Metabolic Profile of [14C]Bendamustine in Rat Urine and Bile: Preliminary Structural Identification of Metabolites

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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 more than 30 years, very limited information is available on its biotransformation. The objective of this investigation was to obtain information on the structures of metabolites excreted into rat urine and bile 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 (MS) in parallel with on-line radioactivity detection in samples obtained after i.v. 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, whereas 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 (Pönisch et al., 2006). Although it has been used in the former East Germany for more than 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 i.v. dosing (Weber et al., 1991). Bendamustine metabolites excreted into rat bile and urine were investigated with 14C-labeled compound using thin-layer chromatography after isolation from cytochrome P450 incubation mixtures (Teichert et al., 2007). Although seven metabolites were tentatively identified in 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 i.v. dose of [14C]bendamustine-HCl was administered, and bile was collected in fractions on wet ice through 2 h 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 AMICS (Bubendorf, Switzerland) for bendamustine hydrochloride, 5-[bis(2-chloroethyl)amino]-1-methyl-1H-benzimidazole-2-carboxylic acid monohydrochloride (purity 97.0–102.0%); a hydroxylated metabolite (M20 in this report), 1-[bis-(2-chloroethyl)amino]-1-methyl-1H-benzimidazol-2-yl-4-hydroxybutyric acid (purity 89.4%); a N-desmethyl metabolite (M21), 1-[bis-(2-chloroethyl)amino]-1H-benzimidazol-2-yl-butyric acid (purity 71.1%); the monohydrolysis metabolite (M6), purity 89.4%; and the dihydrolysis metabolite (M6; purity 92.0%). Naming of metabolites in this report is different from naming in previous publications (e.g., Teichert et al., 2005). [14C]Bendamustine-HCl (purity >98%; specific activity 26.1 μCi/mg) was obtained from BioDynamics.

ABBREVIATIONS: LC, liquid chromatography; MS, mass spectrometry.
Acetonitrile, methanol, and formic acid were high-performance liquid chromatography grade and were obtained from VWR (West Chester, PA) or J.T. Baker (Phillipsburg, NJ). Water was obtained from a Milli-Q Ultrapurification System (Millipore Corporation; Milford, CT). Ultima-Flo liquid scintillation cocktail was purchased from PerkinElmer Life and Analytical Sciences (Shelton, CT). 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 (Raleigh, NC) for the bile studies. After receipt at Cephalon (Cephalon, Inc., West Chester, PA), 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, following Cephalon standard operating procedures. Before dosing, the bile flow was checked, and a predose 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 14C-labeled bendamustine hydrochloride.


FIG. 2. Representative radiochromatograms of bendamustine (P) and its metabolites in 0- to 30-min bile (A), 30- to 60-min bile (B), and 0- to 4-h urine (C) after a 3-mg/kg i.v. dose of [14C]bendamustine·HCl.

Metabolites M1, M2, etc., are designated 1, 2, etc., to save space.

Table 1

<table>
<thead>
<tr>
<th>Peak Identification</th>
<th>Retention Time</th>
<th>[M + H]+ (14C)</th>
<th>Rat Bile</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td>in 0–30 min</td>
<td>30–60 min</td>
</tr>
<tr>
<td>P</td>
<td>36.7</td>
<td>358</td>
<td>✓</td>
</tr>
<tr>
<td>M5</td>
<td>18.4</td>
<td>276</td>
<td>✓</td>
</tr>
<tr>
<td>M12</td>
<td>26.7</td>
<td>501</td>
<td>✓</td>
</tr>
<tr>
<td>M15</td>
<td>28.3</td>
<td>612</td>
<td>✓</td>
</tr>
<tr>
<td>M16</td>
<td>30.8</td>
<td>340</td>
<td>✓</td>
</tr>
<tr>
<td>M17</td>
<td>30.3</td>
<td>354</td>
<td>✓</td>
</tr>
<tr>
<td>M18</td>
<td>31.6</td>
<td>501</td>
<td>✓</td>
</tr>
<tr>
<td>M19</td>
<td>32.9</td>
<td>485</td>
<td>✓</td>
</tr>
<tr>
<td>M20</td>
<td>35.0</td>
<td>374</td>
<td>✓</td>
</tr>
<tr>
<td>M21</td>
<td>36.4</td>
<td>344</td>
<td>✓</td>
</tr>
</tbody>
</table>

P, parent bendamustine; M followed by number, metabolite; ✓, detected; N.D., not detected; N.R., detected by LC-MS, but not by the radiochemical detector.
and nonlabeled 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 to 30, 30 to 60, and 60 to 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.

Table 2

<table>
<thead>
<tr>
<th>Peak Identification</th>
<th>Retention Time (min)</th>
<th>[M + H]+ (Da)</th>
<th>Rat Urine 0–4 h</th>
<th>Rat Urine 4–8 h</th>
<th>Rat Urine 8–24 h</th>
</tr>
</thead>
</table>
Urine Collection. Rat urine samples (0- to 4-, 4- to 8-, and 8- to 24-h fractions) were collected on dry ice after a 3-mg/kg i.v. dose of [14C]bendamustine/HCl during a mass balance study conducted at Charles River Laboratories (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 on dry ice, each sample was thawed individually on wet ice, and a portion was immediately injected (50 μl) without further treatment. The remainder was transferred into vials in 300-μl portions, and 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-MS 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) coupled with an model 4 β-Ram radioactivity detector (IN/US, Tampa, FL). Chromatographic separations were performed on a Phenomenex (Torrance, CA) Synergy Hydro-RP column (4.6 × 250 mm, 4-μm particle size). The mobile phase consisted of 0.1% formic acid in water (mobile phase A) and acetonitrile (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 300°C, sheath gas flow rate of 60 (arbitrary), and an auxiliary gas flow rate of 20 (arbitrary). The MS analyses were performed with relative collision energies of 25% to 45%. The high-performance liquid chromatography 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...
\( \beta \)-Ram detector for radioactivity monitoring. Xcalibur software (version 1.4; ThermoFisher) 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) by adding 10 \( \mu \)l of each sample to 10 ml of Ultima-Flo liquid scintillation cocktail. Similar analyses were conducted by Charles River Laboratories for urine.

**Results**

**Recovery of Radioactivity.** Radioactivity was counted in each fraction of rat bile and urine collected after a single 3-mg/kg i.v. dose of \( ^{14} \)C bendamustine-HCl. In rat bile (1 rat), \( \sim 52\% \) of the total dose was excreted in the 0- to 30-min fraction, \( \sim 17\% \) in the 30- to 60-min fraction, and \( \sim 6\% \) during the 60- to 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- to 4-h fraction, 2.4\% in the 4- to 8-h fraction, and 3.3\% in the 8- to 24-h fraction.

**Metabolite Profile of Rat Bile.** Representative radiochromatograms of 0- to 30-min and 30- to 60-min bile collected after a single 3-mg/kg i.v. dose of bendamustine are shown in Fig. 2, A and B, 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- to 120-min) collection period. Results from LC-MS\(^3\) analysis showed that the major metabolite was M19, with a molecular ion at \( m/z \) 485. This metabolite accounted for approximately 60\% of total peak area in the radiochromatograms of both the 0- to 30-min and 30- to 60-min collections. Levels of radioactivity in bile rapidly declined over the
2-h 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 h after the 3-mg/kg i.v. dose. A representative radiochromatogram of 0- to 4-h 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 to 4 h after administration during the mass balance study. Unchanged drug was not detected above background in the rat urine fractions collected during 4 to 8 and 8 to 24 h. 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 metabolite 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 comparison 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 \( \text{H}_2\text{O}, \text{CO}, \) and \( \text{HCl} \) 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 two 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 one 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 $\text{H}_2\text{O}$ ($m/z$ 302), followed by N-demethylation and cyclizing to a six-membered ring ($m/z$ 287), and loss of $\text{H}_2\text{O}$ followed by losing CO ($m/z$ 274) were three 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 $\text{H}_2\text{O}$ ($m/z$ 260 and 242) and loss of $\text{H}_2\text{O}$ and CO ($m/z$ 232) followed by loss of another $\text{H}_2\text{O}$ ($m/z$ 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 $\text{H}_2\text{O}$) and 230 (loss of $\text{H}_2\text{O}$, 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 \( \text{H}_2\text{O} \) and \( \text{CO} \)) (Table 3). The fragment ions of 246 and 274 in the mass spectra of M2 had masses that were 16 Da 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 \( \text{SO}_3 \)), 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 that of 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. Figure 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. Figure 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 \( \text{CO}_2 \)), 466 (595 – 129), 442 (595 – 133), and 428 (595 – 137).
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 by the molecular ion at \( m/z \) 595 loses H$_2$O 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 CH$_2$CO) to form an intense fragment ion at \( m/z \) 406 in the MS$^2$ 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 H$_2$O 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, 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 (dihydrolysis product), M16 (monohydrolysis), M20 (monohydroxy), and M21 (\( N \)-desmethyl) showed the identical retention times and mass spectral patterns as 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.

Fig. 13. Proposed metabolic pathways for bendamustine in rat urine and bile.
Discussion

The objective of this investigation was to obtain information on the structures of metabolites excreted in rat urine and bile to understand the metabolic fate of bendamustine in vivo. $^{14}$C]Bendamustine was sufficiently stable to analyze in rat urine for up to 4 h 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 three 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 before 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. After a single 3-mg/kg i.v. 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 to 4 h 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, the 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 three 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 dechlorination, 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 nonacetylated 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. On the basis of metabolites identified in rat bile and urine, the major metabolic pathways of bendamustine proposed are shown in Fig. 13.

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References


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