RELATIVE CONTRIBUTIONS OF THE FIVE MAJOR HUMAN CYTOCHROMES P450, 1A2, 2C9, 2C19, 2D6, AND 3A4, TO THE HEPATIC METABOLISM OF THE PROTEASOME INHIBITOR BORTEZOMIB

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ABSTRACT:

VELCADE (bortezomib, PS-341), reversibly inhibits the 20S proteosome and exerts cytotoxic and antitumor activities. Pretreatment of cancer cells with bortezomib increases the chemosensitivity of these cells, suggesting that bortezomib may be used in combination chemotherapy. The relative contributions of the five major human cytochromes P450 (P450s), 1A2, 2C9, 2C19, 2D6, and 3A4 (the focus of the present study), to the metabolism of bortezomib are an important aspect of potential drug interactions. Relative activity factor (RAF), chemical inhibition, and immunoinhibition using monoclonal antibodies were three approaches employed to determine the relative contributions of the major human P450s to the net hepatic metabolism of bortezomib. RAFs for the P450 isoform-selective substrates were determined; the ratio of the rate of metabolism of bortezomib with cDNA-expressed P450s versus rate of metabolism with human liver microsomes was normalized with respect to the RAF for each P450 isoform to determine the percentage contributions of the P450s to the net hepatic metabolism of bortezomib. CYP3A4 followed by CYP2C19 were determined to be the major contributors to the metabolism of bortezomib. Chemical inhibition and immunoinhibition confirmed that CYP3A4 and CYP2C19 were the major P450s responsible for the hepatic metabolism of bortezomib. The studies were conducted with 2 μM bortezomib, and the disappearance of bortezomib, rather than appearance of a specific metabolite, was quantified to determine the contributions of the P450s to the overall hepatic metabolism of bortezomib in humans.

Boronic acids as protease inhibitors were first synthesized in the early 1970s and were demonstrated to act as potent transition state analogs of serine proteases (Koehler and Leinhard, 1971; Philipp and Bender, 1971; Kettner and Shenvi, 1984). Throughout the 1980s, peptide boronate acids were shown to be effective inhibitors of trypsin, chymotrypsin, α-lytic protease, pancreatic elastase, leukocyte elastase, thrombin, and β-lactamases (Kettner and Shenvi, 1984; Crompton et al., 1988) and have been explored for use as therapeutic agents in various disease states (Snow and Bachovchin, 1995; Groziak, 2001). More recently, peptidyl boronic acids were demonstrated as potent proteasome inhibitors, and antitumor and anti-inflammatory efficacy was observed both in vitro and in animal models (Adams et al., 1998). The approval of VELCADE (bortezomib, PS-341; Fig. 1), a dipeptidyl boronic acid, by the United States Food and Drug Administration in 2003 for the treatment of relapsed refractory multiple myeloma made it the first boronic acid and the first in a new class of drugs, proteasome inhibitors, to be marketed as a therapeutic agent.

Bortezomib, an N-pyrazinylcarbonylated derivative of the dipeptide boronic acid Phe-Leu-B(OH)2, is a potent, selective, and reversible inhibitor (Ki ~ 0.62 nM) of the 26S proteasome in mammalian cells (Adams et al., 1998). The molecular mechanisms by which bortezomib exerts its effects include inhibition of proteolysis of inhibitor κB, which in turn leads to inhibition of nuclear factor κB activation and subsequently increases the susceptibility of cells to apoptosis. Studies have shown that pretreatment of cancer cells with bortezomib effectively blocks activation of nuclear factor κB induced by the chemotherapeutic agent CPT-11/SN-38, suggesting that proteasome inhibition may help overcome chemo resistance (Cusack et al., 2001; Hideshima et al., 2001).

The mechanisms of metabolism and the drug-drug interaction potential of boron-containing drugs have not been explored previously. The contribution of the cytochromes P450 (P450), the major drug-metabolizing enzymes in humans, to the hepatic degradation of bortezomib has only been studied recently (Daniels et al., 2003; LaButti et al., 2003; Uttamsingh et al., 2003). Several P450-mediated circulating metabolites of bortezomib have been identified in patients who received a single intravenous dose of bortezomib (Pekol et al., 2005). Knowledge of the metabolism of bortezomib will aid in designing more effective second-generation anticancer agents, as well as help discover and develop boron-containing drugs for other disease states. The study herein discusses the determination of the percentage contribution of the five major human P450s, 1A2, 2C9, 2C19, 2D6, and 3A4, to the hepatic rate of metabolism of bortezomib. This information is also important from the view of drug-drug interactions, espe-
cially since anticancer agents tend to have narrow therapeutic indices and are commonly coadministered to patients with numerous medications. Bortezomib has shown to enhance the chemosensitivity of cancer cells and may be used as an adjuvant to chemotherapy in the future. Three distinct methods, relative activity factor (RAF) (Crespi, 1995; Venkatakrishnan et al., 2001b), chemical inhibition using P450-selective chemical inhibitors, and immunoinhibition using inhibitory monoclonal antibodies (Gelboin et al., 1999), were employed to evaluate the relative contribution of the major human P450s to the net hepatic metabolism of bortezomib. The RAFs as a ratio of clearance (RAFCL) were determined by measuring the kinetic parameters (V\textsubscript{max} and K\textsubscript{m}) for the P450 isofrom-selective substrates using cDNA-expressed P450s and human liver microsomes (HLM). The ratios of the rate of metabolism of bortezomib with the cDNA-expressed P450s and HLM were normalized with respect to the RAFs calculated for the P450 isoforms to determine the percentage contribution of each P450 to the net hepatic metabolism of bortezomib. Results obtained from the RAFCL studies were confirmed by measuring the percentage inhibition of P450-dependent metabolism of bortezomib in HLM using known chemical inhibitors of the P450 isoforms and P450-specific inhibitory monoclonal antibodies.

Materials and Methods

Human liver microsomes (pool of 50 individuals) were purchased from Xenotech LLC (Lenexa, KS). cDNA-expressed CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, insect control Supersomes, 4-OH-tolbutamide, 4-OH-mephenytoin, dextromethorphan, testosterone, 6β-OH-testosterone, furafylline, sulfaphenazole, omeprazole, quinidine, ketoconazole, NADPH, and MgCl\textsubscript{2} were purchased from Sigma-Aldrich (St. Louis, MO). Bortezomib was synthesized at Millennium Pharmaceuticals, Inc. Ascites fluid containing P450-specific monoclonal antibodies were prepared in potassium phosphate buffer, pH 7.4. Four monoclonal antibodies that showed minimum cross-reactivity and maximum inhibition of the human liver microsomal metabolism of the P450 isofrom-selective substrates phenacetin, tobutamide, S-mephenytoin, dextromethorphan, and testosterone were determined with a series of concentrations (1:3 serial dilutions)
of the chemical inhibitors or monoclonal antibodies. HLM were preincubated with the predetermined optimal concentrations of the chemical inhibitors or monoclonal antibodies for 5 min at 37°C in 96-well polypropylene plates; reactions were initiated by the addition of the P450 substrates or bortezomib and prewarmed NADPH/MgCl₂. The reaction mixtures contained 0.5 mg/ml HLM, monoclonal antibodies or chemical inhibitors, P450 substrates (Kₘ concentrations) or bortezomib (2 µM), 2 mM NADPH, and 3 mM MgCl₂ in a final volume of 100 µl and were incubated at 37°C for 15 min. Reactions were stopped by the addition of 100 µl of ice-cold acetonitrile containing carbamazepine as an internal standard. The plates were stored at 4°C for 30 min and then centrifuged at 1800g for 10 min to pellet-precipitated proteins. Supernatants were transferred to another 96-well plate and analyzed for amounts of metabolite formed (for the P450 marker substrates) or amount of bortezomib remaining, as described earlier. The percentage inhibition of metabolism of the P450-selective substrates or bortezomib was calculated as described below.

Data Analyses. LC/MS/MS data were analyzed using Analyst software, version 1.2. Kinetic parameters Kₘ and Vₘₐₓ for each of the P450-catalyzed reactions were determined from velocity versus substrate concentration plots using Prism software (GraphPad Software Inc., San Diego, CA). For the determination of Kₘ for the human liver microsomal metabolism of bortezomib, the amount of metabolite formed was calculated by measuring Δ bortezomib as shown in eq. 1.

Δ Bortezomib = (Amount of bortezomib remaining in reactions in the absence of NADPH) – (Amount of bortezomib remaining in reactions in the presence of bortezomib) (1)

Δ Bortezomib was plotted against substrate concentration, and Kₘ was determined by nonlinear regression using Prism.

Calculation of RAF. The relative contributions of the P450s 1A2, 2C9, 2C19, 2D6, and 3A4 to the rate of metabolism of bortezomib in humans were determined as shown below. The RAF for each of the five P450 isoforms was calculated according to eq. 2.

RAF = (Vₘₐₓ/Kₘ) of CYPn in HLM)/(Vₘₐₓ/Kₘ of cDNA-expressed CYPn) (2)

Contribution of CYPn to metabolism of bortezomib was subsequently calculated using eq. 3.

Contribution of CYPn (%) = RAF × (vₐₜₜₜ/vₐₜₜₜ) (3)

vₐₜₜₜ and vₐₜₜₜ were calculated by linear regression analysis on log (percentage of bortezomib remaining) versus time plots. The amount of metabolite formed at 5 min was determined from the linear regression plots to determine the vₐₜₜₜ and vₐₜₜₜ under initial velocity conditions. The plots of % bortezomib remaining in HLM versus time are shown in Fig. 2.

Calculation of Percent Inhibition of Human Liver Microsomal Metabolism of Bortezomib by P450-Selective Chemical Inhibitors. The percentage inhibition of human liver microsomal metabolism of bortezomib by the P450-selective inhibitors used in the study was calculated as shown in eq. 4.

\[ [1 - (\Delta \text{Bortezomib in the presence of chemical inhibitor})] \times 100 \] (4)

Calculation of Percent Inhibition of Human Liver Microsomal Metabolism of Bortezomib by Inhibitory Monoclonal Antibodies. The percentage inhibition of human liver microsomal metabolism of bortezomib by the CYP-specific monoclonal antibodies used in the study was calculated as shown in eq. 5.

\[ [1 - (\Delta \text{Bortezomib for reactions with anti-P450 antibodies/\Delta \text{Bortezomib for reactions with control antibody}})] \times 100 \] (5)

The Kₘ and Vₘₐₓ for CYP1A2-catalyzed phenacetin O-deethylation, CYP2C9-catalyzed tolbutamide hydroxylation, CYP2C19-catalyzed S-mephenytoin hydroxylation, CYP2D6-catalyzed dextromethorphan O-demethylation, and CYP3A4-catalyzed testosterone 6β-hydroxylation using HLM and cDNA-expressed P450s are shown in Table 1. The Kₘ values obtained were similar to the values reported previously (Pelkonen et al., 1998). The RAFs for the five major human P450, calculated using eq. 2, are also shown in Table 1. The Kₘ for the human liver microsomal metabolism of bortezomib was determined to be approximately 16.6 µM (data not shown). The recovery of bortezomib from samples containing protein was >90% compared with samples containing bortezomib and buffer only, and hence recovery of bortezomib from reaction mixtures was not a concern. The rates of metabolism of bortezomib with HLM and cDNA-expressed CYP1A2, 2C9, 2C19, 2D6, and 3A4 along with the percentage contribution of each P450 isoform, calculated using eq. 3, to the human liver microsomal metabolism of bortezomib are presented in Table 2. The rate of metabolism of bortezomib with HLM was determined to be 438 pmol/min/0.5 mg microsomal protein. The rates of metabolism of bortezomib with the cDNA-expressed P450s followed the order: CYP2C19 > CYP3A4 > CYP2D6 > CYP1A2 > CYP2C9. After normalizing with RAF, the percentage contribution of each P450 isoform in HLM to the rate of metabolism of bortezomib was as follows: CYP3A4 (38.4%) > CYP2C19 (30.1%) > CYP1A2 (10.5%) > CYP2D6 (7.1%) > CYP2C9 (1.2%). Thus, CYP3A4 and CYP2C19 were the major P450s that contributed to the metabolism of bortezomib. The sum of the contributions of the five P450s studied was approximately 87%.

The concentrations of the P450-selective chemical inhibitors furafylline (1A2), sulfaphenazole (2C9), omeprazole (2C19), quinidine (2D6), and ketoconazole (3A4) that inhibit the human liver microsomal metabolism of the P450-specific substrates with a high degree of selectivity were determined. Furafylline (100 µM), sulfaphenazole (10 µM), quinidine (10 µM), and ketoconazole (1 µM) showed >86% inhibition of metabolism of phenacetin, tolbutamide, dextromethorphan, and testosterone, respectively. Omeprazole (11 µM) was the least selective and inhibited the hydroxylation of S-mephenytoin by only 61% while also inhibiting the hydroxylation of tolbutamide by 21%. Furafylline and ketoconazole also exhibited some cross-reactivity by inhibiting 16 and 21% of the S-mephenytoin hydroxylation, respectively.

The percentage inhibitions of bortezomib metabolism in HLM by the P450 inhibitors are shown in Table 3. The percentage inhibition is equivalent to the percentage contribution of the P450 isoforms studied. Based on chemical inhibition studies, the percentage contribution...
of each P450 isoform to human liver microsomal bortezomib metabolism followed the order: CYP3A4 (69.8%) > CYP2C19 (33.5%) > CYP2D6 (23.4%) > CYP1A2 (15.8%) > CYP2C9 (13.6%). The sum of the contributions of the five P450s studied, as determined using inhibitory monoclonal antibodies, was approximately 130%.

**Discussion**

The three in vitro approaches employed to determine the relative contributions of the five major human P450s indicate that CYP3A4 followed by CYP2C19 are the major P450 isoforms that contribute to the net hepatic metabolism of bortezomib. Albeit minor, P450s 1A2, 2C9, and 2D6 also play a role in the hepatic metabolism of bortezomib. The percentage contributions varied according to the methodology used; nonetheless, the conclusions were essentially similar. Furthermore, these results mirrored the qualitative in vitro metabolite profiling studies with bortezomib. Whereas P450s 1A2, 2C9, 2C19, 2D6, and 3A4 mediated the biotransformation of bortezomib to the primary deboronated metabolites, carboxamides, CYP3A4, and CYP2C19 played a more significant role in the generation of secondary metabolites (Pekol et al., 2005), implicating CYP3A4 and CYP2C19 as the major players in the overall hepatic metabolism of bortezomib.

In this study, the relative contributions of the human P450s to the overall hepatic metabolism of the drug of interest (i.e., bortezomib) have been determined rather than evaluating the contributions of the P450s to a specific biotransformation pathway, as has been reported previously by several investigators (Rochat et al., 1997; Venkatakrishnan et al., 2001a; Wójcikowski et al., 2003). The advantage of this approach lies in the fact that it provides valuable information, even if the major metabolite or route of biotransformation for the drug of interest has not been elucidated. Furthermore, these studies were conducted with 2 μM bortezomib, a nonsaturating and clinically relevant concentration. Following a 1 mg/m² dose, the maximum plasma concentration (Cmax) in patients is approximately 150 nM (M Karol, personal communication). Liver concentrations of bortezomib in patients may reach 10× the plasma concentrations, approximately 1.5 μM, although it is unlikely to reach Km concentrations (~16 μM in HLM).

Determination of RAFs for isoforms of Phase I or Phase II drug-metabolizing enzymes was first proposed by Crespi (1995). The availability of HLM and cDNA-expressed P450s enables P450 “reaction mapping,” to be performed on a routine basis. Simple reaction mapping reveals qualitative information on metabolites generated and may not represent an accurate picture of the relative contribution of the individual P450 isoforms to the overall metabolism of drugs because of differences in recombinant systems and microsomal preparations. The RAFs serve as scaling factors that account for differences in relative hepatic abundance and intrinsic activities of the P450 isoforms between the cDNA-expressed enzymes and the human liver microsomal analogs (Rodrigues, 1999; Venkatakrishnan et al., 2001b; Rochat, 2003). The one disadvantage of this approach may be that its accuracy is dependent on the selectivity of the P450 isoform-selective index reaction; the kinetic parameters for these reactions need to be performed with every batch of HLM used, individual or pooled preparations alike. In this respect, inhibitory antibodies offer a distinct advantage and, with increasing availability, provide a convenient way for evaluating the percentage contribution of P450s to the metabolism of a drug, as has been demonstrated in the current study. Highly specific monoclonal antibodies have become available recently, and their utility in conducting such studies is being appreciated (Gelboin et al., 1999; Shou et al., 2000). In the case where inhibitory monoclonal antibodies are unavailable, P450-selective chemical inhibitors can also provide valuable and reliable information, provided that the studies are conducted with appropriate concentrations of the inhibitors. The specificity of the chemical inhibitors used may be a con-
founding factor in the overall accuracy of results (Newton et al., 1995; Zhang et al., 2002; Li et al., 2004); a degree of cross-reactivity was observed with omeprazole and ketoconazole (see Results). Highly selective CYP2C19 and CYP3A4 inhibitors benzylnirvanol and azamulin, respectively, have become available in the recent years and may be used in reaction-phenotyping studies. A multipronged approach that includes 1) recombinant P450s and P450 isoform-selective index reactions, 2) chemical inhibitors, and 3) inhibitory antibodies has been identified as important tools for P450 reaction phenotyping (Lu et al., 2003), and we have employed all three approaches to confirm the relative contribution of P450s to the hepatic metabolism of bortezomib. Although the three approaches yielded similar results in that P450s 3A4 and 2C19 were shown to be the major contributors to the hepatic metabolism of bortezomib, there are discrepancies with regards to the sum of the percentage contributions of the five P450s evaluated. The sum of the percentage contributions of the P450s as determined by the RAF method was 87%, whereas with monoclonal antibodies and chemical inhibitors, the sums were 130 and 150%, respectively. In the case of chemical inhibitors, the high degree of cross-reactivity observed with omeprazole and ketoconazole may contribute to the observed phenomenon. It has been suggested that bortezomib may be susceptible to metal ion-mediated degradation (Wu et al., 2000), which may be exacerbated in the presence of protein (J. S. Daniels, personal communication). In incubations with monoclonal antibodies, nonspecific protein-mediated degradation of monoclonal bortezomib may have contributed to its depletion. Although reactions with a control antibody HyHel-9 were included, a lack of sufficient material prevented equalization of total protein amounts in the reactions with the monoclonal antibodies; reactions with the anti-P450 antibodies were normalized with respect to reactions containing the same dilution of the control antibody.

The knowledge that CYP3A4, the most abundant P450 in the human liver, is the predominant P450 that mediates the hepatic metabolism of bortezomib is valuable for designing clinical studies to evaluate the potential drug interactions that may occur with coadministered chemotherapeutic agents, many of which are substrates or inhibitors of CYP3A4. The CYP3A4 inhibitors ketoconazole and troleandomycin have been shown to increase the exposure of SN-38 in cancer patients and inhibit the metabolism of SN-38 in vitro (Kehrer et al., 2002). Thus, coadministration of bortezomib with potent CYP3A4 inhibitors like ketoconazole and troleandomycin may require attention to the dosage bortezomib. However, clearance of bortezomib in humans at the recommended dose of 1.0 mg/m² is approximately 0.4 l/h/kg, which is one-third the liver blood flow rate; thus, CYP3A4-based drug-drug interactions may not be observed in patients. Clinical trials to determine the effects of ketoconazole coadministration in patients receiving bortezomib are currently underway. Although the effects of CYP3A4/5 polymorphisms on drug clearance are not well understood at present (Lamba et al., 2002; Roy et al., 2005), polymorphisms in the CYP2C subfamily, especially CYP2C19, are known and populations of poor metabolizers with diminished capacity for S-mephenytoin 4′-hydroxylation have been identified (deMorais et al., 1994). CYP2C19 is absent in approximately 15 to 30% of Asians (Wilkinson et al., 1989), and hence, administration of bortezomib to Asian populations may require care. Clinical trials to assess the potential of omeprazole coadministration to cause CYP2C19-mediated drug-drug interactions are also being conducted. The fact that all five major human P450s contribute to the hepatic metabolism of bortezomib may help mitigate drug-drug interactions arising because of concomitantly administered CYP3A4 modulators or substrates or P450 polymorphisms.

TABLE 4

<table>
<thead>
<tr>
<th>P450 Isomorph</th>
<th>Marker Substrate</th>
<th>Reaction Catalyzed</th>
<th>Maximum % Inhibition</th>
<th>Diluted % Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A2</td>
<td>Phenacetin</td>
<td>Phenacetin-O-deethylated</td>
<td>80</td>
<td>1:800</td>
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<tr>
<td>2C9</td>
<td>Tolbutamide</td>
<td>Tolbutamide hydroxylation</td>
<td>65</td>
<td>1:240</td>
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<tr>
<td>2C19</td>
<td>S-Mephenytoin</td>
<td>S-Mephenytoin 4′-hydroxylation</td>
<td>96</td>
<td>1:240</td>
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<tr>
<td>2D6</td>
<td>Dextromethorphan</td>
<td>Dextromethorphan O-demethylation</td>
<td>90</td>
<td>1:40</td>
</tr>
<tr>
<td>3A4</td>
<td>Testosterone</td>
<td>Testosterone 6β-hydroxylation</td>
<td>87</td>
<td>1:20</td>
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</tbody>
</table>

References


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