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

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Running Title: Relative contributions of the major human cytochromes P450 to the hepatic metabolism of bortezomib.

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Abbreviations: RAF, relative activity factor; RAF_{CL}, relative activity factor as a ratio of clearance; CYP, cytochrome P450; MRM, multiple reaction monitoring; HLM, human liver microsomes; HyHel-9, hen egg white lysozyme
ABSTRACT

VELCADE™ (bortezomib, PS-341), reversibly inhibits the 20S proteasome, and exhibits 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 CYPs, 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 CYPs to the net hepatic metabolism of bortezomib. RAFs for the CYP isoform selective substrates were determined; the ratio of the rate of metabolism of bortezomib with cDNA-expressed CYPs versus rate of metabolism with human liver microsomes was normalized with respect to the RAF for each CYP isoform to determine the percent contributions of the CYPs 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 CYPs responsible for the hepatic metabolism of bortezomib. The studies were conducted with 2 μM bortezomib, and disappearance of bortezomib rather than appearance of a specific metabolite was quantified to determine the contributions of the CYPs 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 analogues of serine proteases (Koehler and Leinhard, 1971; Philipp and Bender, 1971; Kettner and Shenvi, 1984). Through the 1980s peptide boronic acids were shown to be effective inhibitors of trypsin, chymotrypsin, α-lytic protease, pancreatic elastase, leukocyte elastase, thrombin, and beta-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, Figure 1), a dipeptidyl boronic acid, by the U.S. 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, [N-(2,3-pyrazine) carbonyl-L-phenylalanine-L-leucine boronic acid], 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 IkB, which in turn leads to inhibition of NF-kB activation, and subsequently increases the susceptibility of cells to apoptosis. Studies have shown that pre-treatment of cancer cells with bortezomib effectively blocks activation of NF-kB induced by the chemotherapeutic agent CPT-11/SN-38 (irinotecan)
suggesting that proteasome inhibition may help overcome chemoresistance (Hideshima et al., 2001; Jr. et al., 2001)).

The mechanisms of metabolism and the drug-drug interaction potential of boron-containing drugs have not been previously explored. The contribution of the cytochromes P450 (CYPs), the major drug metabolizing enzymes in humans, to the hepatic degradation of bortezomib has only recently been studied (Daniels et al., 2003; LaButti et al., 2003; Uttamsingh et al., 2003). Several CYP-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 percent contribution of the five major human CYPs, 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, especially 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, in the future, be used as an adjuvant to chemotherapy. Three distinct methods, relative activity factor (RAF) (Crespi, 1995; Venkatakrishnan et al., 2001a), chemical inhibition using CYP-selective chemical inhibitors, and immunoinhibition using inhibitory monoclonal antibodies (Gelboin et al., 1999) were employed to evaluate the relative contribution of the major human CYPs to the net hepatic metabolism of bortezomib. The RAFs, as a ratio of clearance (RAF_{CL}), were determined by measuring the kinetic parameters (V_{max} and K_{m}) for the CYP
isoform-selective substrates using cDNA-expressed CYPs and human liver microsomes (HLM). The ratios of the rate of metabolism of bortezomib with the cDNA-expressed CYPs and HLM were normalized with respect to the RAFs calculated for the CYP isoforms in order to determine the percent contribution of each CYP to the net hepatic metabolism of bortezomib. Results obtained from the RAF<sub>CL</sub> studies were confirmed by measuring the percent inhibition of CYP-dependent metabolism of bortezomib in HLM using known chemical inhibitors of the CYP isoforms, and CYP-specific inhibitory monoclonal antibodies.
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, dextrorphan, 1’-OH-midazolam were purchased from BD Gentest (Woburn, MA). Phenacetin, acetaminophen, tolbutamide, dextromethorphan, testosterone, 6β-OH-testosterone, furafylline, sulfaphenazole, omeprazole, quinidine, ketoconazole, NADPH, and MgCl₂ were purchased from Sigma (St. Louis, MO). S-Mephenytoin was purchased from BIOMOL Research Labs., Inc. (Plymouth, PA). Bortezomib was synthesized at Millennium Pharmaceuticals, Inc. Ascites fluid containing monoclonal antibodies to CYPs 1A2, 2C9, 2C19, 2D6, 3A4, and hen egg white lysozyme (HyHel-9) were a gift from Dr. H. V. Gelboin at the National Cancer Institute, NIH.

Contribution of CYPs 1A2, 2C9, 2C19, 2D6, and 3A4 to the net hepatic rate of metabolism of bortezomib using the RAF CL approach (RAF as a ratio of clearance).

Determination of Km and Vmax: Kinetic parameters including Km and Vmax for the CYP1A2-catalyzed phenacetin O-dethylation, CYP2C9-catalyzed tolbutamide hydroxylation, CYP2C19-catalyzed S-mephenytoin hydroxylation, CYP2D6-catalyzed dextromethorphan O-demethylation, and CYP3A4-catalyzed testosterone 6β-hydroxylation were determined with HLM and Supersomes™. Stock solutions of phenacetin, tolbutamide, S-mephenytoin, dextromethorphan, and testosterone were prepared in acetonitrile. Dilutions of the substrate stock solutions, HLM, and supersomes to obtain 4x solutions were prepared in 0.1 M potassium phosphate buffer, pH 7.4.
Reaction mixtures contained 0.5 mg/mL total protein (incubations with Supersomes™ contained 10 pmol/mL of each CYP), substrates, 2 mM NADPH, and 3 mM MgCl₂ in a final volume of 100 µL. Incubations were performed in 96-well polypropylene plates. Incubation mixtures containing 0.5 mg/mL total protein and substrate were prewarmed at 37°C for 5 min. Substrate concentrations used were 0, 0.1x, 0.25x, 0.5x, 1x, 2x, 3x, 4x, 6x, and 8x the reported K_m’s for each substrate (Pelkonen et al., 1998). Reactions were initiated by addition of NADPH/MgCl₂ and were incubated for 15 min at 37°C. Reactions were stopped by addition of 100 µL of ice-cold acetonitrile containing 1 µM antipyrine as an internal standard. The plates were stored at 4°C for 30 min, and then centrifuged at 1800 x g for 10 min to pellet precipitated proteins. Supernatants were transferred to another 96-well plate and analyzed for amounts of the CYP-isoform specific metabolite formed using an Agilent 1100/Gilson 215/Applied Biosystems API 4000 LC/MS/MS system. The analytes were separated using a Phenomenex Synergi C18 column (75 x 4.6 mm) and a gradient consisting of 0.1 % formic acid/water (mobile phase A) and 0.1 % formic acid/acetonitrile (mobile phase B) at a flow rate of 1 mL/min. The analytes and internal standard were detected by positive ion spray in the multiple reaction monitoring (MRM) mode. The precursor/product ion mass transitions monitored for each of the metabolites analyzed and the internal standard antipyrine were: m/z 151.9/110.0 for acetaminophen; m/z 287.0/106.9 for 4-OH tolbutamide; m/z 234.9/149.9 for 4-OH mephenytoin; m/z 258.0/156.9 for dextrorphan; m/z 305.1/269.0 for 6β-hydroxytestosterone; and m/z 188.9/77.0 for antipyrine.

The K_m for human liver microsomal metabolism of bortezomib was determined by incubating 1, 2, 5, 10, 20, 40, and 50 µM bortezomib with 0.5 mg/mL HLM in a final
volume of 100 µL in 96-well polypropylene plates. Reactions were initiated by addition of NADPH/MgCl₂ and were incubated for 10 minutes at 37°C; reactions without NADPH were also included. Reactions were stopped by addition of 100 µL of ice-cold acetonitrile containing 1 µM carbutamide as an internal standard. The precipitated proteins were pelleted by centrifugation and supernatants analyzed by LC/MS/MS. Bortezomib versus internal standard (carbutamide) peak area ratios were measured using a LC/MS/MS system that consisted of a Shimadzu LC, a CTC leap auto-sampler, and a SCIEX API 4000 detector. Separation was performed on a Waters Symmetry C18, 2.1 x 3.0 mm HPLC column, and the analytes and internal standard were eluted at a flow rate of 0.4 mL/min. Mobile phase A consisted of 0.1% (v/v) formic acid in water, and the mobile phase B consisted of 0.1 % (v/v) formic acid in acetonitrile. Bortezomib and the internal standard were detected by positive ion spray in the MRM mode. The precursor/product ion mass transitions monitored were m/z 366.9/225.9 for bortezomib, and m/z 271.9/155.7 for the internal standard.

*Determinatation of rate of metabolism.* The rate of metabolism of bortezomib was determined with HLM and individual CYP supersomes. The rate of disappearance of bortezomib was determined by measuring the amounts of bortezomib remaining at 0, 3, 7, 12, 20, and 30 min. Incubations were performed at 37°C and contained 2 µM bortezomib, 0.5 mg/mL total protein (incubations with Supersomes™ contained 10 pmol/mL of each CYP), 2 mM NADPH, and 3 mM MgCl₂ in a final volume of 100 µL. Reactions were stopped with 100 µL acetonitrile containing 1 µM carbutamide as the internal standard at the above mentioned time points. Samples were processed and analyzed by LC/MS/MS to determine bortezomib versus internal standard (carbutamide)
peak area ratios as mentioned earlier. The percent recovery of bortezomib was determined by comparison of peak area ratios of bortezomib versus internal standard in samples containing bortezomib and protein (HLM) and samples containing bortezomib and buffer only.

Contribution of CYPs 1A2, 2C9, 2C19, 2D6, and 3A4 to the net hepatic metabolism of bortezomib using chemical inhibitors and monoclonal antibodies.

Serial dilutions of the CYP-selective chemical inhibitors, furafylline, sulfaphenazole, omeprazole, quinidine, and ketoconazole, and ascites fluids containing CYP-specific monoclonal antibodies were prepared in potassium phosphate buffer, pH 7.4. Concentrations of the chemical inhibitors or monoclonal antibodies that showed minimum cross-reactivity and maximum inhibition of the human liver microsomal metabolism of the CYP isoform selective substrates, phenacetin, tolbutamide, 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 addition of the CYP substrates or bortezomib, and prewarmed NADPH/MgCl₂. The reaction mixtures contained 0.5 mg/mL HLM, monoclonal antibodies or chemical inhibitors, CYP 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 cold acetonitrile containing carbutamide as an internal standard. The plates were stored at 4°C.
for 30 min, and then centrifuged at 1800 x g for 10 min to pellet precipitated proteins. Supernatants were transferred to another 96-well plate and analyzed for amounts of metabolite formed (for the CYP marker substrates) or amount of bortezomib remaining, as described earlier. The percent inhibition of metabolism of the CYP-selective substrates or bortezomib was calculated as described in the data analyses section.

Data analyses

LC/MS/MS data were analyzed using Analyst software version 1.2. Kinetic parameters, $K_m$ and $V_{\text{max}}$, for each of the CYP-catalyzed reactions were determined from velocity versus substrate concentration plots using Prism Software (GraphPad, San Diego, CA). For the determination of $K_m$ for the human liver microsomal metabolism of bortezomib, the amount of metabolite formed was calculated by measuring $\Delta$ bortezomib.

$$\Delta \text{bortezomib} = (\text{amount of bortezomib remaining in reactions in the absence of NADPH}) - (\text{amount of bortezomib remaining in reactions in the presence of bortezomib})$$

Equation 1

$\Delta$ bortezomib was plotted against substrate concentration, and $K_m$ was determined by non-linear regression using Prism Software.

Calculation of RAF_{CL}. The relative contribution of the CYPs 1A2, 2C9, 2C19, 2D6, and 3A4 to the rate of metabolism of bortezomib in humans were determined as shown:

The RAF for each of the 5 CYP isoforms was calculated according to equation 1.

$$\text{RAF} = (V_{\text{max}}/K_m \text{ of CYPn in HLM})/(V_{\text{max}}/K_m \text{ of cDNA-expressed CYPn})$$
Equation 2

\[ n = 12, 2C9, 2C19, 2D6, \text{ or } 3A4 \]

Contribution of CYPn to metabolism of bortezomib was subsequently calculated using equation 2:

\[
\text{Contribution of CYPn (%)} = \text{RAF} \times \frac{v_{CYP}}{v_{HLM}}
\]

Equation 3

\[ v_{CYPn} = \text{Rate of metabolism with cDNA-expressed CYPn} \]

\[ v_{HLM} = \text{Rate of metabolism with HLM} \]

\[ v_{CYPn} \text{ and } v_{HLM} \text{ were calculated by linear regression analysis on log (percentage of bortezomib remaining) vs time plots. The amount of metabolite formed at 5 min was determined from the linear regression plots in order to determine the } v_{CYPn} \text{ and } v_{HLM} \text{ under initial velocity conditions. The plots of % bortezomib remaining in HLM versus time are shown in Fig. 2.} \]

Calculation of percent inhibition of human liver microsomal metabolism of bortezomib by CYP-selective chemical inhibitors. The percent inhibition of human liver microsomal metabolism of bortezomib by the CYP-selective inhibitors used in the study was calculated as shown:

\[
[1 - (\Delta \text{ bortezomib in presence of chemical inhibitor}/\Delta \text{ bortezomib in absence of chemical inhibitor})] \times 100
\]

Equation 4
Calculation of percent inhibition of human liver microsomal metabolism of bortezomib by inhibitory monoclonal antibodies. The percent inhibition of human liver microsomal metabolism of bortezomib by the CYP-specific monoclonal antibodies used in the study was calculated as shown:

\[
\left[1 - \left(\frac{\Delta \text{bortezomib for reactions with anti-CYP antibodies}}{\Delta \text{bortezomib for reactions with control antibody}}\right)\right] \times 100
\]

Equation 5
RESULTS

The $K_m$ and $V_{\text{max}}$ for CYP1A2-catalyzed phenacetin $O$-deethylation, CYP2C9-catalyzed tolbutamide hydroxylation, CYP2C19-catalyzed $S$-mephenytoin hydroxylation, CYP2D6-catalyzed dextromethorphan $O$-demethylation, and CYP3A4-catalyzed testosterone $6\beta$-hydroxylation using HLM and cDNA-expressed CYPs are shown in Table 1. The $K_m$ values obtained were similar to values reported in the literature (Pelkonen et al., 1998). The RAFs for the five major human CYPs, calculated using equation 1, are also shown in Table 1. The $K_m$ for the human liver microsomal metabolism of bortezomib was determined to be approximately 16.6 $\mu$M (data not shown). The recovery of bortezomib from samples containing protein was $>90\%$, compared to samples containing bortezomib and buffer only, and hence recovery of bortezomib from reaction mixtures was not of concern. The rates of metabolism of bortezomib with HLM, and cDNA-expressed CYP1A2, 2C9, 2C19, 2D6, and 3A4 along with the percent contribution of each CYP isoform, calculated using equation 2, 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 CYPs followed the order: CYP2C19 > CYP3A4 > CYP2D6 > CYP1A2 > CYP2C9. After normalizing with RAF, the percent contribution of each CYP isoform in HLM to the rate of metabolism of bortezomib was: CYP3A4 (38.4 %) > CYP2C19 (30.1 %) > CYP1A2 (10.5 %) > CYP2D6 (7.1 %) > CYP2C9 (1.2 %). Thus, CYP3A4 and CYP2C19 were the major CYPs that contributed to the metabolism of bortezomib. The sum of the contributions of the five CYPs studied was approximately 87 %. 

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The concentrations of the CYP-selective chemical inhibitors, furafylline (1A2), sulfaphenazole (2C9), omeprazole (2C19), quinidine (2D6), and ketoconazole (3A4) that inhibit the human liver microsomal metabolism of the CYP 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 percent inhibitions of bortezomib metabolism in HLM by the CYP inhibitors are shown in Table 4. The percent inhibition is equivalent to the percent contribution of the CYP isoforms studied. Based on chemical inhibition studies, the percent contribution of each CYP 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 CYPs studied, as determined using chemical inhibitors, was approximately 150 %.

Dilutions of the ascites fluid containing the CYP-specific monoclonal antibodies that exhibited maximal inhibition of metabolism of the CYP-isoform specific substrates are shown in Table 3. Monoclonal antibodies against CYPs 1A2 (1:800 dilution), 2C19 (1:240 dilution), 2D6 (1:40 dilution), and 3A4 (1:20 dilution) exhibited > 80 % inhibition of metabolism of their respective substrates whereas the monoclonal antibody to
CYP2C9 (1:240) inhibited tolbutamide hydroxylation only about 65%. The percent inhibitions of bortezomib metabolism by CYP-specific inhibitory monoclonal antibodies, at the pre-determined dilutions, are shown in Table 5. The percent inhibition is equivalent to the percent contribution of each CYP isoform considered in this study. Thus, based on studies with the monoclonal antibodies, the percent contribution of each CYP isoform to the human liver microsomal metabolism of bortezomib followed the order: CYP3A4 (79%) > CYP2C19 (23%) > CYP1A2 (18%) > CYP2D6 (6.6%) > CYP2C9 (5.4%). The sum of the contributions of the five CYPs studied, as determined using inhibitory monoclonal antibodies, was approximately 130%.
The three in vitro approaches employed to determine the relative contributions of the five major human CYPs indicate that CYP3A4 followed by CYP2C19 are the major CYP isoforms that contribute to the net hepatic metabolism of bortezomib. Albeit minor, CYPs 1A2, 2C9, and 2D6 also play a role in the hepatic metabolism of bortezomib. The percent 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. While CYPs 1A2, 2C9, 2C19, 2D6, and 3A4 mediated the biotransformation of bortezomib to the primary deboronated metabolites, carbinolamides, CYPs 3A4, and 2C19 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 CYPs to the overall hepatic metabolism of the drug of interest (i.e. bortezomib) has been determined rather than evaluating the contributions of the CYPs to a specific biotransformation pathway as has been reported previously by several investigators (Rochat et al., 1997; Venkatakrishnan et al., 2001b; 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. Further, these studies were conducted with 2 µM bortezomib, a non-saturating and clinically relevant concentration. Following a 1 mg/m² dose, the maximum plasma concentration (C_max) in patients is approximately 150 nM (Karol, M., personal communication). Liver concentrations of bortezomib in patients may reach 10X the plasma concentrations,
approximately 1.5 µM, though it is unlikely to reach $K_m$ concentrations (~16 µM in HLM).

Determination of RAFs for isoforms of Phase I or Phase II drug metabolizing enzymes was first proposed by Crespi et al., (Crespi, 1995). The availability of HLM and cDNA-expressed CYPs enables CYP “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 CYP isoforms to the overall metabolism of drugs due to differences in recombinant systems and microsomal preparations. The RAFs serve as scaling factors which account for differences in relative hepatic abundance and intrinsic activities of the CYP isoforms between the cDNA-expressed enzymes and the human liver microsomal analogs (Rodrigues, 1999; Venkatakrishnan et al., 2001a; Rochat, 2003). The one disadvantage of this approach may be that its accuracy is dependent on the selectivity of the CYP isoform-selective index reaction and 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 percent contribution of CYPs 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, CYP-selective chemical inhibitors can also provide valuable and reliable information, provided the studies are conducted with appropriate concentrations of the inhibitors. The specificity of the
chemical inhibitors used may be a confounding factor in the overall accuracy of results (Newton et al., 1995; Zhang et al., 2002; Li et al., 2004) and a degree of cross-reactivity was observed with omeprazole and ketoconazole (see results). Highly selective CYP2C19 and CYP3A4 inhibitors benzynirvanol and azamulin, respectively, have become available in the recent years and may be used in reaction phenotyping studies. A multi-pronged approach including recombinant CYPs and CYP-isoform selective index reactions, chemical inhibitors, and inhibitory antibodies have been identified to be important tools for CYP reaction phenotyping (Lu et al., 2003) and we have employed all three to confirm the relative contribution of CYPs to the hepatic metabolism of bortezomib. Although the three approaches yielded similar results in that CYPs 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 percent contributions of the five CYPs evaluated. The sum of the percent contributions of the CYPs as determined by the RAF method was 87 %, while 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 (Daniels et al, personal communication). In incubations with monoclonal antibodies, nonspecific protein mediated degradation of bortezomib may have contributed to its depletion. Although reactions with a control antibody, HyHel-9, were included, lack of sufficient material prevented equalization of total protein amounts in the reactions with the monoclonal antibodies; reactions with the anti-CYP antibodies
were normalized with respect to reactions containing the same dilution of the control antibody.

The knowledge that CYP3A4, the most abundant CYP in the human liver, is the predominant CYP which mediates the hepatic metabolism of bortezomib is valuable for designing clinical studies to evaluate the potential drug interactions that may occur with co-administered chemotherapeutic agents, many of which are substrates or inhibitors of CYP3A4. The CYP3A4 inhibitors ketoconazole and troleandomycin have 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, co-administration of bortezomib with potent CYP3A4 inhibitors like ketoconazole and troleandomycin may require attention to the dosage of bortezomib. However, clearance of bortezomib in humans at the recommended dose of 1.0 mg/m² is approximately 0.4 L/hr/kg which is one-third the liver blood flow rate and, thus, CYP3A4 based drug-drug interactions may not be observed in patients. Clinical trials to determine the effects of ketoconazole co-administration in patients receiving bortezomib are currently underway. While 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, specially 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 15-30% 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 co-administration to cause CYP2C19 mediated drug-drug interactions are also being conducted. The fact that all five major human CYPs contribute to the hepatic
metabolism of bortezomib may help mitigate drug-drug interactions arising due to concomitantly administered CYP3A4 modulators or substrates, or CYP polymorphisms.
REFERENCES


OMEPRAZOLE, ESOMEPRAZOLE, LANSOPRAZOLE, PANTOPRAZOLE, AND RABEPRAZOLE ON HUMAN CYTOCHROME P450 ACTIVITIES.

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List of Figures

Figure 1. Structure of bortezomib

Figure 2. Percent bortezomib remaining versus time
Table 1. $K_m$ and $V_{\text{max}}$ values for the CYP -catalyzed reactions with human liver microsomes and Supersomes$^\text{TM}$, and calculated RAFs

<table>
<thead>
<tr>
<th>CYP Isoform-Substrate (Reported $K_m$s)</th>
<th>Human liver microsomes</th>
<th>Supersomes$^\text{TM}$</th>
<th>RAF$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$($\mu$M)</td>
<td>$V_{\text{max}}^a$</td>
<td>$K_m$ ($\mu$M)</td>
</tr>
<tr>
<td>CYP1A2-Phenacetin (30 $\mu$M)</td>
<td>20.0</td>
<td>1.79</td>
<td>15.8</td>
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<td>CYP2C9-Tolbutamide (400 $\mu$M)</td>
<td>317</td>
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<td>113</td>
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<td>CYP2C19-S-Mephenytoin (60 $\mu$M)</td>
<td>32.4</td>
<td>0.05</td>
<td>33.5</td>
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<tr>
<td>CYP2D6-Dextromethorphan (5 $\mu$M)</td>
<td>6.56</td>
<td>2.02</td>
<td>2.50</td>
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<tr>
<td>CYP3A4-Testosterone (47 $\mu$M)</td>
<td>46.6</td>
<td>1.64</td>
<td>28.5</td>
</tr>
</tbody>
</table>

$a$: $V_{\text{max}}$ values are represented as peak area ratio’s of amount of metabolite formed/internal standard

$b$: RAF = ($V_{\text{max}}/K_m$ of CYPn in HLM/$V_{\text{max}}/K_m$ of cDNA-expressed CYPn)
Table 2. In vitro clearance, RAF for CYP Isoforms 1A2, 2C9, 2C19, 2D6, and 3A4, and % contributions to human liver microsomal metabolism of bortezomib

<table>
<thead>
<tr>
<th>CYP Isoform</th>
<th>Rate of metabolism (pmol/min/10 pmol CYP)^a</th>
<th>RAF</th>
<th>% Contribution^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>0.74</td>
<td>0.628</td>
<td>10.5</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>0.07</td>
<td>0.753</td>
<td>1.2</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>3.21</td>
<td>0.411</td>
<td>30.1</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>1.54</td>
<td>0.202</td>
<td>7.1</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>2.92</td>
<td>0.576</td>
<td>38.4</td>
</tr>
</tbody>
</table>

a: pmol/min/10 pmol CYP for isoforms 1A2, 2C9, 2C19, 2D6, and 3A4; in vitro clearance with HLM: 4.38 pmol/min/0.5 mg microsomal protein

b: % contribution = RAF x (CL, CYPn)/(CL, HLM) x 100
Table 3. Dilutions of monoclonal antibodies that exhibited maximum specificity for inhibition of human liver microsomal metabolism of CYP marker substrates

<table>
<thead>
<tr>
<th>CYP Isoform</th>
<th>Marker substrate</th>
<th>Reaction catalyzed</th>
<th>Maximum % inhibition</th>
<th>Dilution of stock solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A2</td>
<td>Phenacetin</td>
<td>Phenacetin-(O)-deethylation</td>
<td>80</td>
<td>1:800</td>
</tr>
<tr>
<td>2C9</td>
<td>Tolbutamide</td>
<td>Tolbutamide hydroxylation</td>
<td>65</td>
<td>1:240</td>
</tr>
<tr>
<td>2C19</td>
<td>S-Mephenytoin</td>
<td>S-Mephenytoin 4’-hydroxylation</td>
<td>96</td>
<td>1:240</td>
</tr>
<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>
</tr>
</tbody>
</table>
Table 4. Percent inhibition of human liver microsomal metabolism of bortezomib using monoclonal antibodies and chemical inhibitors

<table>
<thead>
<tr>
<th>CYP Isoform</th>
<th>% inhibition of bortezomib metabolism*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>inhibitory mAbs</td>
</tr>
<tr>
<td>1A2</td>
<td>18.0</td>
</tr>
<tr>
<td>2C9</td>
<td>5.4</td>
</tr>
<tr>
<td>2C19</td>
<td>23.0</td>
</tr>
<tr>
<td>2D6</td>
<td>6.6</td>
</tr>
<tr>
<td>3A4</td>
<td>79.0</td>
</tr>
</tbody>
</table>

* relative contribution of CYP
Figure 1
Figure 2

% bortezomib remaining in HLM

LN (peak area ratio)

Figure 2