compound was barely detectable.

KEYWORDS
desmethylmoramide, HPLC–HRMS/MS, metabolism, new synthetic opioids

1 | INTRODUCTION

New synthetic opioids (NSO) are an increasing threat to public health.[1] During the last years, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) and United Nations Office on Drugs and Crime (UNODC) registered an elevated share of NSO in seized drugs of abuse.[2,3] Substances that appeared on the drug market were mainly fentanyl analogues, as well as novel entities such as U-47700 and MT-45.[1,4] However, methadone analogues such as dipyanone and desmethylmoramide (DMM) are entering the market as well.[4] DMM is a structural analogue of dextromoramide (DXM) and was developed by Janssen et al in 1957 in order to find new potent analgesics, but was never marketed.[5] Their chemical structures are shown in Figure 1. The analgesic activity of DMM was described by Janssen et al to be slightly lower than that of morphine in mice and rats and about a third of methadone.[5] Additionally, a recent study by

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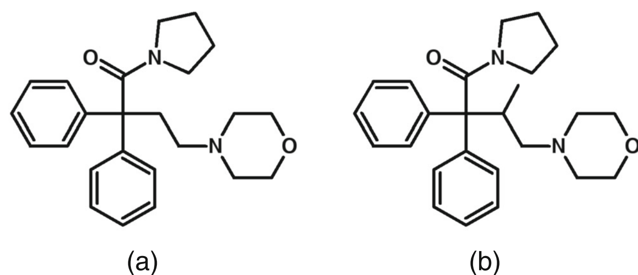

FIGURE 1 Chemical structures of (a) desmethylmoramide and (b) dextromoramide.

Vandeputte et al. performed in vitro functional characterization at the μ-opioid receptor and reported distinctive lower potency and efficacy in comparison to morphine and methadone.[4]

So far, no studies have been published about the metabolism of DMM, and little is known about the metabolism of its archetype DXM. Caddy et al. discussed the hypothetic metabolism of DXM in human and found that numerous metabolites might be formed. Among them were hydroxylations of the morpholine and phenyl ring, as well as *N*-oxide formation.[6] However, they themselves merely described the detection of a metabolite formed after the morpholine ring hydroxylation in human urine and reported a personal communication with colleagues that detected an *N*-oxide.[6] Another metabolite, formed after the hydroxylation of one of the phenyl rings and served as a screening target in human urine using gas chromatography mass spectrometry (MS), was described by Maurer et al.[7]

Therefore, the aim of this study was to elucidate the metabolic fate of DMM in rat, as well as in pooled human liver S9 fraction (pHLS9) to allow DMM detection by high-performance liquid chromatography–high resolution–tandem MS (HPLC–HRMS/MS) in toxicological specimens.

2 | EXPERIMENTAL

2.1 | Chemicals and reagents

DMM was provided by Kaiserslautern University of Applied Sciences from online vendors based in Germany who offer NPS. Nicotinamide adenine dinucleotide phosphate ($NADP^+$), acetonitrile (LC–MS grade), diethyl ether, ethyl acetate, and methanol (LC–MS grade) were obtained from VWR International (Darmstadt, Germany). 3′-phosphoadenosine-5′-phosphosulphate (PAPS), *S*-adenosylmethionine (SAM), dithiothreitol (DTT), reduced glutathione (GSH), acetylcarnitine transferase (AcT), acetylcarnitine, acetyl coenzyme A (AcCoA), magnesium chloride ($MgCl_2$), dipotassium phosphate (K_2HPO_4), monopotassium phosphate (KH_2PO_4), superoxide dismutase, isocitrate dehydrogenase, isocitrate, tromethamine (Tris), ammonium formiate, and formic acid were purchased from Sigma (Taufkirchen, Germany). Purified water was obtained by using a Millipore filtration system (water resistance, 18.2 Ω × cm). Pooled human liver S9 (pHLS9), 20 mg protein/mL, from 30 individual donors; uridine diphosphate (UDP)-glucuronosyltransferase (UGT) reaction mix solution A (25mM UDP-glucuronic acid); and UGT reaction mix solution B (250mM Tris-hydrochloric acid [HCl], 40mM $MgCl_2$, and 0.125 mg/mL alamethicin) were obtained from Corning (Amsterdam, The Netherlands). Following the delivery, the pHLS9 were thawed at 37 °C, aliquoted, quick-frozen using liquid nitrogen, and stored at −80 °C until usage.

2.2 | In vitro drug metabolism studies using pHLS9

The pHLS9 incubations were executed in accordance with previously published papers.[8,9] The total volume of the final incubation mixture was 150 μL. These incubations, containing a final concentration of proteins of 2 mg/mL, were executed after a preincubation of 10 min at 37 °C using 25 μg/mL alamethicin (UGT reaction mix solution B), 90mM phosphate buffer (pH 7.4), 2.5mM Mg^{2+}, 2.5mM isocitrate, 0.6mM $NADP^+$, 0.8 U/mL isocitrate dehydrogenase, 100 U/mL superoxide dismutase, 0.1mM AcCoA, 2.3mM acetylcarnitine, and 8 U/mL AcT. Afterwards, 2.5mM UDP-glucuronic acid (UGT reaction mix solution A), 40 μM aqueous PAPS, 1.2mM SAM, 1mM DTT, 10mM GSH, and 25 μM substrate in phosphate buffer (pH 7.4) were added. All concentrations are final concentrations. The addition of the substrate initiated the reaction. Subsequently, the mixture was set to incubate for a total of 480 min. After an incubation time of 60 min, 60 μL were transferred into a test tube, where the reactions were terminated by adding 20 μL of ice-cold acetonitrile. The rest of the mixture was continuously incubated for the remaining 7 h, and the reactions were again stopped by adding 30 μL of ice-cold acetonitrile. After allowing to precipitate for 30 min at −18 °C, the samples were centrifuged for 2 min at 18,407 × *g*, and 60 μL of the upper phase were transferred into an MS vial. A 1-μL aliquot was finally injected into the high-resolution tandem mass spectrometer coupled to HPLC–HRMS/MS system. To assure the absence of interfering compounds as well as to identify compounds that did not originate from the metabolism of DMM, blank incubations without substrate and control samples without pHLS9 were prepared and analyzed.

2.3 | Rat urine samples for toxicological detectability

Male Wistar rats (Charles River, Sulzfeld, Germany) were used for detectability studies in accordance with the German law for animal protection. This study has been approved by an ethics committee (Landesamt für Verbraucherschutz, Saarbrücken, Germany). DMM was orally administered to the rat in a dose of 2 mg/kg body mass. Rats had water ad libitum during the collection of urine over a period of 24 h. Urine was caught separately from the feces. Before the compound administration, blank urine was collected to confirm the absence of distracting compounds. Creatinine was determined using a P.I.A2 immunoassay (Protzek, Lörrach).

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2.4 | Urine sample preparation

Sample preparation was performed according to a previous study.[10] First, 0.1 mL urine was mixed with 0.5 mL acetonitrile and shaken on a rotary shaker for 2 min at 2000 rpm. After centrifugation for 2 min at 18,407 × *g*, 0.5 mL of the supernatant was transferred into a glass vial and evaporated to dryness under a gentle stream of nitrogen at 70 °C. The residue was reconstituted in 50 μL of a mixture of eluent A and eluent B (1:1; *v/v*). Finally, 10 μL were injected into the HPLC–HRMS/MS system.

2.5 | HPLC–HRMS/MS apparatus

Apparatus conditions were set according to previously published studies.[11,12] Details can be found in the Supporting Information (Data S1).

3 | RESULTS AND DISCUSSION

3.1 | Tentative identification of DMM metabolites

The spectra of each detected DMM metabolite can be found in Figure S1 in the supplementary data. All *m/z* in this section are theoretical (exact) ones.

The parent compound (no. 1 in Figure S1) was detected at a retention time of 5.92 min and a respective precursor ion at *m/z* 379.2380 ($C_{24}H_{31}O_2N_2$). The precursor ion underwent an elimination of the morpholine ring resulting in the fragment ion at *m/z* 292.1701 ($C_{20}H_{22}ON$) that represented the base peak. Subsequently, a benzylic scission led to the fragment ion at *m/z* 264.1388 ($C_{18}H_{18}NO$), and a rather unusual elimination of CO resulted in the fragment ion at *m/z* 248.1439 ($C_{17}H_{18}N$). Such concerted eliminations have already been described by Demarque et al. for cyclic carbonyl compounds, and its occurrence is also observable in the electrospray mass spectra for DXM.[13,14] It is likely that the formation of this ion was favored because of the positive mesomeric effect of the pyrrolidine ring that further stabilized the phenylic carbenium ion, whereas the presence of a carbonyl group destabilized it because of its negative mesomeric effect. At last, the elimination of the pyrrolidine ring led to the formation of a fragment ion at *m/z* 167.0861 ($C_{13}H_{11}$). The lowest detected *m/z* was the fragment ion at *m/z* 98.0606 (C_5H_8NO), representing the amide moiety after an α-elimination. In general, the fragmentation of DMM corresponded well to that of DXM.[14]

The hydroxy metabolite (no. 3 in Figure S1) was identified because of its characteristic shifts of fragments of the MS^2. The parent ion was detected with *m/z* 395.2335 ($C_{24}H_{31}O_3N_2$) and the base peak was again formed after morpholine elimination, resulting in the fragment ion at *m/z* 308.1637 ($C_{20}H_{22}O_2N$). The introduction of the hydroxy group was assumed to have occurred in the ortho position of the pyrrolidine nitrogen for two reasons. First, Vickers et al. investigated the biotransformation of nitrogen-containing xenobiotics and found that most hydroxylations occur in the ortho position of the nitrogen.[15]

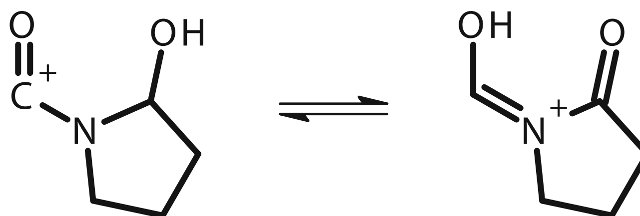

FIGURE 2 Contributing structures to the fragment ions with *m/z* 98.0606 (C_5H_8NO) detected for metabolites after pyrrolidine ring hydroxylation.

Additionally, only pyrrolidine ring-containing fragments displayed a characteristic shift of *m/z* 15.9949 such as the fragment ion at *m/z* 308.1637 ($C_{20}H_{22}O_2N$), *m/z* 252.1388 ($C_{17}H_{18}ON$), and *m/z* 114.0555 ($C_5H_8O_2N$) that were formed after the elimination of the morpholine ring, subsequent CO elimination, or the α-cleavage of the amide moiety, respectively. However, an elimination of water by a shift of *m/z* 18.0106 was not detected, although this fragmentation step is highly characteristic for aliphatic hydroxylations. It is quite likely that the elimination of water was prevented by the delocalization of the electrons over the carbonyl group, the amine moiety, and the hydroxy group. Contributing structures are displayed in Figure 2.

The *N*-oxide metabolite (no. 4 in Figure S1) was detected at a retention time of 5.96 min, and the precursor ion at *m/z* 395.2335 ($C_{24}H_{31}O_3N_2$). Although the *m/z* of the parent ion indicated that oxygen was introduced into this metabolite, the fragmentation of this metabolite corresponded to that of the parent compound. Therefore, the site of the oxidation was likely to be at the morpholine ring. Additionally, the retention time of this metabolite was higher than that of the parent compound, which indicated that this metabolite was in fact an *N*-oxide. Several studies made similar observations with retention times of *N*-oxides being higher than that of the parent compound after analysis using reversed-phase columns.[12,16,17] All other metabolites were identified accordingly.

3.2 | Proposed metabolic pathways of DMM in rat and pHLS9

Metabolic pathways of DMM in rat and pHLS9 are displayed in Figure 3. The blank urine resulted in 73 mg/dL, and the urine after DMM administration resulted in 95 mg/dL creatinine. Five metabolites were detected after the analysis of rat urine. The main metabolic pathway was hydroxylation of the pyrrolidine (no. 3 in Figure 3) or the morpholine ring (no. 6 and 7 in Figure 3), which occurred once or twice and in combination with each other (no. 5, 6, and 7 in Figure 3). The metabolite formed after pyrrolidine ring hydroxylation and *N,N′*-bisdesalkylation (no. 2 in Figure 3) might have been formed in two ways. One way might have been a double retro-hemiaminal addition of the metabolite formed after two hydroxylations in ortho position of the nitrogen (no. 7 in Figure 3); the other way might be a further oxidation of metabolite no. 7 in Figure 3 to lactams and subsequent hydrolyzation. No Phase II metabolites were detected.

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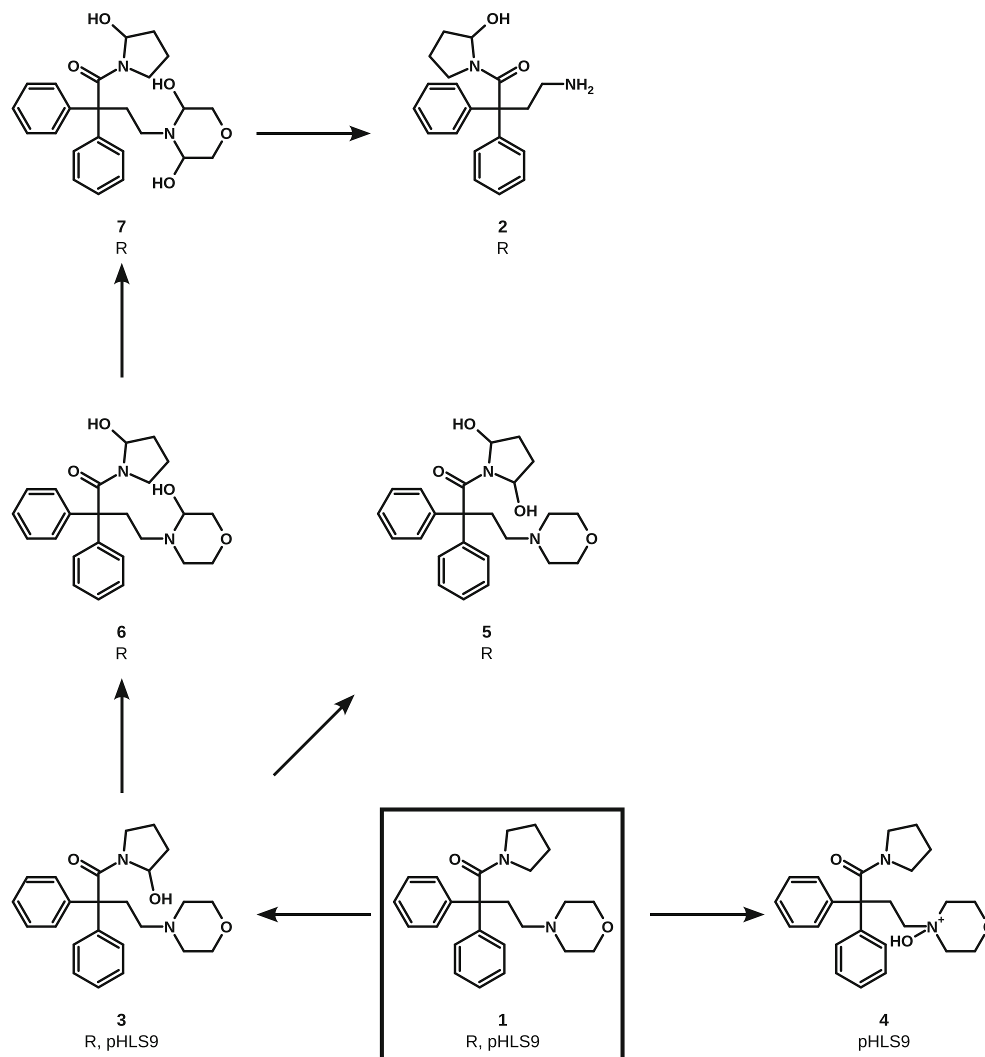

FIGURE 3 Metabolic pathways of desmethylmoramide in vivo and in vitro. Metabolites are numbered according to Figure S1. R, rat urine; pHLS9, pooled human liver S9 fraction.

Incubations using pHLS9 revealed the formation of two metabolites. One metabolite was formed after pyrrolidine ring hydroxylation and another one after *N*-oxidation. Neither glucuronides, nor sulfates were detected.

The metabolites detected in this study merely comply with the metabolites that were described for DXM to some extent.[6,7] No phenylic hydroxylation was found for DMM, and although morpholine hydroxylation was found, the main metabolic pathway appeared to be the hydroxylation of the pyrrolidine ring. However, the *N*-oxidation that was described for DXM was also detected for DMM in incubations using pHLS9. It is notable that no Phase II metabolites were detected within this study. This may be because glucuronidation und sulfation are usually observed for compounds forming aromatic hydroxy groups which were not observed for DMM.[18,19]

4 | CONCLUSION

This study investigated the metabolic fate of DMM in rat, as well as in incubations using pHLS9. In total, six Phase I metabolites were detected, but not Phase II metabolites. Metabolic pathways consisted of hydroxylation of the pyrrolidine ring and the morpholine ring, as well as combinations of them. Additionally, an *N*,*N*′-bisdesalkyl metabolite and an *N*-oxide were detected. The metabolic pathways that were detected within this study do merely comply with that of DXM to some extent. Although phenyl ring hydroxylation was not found, the hydroxylation of the morpholine ring did occur, but is apparently not the main metabolic pathway of DMM. However, *N*-oxidation was described for DXM and detected within this study. Finally, the hydroxy and dihydroxy might be recommended as LC–HRMS/MS screening target, as they were the most

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abundant in rat urine and pHLS9 incubations, especially because the parent compound was scarcely detectable in rat urine.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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