INVESTIGATION OF DRUG-DRUG INTERACTION POTENTIAL OF BORTEZOMIB IN VIVO IN FEMALE SPRAGUE-DAWLEY RATS AND IN VITRO IN HUMAN LIVER MICROSONMES

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List of Abbreviations

P450 (CYP), cytochrome P450; IC_{SO}, concentration that causes 50% inhibition;
LC/MS/MS, liquid chromatography tandem mass spectrometry; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; PBST, phosphate buffered saline with
0.1% Tween 20; TBS, tris buffered saline; Tris buffer, tris (hydroxymethyl) aminomethane.
Abstract

Bortezomib (VELCADE™, PS-341), a dipeptidyl boronic acid, is a first-in-class proteasome inhibitor approved in 2003 for the treatment of multiple myeloma. In a preclinical toxicology study, bortezomib treated rats resulted in liver enlargement (35%). Ex vivo analyses of the liver samples showed an 18% decrease in P450 content, a 60% increase in palmitoyl-CoA β-oxidation activity, and a 41% and 23% decrease in CYP3A protein expression and activity, respectively. Furthermore, liver samples of bortezomib treated rats had little change in CYP2B and CYP4A protein levels and activities. To address the likelihood of clinical drug-drug interactions, the P450 inhibition potential of bortezomib and its major deboronated metabolites M1, M2, and their dealkylated metabolites M3 and M4 was evaluated in human liver microsomes for the major P450 isoforms 1A2, 2C9, 2C19, 2D6, and 3A4/5. Bortezomib, M1, and M2 were found to be mild inhibitors of CYP2C19 (IC₅₀ ~ 18.0, 10.0, 13.2 μM, respectively), and M1 was also a mild inhibitor of CYP2C9 (IC₅₀ ~ 11.5 μM). However, bortezomib, M1, M2, M3, and M4 did not inhibit other P450s (IC₅₀s > 30 μM). There also was no time-dependent inhibition of CYP3A4/5 by bortezomib or its major metabolites. Based on these results, no major P450 mediated clinical drug-drug interactions are anticipated for bortezomib or its major metabolites. To our knowledge, this is the first report on P450 mediated drug-drug interaction potential of proteasome inhibitors or boronic acid containing therapeutics.
The approval of bortezomib (VELCADE™, PS-341) by the U.S. Food and Drug Administration for treatment of multiple myeloma made it the first drug in a new class of medicines called proteasome inhibitors. The proteasome is an enzyme complex found in the nucleus and cytoplasm of all cells in the body. It degrades intracellular proteins, such as IκB and p53, through the ubiquitin proteasome pathway (Ciechanover, 1994) and regulates cell growth, apoptosis, and cell adhesion. In tumor cells, the blockage of degradation of IκB by proteasome inhibitors makes the inflammatory NFκB remain in an inactive form, thus enhancing tumor cell apoptosis (Palombella et al. 1998, Berenson et al. 2001, Garg and Aggarwal 2002). Additionally, proteasome inhibitors block the degradation of the tumor suppressor protein p53. When cells undergo radiation or chemotherapy, the p53 expressed in normal cells allows the arrest of cell proliferation and permits the repair of damaged DNA. In contrast, tumor cells express mutated forms of p53, which hinders cell cycle arrest and the ability to repair damaged DNA (Kuerbitz et al. 1992). Thus proteasome inhibitors help normal cells to recover from DNA damage while allowing tumor cells to undergo apoptosis (Adams 2001, Chanhan et al. 2005).

Bortezomib, a dipeptidyl boronic acid (Figure 1, Wu et al. 2000), is a potent, selective and reversible inhibitor of the proteasome in mammalian cells (Adams 2001, Stinchcombe et al. 2001, Richardson et al. 2003). Bortezomib is approved for intravenous administration of 1.3 mg/m² and has maximum plasma concentration (C_{max}) \sim 150 \text{nM}. In multiple myeloma patients, bortezomib has a mean elimination half-life ranging 9 - 15 hours. The boronic acid group was found to be essential for activity and...
the metabolites are not pharmacologically active. Like many other cancer drugs, bortezomib is a cytotoxic agent with a narrow therapeutic index. Thus, it is important to understand the metabolism of bortezomib and the consequences of inhibition or induction of that metabolism. Furthermore, for cancer patients on multiple drug therapy, adverse effects may arise if bortezomib affects the enzymes metabolizing these co-administered drugs. Therefore, understanding the enzymes which affect the metabolism of bortezomib and the enzymes which are affected by bortezomib would help to predict possible drug-drug interactions.

Bortezomib was found to be primarily metabolized by CYP3A4 and CYP2C19 (Pekol et al. 2005, Uttamsingh et al. 2005). In this study, the effect of bortezomib on P450 enzymes was evaluated \textit{ex vivo} in Sprague-Dawley rats. Rats were selected as the ex vivo model because liver enlargement was observed in preclinical toxicology studies in rats but not in other species such as monkeys. The microsomal activities and protein levels of major inducible P450s (2B1/2, 3A1/2, and 4A1/3) and peroxisomal palmitoyl-CoA oxidation activity in rat liver were determined. The P450 inhibitory potential of bortezomib and its major metabolites, M1, M2, M3, and M4 on the major P450 isoforms 1A2, 2C9, 2C19, 2D6, and 3A4/5 as well as the time-dependent CYP3A4/5 inhibition potential were evaluated in human liver microsomes.
MATERIALS AND METHODS

Bortezomib was obtained from the Process Chemistry Department at Millennium Pharmaceuticals, Inc. Major bortezomib metabolites, M1, M2, M3 and M4 (Figure 1) were synthesized by Albany Molecular Research (Albany, NY). Testosterone, lauric acid, 12-hydroxylauric acid, 10-hydroxydecanoic acid, 16β-hydroxytestosterone, 6β-hydroxytestosterone, 6β-hydroxydianabol, sucrose, imidazole, β-nicotinamide adenine dinucleotide, flavin adenine dinucleotide, Triton-X 100, Tween 20, dithiothreitol, coenzyme A, palmitoyl CoA, sodium dithionite, midazolam, NADPH, phenacetin, tolbutamide, dextromethorphan, furafylline, sulfaphenazole, quinidine, ketoconazole, troleandomycin, and horseradish peroxidase-conjugated rat anti-rabbit and anti-goat polyclonal secondary antibodies were purchased from Sigma-Aldrich (St. Louis, MO). S-mephenytoin was purchased from Biomol, Inc. (Plymouth Meeting, PA). Human, naïve rat, phenobarbital or dexamethasone induced rat liver microsomes, and rabbit anti-rat CYP3A polyclonal antibody were purchased from Xenotech, LLC (Lenesa, KS). Goat anti-rat CYP2B and CYP4A polyclonal antibodies and rat CYP2B1 and CYP3A1 supersomes were purchased from BD Gentest (Woburn, MA). [14C] palmitoyl-CoA, ECL plus Western blotting detection reagents were purchased from Amersham (Piscataway, NJ). Nupage Novex Bis-tris gels and electrophoresis reagents kit were purchased from Invitrogen (Carlsbad, CA).
Female Sprague-Dawley rats (8 weeks old with body weights ~ 200 g) were dosed intravenously with 0.15 mg/kg bortezomib or the vehicle control (mannitol saline, 1.5 mL) on days 1, 4, 8, and 12 at Charles River Laboratories (Worcester, MA). This single dose was the highest dose that could be administered to rats without causing any adverse effects, such as neuropathy. Twenty-four hours after the last dose the animals were sacrificed and livers were removed, frozen in liquid nitrogen, and stored at -80°C. Microsomes and peroxisomes enriched fractions were prepared in house following published protocols (Guengerich 1989, Small et al. 1985). In the peroxisomes preparation, the liver homogenates were first centrifuged at 6,000g for 10 min to remove mitochondria and other heavy cellular fragments. Then a 30 min centrifugation at 30,000g was applied to collect the peroxisomes enriched fractions. Microsomes and peroxisomes enriched fractions prepared from the livers of female rats treated with 200 mg/kg clofibric acid in 2.5 mL Mazola® oil in house for 4 days were used as positive controls in the CYP4A1 and peroxisomal activity assay, respectively. The total P450 content in microsomes was determined using the method described by Omura and Sato (1964). Protein concentrations in microsomes or peroxisomal fractions were determined using the BCA Protein Assay kit (Pierce, Rockford, IL).

**Testosterone and lauric acid oxidation assays.** Rat liver microsomes (0.25 mg/mL), in triplicate, were pre-warmed for 5 min at 37°C in 0.1 M potassium phosphate buffer (pH 7.4). The microsomes were then incubated with testosterone (200 µM) or lauric acid (100 µM) with 2 mM NADPH and 3 mM MgCl₂ for 30 min at 37°C. The reactions were
stopped by the addition of equal volume of acetonitrile containing 1 µM internal standards (6β-hydroxydianabol and 10-hydroxydecanoic acid for the testosterone and lauric acid assay, respectively). After placing the sample on ice for 30 min, the precipitated proteins were removed via a 10 min centrifugation at 3,000g. Metabolites 16β- and 6β-hydroxytestosterone, and 12-hydroxylauric acid were analyzed using an Applied Biosystems API 4000 LC/MS/MS system and a Phenomenex Synergi C18 column (75 x 4.6 mm). Metabolite separation was achieved with 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 1.0 mL/min, specifically, 5% of mobile phase B was applied for 0.5 min after injection and increased linearly to 95% B from 0.5 to 3.5 min. Mobile phase B was held at 90% from 3.5 to 3.6 min and the column was re-equilibrated to 5% B from 3.6 to 5.0 min. Positive and negative ion spray modes were applied to 6β-, 16β-hydroxytestosterone and 12-hydroxylauric acid, respectively. The following precursor/product ion pairs were monitored: 305.3/269.1 (6β-hydroxytestosterone), 305.3/97.1 (16β-hydroxytestosterone), 215.2/169.0 (12-hydroxylauric acid), 187.1/140.9 (10-hydroxydecanoic), and 317.0/281.0 (6β-hydroxydianabol). Enzyme catalytic activities were calculated based on external standard curves of the analytes prepared in the same matrix.

**[14C] Palmitoyl-CoA oxidation assay.** Palmitoyl CoA β-oxidation is a widely used assay to determine peroxisomal activity. Palmitoyl CoA is an acid-insoluble substrate; however its metabolite, acetyl CoA, is acid-soluble. By incubating a [14C]-labeled
palmitoyl CoA with peroxisomes and then stopping the reaction with acid, the metabolite formation rate can be measured by counting the radioactivity in the supernatants. Reaction mixtures were prepared with 50 mM Tris-HCl (pH 8.0), 0.2 mM β-nicotinamide adenine dinucleotide, 0.01 mM flavin adenine dinucleotide, 0.0075% bovine serum albumin, 0.01% Triton-X 100, 1 mM dithiothreitol, 0.1 mM coenzyme A (Co-A), 0.01 mM palmitoyl CoA, and 20 nCi/mL \( ^{14} \text{C} \) palmitoyl-CoA. Aliquots of 10 µL of peroxisomes (0.25 mg/mL in sucrose imidazole buffer) were incubated in triplicate with 500 µL of the reaction mixture solution for 10 min in a 37°C water bath. The reactions were stopped by adding 250 µL of cold 6% perchloric acid and the samples were kept on ice for 1 hour before being centrifuged at 14,000g for 10 min. Supernatants (500 µL) were transferred into scintillation vials containing 5 mL of Ultima Flo M cocktail (Perkin Elmer, Boston, MA), and the total radioactivity was counted using a Beckman LS6500 scintillation counter. An identical control set of samples was incubated on ice (Lazarow 1981). Specific activity (DPM/pmol) of \( ^{14} \text{C} \) palmitoyl-CoA was determined by counting 100 µL of the reaction mixture. Due to the limitation of our laboratory set up for safety concern, potassium cyanide (KCN), an inhibitor of residual mitochondrial activity, was not used in this experiment. Thus, minor contribution of mitochondria to the palmitoyl-CoA oxidation activity is possible. The palmitoyl-CoA oxidation activity was calculated using the following formula:
Activity (pmol/min/mg protein) = (DPM of incubation sample – DPM of control samples) x 1.5/incubation time (min) / protein amount (mg) / specific activity of reaction mixture (DPM/pmol)

Immunoblotting (Western Blot) assays for CYP2B1/2, 3A1/2, and 4A1/3 proteins

Liver microsomes from vehicle- and bortezomib-treated rats, P450-induced rats, naïve rats, and Supersomes were diluted in deionized water to 0.5 mg/mL, 0.05 mg/mL, and 10 pmol/mL, respectively. The diluted proteins (100 µL) were incubated with 20 µL NuPage reducing agent (10x), 50 µL of NuPage SDS sample buffer (4x), and 30 µL of deionized water for 10 min at 70°C, then kept on ice for at least 5 min. Aliquots of 20 µL of the reduced proteins were loaded on NuPage Novex Bis-Tris gels. Electrophoresis was applied at 60-volt constant for 4 hours. The proteins were then transferred from the gels to PVDF membranes by applying a 25-volt constant for 1.5 hours. The membranes were blocked in 5% non-fat dry milk/PBST for 1 hour before being incubated with primary antibodies (1:1000 diluted in 5% non-fat dry milk/PBST) overnight at 4°C. After washing three times in PBST, the membranes were incubated with the secondary antibodies for 1 hour, followed by three washes in PBST and once in TBS to remove the excess second antibodies. ECL plus Western Blotting Detection Reagents were then used to detect proteins and the chemiluminescent signals were captured on Kodak Biomax films (Rochester, NY). Images were quantitatively analyzed using the NIH Images software (NIH, Bethesda, MD).
Microsomal CYP inhibition assay. Serial dilutions of bortezomib, M1, M2, M3, and M4 solutions (triplicate in 0.1 M potassium phosphate buffer, pH 7.4) were prepared in a 96-well plate. Pooled human liver microsomes (0.5 mg/mL) and CYP substrates (30 µM phenacetin, 150 µM tolbutamide, 100 µM S-mephenytoin, 8 µM dextromethorphan, 50 µM testosterone, and 5 µM midazolam) were added and the samples were pre-incubated for 5 min at 37°C. The reactions were initiated by the addition of pre-warmed NADPH (2 mM) and MgCl₂ (3 mM) solution. After 15 min incubation at 37°C, the reactions were quenched with 100 µL of ACN containing 1 µM antipyrine (internal standard). The samples were centrifuged at 800g for 10 min and the supernatants were transferred to another 96-well plate for LC/MS/MS analyses of the metabolite formation of the probe substrates. The selective CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4/5 inhibitors furafylline (0 to 10 µM), sulfaphenazole (0 to 10 µM), omeprazole (0 to 100 µM), quinidine (0 to 10 µM) and ketoconazole (0 to 10 µM), respectively, were included in the experiments as positive controls. The IC₅₀s were determined using 12-point inhibition curves for bortezomib, M1, M2, M3, and M4 (0, 0.000508, 0.00152, 0.00457, 0.0137, 0.0412, 0.123, 0.370, 1.11, 3.33, 10, and 30 µM). Data were analyzed using Prism software (GraphPad, San Diego, CA).

CYP3A4/5 time-dependent assay In 96-well plates, bortezomib, M1, M2, M3, and M4 (0, 1, 10, 30 µM final concentrations) were preincubated with 2.5 mg/mL of human liver microsomal protein in the presence or absence of 2 mM NADPH / 3 mM MgCl₂ in 100 µL of 0.1 M potassium phosphate buffer, pH 7.4. The reactions (often referred as pre-
incubation) were initiated with the addition of microsomes. The residual activities after 0, 15, and 30 min pre-incubation were measured in secondary incubations by transferring 15 µL of the incubation mixture solutions into another 96-well plate containing 135 µL of testosterone and NADPH / MgCl₂ in 0.1 M potassium phosphate buffer, pH 7.4. The final concentrations of testosterone, NADPH, and MgCl₂ were 200 µM, 2 mM, and 3 mM, respectively. After a 10 min incubation at 37°C, reactions were terminated by the addition of 100 µL ACN containing 1 µM antipyrine (internal standard) and chilled at 4°C for 30 min. The samples were centrifuged at 800g for 10 min. The supernatants were analyzed using LC/MS/MS for the 6β-hydroxytestosterone formation.

Troleandomycin (10 µM) was included as a positive control. The results were processed following a method described by Kitz and Wilson (1962).

**Statistical analysis** Data were processed using Microsoft Excel (Microsoft, Redwood, WA) to calculate means and standard deviations. Student t-test with 95% confidence (p<0.05) was performed on data from different treatment groups to determine any significant differences between the means of the treatment groups.
RESULTS

*Rat ex vivo liver enzyme characterization*

**Total P450 content in rat liver microsomes.** Body weights, liver weights, and P450 contents were measured. Results in Table 1 showed that bortezomib-treated rats had an average liver weight of 13.8 g compared to the vehicle control group of 10.2 g. This represented a 35% increase in liver weight whereas the body weights were barely changed (291 g vs. 302 g in the bortezomib-treated rats). On the other hand, microsomes prepared from bortezomib-treated rats had an average total P450 content of 0.503 nmol/mg protein compared to 0.614 nmol/mg protein for the vehicle control group, resulting in an 18% decrease in P450 content.

**CYP2B1/2, CYP3A1/2, CYP4A1/3 activities and protein expression levels in rat liver microsomes.** Testosterone 16β-hydroxylation, 6β-hydroxylation, and lauric acid 12-hydroxylation are mediated by CYP2B1/2, CYP3A1/2, and CYP4A1/3 in rats, respectively. The rates of formation of these metabolites were measured to evaluate the effect of bortezomib on rat P450s. Results from this study showed little difference in CYP2B1/2 activities between the control- and the bortezomib-treated rats (97.5 ± 9.5 vs. 96.9 ± 12.8 pmol/min/mg protein, Table 1). In addition, quantitative image analysis of CYP2B1/2 Western blots (Table 1 and Figure 2) showed that the microsomes from the vehicle control group had an average band intensity of 1.49 ± 0.08 compared to 1.23 ±
0.16 of the bortezomib-treated group. This 17% decrease in CYP2B1/2 protein expression level was statistically insignificant (p<0.05). Regarding CYP3A1/2, while the vehicle control rats had an average activity of 217 ± 92 pmol/min/mg protein, the bortezomib-treated rats showed an average activity of 167 ± 65 pmol/min/mg protein (Table 1). This represented a 23% decrease in CYP3A1/2 activity, but it was found not statistically significant (p<0.05). The Western blots analysis (Table 1 and Figure 2) showed that the control group had an average CYP3A1/2 band intensity of 1.64 ± 0.04 compared to that of 0.97 ± 0.34 in the bortezomib-treated group. This was a 41% decrease in 3A1/2 protein expression level which could be attributed to the bortezomib treatment in rats. The CYP4A1/3 activity in the vehicle control rats was 1286 ± 236 pmol/min/mg protein, whereas the bortezomib-treated rats had an average specific activity of 1418 ± 379 pmol/min/mg protein (Table 1). In the Western blots (Table 1 and Figure 2), the vehicle control rats showed an average CYP4A1/3 band intensity of 2.24 ± 0.58 and the bortezomib-treated rats showed an average band intensity of 1.93 ± 0.38. This 10% increase in CYP4A1/3 activity and 14% decrease in the CYP4A1/3 protein expression in bortezomib treated rats was statistically insignificant (Student t test, p<0.05).

**Fatty acid β-oxidation activity in rat liver peroxisomal fractions.** Results from this study showed that vehicle control rats had an average peroxisomal activity of 1.57 ± 0.43 nmol/min/mg protein, whereas the bortezomib-treated rats had an average activity of 2.57
± 1.04 nmol/min/mg protein (Table 1), representing a 60% increase in peroxisomal activity in the bortezomib treated rats. Female rats treated in house with clofibric acid, the prototypical CYP4A and peroxisomal acyl-CoA oxidase inducer, were included in this study as a positive control. An average peroxisomal activity of 21.7 nmol/min/mg protein was observed in the clofibric acid treated rats as compared to the oil-diet vehicle control rats which had a specific activity of 2.42 nmol/min/mg protein - a 9-fold increase. It has been suggested that rats treated with an oil diet have slightly higher (50% increase) basal peroxisomal activity compared to rats fed a standard laboratory diet (Huber et al. 1997). In our study, it was indeed the oil-diet control rats that had higher peroxisomal activity than the chow-diet control rats.

**CYP inhibition studies in human liver microsomes**

The CYP inhibition potential of bortezomib and its major metabolites M1 - M4 was studied in human liver microsomes using drug-like probe substrates. The results presented in Table 2 and Figures 3-5 indicated that bortezomib was a mild inhibitor of CYP2C19 (IC$_{50}$ ~ 18 µM); its deboronated metabolite M1 was a mild inhibitor of CYP2C9 and CYP2C19 (IC$_{50}$ ~ 11.5 and 10.0 µM, respectively); and another deboronated metabolite M2 was a mild inhibitor of CYP2C19 (IC$_{50}$ ~ 13.2 µM). Furthermore bortezomib, M1 and M2 showed little inhibition of the other CYP isozymes (IC$_{50}$s > 30 µM). In addition, the secondary dealkylation metabolites M3 and M4 did not inhibit CYP isozymes 1A2, 2C9, 2C19, 2D6, or 3A4/5 (IC$_{50}$s > 30 µM). IC$_{50}$ values of
the known selective CYP inhibitors are also presented in Table 2 for comparison. Since
the CYP3A substrates used in this study (testosterone and midazolam) could not
distinguish the CYP3A4 from the CYP3A5, the CYP3A activities in human liver
microsomes were expressed as CYP3A4/5, although CYP3A4 is the major CYP3A
isoform in liver.

In the time-dependent inhibition study, the percent of CYP3A4/5 activities remaining in
various pre-incubation groups (in the presence of different concentrations of bortezomib
or its metabolites) were plotted (in log scale) vs. the pre-incubation time. A negative
slope of the plot ($K_{\text{obs}}$) would usually indicate enzyme inactivation. If enzymes became
inactivated during pre-incubation, the slope ($K_{\text{obs}}$) would also increase as the test
compound concentration increased. Plotting of our data resulted in a set of flat regression
lines (slopes $\sim 0$) suggesting there was no time-dependent or concentration-dependent
loss of CYP3A4/5 activities. This indicated that bortezomib and its metabolites, M1, M2,
M3, and M4 were unlikely to cause time-dependent inactivation of CYP3A4/5 (data not
shown).
DISCUSSION

CYP2B1/2, 3A1/2, and 4A1/3 are the major inducible CYP isozymes in rat liver. Peroxisomal acyl-CoA oxidase (β-oxidation of fatty acids) is also an inducible enzyme which often attributes to liver enlargement. In our preclinical in vivo toxicity study, an increase in liver weight (35%) following a repeat dose of bortezomib was observed in both male and female rats. Thus, the primary goal for this study was to investigate if the liver enlargement was caused by the induction of CYP2B1/2, 3A1/2, 4A1/3, and/or peroxisomal acyl-CoA oxidase. Female rats were chosen because their lower hepatic CYP3A expression makes them a more sensitive gender for studying P450 induction, especially for CYP3A. Compared to the vehicle control group, the bortezomib treated rats showed no changes in CYP2B1/2 and 4A1/3 activities and protein levels. However, a 23% decrease in CYP3A1/2 activity was observed but it was not statistically significant. This could be due to the variability of the data and the small size of the data set. A decrease in the P450 content and the CYP3A1/2 protein expression, and an increase in the palmitoyl CoA β-oxidation activity were also observed in bortezomib-treated rats. If these changes were normalized to the total liver weight by assuming that the yields of microsomes or peroxisomes (mg protein / gram of liver) were similar across all of the treatment groups, the changes in the total P450 content would become insignificant while the increase of palmitoyl CoA β-oxidation activity would become apparent. Because the microsomal content may vary upon drug treatment in rats (Carlile et al. 1999) and the recovery of microsomes preparation was not tracked in this study, our
data were not expressed in per liver basis. The increase in the palmitoyl CoA β-oxidation activity may correlate with the increased liver weight in this study. However, compared to the 9-fold induction observed with the positive control compound clofibric acid, the 60% increase by bortezomib is mild. The increase in liver weight may also be attributed to the accumulation of ubiquitinated protein in hepatocytes, as hypertrophy not hyperplasia was observed in these liver samples.

It is known that proteasomes mediate degradation of some P450 isoforms including 1A2, 2E1, 3A, and 4A but not 1A1, 2B1/2, or NADPH reductase (Roberts, 1997, Correia et al. 2004). In our study the CYP2B1/2 activity and the protein expression level in rats were indeed not affected by the proteasome inhibitor bortezomib. CYP1A and 2E were not included in the present study because they are relatively minor enzymes compared to the CYP2B and 3A in rats. It is interesting to note that the CYP4A1/3 activities and protein expression levels in our study were unchanged, whereas Roberts (1997) showed that proteasome inhibitors could enhance CYP4A degradation in rat hepatocyte cultures. Studies by Zangar and colleagues (2003) also showed that rat hepatocytes treated with proteasome inhibitors resulted in a decrease of CYP3A protein and mRNA levels. However this is contrary to the CYP3A-ubiquitin-proteasome pathway, thus suggesting CYP3A may be degraded by multiple pathways, or proteasomes could be responsible for the degradation of proteins which suppress CYP3A expression. In our study we also observed a 41% decrease in CYP3A protein expression and a 23% decrease in CYP3A activity although the latter is not statistically significant. Although a change in
bortezomib exposure has not been observed in clinical trials or pre-clinical studies, the
effect of bortezomib on CYP activities, mRNA levels, transcriptional factors and protein
expression, and ubiquitination of CYPs is being conducted in human cultured hepatocytes
to investigate if the ubiquitination pathway could mediate CYP degradation and de novo
synthesis in humans.

CYP1A2, 2C9, 2C19, 2D6, and 3A4 are the five major CYP isozymes in the human liver
responsible for metabolism of > 90% of drugs (Kwon 2001). Understanding the P450
inhibition potential of a new chemical entity (NCE) to these five major CYPs helps in
predicting possible drug-drug interactions in clinical trials (Bjornsson et al. 2003). To
our knowledge, the CYP inhibition potential has not been reported for any boronic acid-
containing molecules or proteasome inhibitors. In this study, up to 30 µM of bortezomib
and its major metabolites were tested. These concentrations are much higher compared
to their plasma C_{max} which is approximately 150 nM (unpublished data). Besides being a
mild inhibitor of CYP2C19, bortezomib also inhibited CYP3A4/5 and 2C9 to a lesser
extent (about 20% at 30 µM, Figure 3). One of its deboronated metabolites (M1) was a
mild inhibitor of both CYP2C9 and CYP2C19, but its diastereoisomer (M2) was only a
mild inhibitor of CYP2C19 and showed little inhibition toward CYP2C9. M1 and M2
also showed mild inhibition of CYP3A4/5 as determined by the testosterone 6β-
hydroxylation (about 40% at 30 µM), while midazolam 1’-hydroxylation was unaffected
(Figure 5). It is generally acknowledged that CYP3A4 is an enzyme with multiple active
sites (Kenworthy et al. 1999), and midazolam and testosterone are substrates that bind
selectively to two different sites of CYP3A4/5 enzymes (Tucker et al. 2001). Therefore, M1 and M2 could interact with one CYP3A4/5 site, but not the other. In metabolite profiling and CYP phenotyping studies, bortezomib was found to be primarily metabolized by CYP3A4/5 and 2C19 (Pekol et al. 2005, Uttamsingh et al. 2005). This explains that bortezomib, being a substrate of CYP3A4/5 and 2C19, is also a mild reversible inhibitor of these two enzymes. The secondary dealkylated metabolites M3 and M4 did not inhibit any of the five CYPs suggesting that the boron containing chain of bortezomib is responsible for the mild CYP inhibition.

Reactive metabolites are often generated by P450-mediated bioactivation of drugs. These reactive metabolites could covalently bind to microsomes or form a metabolite intermediate complex (MIC) to inactivate microsomal activity and thus result in an increase in exposure of that compound. Bortezomib was found not to cause exposure change in our preclinical animal species, thus in the present study time-dependent inhibition was focused on CYP3A4/5 - the primary CYP metabolizing bortezomib as well as over 50% of drugs on the market (Uttamsingh et al. 2005, Kwon 2001). In our study, the observation of no time-dependent inhibition of CYP3A4/5 by bortezomib and its major metabolites suggests that there were no reactive metabolites being generated that would interact with CYP3A4/5. Consistent with that, bortezomib was found not to cause exposure change in preclinical species.
In conclusion, there was no change in microsomal CYP2B1/2, and 4A1/3 specific activities and protein expression in bortezomib-treated rats, however, there were slight decreases in the CYP3A1/2 protein expression (possibly activity) as well as the total P450 content. A mild liver weight increase was observed in this study which may be attributed to ubiquitin/protein accumulation in hepatocytes. The liver weight increase seemed to have direct correlation with the increase of peroxisomal activity. In human liver microsomes bortezomib and its deboronated metabolites M1 and M2 were mild inhibitors of CYP2C19, while the dealkylated metabolites M3 and M4 did not show any inhibition to CYPs. Inhibition of CYP3A4/5 by bortezomib was apparent only at concentrations above 30 µM, which is much higher than the average plasma maximum concentration (C_{max}) observed in humans (~150 nM). No time-dependent inhibition of CYP3A4/5 by bortezomib or its major metabolites M1, M2, M3, and M4 was observed in human liver microsomes. Based on these results, no major bortezomib- or its metabolite-mediated clinical drug-drug interactions are anticipated.
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Legends for Figures

Figure 1    Bortezomib and its major metabolites, M1, M2, M3, and M4

Figure 2    Electrophoresis and Immunoblot (Western Blot) of CYP Proteins in Control and Bortezomib-Treated Female Sprague-Dawley Rats

In all three gels, Lane 1, 2, 3: vehicle control rat microsomes (5 µg); Lane 4, 5, 6: bortezomib-treated rat microsomes (5 µg); Lane 7, 8: Xenotech female and male rat microsomes (5 µg). In CYP2B gel, Lane 9: Phenobarbital-treated rat microsomes (0.5 µg), Lane 10: Gentest CYP2B1 supersomes (0.1 pmol). In CYP3A gel, Lane 9: Dexamethasone-treated rat microsomes (0.5 µg), Lane 10: Gentest CYP3A1 supersomes (0.1 pmol). In CYP4A gel, Lane 9: clofibric acid-treated rat microsomes (0.5 µg).

Figure 3    Inhibition of CYP2C9, 2C19, and 3A4 by Bortezomib

Figure 4    Inhibition of CYP2C9 and 2C19 by Bortezomib Metabolites M1 and M2

Figure 5    Selective Inhibition of CYP3A4/5 Substrate Testosterone Metabolism (not Midazolam) by Bortezomib Metabolites M1 and M2
Table 1: Effect of Bortezomib on Hepatic Oxidative Enzyme Activity in Female Sprague-Dawley Rats

<table>
<thead>
<tr>
<th>Group(^a)</th>
<th>Body Weight (\text{(g)})</th>
<th>Liver Weight (\text{(g)})</th>
<th>P450 Content (\text{nmol/mg microsomal protein})</th>
<th>CYP2B1/2 (\text{pmol/min/mg protein})</th>
<th>CYP3A1/2</th>
<th>CYP4A1/3</th>
<th>Palmitoyl CoA β-Oxidation</th>
<th>CYP 2B1/2</th>
<th>CYP 3A1/2</th>
<th>CYP 4A1/3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle-treated</td>
<td>291 ± 13</td>
<td>10.2 ± 1.9</td>
<td>0.614 ± 0.026</td>
<td>97.5 ± 9.5</td>
<td>217 ± 92</td>
<td>1286 ± 236</td>
<td>1.57 ± 0.43</td>
<td>1.49 ± 0.08</td>
<td>1.64 ± 0.04</td>
<td>2.24 ± 0.58</td>
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<tr>
<td>Bortezomib-treated</td>
<td>302 ± 23</td>
<td>13.8 ± 1.4(^c)</td>
<td>0.503 ± 0.100(^c)</td>
<td>96.9 ± 12.8</td>
<td>167 ± 65</td>
<td>1418 ± 379</td>
<td>2.57 ± 1.04(^c)</td>
<td>1.23 ± 0.16</td>
<td>0.97 ± 0.34(^c)</td>
<td>1.93 ± 0.38</td>
</tr>
</tbody>
</table>

\(^{a}\) All data are presented as Mean ± SD, n = 3 rats, except for body and liver weights where n=6 rats. Each rat sample had 3 measurements.

\(^{b}\) Arbitrary unit

\(^{c}\) Statistically different from the vehicle-treated group (p<0.05)
Table 2  Inhibition of CYP Activities by Bortezomib, Major Metabolites M1 - M4, and Known CYP Inhibitors in Human Liver Microsomes

<table>
<thead>
<tr>
<th>CYP</th>
<th>1A2</th>
<th>2C9</th>
<th>2C19</th>
<th>2D6</th>
<th>3A4&lt;sup&gt;a&lt;/sup&gt;</th>
<th>3A4&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Bortezomib</td>
<td>&gt;30</td>
<td>&gt;30</td>
<td>18.0</td>
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<tr>
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<td>10.0</td>
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<td>13.2</td>
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<td>M3</td>
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<tr>
<td>M4</td>
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<td>Furafylline</td>
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<td>0.10</td>
<td>0.15</td>
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</table>

<sup>a</sup>: Midazolam as the substrate; <sup>b</sup>: testosterone as the substrate
Figure 1

[Chemical structure diagram showing the transformation of Bortezomib into M1, M2, M3, and M4.]
Figure 2

CYP2B

CYP3A

CYP4A
Figure 3

IC₅₀ > 30 µM [Bortezomib], % CYP3A4 activity (testosterone)

IC₅₀ = 18 µM [Bortezomib], % CYP2C19 activity

IC₅₀ > 30 µM [Bortezomib], % CYP2C9 activity

IC₅₀ > 30 µM [Bortezomib], % CYP3A4 activity (midazolam)

IC₅₀ > 30 µM [Bortezomib], % CYP2C9 activity (testosterone)
Figure 4

IC₅₀ = 11.5 µM

IC₅₀ > 30 µM

IC₅₀ = 10.0 µM

IC₅₀ = 13.2 µM
Figure 5

IC₅₀ > 30 μM

% of CYP3A4 activity (midazolam)

[M1], μM

IC₅₀ > 30 μM

% of CYP3A4 activity (midazolam)

[M2], μM

IC₅₀ > 30 μM

% of CYP3A4 activity (testosterone)

[M1], μM

IC₅₀ > 30 μM

% of CYP3A4 activity (testosterone)

[M2], μM