Metabolic Profile of $^{14}$C-Bendamustine in Rat Urine and Bile:
Preliminary Structural Identification of Metabolites

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Running Title

Metabolites of bendamustine in rats

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Abbreviations used are: TLC, thin layer chromatography; LC-MS, liquid chromatography-mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; SOPs, standard operating procedures; ACN, acetonitrile; MeOH, methanol.
ABSTRACT

Bendamustine, a bifunctional alkylating agent, is currently in clinical trials for the treatment of hematological and other malignancies. Although it has been used in the former East Germany for over 30 years, very limited information is available on biotransformation. The objective of this investigation was to obtain information on the structures of metabolites excreted into rat urine and bile in order to understand the metabolic fate of bendamustine in vivo. Metabolites of [14C]-bendamustine hydrochloride in rat urine and bile were determined using liquid chromatography-mass spectrometry in parallel with on-line radioactivity detection in samples obtained after intravenous dosing of 3 mg/kg. A total of 17 radioactive peaks were identified in rat urine and 10 in rat bile (2 were unique to bile). Four of these metabolites had been previously reported, while 15 are novel. Proposed structures of all metabolites detected are based on MS^n spectra generated from a linear ion trap mass spectrometer. These results suggest that the major metabolic pathways in rat are oxidative and/or hydrolytic dehalogenation, oxidation, carboxylic acid formation, N-dealkylation, sulfation and glutathione and cysteine (probably via glutathione) conjugation. The cysteine-conjugated compounds are observed in their N-acetylated cysteine (mercapturic acid) forms.
Bendamustine, a bifunctional alkylating agent (Fig. 1), is currently in late stage clinical trials for the treatment of hematological and other malignancies (Ponisch et al., 2006). Although it has been used in the former East Germany for over 30 years (Teichert et al., 2005; Gandhi, 2002), limited information is available on its biotransformation. Pharmacokinetic studies in mice indicated that bendamustine concentrations in plasma rapidly decreased after intravenous dosing (Weber et al., 1991). Bendamustine metabolites excreted into rat bile and urine was investigated with [14C]-labeled compound using TLC (thin layer chromatography) analysis, and conjugated and hydroxylated metabolites were observed (Sezek et al., 1991). Hydroxylated, N-demethylated and cysteine conjugated metabolites of bendamustine were identified in human bile, urine, and plasma (Teichert et al., 2005), and two phase I metabolites (γ-hydroxy- and N-desmethyl-bendamustine) were further characterized by LC-MS and NMR after isolation from cytochrome P450 incubation mixtures (Teichert et al., 2007). Although 7 metabolites were tentatively identified from the above investigations, 4 of which were also observed in the studies reported herein and designated M6, M16, M20 and M21 (see below), the complete metabolic fate of bendamustine in vivo remains to be fully elucidated. The other 3 metabolites previously observed were mono- (with or without hydrolysis of the opposite chlorine) or di-cysteine conjugates of the N-chloroethyl groups.

The objective of this investigation was to obtain information on the structures of metabolites excreted into rat urine and bile in order to understand the routes of metabolic clearance of bendamustine in vivo. The rat is the principle rodent species used for safety evaluation of bendamustine. In a mass balance study in rats, approximately 90% of the
dose was recovered in excreta during 7 days after a single 3-mg/kg intravenous dose of
$[^{14}\text{C}]$-bendamustine.HCl, with most (~77%) of the radioactivity recovered during the first
24 hours. Urine fractions (0-4, 4-8 and 8-24 hr) were obtained on dry ice during this
mass balance study. After analysis for total radioactivity, the samples were refrozen at
approximately -80°C for later LC-MS analysis. Since substantial radioactivity (~49%)
had been recovered in the feces during this study, biliary excretion of compound–derived
radioactivity was presumed. Therefore, the bile duct of a male Sprague Dawley rat was
cannulated, a 3-mg/kg intravenous dose of $[^{14}\text{C}]$-bendamustine.HCl was administered,
and bile was collected in fractions on wet ice through 2 hours after dosing and analyzed
immediately by LC-MS. The preliminary results for the identification of the radioactive
components observed in rat urine and bile are reported herein. Besides the previously
characterized metabolites (Teichert et al., 2005), 15 novel metabolites were identified in
this study.
Materials and Methods

Materials

Reference standards were obtained from AMCIS (Bubendorf, Switzerland) for bendamustine hydrochloride, 5-[bis(2-chloroethyl)amino]-1-methyl-1H-benzimidazole-2-butanoic acid monohydrochloride (purity 97.0-102.0%); a hydroxylated metabolite (M20 in this report), 4{5-[bis-(2-chloroethyl)amino]-1-methyl-1H-benzoimidazol-2-yl}-4-hydroxybutyric acid (purity 89.4%); a N-desmethyl metabolite (M21), 4{5-[bis-(2-chloroethyl)-amino]-1H-benzoimidazol-2-yl}-butyric acid (purity 71.1%); the monohydrolysis metabolite (M16; purity 82.5%); and the dihydrolysis metabolite (M6; purity 92.0%). Naming of metabolites in this report is different from previous publications (eg, Teichert et al., 2005). [14C]-Bendamustine.HCl (purity >98%; specific activity 26.1 µCi/mg) was obtained from BioDynamics (Rushden, UK). Acetonitrile (ACN), methanol (MeOH) and formic acid were HPLC grade and were obtained from VWR (West Chester, PA, USA) or J.T. Baker (Phillipsburg, NJ, USA). Water was obtained from a Milli-Q Ultrapurification System (Millipore Corporation; Milford, CT, USA). Ultima-Flo™ liquid scintillation cocktail was purchased from PerkinElmer (Shelton, CT, USA). Other chemicals used were generally of reagent grade or better.

Animals and Sampling Procedures

A male Sprague Dawley rat was commercially obtained from Charles River Laboratories (CRL; Raleigh, NC, USA) for the bile studies. After receipt at Cephalon WC (Cephalon,
Inc., West Chester, PA, USA), the animal was housed under standard conditions with food and water provided ad libitum.

**Bile Collection:** Bile duct cannulation and bile collection from a male Sprague Dawley rat was conducted at Cephalon WC, following Cephalon WC Standard Operating Procedures (SOPs). Prior to dosing, the bile flow was checked and a pre-dose sample (~200 µL) was collected. The dose was prepared in a 1:10 mixture of MeOH:0.9% (w/v) sodium chloride containing an equal amount of $^{14}$C-labeled and non-labeled bendamustine.HCl at a total concentration of 3 mg/mL. The rat was intravenously dosed via a lateral tail vein at a dose volume of 1 mL/kg. Bile was then collected on wet ice in fractions during 0-30, 30-60 and 60-120 min after administration. Portions of 100 µL from each fraction were transferred into autosampler vials for immediate analysis or for storage at approximately -80°C. A sample from each fraction was injected into the LC-MS system for analysis as soon as possible (within 60 min) after collection; all samples were maintained on wet ice or in the autosampler at 6°C until analyzed.

**Urine Collection:** Rat urine samples (0-4-, 4-8- and 8-24-hr fractions from 5 Sprague Dawley rats) were collected on dry ice after a 3-mg/kg intravenous dose of $[^{14}$C]-bendamustine.HCl during a mass balance study conducted at CRL (Edinburgh, UK). The samples were thawed on wet ice for analysis of total radioactivity, and portions were immediately refrozen at approximately -80°C. After shipment to Cephalon WC on dry ice, each sample was thawed individually on wet ice, a portion immediately injected (50 µL) without further treatment, and the remainder was transferred into vials in 300-µL portions, and then these samples were stored at approximately -80°C. Each fraction was thawed on wet ice only one additional time, if needed, for repeat analysis.
All urine samples, including predose control urine, were analyzed by LC-MS³ with a 50-µL injection volume.

Analytical Methods

The LC-MS system consisted of a ThermoFinnigan Surveyor MS pump, autosampler and LTQ linear ion trap mass spectrometer (ThermoFisher Scientific, San Jose, CA, USA) coupled with an IN/US model 4 β-Ram radioactivity detector (Tampa, FL, USA). Chromatographic separations were performed on a Phenomenex (Torrance, CA, USA) Synergy Hydro-RP column (4.6 x 250 mm, 4-µm particle size). The mobile phase consisted of 0.1% formic acid in water (mobile phase A) and ACN (mobile phase B). Metabolites were eluted using the following gradient scheme (segments were all linear changes): 0 min-5% B, 5 min-5% B, 15 min-10% B, 25 min-20% B, 35 min-40% B, 45 min-60% B, 50 min-80% B, 54.9 min-80% B, 55 min-5% B and 60 min-5% B. The samples were analyzed by positive electrospray ionization mode with a spray voltage of 4.5 kV, capillary temperature of 300ºC, sheath gas flow rate of 60 (arbitrary), and an auxiliary gas flow rate of 20 (arbitrary). The MSⁿ analyses were performed with relative collision energies of 25% to 45%. The HPLC effluent was directed through a diversion valve set to divert the flow to waste from 0 to 3 min, and then split at a 1:9 ratio to allow 100 µL/min into the MS source and 900 µL/min to mix with scintillation liquid at 1.5 mL/min. The latter was directed through the β-Ram detector for radioactivity monitoring. Xcalibur v1.4 software (Thermo) was used to control the radioactivity detector and the LC-MS system. Xcalibur software also provided simultaneous acquisition and processing of radiochemical and mass spectral data.
Analysis of Radioactivity

Radioactivity in rat bile was determined by liquid scintillation counting on a Packard, model 2700 TR liquid scintillation analyzer (PerkinElmer Life and Analytical Sciences, Boston, MA, USA) by adding 10 µL of each sample to 10 mL of Ultima-Flo™ liquid scintillation cocktail. Similar analyses were conducted by CRL for urine.
Results

Recovery of Radioactivity

Radioactivity was counted in each fraction of rat bile and urine collected following a single 3-mg/kg intravenous dose of [14C]-bendamustine.HCl. In rat bile (1 rat), ~52% of the total dose was excreted in the 0-30-min fraction, ~17% in the 30-60-min fraction and ~6% during the 60-120-min collection period. In rat urine (N=5 separate animals), a mean of 28.5% of the total dose was excreted in the 0-4-hr fraction, 2.4% during 4-8 hr and 3.3% in the 8-24-hr fraction.

Metabolite Profile of Rat Bile

Representative radiochromatograms of 0-30-min and 30-60-min bile collected after a single 3-mg/kg intravenous dose of bendamustine are shown in Fig. 2A and 2B, respectively. Between these two fractions, a total of 10 radioactive peaks, including one for parent (P) bendamustine, were detected in the bile. No additional metabolites were observed in the final (60-120-min) collection period. Results from LC-MS3 analysis showed that the major metabolite was M19, with a molecular ion at m/z 485. This metabolite accounted for about 60% of total peak area in the radiochromatograms of both the 0-30-min and 30-60-min collections. Levels of radioactivity in bile rapidly declined over the 2-hr collection period. With regard to increasing retention time, and including all of the results in bile and urine, the other metabolites detected were designated M5, M12, M15, M16, M17, M18, M20 and M21. The relative retention time, molecular ions
and whether or not each was detected in a particular bile fraction are summarized in Table 1.

**Metabolite Profile of Rat Urine**

A total of 17 radioactive peaks were identified (not necessarily in every individual rat sample), mainly in the samples collected from 0 to 4 hr after the 3-mg/kg intravenous dose. A representative radiochromatogram of 0-4-hr urine from rat 004M, showing 16 radioactive peaks (including P), is shown in Fig. 2C. Metabolite M3 was not detected in this specific sample, and M7 and M9 were observed in other urine samples, but were not characterized. Approximately 78% of the total radioactivity excreted into urine had been collected during 0-4 hr after administration during the mass balance study. Unchanged drug was not detected above background in the rat urine fractions collected during 4-8 and 8-24 hr. M5 (molecular ion m/z = 276) and M19 were found to be the major metabolites in rat urine. The relative retention times, molecular ions and whether or not each was detected in a particular urine fraction are summarized in Table 2.

**Identification of Metabolites**

Structures of metabolites were proposed primarily based on MS^n (usually MS^3) spectra of each radioactive peak and by comparing to results from the available reference standards M6, M16, M20 and M21. LC-MS^n experiments of unknowns were carried out using the matrix that showed the most substantial radioactive peak of the metabolite of interest. A total of 19 metabolites were identified in rat bile and urine. The major fragment ions, the metabolite retention time and the matrix that was used for the analysis are summarized in
Table 3. The proposed structures of the metabolites detected in this study are shown in Fig. 13.

The MS\(^n\) spectra of bendamustine, shown in Fig. 3, indicated that loss of H\(_2\)O, CO and HCl accounted for the major fragment ions. Cleavage at the chloroethylamino C-N bond appeared to be common for bendamustine, and these fragmentation patterns were also observed in the MS\(^n\) spectra of several of its metabolites. A chlorine isotope pattern in a full scan MS spectrum was the signature pattern for bendamustine and its metabolites. Bendamustine, M20 and M21 have 2 chlorines on the molecule; including the \(^{14}\text{C}/^{12}\text{C}\) and the chlorine ratios, [M+2]/M is approximately 85%. M12 and M16 to M19 each have 1 chlorine; in this case, the isotope ratio observed was approximately 60%. No such chlorine isotopic pattern was observed in the full scan MS spectra for metabolites M1 to M11 and M13 to M15 (\(^{14}\text{C}/^{12}\text{C}\) ratio of approximately 25%).

M1 was a minor metabolite in urine. The MS\(^n\) spectra showed a molecular ion at m/z 320 and major fragment ions at m/z 302, 287, 274, 260, 247, 228 and 213 (Table 3). Loss of H\(_2\)O (m/z 302), followed by N-demethylation and cyclizing to a six-membered ring (m/z 287), and loss of H\(_2\)O followed by losing CO (m/z 274) were 3 significant fragment ions. M1 was proposed to be a di-dechlorinated metabolite. The proposed structure of M1 and its fragmentation interpretation are shown in Fig. 4.

M3 had a molecular ion at m/z 278 and fragment ions at m/z 260, 245, 242, 232 and 214 (Table 3). The fragmentation pattern of M3 was similar to that of bendamustine; loss of H\(_2\)O (m/z 260 and 242) and loss of H\(_2\)O and CO (m/z 232) followed by loss of another H\(_2\)O (m/z 214) are major fragments. A fragment ion at m/z 245 indicated that there was ring formation similar to the ion at m/z 287 in M1. M3 was therefore proposed to be a
metabolite with \( N \)-dealkylation of the chloroethyl side chain and dechlorination from the other chloroethyl group to form a 2-hydroxyethyl derivative. The molecular ion matches the assigned structure (Fig. 13).

M5 was the major metabolite in urine, with a molecular ion at \( m/z \) 276 and major fragment ions at \( m/z \) 258 (loss of H\(_2\)O) and 230 (loss of H\(_2\)O, then CO). It appeared to be a further oxidation product of M3, with the 2-hydroxyethyl moiety oxidized to an aldehyde or enol, which would be in tautomeric equilibrium (Smith and March, 2001). M5 was therefore proposed to be an oxidized \( N \)-dealkylated metabolite whose spectra and proposed structure are shown in Fig. 5.

M2 and M4 had the same molecular ion at \( m/z \) 292 (276 + 16); both were detected in urine. Major fragment ions were at \( m/z \) 274, 256, 246 and 228 (losses of H\(_2\)O and CO; Table 3). The fragment ions of 246 and 274 in the mass spectra of M2 had masses that were 16 Daltons above the M5 fragment ions of 230 and 258, respectively. This result suggests that M2 and M4 were mono-oxidation metabolites of M5 with the hydroxyl group on different sites in the molecule.

M8 showed a molecular ion at \( m/z \) 547 and major fragment ions at \( m/z \) 529 (547 – 18), 467 (547 – 80, loss of SO\(_3\)), 449, 358, 320, 302 and 260, and a minor ion at \( m/z \) 162 (Table 3); the latter indicated the presence of mercapturic acid. M8 was proposed to be a di-dechlorinated, sulfated, mercapturic acid conjugate. The proposed structure of M8 and the fragmentation interpretation are shown in Fig. 6. M10 showed a molecular ion at \( m/z \) 467, which was the same as one of the fragment ions in M8. This suggested mercapturic acid conjugation on one of the ethyl amino chains in the molecule. The proposed structure was similar to M8, except without sulfate (Fig. 13).
M11 and M13 each showed the same molecular ion at m/z 641, with major fragment ions at m/z 623 (641 - 18), 512 (641 - 129, loss of glutamate from glutathione), 452, 434 and 348 (Table 3). The neutral loss of mass 129 from the molecular ion of m/z 641 indicated that M11 and M13 were glutathione conjugates. The metabolic route to their formation was proposed to be di-dechlorination, oxidation (at different sites) and glutathione conjugation. Fig. 7 shows the structure and fragmentation interpretation of M11.

M12 and M18 had the same molecular ion at m/z 501 and similar major fragment ions at m/z 483 (501 - 18), 372 (501 - 129), 354 (501 – 129 – 18), and a minor ion at m/z 162 (Table 3). They were proposed to be mercapturic acid conjugates with hydroxylation at different sites in the molecule. Fig. 8 shows the proposed structure and fragmentation interpretation of M18.

M14 showed a molecular ion at m/z 595 and major fragment ions at m/z 577 (595 – 18), 551 (595 – 44, loss of CO₂), 466 (595 – 129), 422, 406, 362 and 344 (Table 3). The neutral loss of mass 129 from the molecular ion suggested a glutathione conjugate. Fragmentation interpretation and structure assignment are shown in Fig. 9. The fragmentation can also be explained that the molecular ion at m/z 595 loses H₂O first (m/z 577), then there is simultaneous cleavage of glutamic acid from glutathione (loss of 129) and one of the C-N bond (loss of CH₂CO) to form an intense fragment ion at m/z 406 in the MS² spectrum.

M15 showed a molecular ion at m/z 612 and major fragment ions at m/z 594 (612 – 18), 483 (612 – 129), 423, 405, 294, 234 and a minor ion at m/z 162 (Table 3). The ion at m/z 162 indicated that a mercapturic acid moiety is on the molecule. The ion at m/z 483 showed loss of 129 from molecule, in this case suggesting loss of the acetyl-cysteine.
moiety except for the sulfur. M15 was proposed to be a di-mercapturic acid conjugate. The fragmentation interpretation and structure assignment are shown in Fig. 10.

M17 showed a molecular ion at m/z 354 and major fragment ions at m/z 336 (354 – 18), 308 (354 – 46, loss of HCOOH), 300 (354 – 18 – 36, loss of H₂O and HCl), 292, 278, 263, 256, 250, 242, 228 and 214 (Table 3). The isotope pattern in the full scan MS spectrum indicated that chlorine was present, and that one of the two chloroethyl chains remained intact and the other chain was oxidized to a carboxylic acid, consistent with the molecular ion. Fragmentation interpretation and structure assignment are shown in Fig. 11.

M19 showed a molecular ion at m/z 485 and major fragment ions at m/z 467 (485 – 18), 356, 320, 296, 278 and 162 (Table 3). The fragment ion of 162 suggested a mercapturic acid conjugate. The isotope pattern in the full scan MS spectrum indicated that chlorine was present, and that one of two chloroethyl chains remained intact while the other chain was conjugated with mercapturic acid. Fragmentation interpretation and structure assignment are shown in Fig. 12.

M6 (di-hydrolysis product), M16 (mono-hydrolysis), M20 (monohydroxy) and M21 (N-desmethyl) showed the identical retention times and mass spectral patterns to those of the reference standards. These metabolites were also described by Teichert et al. (2005; 2007).

Two additional minor radioactive peaks, M7 and M9, were detected in some of the rat urine samples, but structural elucidation has not been completed.
Discussion

The objective of this investigation was to obtain information on the structures of metabolites excreted into rat urine and bile in order to understand the metabolic fate of bendamustine in vivo. $^{14}$C-Bendamustine was sufficiently stable to analyze in rat urine for up to 4 hours at 6°C in the autosampler, but was gradually degraded to several products thereafter. $^{14}$C-Bendamustine was stable in spiked control rat urine during 3 freeze (at -80°C) and thaw cycles, if thawing was conducted at ~4°C. Therefore, urine samples were stored at approximately -80°C and individually thawed on wet ice for analysis. Stability of the compounds in bile was not directly examined. To ensure the minimum possible degradation of metabolites, bile was collected on wet ice and a portion was injected onto the analytical system as soon as possible after collection, but in no case was a sample on wet ice for longer than 60 min prior to injection. The remainder from each fraction was apportioned into vials for storage at -80°C, so that no sample would have to be thawed (on wet ice) more than once for reanalysis (if necessary).

Bendamustine was extensively metabolized and rapidly cleared in rats. Following a single 3-mg/kg intravenous dose of $^{14}$C-bendamustine.HCl, a substantial fraction of the dose was excreted in the bile during the first 60 min. Parent drug was not detected in bile collected from 60 to 120 min, and the total radioactivity in this fraction was less than 10% of that collected from 0 to 30 min. Urine collected during 0-4 hr contained nearly 30% of the dose, which represented more than half of the radioactivity excreted into urine during a 7-day mass balance study. This matrix also showed minimal parent bendamustine. It was evident from these results that a major portion of the radioactivity
detected in urine and bile was accounted for by mercapturic acid conjugation and the
N-dealkylation metabolites.

Metabolites have been characterized using LC-MS$^n$ analysis with on-line radioactivity
detection and by comparison with retention times and MS$^n$ spectra of available reference
standards. Molecular ion data, isotopic patterns of chlorine, ratio of $^{14}$C/$^{12}$C, fragment
ions and/or characteristic mass loss and molecular weight information, have all been used
to make these assignments.

Six metabolites were proposed to be mercapturic acid conjugates and 3 metabolites
appeared to be glutathione conjugates. Metabolite M19, a mercapturic acid conjugate,
was the major metabolite in both rat bile and urine. Therefore, glutathione conjugation
was suggested to be a major detoxification pathway in rats.

The major metabolic routes of clearance were proposed to be oxidative and/or hydrolytic
dehlorination, glutathione conjugation and N-dealkylation. Cysteine conjugates were
observed in their mercapturic acid forms. One route to a mercapturic acid derivative
could be via glutathione conjugation, followed by cleavage of the glutamate moiety and
acetylation, resulting in the excretion of mercapturic acid (N-acetylcysteine) conjugates,
although direct conjugation with cysteine, followed by N-acetylation cannot be excluded
(Hinchman and Ballatori, 1994). Both mono- (M8, M10, M12, M18 and M19) and
di-mercapturic acids (M15) were detected, consistent with the non-acetylated pair of
cysteine conjugates detected in human bile (Teichert et al., 2005). Minor routes of
metabolism included oxidation, carboxylic acid formation and sulfate conjugation.

In conclusion, in the present study 15 novel metabolites were detected in rat bile and
urine. Among these metabolites, 3 were identified as glutathione conjugates, 6 as
mercapturic acid conjugates and 4 of the 6 phase I metabolites were $N$-dealkylation products. Chlorines on the bendamustine molecule were extensively metabolized through either oxidative or hydrolytic processes. Based on metabolites identified in rat bile and urine, the major metabolic pathways of bendamustine proposed are shown in Fig. 13.

Acknowledgments

We thank Dr. Philmore Robertson, Jr., and Dr. George C. McCormick of Cephalon, Inc., for review of the data and helpful discussions.
References


Legends for Figures

Figure 1  Structure of [$^{14}$C]-bendamustine

Figure 2  Representative radiochromatograms of bendamustine (P) and its metabolites in 0-30-min bile (A), 30-60 min-bile (B) and 0-4-hr urine (C) after a 3-mg/kg intravenous dose of [$^{14}$C]-bendamustine.HCl. Metabolites M1, M2, etc., are designated 1, 2, etc., to save space.

Figure 3  Representative LC-MS$^n$ spectra of bendamustine in rat bile

Figure 4  Representative LC-MS$^n$ spectra of metabolite M1 in rat urine

Figure 5  Representative LC-MS$^n$ spectra of metabolite M5 in rat urine

Figure 6  Representative LC-MS$^n$ spectra of metabolite M8 in rat urine

Figure 7  Representative LC-MS$^n$ spectra of metabolite M11 in rat urine

Figure 8  Representative LC-MS$^n$ spectra of metabolite M18 in rat urine

Figure 9  Representative LC-MS$^n$ spectra of metabolite M14 in rat urine

Figure 10 Representative LC-MS$^n$ spectra of metabolite M15 in rat urine

Figure 11 Representative LC-MS$^n$ spectra of metabolite M17 in rat bile

Figure 12 Representative LC-MS$^n$ spectra of metabolite M19 in rat urine

Figure 13 Proposed Metabolic Pathways for Bendamustine in rat urine and bile
Table 1  Summary of bendamustine metabolites detected in rat bile using LC-MS

<table>
<thead>
<tr>
<th>Peak ID</th>
<th>Retention Time (min)</th>
<th>[M+H]+ (12C)</th>
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√ = detected; ND = not detected.
NR = detected by LC-MS, but not by the radiochemical detector.
P = parent bendamustine; M followed with number = metabolite
Table 2  Summary of bendamustine metabolites detected in rat urine using LC-MS<sup>n</sup>

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<td>√</td>
<td>ND</td>
<td>ND</td>
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<tr>
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<td>24.9</td>
<td>467</td>
<td>√</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
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<td>641</td>
<td>√</td>
<td>ND</td>
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<tr>
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<td>501</td>
<td>√</td>
<td>ND</td>
<td>ND</td>
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<td>641</td>
<td>√</td>
<td>ND</td>
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<td>√</td>
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<td>ND</td>
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</tr>
<tr>
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<td>340</td>
<td>√</td>
<td>ND</td>
<td>ND</td>
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<tr>
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<td>501</td>
<td>√</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>M19</td>
<td>31.7</td>
<td>485</td>
<td>√</td>
<td>√</td>
<td>√</td>
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</table>

√ = detected; ND = not detected.

P = parent bendamustine; M followed with number = metabolite

Metabolites M7 and M9 were observed at low levels in some samples, but were not characterized.
Table 3: Summary of structural information obtained by LC-MS\textsuperscript{n} for metabolites of bendamustine in rat urine and bile

<table>
<thead>
<tr>
<th>Peak</th>
<th>Matrix</th>
<th>RT (min)</th>
<th>[M+H]\textsuperscript{+}</th>
<th>Major fragment ions (m/z)</th>
<th>Metabolite identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>Bile</td>
<td>35.6</td>
<td>358</td>
<td>228, 268, 276, 304, 312, 340\textsuperscript{a}</td>
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<tr>
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<td>Urine</td>
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<td>320</td>
<td>213, 228, 247, 260, 274, 287, 302</td>
<td>Di-dechlorination, oxidation</td>
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<tr>
<td>M2</td>
<td>Urine</td>
<td>13.6</td>
<td>292</td>
<td>216, 228, 246, 256, 274</td>
<td>Di-dechlorination, N-dealkylation and oxidation</td>
</tr>
<tr>
<td>M3</td>
<td>Urine</td>
<td>15.6</td>
<td>278</td>
<td>214, 232, 242, 245, 260</td>
<td>Di-dechlorination, N-dealkylation and oxidation</td>
</tr>
<tr>
<td>M4</td>
<td>Urine</td>
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<td>292</td>
<td>228, 256, 274</td>
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<tr>
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<td>189, 203, 217, 230, 258</td>
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<tr>
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<td>Sample Name</td>
<td>Mass Range (Da)</td>
<td>Matched Masses (Da)</td>
<td>Conjugation Type</td>
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<td>------------------</td>
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<tr>
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<td>mercapturic acid conjugation</td>
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<td>mercapturic acid conjugation</td>
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<tr>
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<td>mercapturic acid conjugation</td>
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<tr>
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<td>mercapturic acid conjugation</td>
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<td>Urine</td>
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<td>Oxidation</td>
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<td>N-Demethylation</td>
</tr>
</tbody>
</table>

* The most intensive fragment ions are bolded in the major fragment ion column.

P = parent bendamustine; M followed with number = metabolite.
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6
Fig. 7
Fig. 8
Fig. 10
Fig. 11
Fig. 12