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RESEARCH ARTICLE

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# Metabolism of highly potent synthetic opioid nitazene analogs: *N*-ethyl-*N*-(1-glucuronyloxyethyl) metabolite formation and degradation to *N*-desethyl metabolites during enzymatic hydrolysis

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## Abstract

The metabolism of the highly potent synthetic opioids metonitazene, etonitazene, and protonitazene was investigated in fresh human hepatocytes. In the hydrolyzed culture medium, *N*-desethyl-, *N*,*N*-di-desethyl-, *O*-desalkyl-, *N*-desethyl-*O*-desalkyl-, *N*,*N*-di-desethyl-*O*-desalkyl-, and *N*-oxidated metabolites were detected as phase I metabolites, whereas in the unhydrolyzed culture medium, *O*-glucuronides of phase I metabolites with *O*-dealkylation were detected as phase II metabolites. The detected phase I metabolites were identified by comparing their analytical data with those of synthesized authentic standards. In contrast, phase II metabolites were identified by comparing their analytical data with those of the glucuronidated products formed by the incubation of the corresponding substrates with human liver microsomes in the presence of uridine diphosphate glucuronic acid. In addition to the aforementioned metabolites, some putative *N*-ethyl-*N*-(1-glucuronyloxyethyl) metabolites were detected in the unhydrolyzed culture medium. Purification and hydrolysis experiments revealed that *N*-ethyl-*N*-(1-glucuronyloxyethyl) metabolites formed the corresponding *N*-desethyl metabolites via unstable *N*-ethyl-*N*-(1-hydroxyethyl) metabolites during enzymatic hydrolysis.

**KEYWORDS**
human hepatocytes, metabolism, nitazene analogs, synthetic opioid

## 1 | INTRODUCTION

In the last decade, opioid poisoning has become a serious social problem, especially in North America. According to the National Institute on Drug Abuse (NIDA), more than 80,000 people died from opioid poisoning in 2021 in the USA, and approximately 88% of those deaths were attributed to synthetic opioid overdoses, primarily fentanyl and its analogs.[1] In addition, Centers for Disease Control and Prevention (CDC) reported that the age-adjusted rate of drug overdose deaths involving synthetic opioids other than methadone still remained significantly high in 2022.[2] With the tightening of regulations on fentanyl analogs, a new type of synthetic opioids, called nitazene analogs (nitazenes), has appeared in the illicit drug market recently.[3,4] Nitazenes were developed as powerful analgesics by the Swiss chemical company CIBA Aktiengesellschaft in the 1950s.[4] Some nitazenes, e.g., etonitazene (EtN, Figure 1), reportedly had higher analgesic potency than fentanyl in animal and in vitro experiments[3,5]; however, they have never been distributed as pharmaceutical drugs to date. In

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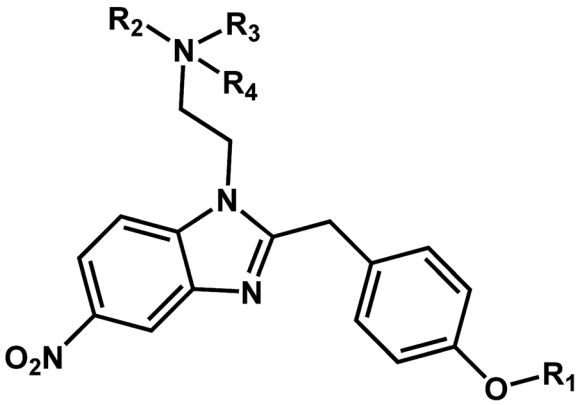


| Compound | $R_1$ | $R_2$ | $R_3$ | $R_4$ | Peak No. |
|---|---|---|---|---|---|
| Metonitazene (MetN) | $CH_3$ | $CH_2CH_3$ | $CH_2CH_3$ | H | - |
| *N*-desethyl-MetN (MetN-dEt) | $CH_3$ | H | $CH_2CH_3$ | H | 1 |
| *N*,*N*-di-desethyl-MetN (MetN-ddEt) | $CH_3$ | H | H | H | 2 |
| MetN-*N*-oxide (MetN-NOX) | $CH_3$ | $CH_2CH_3$ | $CH_2CH_3$ | O | 3 |
| *N*-(2-hydroxyethyl)-MetN (MetN-2OHEt) | $CH_3$ | $-CH_2CH_2OH$ | $CH_2CH_3$ | H | - |
| Etonitazene (EtN) | $CH_2CH_3$ | $CH_2CH_3$ | $CH_2CH_3$ | H | - |
| *N*-desethyl-EtN (EtN-dEt) | $CH_2CH_3$ | H | $CH_2CH_3$ | H | 12 |
| *N*,*N*-di-desethyl-EtN (EtN-ddEt) | $CH_2CH_3$ | H | H | H | 13 |
| EtN-*N*-oxide (EtN-NOX) | $CH_2CH_3$ | $CH_2CH_3$ | $CH_2CH_3$ | O | 14 |
| Protonitazene (ProtN) | $CH_2CH_2CH_3$ | $CH_2CH_3$ | $CH_2CH_3$ | H | - |
| *N*-desethyl-ProtN (ProtN-dEt) | $CH_2CH_2CH_3$ | H | $CH_2CH_3$ | H | 16 |
| *N*,*N*-di-desethyl-ProtN (ProtN-ddEt) | $CH_2CH_2CH_3$ | H | H | H | 17 |
| ProtN-*N*-oxide (ProtN-NOX) | $CH_2CH_2CH_3$ | $CH_2CH_3$ | $CH_2CH_3$ | O | 18 |
| *O*-desalkyl-nitazene (OHN) | H | $CH_2CH_3$ | $CH_2CH_3$ | H | 4 |
| *N*-desethyl-OHN (OHN-dEt) | H | H | $CH_2CH_3$ | H | 5 |
| *N*,*N*-di-desethyl-OHN (OHN-ddEt) | H | H | H | H | 6 |

**FIGURE 1** Chemical structures of nitazene analogs and their metabolites.

response to the recent re-emergence of nitazenes, probably produced mainly in clandestine laboratories as recreational synthetic opioids, some nitazenes have been placed under international drug control.[6]

Although reports on nitazenes have increased in the last few years, reports on the metabolism of nitazenes have been limited. Krotulski et al. detected *N*-desethyl, *O*-desalkyl, *N*-desethyl-*O*-desalkyl, and 5-amino metabolites of metonitazene (MetN, Figure 1) and isotonitazene (IsotoN, *O*-isopropyl derivative of MetN) in urine and blood samples.[7,8] Interestingly, *N*-desethyl metabolites of EtN and IsotoN were reported to have similar agonistic activity on μ-opioid receptors with their parent drugs, whereas *O*-dealkylation caused drastic decrease of the activity.[3] Murari et al. also investigated the metabolism of nine synthetic opioids including five nitazenes using a zebrafish larvae model and reported that the nitazenes underwent similar metabolism to that mentioned above (refs. 7 and 8).[9] However, in the reports of Krotulski et al. and Murari et al., the tentative identification of the metabolites was based only on mass spectral data alone, and not on comparison with synthesized authentic standards. Accurate mass spectral data are useful for estimating the chemical structures of unknown compounds, but chromatographic comparison with authentic standards is needed for unambiguous identification. For example, it was recently reported that furanylfentanyl formed a ring-opened carboxylic acid metabolite by using a chemically synthesized authentic standard.[10] Until then, furanylfentanyl was believed to form a dihydrodiol metabolite,[11] which had the same mass as the ring-opened carboxylic acid metabolite. These results indicated that the ring-opened carboxylic acid metabolite could have been misidentified as a dihydrodiol metabolite in an earlier study, because it had not been identified with an authentic standard.

In this study, we investigated the metabolism of three nitazenes, MetN, EtN, and protonitazene (ProtN; Figure 1), in fresh human hepatocytes. The metabolites formed in the culture medium were identified by comparing their analytical data with those of authentic synthesized standards. Additionally, phase II metabolites (glucuronides) in the culture medium were identified by confirming that glucuronides were formed by incubating the corresponding substrates with human liver microsomes in the presence of uridine diphosphate glucuronic acid (UDP-GlucA).

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## 2 | EXPERIMENTAL

### 2.1 | Chemicals and reagents

Authentic standards of nitazenes and their metabolites were synthesized in our laboratory based on previously reported methods.[12] Fresh human hepatocytes (PXB-cells®) and culture medium for PXB-cells were purchased from PhoenixBio Co., Ltd. (Higashi-Hiroshima, Japan). Human liver microsomes (HLM; pool of 150 donors) were purchased from Corning Inc. (Corning, NY, USA). B-One® β-glucuronidase was purchased from KURA biotech, Inc. (Atlanta, GA, USA). All the other reagents were of analytical grade, and were purchased from Fujifilm Wako Pure Chemical Corp. (Osaka, Japan) and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

### 2.2 | Synthesis of nitazene metabolite authentic standards

The chemical structures of the nitazene metabolites were confirmed using accurate mass and nuclear magnetic resonance (NMR) data (Table S1, Figures S1 and S2). Accurate mass data were obtained using a Thermo Q Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). NMR data ($^1$H, $^1$H–$^1$H correlation spectroscopy [COSY], heteronuclear multiple quantum coherence [HMQC], and heteronuclear multiple bond correlation [HMBC]) were obtained using a JNM-ECA600 NMR spectrometer equipped with an auto-tune 5 mm FG/TH tunable probe (JEOL, Akishima, Japan). A 1 mg sample of each compound was dissolved in 0.53 ml of methanol-d4 and transferred to an NMR tube. The parameters used for the NMR experiments are summarized in Table S2.

#### 2.2.1 | *N*,*N*-Diethyl-2-[2-(4-hydroxybenzyl)-5-nitro-1*H*-benzimidazole-1-yl]ethanamine (OHN, **4**)

To a solution of 670 mg of 4-benzyloxyphenylacetonitrile (3.0 mmol) in 2.1 ml of ethanol (36 mmol) in an ice bath, 1.7 ml of acetyl chloride (24 mmol) was added, followed by stirring for 3 h at room temperature. The solvent was evaporated to dryness under vacuum, and 4 ml of 1-butanol and 413 mg of 4-nitro-1,2-phenylenediamine (2.7 mmol) were added and refluxed for 20 h. The solvent was evaporated to dryness under vacuum, and the residue was purified by flash chromatography (column, silica gel; solvent, chloroform/methanol) to obtain 330 mg of 2-(4-benzyloxybenzyl)-5-nitro-1*H*-benzimidazole (0.92 mmol, 34%).

To a solution of 330 mg of 2-(4-benzyloxybenzyl)-5-nitro-1*H*-benzimidazole (0.92 mmol) in 10 ml of dry tetrahydrofuran in an ice bath, 74 mg of sodium hydride (60%; dispersed in paraffin liquid; 1.9 mmol) was added, and then, the solution was refluxed for 1 h. After cooling the reaction mixture in an ice bath, 160 mg of 2-(diethylamino)ethyl chloride hydrochloride (0.93 mmol) was added, and the mixture was refluxed for 2 h. The solvent was evaporated to dryness under vacuum, and the residue was purified by flash chromatography (column, silica gel; solvent, *n*-hexane/ethyl acetate) to obtain 112 mg of *N*,*N*-diethyl-2-[2-(4-benzyloxybenzyl)-5-nitro-1*H*-benzimidazole-1-yl]ethanamine (0.24 mmol, 26%).

A 50 mg sample of *N*,*N*-diethyl-2-[2-(4-benzyloxybenzyl)-5-nitro-1*H*-benzimidazole-1-yl]ethanamine (0.11 mmol) was dissolved in a mixture of 0.64 ml of thioanisole and 2.1 ml of trifluoroacetic acid and stirred for 3 h at room temperature. Water was added to the reaction mixture, the pH of the solution was adjusted to 9 using ammonium hydroxide (28% in water; aq. ammonia), and the solution was extracted using chloroform/2-propanol (3:1). The solvent was evaporated to dryness under vacuum, and the residue was purified by flash chromatography (column, silica gel; solvent, chloroform/methanol). The obtained residue was converted to a hydrochloride salt by the addition of concentrated hydrochloric acid/methanol (1:9), and the hydrochloride salt was precipitated by adding diethylether to obtain 32 mg of OHN hydrochloride (**4**) as a white powder (0.080 mmol, 73%; melting point, 236°C).

#### 2.2.2 | *N*-Ethyl-2-[2-(4-methoxybenzyl)-5-nitro-1*H*-benzimidazole-1-yl]ethanamine (MetN-dEt, **1**)

To a solution of 5.0 g of 2-(ethylamino)ethanol (56 mmol) in 42 ml of chloroform in an ice bath, 13.3 g of thionyl chloride (112 mmol) was added dropwise, and then, the solution was refluxed for 6 h. After cooling the solution to room temperature, it was poured stepwise into 70 ml of diethyl ether and stirred for 18 h at room temperature. The solution was cooled in an ice bath, and 3.5 ml of ethanol was added, followed by stirring for 30 min at room temperature. The resulting powder was collected by filtration and washed with diethylether to obtain 7.1 g of *N*-(2-chloroethyl)ethylamine hydrochloride (49 mmol, 88%) as a slightly colored (rosy brown) powder.

To a solution of 1.0 g of *N*-(2-chloroethyl)ethylamine hydrochloride (6.9 mmol) in 30 ml of tetrahydrofuran, 6 ml of 4 M sodium hydroxide solution was added, and the mixture was cooled in an ice bath. To the solution, 1.8 g of benzyl chloroformate (10 mmol) was then added dropwise, and the solution was stirred for 20 h at room temperature. The solution was concentrated under vacuum and water was added, followed by extraction with chloroform. The solvent was evaporated to dryness under vacuum, and the residue was purified by flash chromatography (column, silica gel; solvent, *n*-hexane/ethyl acetate) to obtain 1.64 g of *N*-benzyloxycarbonyl-*N*-(2-chloroethyl)ethylamine as a slightly colored oil (6.8 mmol, 99%).

To a solution of 283 mg of 2-(4-methoxybenzyl)-5-nitro-1*H*-benzimidazole[12] (1.0 mmol) in 15 ml of dry 1,4-dioxane, 60 mg of sodium hydride (60%; dispersion in paraffin liquid; 1.5 mmol) was added, and then, the solution was refluxed for 1 h. After cooling the reaction mixture in an ice bath, 363 mg of *N*-benzyloxycarbonyl-*N*-(2-chloroethyl)ethylamine (1.5 mmol) in 1 ml of 1,4-dioxane was added, and the solution was refluxed for 20 h. The solvent was

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evaporated to dryness under vacuum, 0.3 ml of thioanisole and 3.0 ml of trifluoroacetic acid were added to the residue, and the solution was heated for 100 min at 80°C to remove the benzyloxycarbonyl group. Water was added to the solution, which was basified using aq. ammonia, and then extracted with chloroform/2-propanol (3:1). The solvent was evaporated to dryness under vacuum, and the residue was purified by flash chromatography (column, silica gel; solvent, chloroform/methanol). The fraction containing the target compound was further purified using preparative thin-layer chromatography (plate, silica gel; solvent, ethyl acetate/2-propanol/aq. ammonia [90:10:1]) to obtain 30 mg of MetN-dEt (**1**) as an oil (0.086 mmol, 8.6%).

### 2.2.3 | *N*-Ethyl-2-[2-(4-ethoxybenzyl)-5-nitro-1*H*-benzimidazole-1-yl]ethanamine (EtN-dEt, **12**)

EtN-dEt (**12**) was synthesized using the same procedure as that used to synthesize MetN-dEt. A 11.7 mg sample of EtN-dEt (oil, 0.032 mmol, 6.5%) was obtained from 147 mg of 2-(4-ethoxybenzyl)-5-nitro-1*H*-benzimidazole[12] (0.49 mmol).

### 2.2.4 | *N*-Ethyl-2-[2-(4-propoxybenzyl)-5-nitro-1*H*-benzimidazole-1-yl]ethanamine (ProtN-dEt, **16**)

ProtN-dEt (**16**) was synthesized using the same procedure as that used to synthesize MetN-dEt. A 5.9 mg sample of ProtN-dEt (oil, 0.015 mmol, 5.6%) was obtained from 82.7 mg of 2-(4-propoxybenzyl)-5-nitro-1*H*-benzimidazole[12] (0.27 mmol).

### 2.2.5 | *N*-Ethyl-2-[2-(4-hydroxybenzyl)-5-nitro-1*H*-benzimidazole-1-yl]ethanamine (OHN-dEt, **5**)

OHN-dEt (**5**) was synthesized using the same procedure as that used to synthesize MetN-dEt. A 5.8 mg sample of OHN-dEt (oil, 0.017 mmol, 8.5%) was obtained from 72.5 mg of 2-(4-benzyloxybenzyl)-5-nitro-1*H*-benzimidazole (0.20 mmol). Benzyl and benzyloxycarbonyl groups were removed concurrently by treating the intermediate *N*-ethyl-*N*-benzyloxycarbonyl-2-[2-(4-benzyloxybenzyl)-5-nitro-1*H*-benzimidazole-1-yl]ethanamine with thioanisole and trifluoroacetic acid.

### 2.2.6 | 2-[2-(4-Methoxybenzyl)-5-nitro-1*H*-benzimidazole-1-yl]ethanamine (MetN-ddEt, **2**)

To a solution of 50 mg of 2-(4-methoxybenzyl)-5-nitro-1*H*-benzimidazole (0.18 mmol) in 5 ml of dry tetrahydrofuran, 10 mg of sodium hydride (60%, dispersion in paraffin liquid) (0.25 mmol) was added, and then the solution was refluxed for 1 h. After cooling the reaction mixture in an ice bath, 56 mg of 2-(*tert*-butoxycarbonylamino)ethyl bromide (0.25 mmol) was added, and the mixture was refluxed for 3 h. The solvent was evaporated to dryness under vacuum, and then, 0.3 ml of thioanisole and 1.0 ml of trifluoroacetic acid were added to the residue, followed by stirring for 2 h at room temperature to remove the *tert*-butoxycarbonyl group. The reaction mixture was processed in the same manner as in MetN-dEt synthesis to obtain 5.9 mg of MetN-ddEt (**2**) as an oil (0.018 mmol, 10%).

### 2.2.7 | 2-[2-(4-Ethoxybenzyl)-5-nitro-1*H*-benzimidazole-1-yl]ethanamine (EtN-ddEt, **13**)

EtN-ddEt (**13**) was synthesized using the same procedure as that used to synthesize MetN-ddEt. A 20.6 mg sample of EtN-ddEt (oil, 0.061 mmol, 36%) was obtained from 51 mg of 2-(4-ethoxybenzyl)-5-nitro-1*H*-benzimidazole (0.17 mmol).

### 2.2.8 | 2-[2-(4-Propoxybenzyl)-5-nitro-1*H*-benzimidazole-1-yl]ethanamine (ProtN-ddEt, **17**)

ProtN-ddEt (**17**) was synthesized using the same procedure as that used to synthesize MetN-ddEt. An 18.1 mg sample of ProtN-ddEt (oil, 0.051 mmol, 32%) was obtained from 50 mg of 2-(4-propoxybenzyl)-5-nitro-1*H*-benzimidazole (0.16 mmol).

### 2.2.9 | 2-[2-(4-Hydroxybenzyl)-5-nitro-1*H*-benzimidazole-1-yl]ethanamine (OHN-ddEt, **6**)

OHN-ddEt (**6**) was synthesized using the same procedure as that used to synthesize MetN-ddEt. A 9.2 mg sample of OHN-ddEt (oil, 0.029 mmol, 16%) was obtained from 66 mg of 2-(4-benzyloxybenzyl)-5-nitro-1*H*-benzimidazole (0.18 mmol).

### 2.2.10 | *N*,*N*-Diethyl-2-[2-(4-methoxybenzyl)-5-nitro-1*H*-benzimidazole-1-yl]ethanamine *N*-oxide (MetN-NOX, **3**)

A 5 mg sample of MetN hydrochloride (0.012 mmol) was dissolved in 1 ml of water, and the solution was basified with aq. ammonia and extracted with chloroform/2-propanol (3:1). The solvent was evaporated to dryness under vacuum, and then, 2 ml of chloroform and 3.1 mg of *m*-chloroperoxybenzoic acid (0.018 mmol) were added to the residue, followed by stirring for 3 h at room temperature. The solvent was evaporated to dryness under vacuum, and the residue was purified by preparative thin-layer chromatography (plate, silica gel; solvent, methanol) to obtain 4.1 mg of MetN-NOX (**3**) as an oil (0.010 mmol, 83%).

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#### 2.2.11 | *N*,*N*-Diethyl-2-[2-(4-ethoxybenzyl)-5-nitro-1*H*-benzimidazole-1-yl]ethanamine *N*-oxide (EtN-NOX, **14**)

EtN-NOX (**14**) was synthesized using the same procedure as that used to synthesize MetN-NOX. A 6.1 mg sample of EtN-NOX (oil, 0.015 mmol, 79%) was obtained from 8 mg of EtN hydrochloride (0.019 mmol).

#### 2.2.12 | *N*,*N*-Diethyl-2-[2-(4-propoxybenzyl)-5-nitro-1*H*-benzimidazole-1-yl]ethanamine *N*-oxide (ProtN-NOX, **18**)

ProtN-NOX (**18**) was synthesized using the same procedure as that used to synthesize MetN-NOX. A 6.8 mg sample of ProtN-NOX (oil, 0.016 mmol, 89%) was obtained from 8 mg of ProtN hydrochloride (0.018 mmol).

#### 2.2.13 | *N*-ethyl-*N*-(2-hydroxyethyl)-2-[2-(4-methoxybenzyl)-5-nitro-1*H*-benzimidazole-1-yl] ethanamine (MetN-2OHEt)

A mixture of 6 mg of MetN-dEt (**1**, 0.017 mmol), 15 mg of 2-chloroethanol (0.19 mmol), 89 mg of sodium carbonate (0.84 mmol), and 5 mg of sodium iodide (0.033 mmol) in 0.3 ml of 1-butanol was heated for 20 h at 110°C. Water was added to the reaction mixture, and the mixture was extracted using chloroform/2-propanol (3:1). The solvent was evaporated to dryness under vacuum, and the residue was purified by flash chromatography (column, silica gel; solvent, chloroform/methanol). The fraction containing the target compound was further purified using preparative thin-layer chromatography (plate, silica gel; solvent, ethyl acetate/2-propanol/aq. ammonia [80:20:1]) to obtain 3.9 mg of MetN-2OHEt as an oil (0.010 mmol, 59%).

### 2.3 | Incubation of fresh human hepatocytes with nitazenes

PXB-cells seeded in a 24-well plate were incubated for 7 days at 37°C in 5% $CO_2$ before addition of the drug (culture medium was replaced on day 4). Drug (MetN hydrochloride, EtN hydrochloride, or ProtN hydrochloride, 2mM, dissolved in dimethylsulfoxide) was added to the cells at a final concentration of 10 μM, and then, the cells were continuously incubated. The medium was sampled 48 h after the addition of the drug and stored at −20°C until analysis.

To identify nitazene metabolites, the culture medium was hydrolyzed, deproteinized, and analyzed using liquid chromatography–mass spectrometry (LC–MS). To 20 μl of the culture medium, 40 μl of B-One β-glucuronidase was added, and the solution was left to stand for 1 h at room temperature. Acetonitrile (120 μl) was added to the hydrolyzed sample; then, the mixture was vortexed for 10 s and centrifuged at 10,000 × g for 1 min. A 50 μl volume of the supernatant was diluted with 150 μl of 0.1% formic acid and centrifuged at 10,000 × g for 5 min. A 10 μl volume of the supernatant was injected into the liquid chromatograph. The unhydrolyzed culture medium was also analyzed to detect conjugated metabolites.

### 2.4 | Incubation of liver microsomes with substrate

After treatment of HLM with alamethicin in phosphate buffer (pH of 7.1) at 0°C for 15 min, magnesium chloride and substrate were added and incubated for 3 min at 37°C. Then, UDP-GlucA was added and incubated for 180 min at 37°C. The volume of the reaction mixture was 200 μl, and the final concentrations of each component were as follows (the concentrations in parentheses are the stock solution concentrations): substrate (OHN [**4**], OHN-dEt [**5**], OHN-ddEt [**6**], or MetN-2OHEt), 50 μM (1mM in water); HLM, 0.5 mg protein/mL (20 mg protein/mL); phosphate buffer, 0.1 M (1 M); alamethicin, 25 μg/ml (1 mg/ml in dimethylsulfoxide); magnesium chloride, 1mM (20mM in water); and UDP-GlucA, 2mM (40mM in water). The free base of the substrate (OHN-dEt [**5**], OHN-ddEt [**6**], and MetN-2OHEt) was converted to a hydrochloride salt by adding concentrated hydrochloric acid/methanol (1:9) prior to preparing the stock solution.

After incubation, 400 μl of acetonitrile was added to the reaction mixture, which was then vortexed for 10 s and centrifuged at 1,600 ×g for 3 min. A 5 μl volume of the supernatant was diluted with 495 μl of the initial mobile phase and centrifuged at 10,000 ×g for 5 min. A 10 μl volume of the supernatant was injected into the liquid chromatograph.

### 2.5 | Liquid chromatography-mass spectrometry (LC–MS)

LC–MS analysis was performed using an Exion LC system connected to a QTRAP 4500 mass spectrometer (SCIEX, Framingham, MA). The conditions were as follows: column, XBridge BEH C18 (2.1 × 150 mm; particle diameter, 3.5 μm; Waters, Milford, MA) maintained at 40°C; mobile phase composition, 10mM ammonium acetate (A) and methanol (B); linear gradient mode, 20% to 90% B over 15 min, 90% B for 5 min, and 90% to 20% B over 0.1 min; flow rate, 0.2 ml/min; MS interface, electrospray ionization (positive); analysis mode, scan (*m/z* 100–650), and product ion analysis (collision energy, 40 V; precursor ions, protonated molecule of each compound).

### 2.6 | Purification, hydrolysis, and analysis of the putative *N*-ethyl-*N*-(1-glucuronyloxyethyl) metabolite of metonitazene

To a 200 μl sample of the culture medium of hepatocytes incubated with MetN, 400 μl of acetonitrile was added, followed by

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centrifugation at 1,600 × g for 3 min. The supernatant was diluted with 1.4 ml of water and loaded onto a solid-phase extraction cartridge (Oasis® HLB, 3 cc, 60 mg, Waters) preconditioned with 2 ml of methanol and 2 ml of water. After washing with 2 ml of water, the metabolites were eluted with 2 ml of methanol. The eluate was evaporated to dryness under a nitrogen stream, and then the residue was dissolved in 100 µl of the initial mobile phase. The solution was centrifuged at 10,000 × g for 5 min, and then, 10 µl of the supernatant was injected into the liquid chromatograph. The LC conditions were identical to those described in the previous section. For peaks **10** and **11**, eluates of 10.2–10.7 min and 7.8–8.2 min were collected, respectively. This injection and collection was repeated six times to collect sufficient samples. Each fraction of peaks **10** and **11** was evaporated to dryness under a nitrogen stream and dissolved in 40 µl of water. Half of the sample was hydrolyzed, deproteinized, and analyzed as described in Section 2.3. The remaining sample was analyzed without hydrolysis.

## 3 | RESULTS AND DISCUSSION

### 3.1 | Synthesis of nitazene metabolites

The *N*-desethyl and *N*,*N*-di-desethyl metabolites of nitazenes were obtained by the condensation of the corresponding benzimidazole intermediate and *N*-substituted-2-halogenated-ethyl amine, followed by the removal of the protecting group (see Figure S3). In the condensation reaction, the phenolic hydroxyl and amino groups of the chemical material should be protected; therefore, the *O*-benzyloxy-benzimidazole intermediate and *N*-*tert*-butoxycarbonyl-2-bromoethyl amine were used for the synthesis of OHN-ddEt (**6**). The *O*-benzyl and *N*-*tert*-butoxycarbonyl groups were efficiently removed at room temperature by using a mixture of thioanisole and trifluoroacetic acid. The benzyloxycarbonyl group, used for the protection of the amino group in the synthesis of *N*-desethyl metabolites, also could be removed by the same mixture[13]; however, the reaction solution had to be heated at 80°C. The condensation reaction produced as much positional isomer (6-nitro compound) as desired product (5-nitro compound); however, they could be separated by preparative thin-layer chromatography, although their spots were close to each other (higher spot, 6-nitro compound; lower spot, 5-nitro compound). The yields of *N*-desethyl metabolites (5.8–8.6%) from benzimidazole intermediates were low compared to those of *N*,*N*-di-desethyl metabolites (10–36%), probably due to the formation of unknown byproducts.

The *N*-oxides of nitazenes (**3**, **14**, **18**) were successfully synthesized by treatment of the free base of nitazenes with *m*-chloroperoxybenzoic acid in chloroform. The NMR data of the synthesized *N*-oxides indicated that the nitrogen atom in the side chain was surely oxidized; i.e., drastic chemical shift changes at carbon and proton atoms adjacent to *N*-oxide (e.g., H-17, H-23, C-17, and C-23 of MetN-NOX) were observed (see Table S1 and Figure S2).

### 3.2 | Identification of nitazene metabolites

Figure 2 shows the total ion current chromatograms (TICs) and extracted ion chromatograms (EICs) obtained from the unhydrolyzed (a) and hydrolyzed (b) media of hepatocytes cultured with MetN. As the presumed phase I metabolites of MetN, peaks **1**–**6** were detected on the chromatogram. The electrospray ionization (ESI) product ion spectra of peaks **1**–**6** detected in Figure 2(b) are shown in Figure 3. The proposed fragmentation pathways are shown in Figure S4. The retention times and mass spectra of peaks **1**–**6** were identical to those of the synthetic standards of MetN-dEt (**1**, *N*-desethyl metabolite), MetN-ddEt (**2**, *N*,*N*-di-desethyl metabolite), MetN-NOX (**3**, *N*-oxide), OHN (**4**, *O*-desalkyl metabolite), OHN-dEt (**5**), and OHN-ddEt (**6**), respectively. This is the first report of *N*-oxide and OHN-ddEt formation of nitazenes. Peak **1** was detected with the largest intensity (6.9e7) among the metabolites, suggesting that this metabolite was the main metabolite of MetN; however, it should be noted that the peak intensity ratio did not always reflect the quantity ratio of the analytes due to potential differences in ionization. Peaks except for peak **3** (MetN-NOX) were not detected in the unhydrolyzed medium (Figure 2[a]), indicating that these metabolites underwent glucuronidation. Therefore, the phase II metabolites of MetN were subsequently searched for.

Figure 4(a) shows the EICs and multiple reaction monitoring (MRM) chromatogram obtained from unhydrolyzed MetN culture medium. As presumed phase II metabolites, peaks **7**–**11** were detected. Peak **9** could be detected in the MRM mode but not in the scan mode owing to its extremely low concentration. The product ion spectra of peaks **7**–**11** are shown in Figure 4(b). The protonated molecule of peak **7** was *m/z* 545, and fragment ions were observed at *m/z* 100 and 369 in its product ion spectrum, suggesting that peak **7** was the glucuronide of peak **4** (OHN) (see Figure S5). Similarly, peaks **8** and **9** were presumed to be the glucuronides of peaks **5** (OHN-dEt) and **6** (OHN-ddEt), respectively. To identify these peaks, the formation of glucuronides from the corresponding substrates OHN (**4**), OHN-dEt (**5**), and OHN-ddEt (**6**) was investigated by incubating these substrates with HLM in the presence of UDP-GlucA. Figure 5 shows the EICs and product-ion spectra obtained from the reaction mixture of the substrate and HLM. Peaks a–c were formed from OHN (**4**), OHN-dEt (**5**), and OHN-ddEt (**6**), and their retention times and mass spectra were identical to those of peaks **7**–**9** (Figure 4), indicating that peaks **7**–**9** were the glucuronides of OHN (**4**), OHN-dEt (**5**), and OHN-ddEt (**6**), respectively. As mentioned above, MetN-dEt (**1**) and MetN-ddEt (**2**) were also expected to be glucuronidated, and considering their chemical structures, they were likely to be *N*-glucuronides. Therefore, the putative *N*-glucuronides were searched for based on their protonated molecules (*m/z* 531 and 503); however, no peaks of the desired *N*-glucuronides were found, and the identities of the glucuronides of MetN-dEt (**1**) and MetN-ddEt (**2**) remained unclear.

In contrast, the protonated molecule of peak **10** was *m/z* 575, indicating that one oxygen atom and one glucuronic acid moiety were introduced into the MetN molecule to form this metabolite, and

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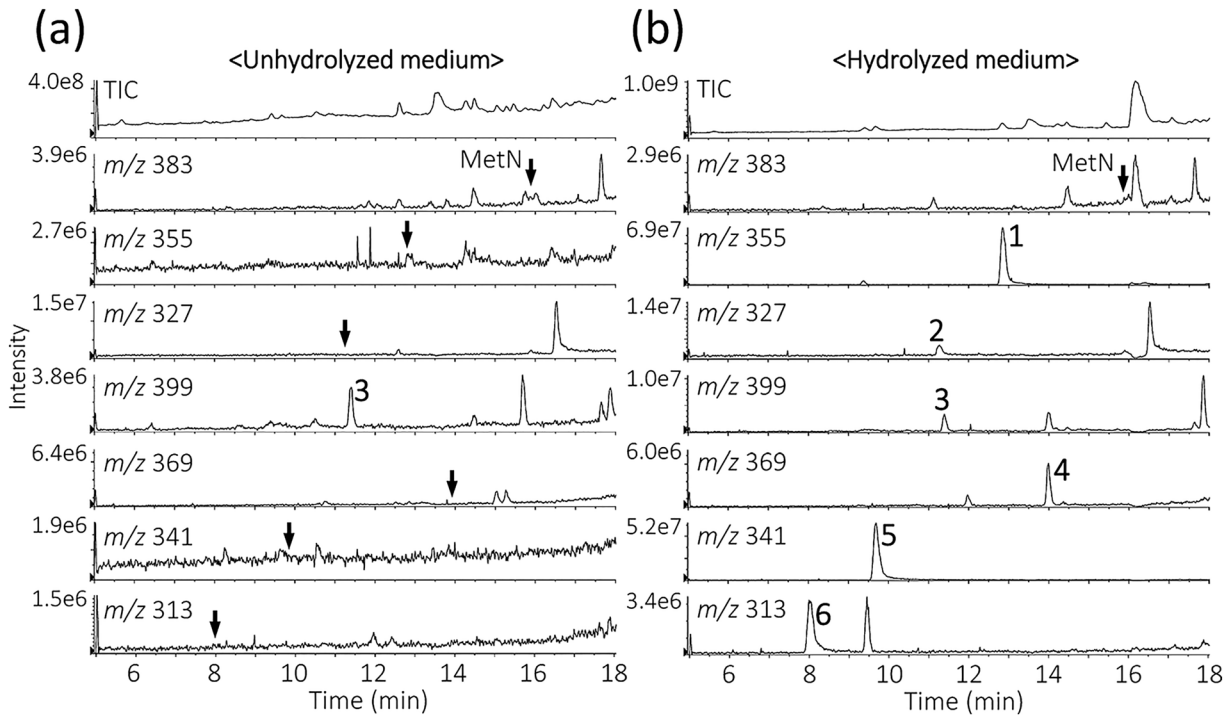


**FIGURE 2** TICs and EICs obtained from the medium of hepatocytes cultured with MetN. (a) Unhydrolyzed medium, (b) hydrolyzed medium. 1, MetN-dEt; 2, MetN-ddEt; 3, MetN-NOX; 4, OHN; 5, OHN-dEt; 6, OHN-ddEt.

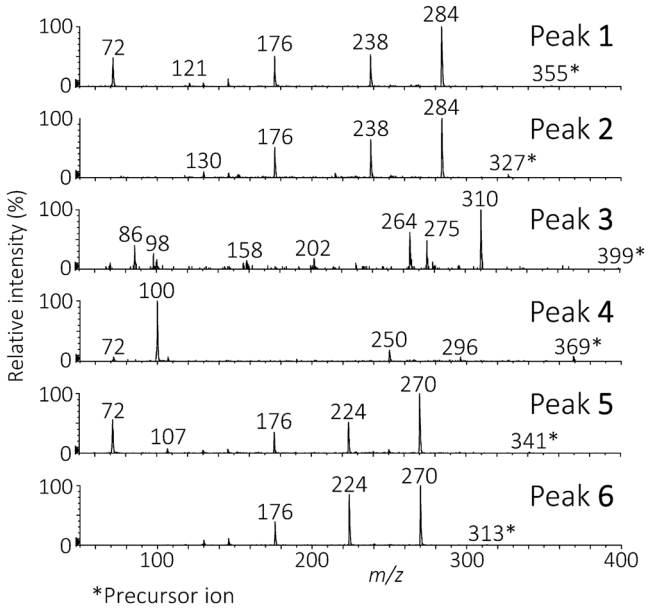


**FIGURE 3** ESI product ion spectra of peaks 1–6 detected in Figure 2. 1, MetN-dEt; 2, MetN-ddEt; 3, MetN-NOX; 4, OHN; 5, OHN-dEt; 6, OHN-ddEt.

the detection of an ion at *m/z* 284 in its product ion spectrum suggested that the benzimidazole core and 4-methoxybenzyl moiety were intact (Figure 4). This implied that oxygen atoms were introduced into the *N*-ethyl chains of MetN. The *N*-1-hydroxylation of the *N*-alkyl chain is known to cause prompt *N*-dealkylation[14]; therefore, peak **10** is likely the *N*-ethyl-*N*-(2-glucuronyloxyethyl) metabolite of MetN (MetN-2OHEt-GlucA). An authentic standard of the *N*-ethyl-*N*-(2-hydroxyethyl) metabolite of MetN (MetN-2OHEt) was chemically synthesized, then glucuronidated by HLM in the presence of UDP-GlucA to form MetN-2OHEt-GlucA (protonated molecule, *m/z* 575), and analyzed by LC–MS. However, the retention time and mass spectrum of MetN-2OHEt-GlucA (peak d, Figure 5) were not identical to those of peak **10** (Figure 4). Although peak **11** was considered to be the *O*-desmethyl metabolite of peak **10** according to its product ion spectrum, the identity of this peak also remained unclear.

The LC–MS data for the EtN and ProtN metabolites were obtained in the same manner. Similar to MetN, the *N*-desethyl metabolite (**12**, **16**), *N*,*N*-di-desethyl metabolite (**13**, **17**), *O*-desalkyl metabolite (**4**), *N*-desethyl-*O*-desalkyl metabolite (**5**), *N*,*N*-di-desethyl-*O*-desalkyl metabolite (**6**), and *N*-oxide (**14**, **18**) were detected in the hydrolyzed media of EtN and ProtN (Figure S6–S9). Metabolites with *O*-dealkylation (**4**–**6**) were common metabolites of MetN, EtN, and ProtN. In addition, the *O*-desalkyl-*O*-glucuronidated metabolites detected in the case of MetN, i.e., peaks **7**–**9**, were detected in the unhydrolyzed media of EtN and ProtN (Figures S10 and S11). Other glucuronidated metabolites, that is, peaks **15** and **19**, which correspond to peak **10** in the case of MetN, and peak **11**, were also detected; however, the identity of these peaks remained unclear.

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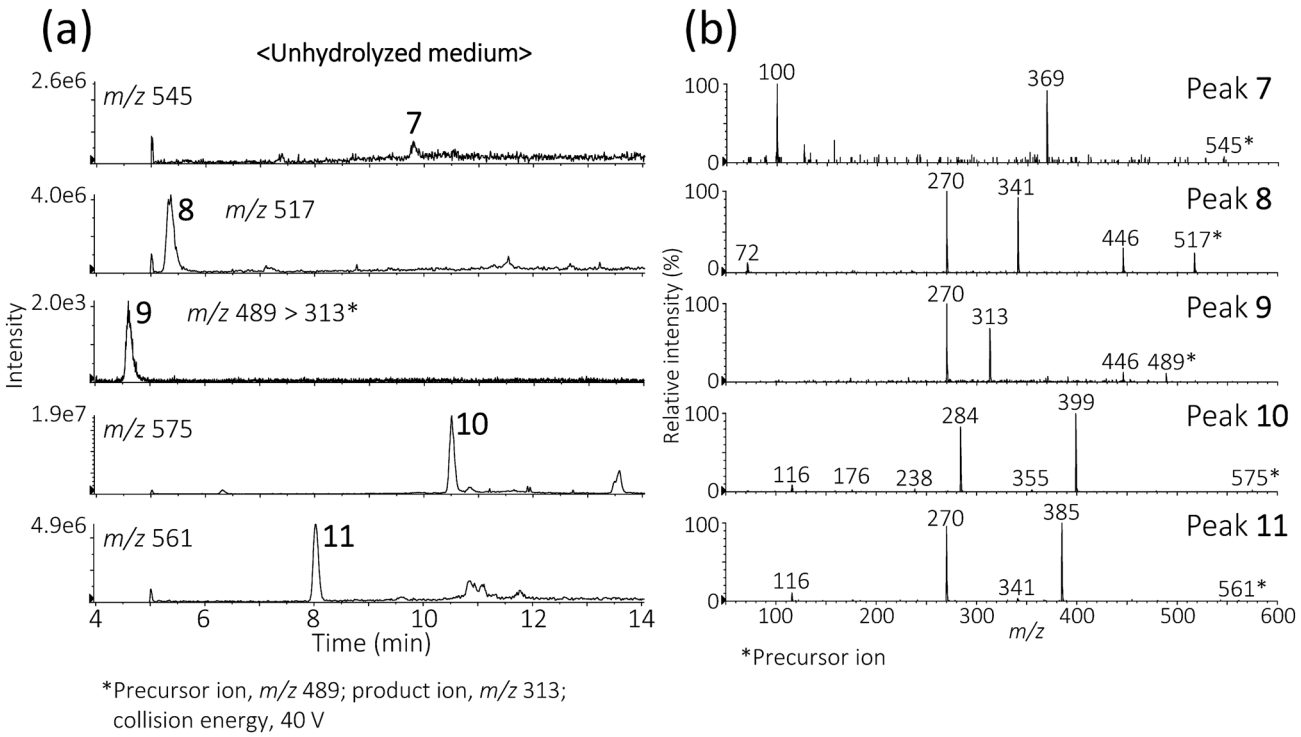


**FIGURE 4** Detection of the glucuronidated metabolites of MetN. (a) EICs and MRM chromatogram, (b) ESI product ion spectra of peaks 7–11. 7, OHN-GlucA; 8, OHN-dEt-GlucA; 9, OHN-ddEt-GlucA; 10, putative *N*-ethyl-*N*-(1-glucuronyloxyethyl) metabolite; 11, putative *N*-ethyl-*N*-(1-glucuronyloxyethyl)-*O*-desmethyl metabolite.

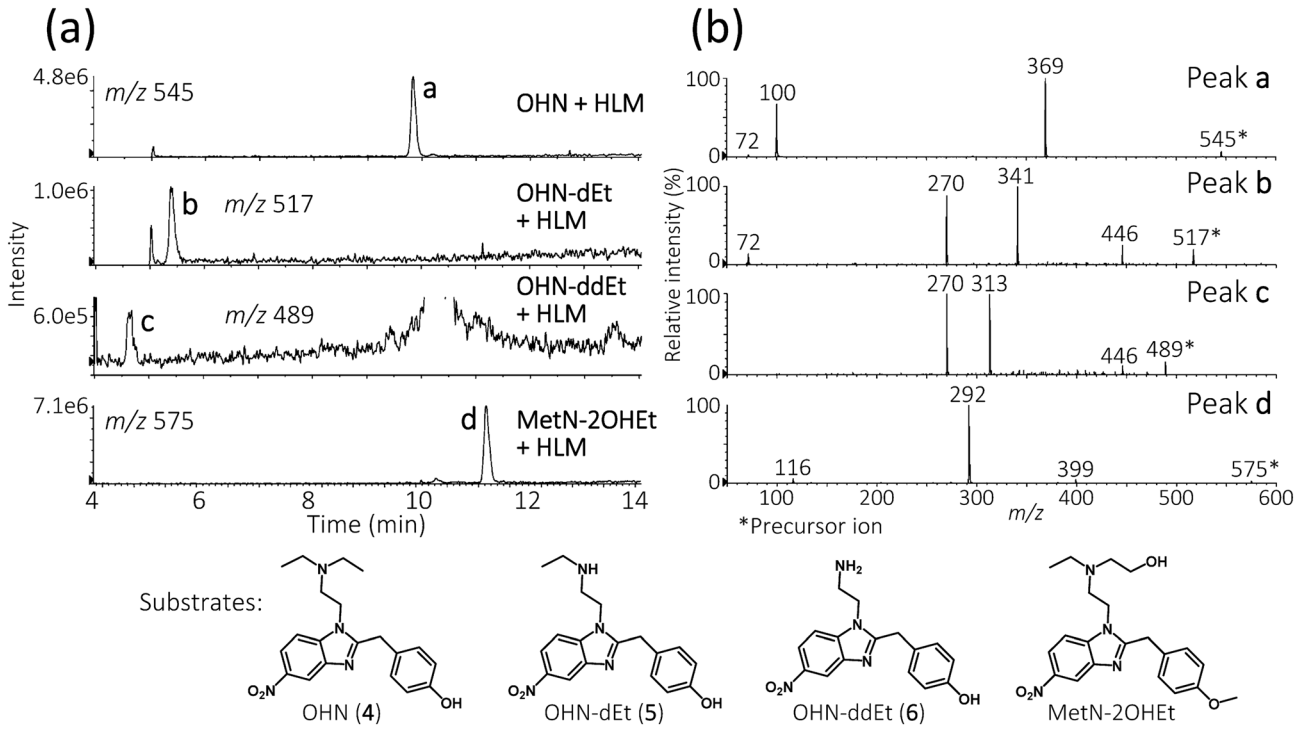


**FIGURE 5** EICs and ESI product ion spectra obtained from the reaction mixture of HLM and substrate in the presence of uridine diphosphate glucuronic acid. (a) EICs, (b) ESI product ion spectra of peaks a–d. a, OHN-GlucA; b, OHN-dEt-GlucA; c, OHN-ddEt-GlucA; d, MetN-2OHEt-GlucA.

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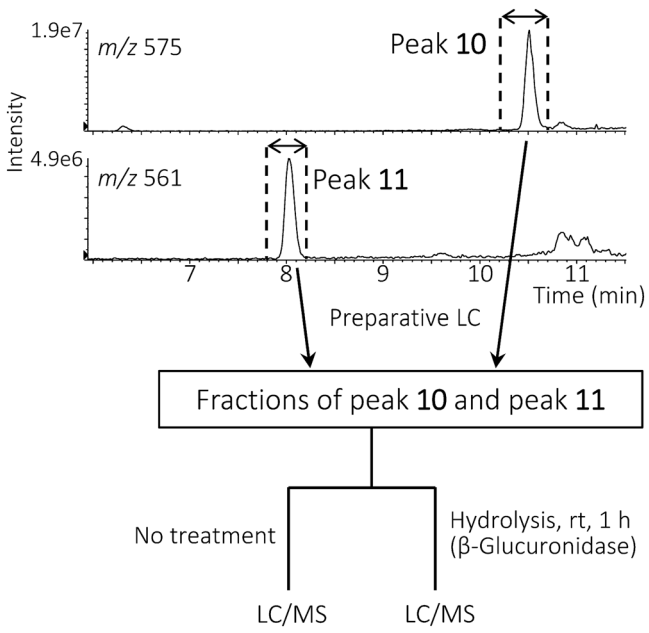


**FIGURE 6** Scheme for the detection of hydrolyzed products of peaks 10 and 11.

### 3.3 | Estimation of *N*-ethyl-*N*-(1-glucuronyloxyethyl) metabolites in the culture medium

To identify peaks **10** and **11**, the putative glucuronidated metabolites of MetN, detecting the hydrolyzed products of these metabolites seemed to be reasonable. Therefore, peaks **10** and **11** were purified by LC, and the peak **10** and **11** fractions were analyzed by LC–MS without any treatment or after enzymatic hydrolysis (Figure 6). Figure 7(a) clearly indicates that peak **10** disappeared after treatment of the peak **10** fraction with β-glucuronidase, and instead of peak **10**, peak **1** (MetN-dEt) appeared with a relatively large intensity. Similarly, peak **5** (OHN-dEt) appeared instead of peak **11** after the enzymatic hydrolysis of the peak **11** fraction (Figure 7[b]). These results indicated that Peak **10** was the *N*-ethyl-*N*-(1-glucuronyloxyethyl) metabolite of MetN, it was hydrolyzed by β-glucuronidase to form the *N*-ethyl-*N*-(1-hydroxyethyl) metabolite, and then, it spontaneously decomposed into MetN-dEt (**1**) due to the extreme instability of the *N*-ethyl-*N*-(1-hydroxyethyl) metabolite (Figure 7, bottom). The same was true for peak **11** (*O*-desmethyl metabolite of peak **10**), which formed OHN-dEt (**5**) after hydrolysis.

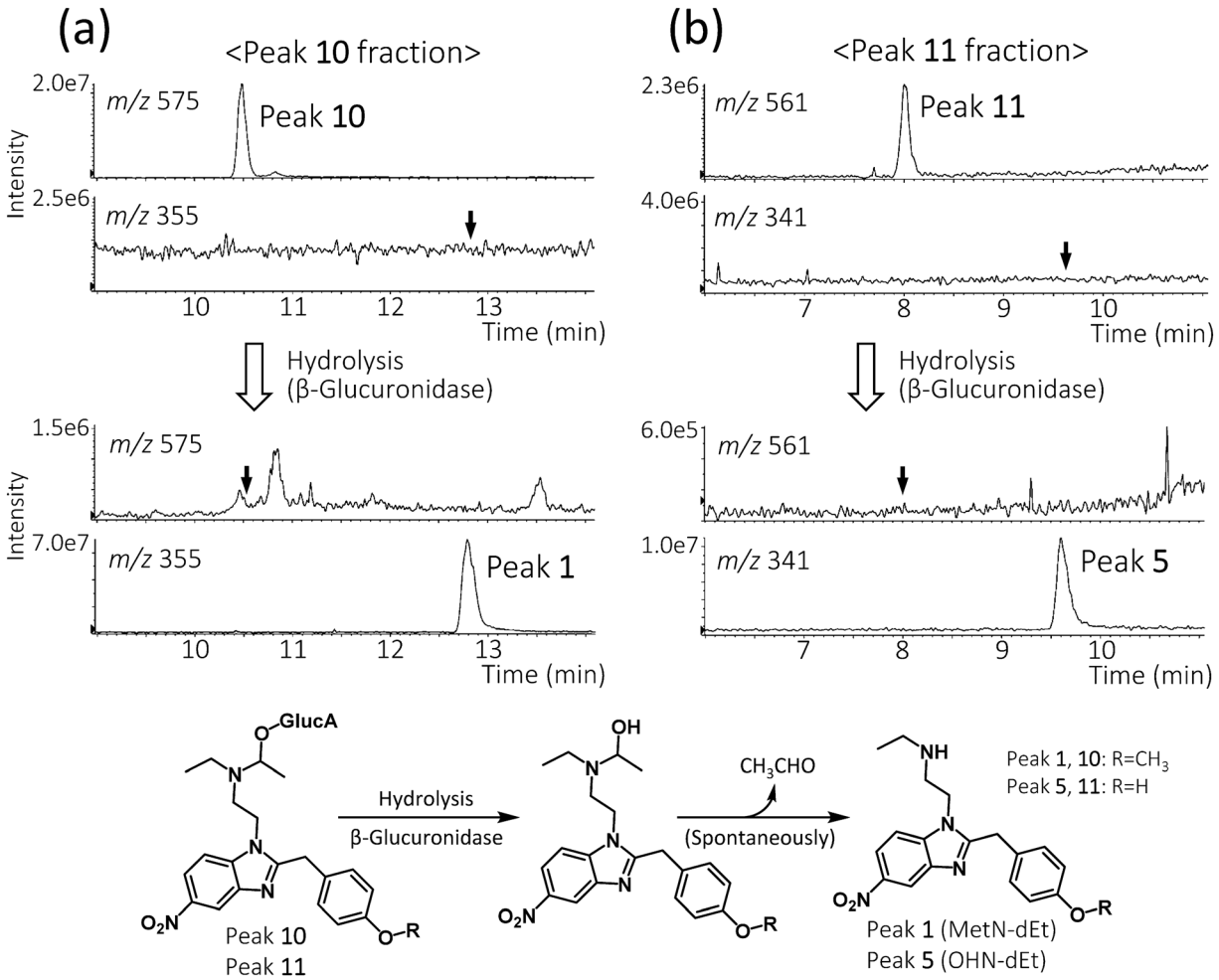


**FIGURE 7** EICs obtained from untreated and β-glucuronidase-treated fractions of peaks 10 and 11. (a) Peak 10 fraction, (b) peak 11 fraction. GlucA, glucuronic acid.

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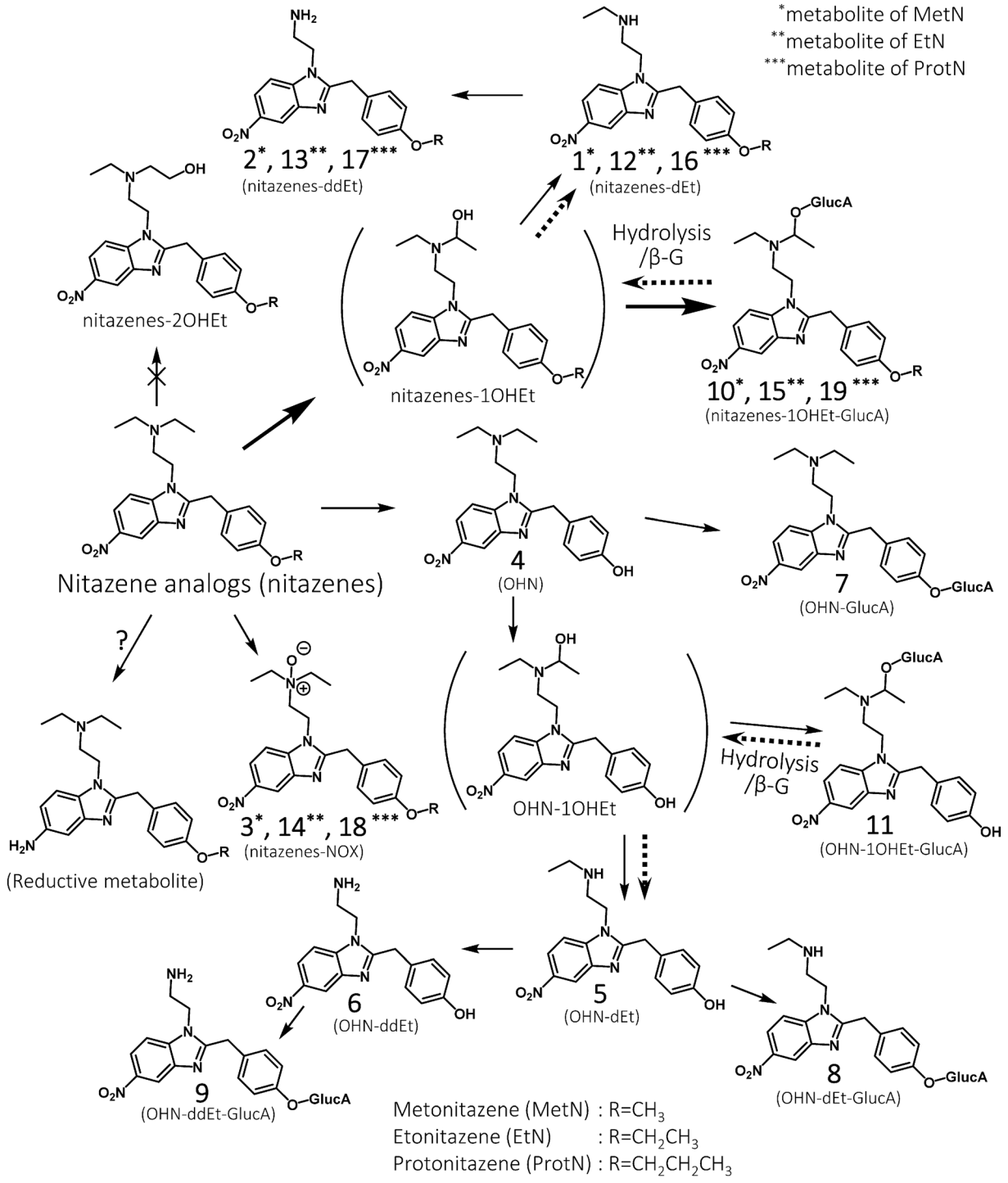


**FIGURE 8** Proposed metabolic pathways for nitazenes. GlucA, glucuronic acid; β-G, β-glucuronidase.

This clearly explains why MetN-dEt (**1**) and its putative *N*-glucuronide were not detected in the unhydrolyzed medium. The precursor compound of MetN-dEt (**1**) is *N*-ethyl-*N*-(1-glucuronyloxyethyl) metabolite and not *N*-glucuronide. In addition, the similarity between the product ion spectra of peak **10** and MetN-dEt (**1**) corroborates this hypothesis. The ion at *m/z* 355 in the spectrum of peak **10**, which could have been formed by the elimination of GlucA and *N*-(1-hydroxyethyl) moieties, seemed to be identical to the protonated molecule of MetN-dEt (Figure S5) and decomposed into several ions, such as *m/z* 284, 238, and 176, which were commonly observed in the

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spectra of peak **10** and MetN-dEt (**1**) (Figures 3 and 4). Although purification/hydrolysis experiments (Figure 6) for medium samples of EtN and ProtN were not conducted, the same may be expected for these drugs; that is, peaks **15** and **19**, which corresponded to peak **10**, formed peaks **12** and **16**, the *N*-desethyl metabolites of EtN and ProtN, respectively, during enzymatic hydrolysis (Figures S6–S11). In contrast, MetN-ddEt (**2**) and its putative *N*-glucuronide were not detected in the unhydrolyzed medium, implying the presence of the *N*-(1-glucuronyloxyethyl) metabolite of MetN (*N*-desethyl metabolite of peak **10**); however, the desired peak was not observed. This may have been due to the low concentration of the target glucuronide.

There is little doubt regarding the presence of the *N*-ethyl-*N*-(1-glucuronyloxyethyl) metabolite of nitazenes. However, the question arises as to how this metabolite forms, considering the extreme instability of its precursor, the *N*-ethyl-*N*-(1-hydroxyethyl) metabolite, and this may be answered to some degree by the observation that cytochrome P450 (P450) and UDP-glucuronosyltransferase (UGT) form a functional complex on the endoplasmic reticulum membrane.[15] Continuous *N*-1-hydroxylation and glucuronidation by the P450-UGT complex may enable the formation of *N*-ethyl-*N*-(1-glucuronyloxyethyl) metabolites of nitazenes. According to the review article of Eh-Haj, CYP3A4 and/or CYP2C8 involve in the *N*-deethylation (caused by *N*-1-hydroxylation) of some drugs (amiodarone, lidocaine, oxybutynin), which have *N*,*N*-diethylamino moiety. As nitazenes also have the same moiety, above CYP isozymes may involve in the *N*-deethylation of nitazenes.[14]

The proposed metabolic pathways for nitazenes are summarized in Figure 8. The main metabolic pathways were *N*-1-hydroxylation and *O*-glucuronidation, which formed *N*-ethyl-*N*-(1-glucuronyloxyethyl) metabolites (**10**, **15**, and **19**). A small portion of the intermediate, the *N*-ethyl-*N*-(1-hydroxyethyl) metabolite (nitazenes-1OHEt), probably formed an *N*-desethyl metabolite (**1**, **12**, and **16**), which was *N*-de-ethylated to form an *N*,*N*-di-desethyl metabolite (**2**, **13**, and **17**). *O*-Dealkylation, another important metabolic reaction for nitazenes, formed an *O*-dealkylated metabolite (**4**, OHN). This metabolite was further metabolized in a manner similar to the one described above. The formation of *N*-oxide (**3**, **14**, and **18**) was a minor route. Reductive metabolites (5-amino metabolites) were not clearly detected in this study, although they have been reported as in vivo metabolites of nitazenes in previous studies.[7,8] One possible reason for this is that intestinal microorganisms may play an essential role in the reduction of nitro groups during nitazene metabolism in vivo.

## 4 | CONCLUSION

This study revealed that nitazene analogs were mainly metabolized by *N*-1-hydroxylation followed by glucuronidation, *N*-deethylation, *O*-dealkylation followed by glucuronidation, and *N*-oxidation. Interestingly, the *N*-ethyl-*N*-(1-glucuronyloxyethyl) metabolites of nitazenes were converted into their corresponding *N*-desethyl metabolites during enzymatic hydrolysis. This is the first report indicating that the *N*-(1-hydroxyalkyl) metabolite, which is generally deemed extremely unstable, can be stabilized by glucuronate conjugation.

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