HUMAN METABOLISM OF THE PROTEASOME INHIBITOR BORTEZOMIB: IDENTIFICATION OF CIRCULATING METABOLITES

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ABSTRACT:

Bortezomib [3-(2,3-pyrazine)carbonyl-L-phenylalanine-L-leucine boronic acid] is a potent first-in-class dipeptidyl boronic acid proteasome inhibitor that was approved in May 2003 in the United States for the treatment of patients with relapsed multiple myeloma where the disease is refractory to conventional lines of therapy. Bortezomib binds the proteasome via the boronic acid moiety, and therefore, the presence of this moiety is necessary to achieve proteasome inhibition. Metabolites in plasma obtained from patients receiving a single intravenous dose of bortezomib were identified and characterized by liquid chromatography/mass spectrometry (LC/MS) and liquid chromatography/tandem mass spectrometry (LC/MS/MS). Metabolite standards that were synthesized and characterized by LC/MS/MS and high field nuclear magnetic resonance spectroscopy (NMR) were used to confirm metabolite structures. The principal biotransformation pathway observed was oxidative deboronation, most notably to a pair of diastereomeric carbinolamide metabolites. Further metabolism of the leucine and phenylalanine moieties produced tertiary hydroxylated metabolites and a metabolite hydroxylated at the benzylic position, respectively. Conversion of the carbinolamides to the corresponding amide and carboxylic acid was also observed. Human liver microsomes adequately modeled the in vivo metabolism of bortezomib, as the principal circulating metabolites were observed in vitro. Using cDNA-expressed cytochrome P450 isoenzymes, it was determined that several isoforms contributed to the metabolism of bortezomib, including CYP3A4, CYP2C19, CYP1A2, CYP2D6, and CYP2C9. The development of bortezomib has provided an opportunity to describe the metabolism of a novel boronic acid pharmacophore.

The 26S proteasome is a multicatalytic proteolytic complex found in both the nuclei and cytoplasm of eukaryotic cells (Dahlmann et al., 1989). The center of the proteasome, designated the 20S proteasome, comprises two α-subunits and two proteolytic β-subunits, each having chymotryptic, trypsinic, and postglutamyl endopeptidase activities (Lupas et al., 1994). The proteasome hydrolyzes proteins marked for degradation through the ubiquitin pathway and, in doing so, plays a critical role in cellular homeostasis and cell cycle regulation (Hideshima et al., 2003). Recent data indicate that various hematologic cancers are highly dependent on amplified proteasome activity for cell survival by way of promoting nuclear transcription and activation of anti-apoptotic proteins (Hideshima et al., 2001). These proteins are essential in the promotion of unregulated cell division, a common feature of malignant cells. Although the reasons are not completely understood, healthy cells do not exhibit this same dependence, and therefore, inhibition of the proteasome may represent a therapeutic advantage in targeted cancer therapy.

Bortezomib (VELCADE, formerly known as PS-341; Fig. 1) is a peptide boronic acid that reversibly inhibits the 26S proteasome. Compared with other targeted serine proteases, such as chymotrypsin, leukocyte elastase, and thrombin (Kᵢ values of 320, 2300, and 13,000 nM, respectively), bortezomib has exceptional specificity for the 26S proteasome (Kᵢ ≈1 nM) (Adams et al., 1998). Inhibition of the 26S proteasome involves the formation of a reversible dative bond between the N-terminal threonine residue of the chymotryptic-like site and the boron atom of bortezomib (McCormack et al., 1997).

The sensitivity of tumor cells to proteasome inhibition was evident in a number of nonclinical studies in which the initial indications of efficacy were demonstrated. Early studies showed that bortezomib induced apoptosis in tumor cells in vitro, as well as in a range of in vivo tumor models (Adams et al., 1999; Bold et al., 2001; Cusack et al., 2001; Hideshima et al., 2001; Shah et al., 2001). Likewise, the initial clinical trials indicated efficacy in the treatment of relapsed and refractory multiple myeloma (Richardson et al., 2003). Bortezomib was approved in May 2003 in the United States for the treatment of patients with relapsed multiple myeloma where the disease is refractory to conventional lines of therapy.

Although 30 years of research has generated a wealth of information regarding the inhibition of various proteases by boronic acids, little has been reported regarding the general disposition of this class of compounds in humans (Kettner and Shenvi, 1984; Hussain et al., 1991; Kelly et al., 1993; Priestley and Deciccio, 2000; London and Gabel, 2001; Yang et al., 2003). Moreover, the metabolic fate of boronic acids remains an unexplored area of biotransformation. The objective of the present study was to identify the circulating metabolites in the plasma from patients

ABBREVIATIONS: LC/MS/MS, liquid chromatography/tandem mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; P450, cytochrome P450; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; MS/MS, tandem mass spectrometry; NOESY, Nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; MeCN, acetonitrile; SRM, selected reaction monitoring.

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receiving a single intravenous dose of bortezomib. Liquid chromatography/tandem mass spectrometry (LC/MS/MS) was used to identify the metabolites of bortezomib. Confirmation of several metabolite structures was achieved by direct comparison of their LC/MS/MS data to that obtained from synthesized and isolated metabolites that were fully characterized by high-field NMR. Human liver microsomes and cDNA-expressed P450 isoenzymes were used to investigate the hepatic contribution to the metabolism of bortezomib. Additionally, two principal metabolites were synthesized to investigate secondary pathways of metabolism in the overall metabolism profile.

Materials and Methods

Reagents. Bortezomib [(1R)-3-methyl-1-[(2S)-1-oxo-3-phenyl-2-[pyrazin-nylcarbonyl] amino]propyl]butyl]boronic acid, pyrazine-2-carboxylic acid [1-(1-hydroxy-3-methyl-butylcarbamoxy)-2-phenyl-ethyl]-amide (M1 and M2), pyrazine-2-carboxylic acid (1-carbomethoxy-2-phenyl-ethyl)-amide (M3), 3-phenyl-2-[pyrazine-2-carbonyl]-amino-propionic acid (M4), and pyrazine-2-carboxylic acid [1-benzyl-2-(3-methyl-butyramino)-2-oxo-ethyl]-amide (M34) were synthesized and characterized by Albany Molecular Research, Inc. (Albany, NY). Potassium phosphate, ammonium formate, NADPH, trifluoroacetic acid (TFA), formic acid, and magnesium chloride (MgCl₂) were purchased from Sigma-Aldrich (St. Louis, MO). Human liver microsomes were obtained from Xenotech LLC (Lenexa, KS), and human cDNA-expressed Supersomes CYP3A4, CYP2C19, CYP1A2, CYP2D6, and CYP2C9 were purchased from BD Gentest (Woburn, MA). HPLC-grade solvents were purchased from Burdick and Jackson (Muskegon, MI). All reagents and solvents were obtained at the highest purity available.

In Vitro Metabolite Profiling. Human Microsomal and cDNA-Expressed P450 Isoform Incubations. The in vitro metabolism of bortezomib was studied using human liver microsomes and human cDNA-expressed Supersomes, which are derived from baculovirus expressed insect cells that produce both P450 and P450 reductase. A potassium phosphate-buffered reaction (0.1 M, pH 7.4) of bortezomib (1 or 50 μM), human liver microsomes (2 mg/ml), and MgCl₂ (3 mM) was initiated by the addition of NADPH (2 mM) and incubated at 37°C under ambient oxygenation for 1 h. Protein was precipitated by the addition of 1 volume of MeCN, and the resulting mixture was chilled at 4°C for 20 min followed by centrifugation. The supernatant was dried under a stream of nitrogen and reconstituted in 90:10 H₂O/MeCN in preparation for LC/MS analysis. Incubations with cDNA-expressed P450 isoforms (Supersomes) were performed in an analogous manner, substituting a P450 isoform (150 pmol/ml) for human liver microsomes.

LC/MS and LC/MS/MS of Human Microsomal Extracts. An Agilent 1100 HPLC system was coupled to a Symmetry C₁₈ column (5 μ, 3.8 × 150 mm; Waters Corporation, Milford, MA). Solvent A was 10 mM (pH 4.1) ammonium formate (aq.), and solvent B was MeCN. The initial mobile phase was 85:15 A/B (v/v) and by linear gradient transitioned to 20:80 A/B over 20 min. The flow rate was 0.400 ml/min. The HPLC eluent was via electrospray ionization directly into a Finnigan LCQ Deca XP™ ion trap mass spectrometer (Thermo Electron Corporation, San Jose, CA) operated in the positive ion mode. Ionization was assisted with sheath and auxiliary gas (nitrogen) set at 60 and 40 psi, respectively. The electrospray voltage was set at 5 kV with the heated ion transfer capillary set at 300°C and 30 V. Relative collision energies of 25 to 30% were used when operating in the MS/MS mode of the ion trap.

In Vitro Isolation of Metabolites from Human Liver Microsomes. Metabolites were isolated from reactions of bortezomib (1 mM) with human liver microsomes as previously described. Metabolites were separated on a Symmetry C₁₈ column (5 μ, 3.8 × 150 mm; Waters Corporation) coupled to an Agilent 1100 HPLC system including a variable wavelength ultraviolet/visible spectrophotometry detector (254 nm; Agilent Technologies, Palo Alto, CA) and an automated fraction collector. Solvent A was 0.05% TFA (aq.), and solvent B was 0.05% TFA in MeCN. The initial mobile phase was 85:15 A/B (v/v) and by linear gradient transitioned to 20:80 A/B over 20 min. The flow rate was 0.400 ml/min.

High-Field NMR. NMR data were acquired on a 600 MHz INOVA spectrometer (Varian Inc., Palo Alto, CA). NMR data for the isolated metabolites M5, M6, and M33 were obtained with a proton-only Prodigy capillary flow-probe by direct injection of the isolated metabolite in 1 mM MeCN-d₄/D₂O. Nuclear Overhauser Effect Spectroscopy (NOESY) and Total Correlation Spectroscopy (TOCSY) experiments were also performed with these samples. In a typical experiment, 64 complex increments of data were acquired, with 32 (TOCSY) or 128 (NOESY) scans per increment and mixing times of 30 (TOCSY) or 700 ms (NOESY).

In Vivo Metabolite Profiling. Study Design. Plasma samples from eight solid tumor patients in a phase 1 clinical study (DM98–194) conducted at the University of Texas MD Anderson Cancer Center (Houston, TX) were profiled to identify circulating metabolites. Blood was collected from the subjects prior to and at selected time points after the administration of a bortezomib intravenous bolus dose (1.6–2.0 mg/kg). Plasma was shipped frozen to Millennium Pharmaceuticals, Inc. (Cambridge, MA).

Sample Preparation. Due to low total dose, aliquots (250 μl) of plasma from each of the eight subjects were pooled from samples obtained at predose and 10- and 30-min postdose. Four volumes of chilled 0.1% formic acid in MeCN were added to precipitate protein, and the resulting mixture was centrifuged for 10 min at 15°C. The supernatant was removed and concentrated to approximately 225 μl under reduced pressure. The sample (200 μl) was injected into an LC/MS system as described below.

LC/MS and LC/MS/MS of Human Plasma Extracts. An Agilent 1100 HPLC (Agilent Technologies) was coupled to a YMC ODS-AQ column (3 μm; 2 × 100 mm; Waters Corporation). Solvent A was 0.1% formic acid (aq.) and solvent B was 0.1% formic acid in 90:10 MeCN/H₂O (v/v). The initial mobile phase was 100% A and by linear gradient transitioned to 100% B over 95 min. The flow rate was 0.350 ml/min. The HPLC eluent was introduced via electrospray ionization directly into an MDS Sciex API QStar Pulsar (QqTOF) mass spectrometer (Applied Biosystems, Foster City, CA) using a Turbo IonSpray interface set at 400°C. An IonSpray voltage of 5 kV was maintained along with a declustering potential of 30 V. Ionization was assisted with a nebulizer and ionspray gas (nitrogen) set at 55 and 75 (arbitrary units), respectively. Data were acquired in full-scan ion mode (time of flight mass spectrometry).

Because of the low total dose administered, selected reaction monitoring (SRM) of plasma extracts was performed to optimize the detection of in vivo metabolites as well as any potential metabolites that may have been at levels below the detection limit of a full scan acquisition.

Supporting LC/MS/MS analysis of plasma extracts was performed with an Agilent 1100 HPLC (Agilent Technologies) coupled to a LUNA C₁₈ column (3 μm, 2 × 100 mm; Phenomenex, Torrance, CA). Solvent A was 0.1% formic acid (aq.) and solvent B was 0.1% formic acid in 90:10 MeCN/H₂O (v/v). The initial mobile phase was 100% A and transitioned by linear gradient to 100% B over 50 min. The flow rate was 0.300 ml/min. The HPLC eluent was split and approximately 0.100 ml/min was introduced via electrospray ionization directly into an MDS Sciex API 3000 triple quadrupole mass spectrometer (Applied Biosystems) using a Turbo IonSpray interface set at 325°C. Ionization was assisted with nebulizer and Ionspray gas (nitrogen) at 8 (arbitrary units) and 7 l/min, respectively. The IonSpray potential was maintained at 5 kV. During MS/MS analysis, the collision energies used ranged from 18 to 25 V. SRM analysis of the circulating metabolites was performed using the following transitions: m/z 339.2 to 226.1 (M1, M2, M23, and M24), m/z 271.2 to 226.1 (M3), m/z 272.2 to 226.1 (M4), m/z 297.2 to 226.1 (M5/M6), m/z 355.2 to 242.1 (M8), m/z 395.2 to 293.2 (M25/M28), and m/z 567.2 to 226.1 (Bortezomib).

Results

Characterization of Bortezomib and Metabolites Observed in Plasma and Human Liver Microsomes. LC/MS/MS was used to characterize the circulating metabolites of bortezomib in human
plasma and in vitro in humans. The metabolites of bortezomib are depicted in Scheme 1.

**Bortezomib.** The protonated molecular ion [M + H]^+ of bortezomib is labile and undergoes in-source dehydration (−18 Da) to yield an observed [M + H-H2O]^+ at m/z 367. Sodium and potassium adducts of the parent molecule, [M + Na]^+ and [M + K]^+, were observed at m/z 407 and 423, respectively. In the negative ion mode, both the [M-H]^− at m/z 383 and [M-H-H2O]^− at m/z 365 were observed. Interestingly, in methanolic mobile phase, gas-phase formation of boronic acid methyl esters was observed. In positive ion mode, the ester of dehydrated bortezomib was observed at m/z 381; the parent ester was observed in the negative ion mode at m/z 397. The proposed gas-phase rearrangement of bortezomib leading to dehydration is depicted in Fig. 2 along with MS/MS fragment assignments. Fragmentation of the parent compound in positive ion mode produced the key fragments at m/z 226 and 208. These fragments were observed for nearly all the bortezomib metabolites and were generally indicative of an unmodified pyrazinyl-phenylalanine structure. The presence or absence of the characteristic boron isotope pattern was also useful in assigning structures, as it clearly indicated the presence or absence of the boron atom. The natural abundances of^{10}B and^{11}B are 20 and 80%, respectively.

**Metabolites M1 and M2.** Oxidative deboronation resulted in the carbinolamide diastereomers M1 and M2, the principal metabolites detected in both plasma and microsomes. In the gas phase, the protonated molecular ions [M + H]^+ are labile and undergo in-source dehydration to form [M + H-H2O]^+ ions at m/z 339. Sodium and potassium adducts of the parent molecules, [M + Na]^+ and [M + K]^+, were observed at m/z 379 and 395, respectively. An MS/MS fragment at m/z 271 was observed and represents the amide species resulting from cleavage of the carbinolamide carbon-nitrogen bond. Loss of the iminoleucine moiety produced an m/z 254 fragment; the iminoleucine ion was observed at m/z 86. Subsequent loss of carbon monoxide produced the characteristic m/z 226 ion, which undergoes facile dehydration to yield the m/z 208 ion (Table 1). The structures of metabolites M1 and M2 were confirmed by comparing their chromatographic and MS/MS fragmentation properties with those of authentic standards. Importantly, the fragmentation patterns of metabolites M1 and M2 established a carbinolamide signature (m/z 339, 271, 254, 226, and 208) that was useful in indicating the site of metabolism in subsequent structure assignments.

**Metabolite M3.** The amide metabolite M3 was detected in plasma and microsomes as the protonated molecular ion [M + H]^+ at m/z 271. Fragmentation of the parent ion produced fragments at m/z 254, 226, and 208 (Table 1). The structure of the amide metabolite M3 was confirmed by comparison of its chromatographic and MS/MS fragmentation properties to those of an authentic standard.

**Metabolite M4.** The carboxylic acid metabolite M4 is another important circulating metabolite that was detected in plasma and microsomes as the protonated molecular ion [M + H]^+ at m/z 272. MS/MS fragmentation yielded the characteristic m/z 226 and 208 ions (Table 1). The structure of the carboxylic acid metabolite M4 was confirmed by comparison of its chromatographic and MS/MS fragmentation properties to those of an authentic standard.

**Metabolites M5 and M6.** The hydroxylated carbinolamides M5 and M6 were observed in plasma and microsomes and are proposed to be diastereomers derived from M1 and M2, respectively. In the gas phase, the protonated molecular ions [M + H]^+ are labile and undergo in-source fragmentation to produce m/z 297, which results from a concerted loss of acetone. Sodium and potassium adducts of the

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**Scheme 1.** Proposed metabolism of bortezomib in humans.
parent molecule, [M + Na]⁺ and [M + K]⁺, were observed at *m/z* 395 and 411, respectively. MS/MS fragment ions at *m/z* 271, 254, 226, and 208 were observed (Table 1), indicating hydroxylation on the leucine moiety. The absence of additional diastereomers was consistent with hydroxylation on the tertiary carbon.

Metabolites M5 and M6 were isolated from in vitro incubations of bortezomib with human liver microsomes and characterized by NMR. 

1H NMR M5 (600 MHz, 1:1 MeCN-d₃/D₂O) 1.07 Me-1 (s, 3H), 1.10 Me-1 (s, 3H), 1.65 H-3a (dd, J = 14.4, 7.1 Hz, 1H), 5.38 H-4 (dd, J = 5.5, 7.1 Hz, 1H), 4.67 H-6 (dd, J = 5.1, 9.0 Hz, 1H), 3.17 H-7a (dd, J = 5.1, 14.0 Hz, 1H), 2.99 H-7b (dd, J = 9.0, 14.0 Hz, 1H), 7.12–7.21 (m, 5H), 8.99 (br s, 1H), 8.58 (br m, 1H), 8.66 (br m, 1H). 1H NMR M6 (600 MHz, 1:1 ACN-d₃/D₂O) 1.04 Me-1 (s, 3H), 1.07 Me-1 (s, 3H), 1.48 H-3a (dd, J = 14.4, 5.2 Hz, 1H), 1.64 H-3b (dd, J = 14.4, ca. 7.2 Hz, 1H), 5.32 H-4 (dd, J = 5.2, 7.2 Hz, 1H), 4.65 H-6 (dd, J = 5.9, ca. 8.5 Hz, 1H), 3.12 H-7a (dd, J = 5.9, 14.0 Hz, 1H), 2.99 H-7b (dd, J = ca. 8.5, 14.0 Hz, 1H), 7.12–7.21 (m, 5H), 8.99 (br s, 1H), 8.58 (br m, 1H), 8.66 (br m, 1H). The NMR data indicated that the sample contained a ca. 2:1 mixture of M5 and M6. Key features of the 1H NMR spectra for the major component were the AB components of an ABₜ system at 1.73 and 1.65 ppm and the presence of two methyl singlets at 1.10 and 1.07 ppm. The singlet nature and chemical shift of these geminal H-1 methyls is consistent with oxygenation at C-2, and the multiplicity of the AB protons is also consistent with their assignment as H-3a and H-3b and the absence of an H-2 proton. The minor component of the isolated metabolite sample showed corresponding signals at 1.64 and 1.48 ppm (AB components of ABₜ system) and 1.07 and 1.04 ppm (methyl signals).

**Metabolite M8.** The hydroxylated carbinolamide metabolite M8 was detected in plasma and microsomes. In the gas phase, the protonated molecular ion [M + H]⁺ is labile and undergoes in-source

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**TABLE 1**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>[M + H]⁺</th>
<th>Mass Fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bortezomib</td>
<td>367⁺</td>
<td>271, 254, 226, 208</td>
</tr>
<tr>
<td>M1</td>
<td>379, 395⁺</td>
<td>339, 254, 226, 208, 86</td>
</tr>
<tr>
<td>M2</td>
<td>379, 395⁺</td>
<td>339, 254, 226, 208, 86</td>
</tr>
<tr>
<td>M3</td>
<td>271</td>
<td>254, 226, 208</td>
</tr>
<tr>
<td>M4</td>
<td>272</td>
<td>226, 208</td>
</tr>
<tr>
<td>M5</td>
<td>395, 411⁺</td>
<td>297, 271, 254, 226, 208</td>
</tr>
<tr>
<td>M6</td>
<td>395, 411⁺</td>
<td>297, 271, 254, 226, 208</td>
</tr>
<tr>
<td>M7</td>
<td>411⁺</td>
<td>355, 269, 242, 226, 208</td>
</tr>
<tr>
<td>M8</td>
<td>339</td>
<td>321, 303, 254, 226</td>
</tr>
<tr>
<td>M23</td>
<td>339</td>
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</tr>
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<td>M24</td>
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</tr>
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<td>M25</td>
<td>395, 411⁺</td>
<td>293, 271, 254, 226, 208</td>
</tr>
<tr>
<td>M26</td>
<td>395, 411⁺</td>
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<td>M27</td>
<td>395, 411⁺</td>
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<td>M28</td>
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</tr>
<tr>
<td>M29</td>
<td>287</td>
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</tr>
<tr>
<td>M30</td>
<td>355</td>
<td>337, 308, 284, 271, 254, 226</td>
</tr>
</tbody>
</table>

*Observed as dehydrated molecular ion.

dehydration to form \([M + H-H_2O]^+\) ions at \(m/z\) 355. Sodium and potassium adducts of the parent molecules, \([M + Na]^+\) and \([M + K]^+\), were observed at \(m/z\) 395 and 411, respectively. MS/MS fragment ions observed at \(m/z\) 287, 270, 242, and 224 (Table 1) correspond to the carboxylamine signature +16 Da, indicative of hydrolysis on the pyrazinyl-phenylalanine moiety. The fragment at \(m/z\) 269 results from the loss of isopentenyldehydro (86 Da).

**Metabolites M23 and M24.** Metabolites M23 and M24 were detected in plasma and microsomes as the protonated molecular ion, \([M + H]^+\), at \(m/z\) 339 and are proposed to be structural isomers resulting from a net deboronation and dehydrogenation. The characteristic MS/MS fragments at \(m/z\) 254, 226, and 208 (Table 1) were observed, indicating a pyrazinyl-phenylalanine structure that was not modified.

**Metabolites M25, M26 and M27, and M28.** The hydroxylated carbinolamides M25, M26 and M27, and M28 were observed in plasma and microsomes and are proposed to be two pairs of diastereomers derived from M1 and M2, respectively. The protonated molecular ions \([M + H]^+\) are labile and undergo in-source fragmentation to produce \(m/z\) 355. Sodium and potassium adducts of the parent molecule, \([M + Na]^+\) and \([M + K]^+\), were observed at \(m/z\) 395 and 411, respectively. The MS/MS carbinolamine signature fragments at \(m/z\) 271, 254, 226, and 208 were observed and suggested hydroxylation on the prochiral carbon of the leucine moiety. A stereoisomeric relationship is proposed for M25 to M28 and M5/M6.

**Metabolite M33.** The hydroxylated amide metabolite M33 was observed in microsomes as the protonated molecular ion \([M + H]^+\) at \(m/z\) 287. This metabolite, which is proposed to originate from M8, produces MS/MS fragment ions at \(m/z\) 242 and 224. As in the case of M8, the observed fragment ions suggest hydroxylation on the pyrazinyl-phenylalanine moiety. \(^1\)H NMR data were obtained for M33 isolated from microsomes. \(^1\)H NMR M33 (600 MHz, 1:1 MeCN-d6/D2O) δ 4.82 H6 (d, J = 7.3 Hz), 5.06 H7 (d, J = 7.3, 1H), 7.36 (br d, J = ca. 7.5, 2H), 7.29 (br t, J = ca. 7.5, 2H), 5.25 (br t, J = ca. 7.5, 1H), 9.00 (d, J = 1.4, 1H), 8.61 (dd, J = 1.4, 2.4, 1H), 8.70 (d, J = 2.4, 1H). Although the H-6 and H-7 signal assignments may be interchangeable, the \(^1\)H NMR spectrum clearly showed that the H-6, H-7a, and H-7b protons of the phenylalanine moiety had been replaced by two 1H doublets at 4.82 and 5.05 ppm, with a mutual 7.3 Hz coupling. NOESY data using the same probe then showed correlations of both these protons to the phenylalanine aromatic protons, consistent with oxidation at the C-7 carbon.

**Metabolite M34.** The metabolite M34 was observed in microsomes resulting from the oxidative deboronation of bortezomib. The protonated molecular ion, \([M + H]^+\), was observed at \(m/z\) 355. The characteristic MS/MS fragments at \(m/z\) 271, 254, and 226 (Table 1) were observed, indicating a pyrazinyl-phenylalanine structure that was unmodified. Structural confirmation was provided by comparison with an authentic standard.

**Metabolism of Bortezomib in Humans.** The circulating metabolites of bortezomib were determined in the plasma of human subjects that were administered a single intravenous dose of bortezomib (1.6–2.0 mg/m²). The results from LC/MS and LC/MS/MS analyses revealed that oxidative deboronation to form the diastereomeric carbinolamide metabolites M1 and M2 was the principal biotransformation pathway observed for bortezomib. The structure and configurations of M1 and M2 were confirmed using authentic standards. Acidic conditions (0.1% formic acid/MeCN) were required to stabilize bortezomib during protein precipitation of plasma samples in preparation for LC/MS analysis. Consequently, degradation of metabolites M1 and M2 to their amide derivative M3 was observed in plasma. The carboxylic acid M4 resulting from the hydrolysis of M3 was also observed. The presence of the esterase inhibitor phenylmethanesulfonyl fluoride (100 μM) had no effect on the levels of M3 formation when bortezomib, M1, or M2 were incubated in naïve human plasma (data not shown), suggesting that M3 is produced as a result of nonenzymatic hydrolysis (Morgan et al., 1994; Satoh and Hosokawa, 1998). Oxidation to M8 was observed as the only hydroxylation of the phenylalanine moiety. Hydroxylation of the leucine moiety resulted in the formation of the hydroxylated metabolites M5 and M6, with relative stereochemistry corresponding to that of M1 and M2, respectively. Additional metabolites hydroxylated at the leucine residue were detected at lower levels in the plasma (M25–M28). Fragmentation data from the LC/MS/MS analysis of metabolites M23 and M24 indicated a site of unsaturation on the leucine moiety, suggesting deboronation and dehydrogenation as a pathway of bortezomib metabolism.

**Metabolism of Bortezomib in Vitro in Human Microsomes and cDNA-Expressed Cytochrome P450.** Human Liver Microsomal Metabolism. Incubations of bortezomib in human liver microsomes were performed to determine the hepatic contribution to the overall disposition of the compound in humans. The bortezomib metabolite profile in microsomes was qualitatively similar to the profile observed in human plasma. Metabolites M1 and M2 were the principal species observed in microsomes and were produced in nearly equimolar quantities, as indicated by LC/MS detection (Fig. 3). Importantly, deboronation to carbinolamides M1 and M2 remained the principal pathway of metabolism when a more relevant therapeutic concentration of bortezomib (1 μM) was incubated with microsomes (data not shown). Hydrolysis of M1 and M2 to the amide M3 was also observed in microsomes. The range of leucine-hydroxylated carbinolamide metabolites detected in plasma was also produced in microsomes. Consistent with the in vivo findings, metabolites M5 and M6 represented the principal hydroxylated carbinolamides, whereas metabolites M25 to M28 were detected at lower levels in microsomes. The phenylalanine hydroxylated carbinolamide M8 was also produced in microsomes. Subsequent degradation of M8 to its respective amide, M33, was also observed and is consistent with carbinolamide degradation, e.g., M1 and M2 degradation to M3. The oxidative deboronation of bortezomib to metabolite M34 was also detected in human microsomes.

cDNA-Expressed P450 Metabolism of Bortezomib and Metabolites M1 and M2. Individual cDNA-expressed P450 reactions were performed to determine the isoforms contributing to the metabolism of bortezomib and the formation of the observed metabolites. The isoforms investigated were CYP1A2, CYP3A4, CYP2C9, CYP2C19, and CYP2D6, and the incubations with each of these isoforms were analyzed by LC/MS. The major circulating carbinolamide metabolites, M1 and M2, were found to be formed by each of the five isoforms investigated and were observed in vitro as the principal biotransformation pathway. Consistent with the metabolism in microsomes, the ratio of M1/M2 observed in each individual P450 isoform reaction was found to be nearly 1:1. Multiple isoforms, including CYP1A2, CYP2C9, CYP2D6, and CYP3A4, were found to produce metabolites M23 and M24.

The potential of the predominant circulating metabolites M1 and M2 accounting for the formation of the remaining deboronated metabolites was also investigated with individual P450 isoforms. Authentic standards of metabolites M1 and M2 were incubated with specific P450 isoforms and subjected to LC/MS analysis. The results of these in vitro reactions indicated that CYP3A4 and CYP2C19 were responsible for the hydroxylation of M1 and M2 to metabolites M5 and M6, respectively. The remaining hydroxycarbinolamide metabolites M25/26 and M27/28 were found to be formed by CYP2C19 metabolism of M1 and M2, respectively. Biotransformation of M2 to
its corresponding hydroxyphenylalanine metabolite M8 was determined to be mediated by CYP3A4.

Discussion

Characterization of circulating metabolites from patients administered an intravenous dose of the proteasome inhibitor bortezomib revealed two biotransformation pathways for this chemotherapeutic agent (Scheme 1). The primary route of metabolism observed in humans was deboronation, yielding a pair of diastereomeric carbinalamide metabolites (M1 and M2). Perhaps this finding is not surprising given the well established lability of boronates toward oxidative deboronation. Attributed in part to the vacant $p$-orbital of the boron atom, boronates are susceptible to Baeyer-Villiger-type oxidations, which ultimately result in deboronation (Mirviss, 1967; Brown et al., 1971; Wu et al., 2000; Brot et al., 2003). Considering the crucial role of the boron atom in the present class of inhibitors, it is noteworthy that deboronation of bortezomib renders the agent and its metabolites inactive against the proteasome.

In vitro metabolism of bortezomib in human liver microsomes adequately modeled the in vivo metabolism of bortezomib, as the principal metabolites observed in vivo were also observed in vitro. Human cDNA-expressed P450 isoforms were used to determine the P450(s) contributing to the deboronation of bortezomib. Results from these experiments indicated that several isoforms, including CYP3A4, CYP2C19, CYP1A2, CYP2D6, and CYP2C9, mediated the biotransformation of bortezomib to the carbinalamides M1 and M2 (Daniels et al., 2003; Uttamsingh et al., 2003).

Secondary metabolism of the carbinalamides M1 and M2 accounted for a large number of the metabolites observed in plasma and in microsomes. Hydroxylation at the C-2 and C-3 positions of M1 and M2 resulted in the formation of hydroxycarbinalamides, e.g., M5 and M6. Although the relative stereochemistry has yet to be determined, metabolites M25, M26, M27, and M28 implicate the leucine moiety as a metabolic soft spot, secondary to oxidative deboronation. Metabolism of the phenylalanine moiety of M2 (C-7) by CYP3A4 produced metabolite M8 and represented another site for oxidative metabolism. Phenylalanine oxidation by CYP3A4 is consistent with the metabolism of peptide protease inhibitors bearing phenylalanine substituents (Balani et al., 1996). Hydrolysis of M1 and M2 to the amide metabolite M3 was observed in human microsomes and was subsequently identified as a major metabolite in plasma. The apparent hydrolytic lability of M1 and M2 was consistent with a previous account of pH-dependent hydrolysis of M1 and M2 to the corresponding amide M3 and the carboxylic acid M4 (Wu et al., 2000).

Desaturation of the leucine moiety represents the second route of metabolism for bortezomib. Mediated by CYP1A2, CYP2C9, CYP2D6, and CYP3A4, this biotransformation resulted in the production of two metabolites (M23 and M24) proposed to be regioisomers (Scheme 1). The low levels, detected in vivo and in vitro, prevented the isolation of M23 and M24 for regiochemical assignment, and tentative structures for M23 and M24 are therefore depicted by an unsaturation of the C1 to C3 alkyl system. Although P450-catalyzed dehydrogenation is a precedent biotransformation (Rettie et al., 1987, 1988; Fisher et al., 1998) and may be relevant to the boronate-leucine moiety, the possibility remains that M23 and M24 are a result of multiple biotransformation events, for example, deboronation followed by desaturation. Importantly, incubation of M1 or M2 in buffered solution failed to produce metabolites M23 and M24, suggesting that these metabolites were not formed as a result of carbinalamide dehydration (data not shown).

Although literature accounts are replete with descriptions of boronate synthesis and reactivity, very little is known regarding boronic acid metabolism. The present investigation has served to broaden our understanding of an unexplored area of drug metabolism and biotransformation, ultimately contributing to the design of boronic acids with improved pharmacodynamic and pharmacokinetic properties.

FIG. 3. Representative LC/MS (A) and HPLC/UV (B) profiles of bortezomib metabolites obtained from human liver microsomes.

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