The relationship among tumor architecture, pharmacokinetics, pharmacodynamics, and efficacy of bortezomib in mouse xenograft models

Mark J. Williamson, Matthew D. Silva, Jennifer Terkelsen, Robbie Robertson, Li Yu, Cindy Xia, Panos Hatsis, Bret Bannerman, Trisha Babcock, Yueying Cao, and Erik Kupperman

Millennium Pharmaceuticals, Inc., Cambridge, Massachusetts

Abstract
Understanding a compound’s preclinical pharmacokinetic, pharmacodynamic, and efficacy relationship can greatly facilitate its clinical development. Bortezomib is a first-in-class proteasome inhibitor whose pharmacokinetic/pharmacodynamic parameters are poorly understood in terms of their relationship with efficacy. Here we characterized the bortezomib pharmacokinetic/pharmacodynamic/efficacy relationship in the CWR22 and H460 xenograft models. These studies allowed us to specifically address the question of whether the lack of broad bortezomib activity in solid tumor xenografts was due to insufficient tumor penetration. In vivo studies showed that bortezomib treatment resulted in tumor growth inhibition in CWR22 xenografts, but not in H460 xenografts. Using 20S proteasome inhibition as a pharmacodynamic marker and analyzing bortezomib tumor exposures, we show that efficacy was achieved only when suitable drug exposures drove proteasome inhibition that was sustained over time. This suggested that both the magnitude and duration of proteasome inhibition were important drivers of efficacy. Using dynamic contrast-enhanced magnetic resonance imaging and high-resolution computed tomographic imaging of vascular casts, we characterized the vasculature of CWR22 and H460 xenograft tumors and identified prominent differences in vessel perfusion, permeability, and architecture that ultimately resulted in variations in bortezomib tumor exposure. Comparing and contrasting the differences between a bortezomib-responsive and a bortezomib-resistant model with these techniques allowed us to establish a relationship among tumor perfusion, drug exposure, pharmacodynamic response and efficacy, and provided an explanation for why some solid tumor models do not respond to bortezomib treatment. [Mol Cancer Ther 2009;8(12):3234–43]

Introduction
The therapeutic potential for targeting the proteasome for cancer treatment has been shown by the clinical success of bortezomib (VELCADE, Millennium Pharmaceuticals, Inc.), a Food and Drug Administration–approved treatment for multiple myeloma patients and mantle cell lymphoma patients who have received at least one prior therapy (1–7). Evaluation of bortezomib in phase I and II clinical trials for a variety of advanced hematologic malignancies and non–small cell lung cancer have suggested potentially expanded antitumor activity of this drug and have confirmed its manageable toxicities (8, 9).

The molecular target of bortezomib, the 20S proteasome, is a multicatalytic protease responsible for the majority of intracellular protein turnover in eukaryotic cells (10). Because many cellular processes rely on the proteasome as a regulatory mechanism, proteasome inhibition is expected to have a significant impact on the pathogenesis of proteasome-dependent human malignancies (11–13). Broadly speaking, this hypothesis has been confirmed as nearly all human tumor cell lines were sensitive to the effects of proteasome inhibition in vitro, ultimately succumbing to apoptosis and cell death (14). However, this potent in vitro activity has not translated to wide in vivo activity in preclinical xenograft models. Furthermore, although clinical trials in multiple myeloma and refractory mantle cell lymphoma have shown remarkable efficacy, trials in other solid tumor indications have not shown similar activity (15). Attempts to understand why bortezomib had variable effects in vivo led to the observation that bortezomib exposures and pharmacodynamic profiles can vary across different xenograft models, resulting in variable levels of drug activity. Bortezomib is not a substrate of the efflux transporters P-gp or MRP2 based on Caco-2 results. Therefore, we examined two models, one bortezomib-responsive and the other bortezomib-refractory, to better understand the relationship that exists among tumor perfusion, pharmacokinetics, pharmacodynamics, and efficacy.

Understanding pharmacokinetic/pharmacodynamic relationships may enhance success in the clinic because it facilitates human dose projections and helps define the compound’s anticipated therapeutic index (16–18). Although the
physiochemical properties of a compound have an effect on its pharmacokinetic/pharmacodynamic profile, one must also consider the influence of the tumor environment on drug distribution. Rapidly growing tumors require an adequate blood supply to provide both nutrients and oxygen to sustain their high rate of cellular proliferation. These tumors commonly have abnormal vasculature and are typically characterized by either an extensive network of vessel beds throughout the entire tumor or a simple peripheral supply of blood vessels that provide oxygen and nutrients to only the outer ring of tumor cells, leaving the tumor core hypoxic and necrotic.

Measurement of tumor vascular growth typically relies on assessing circulating levels of fibroblast growth factor and vascular endothelial growth factor. However, meaningful correlation with response is difficult to achieve, as treatment with chemotherapy and angiogenic inhibitors can often themselves manipulate the expression of these proangiogenic factors. More recently, dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) has been implemented as a noninvasive means to acquire images of tumor blood perfusion and predict response of tumors to therapeutic treatments (20–24). To complement the functional MRI readout, micro–computed tomographic (microCT) imaging of vascular casts can provide a high-resolution, three-dimensional (3D) view of the tumor vessel architecture (25, 26). Using pharmacodynamic (20S proteasome inhibition; ref. 27), pharmacokinetic, DCE-MRI, microCT, and efficacy analyses, we present here the pharmacokinetic/pharmacodynamic/efficacy relationship of bortezomib in the CWR22 prostate and H460 NSCLC xenograft models (28–30). The use of this integrated approach and the interpretation of results provide insight into the observed efficacy of bortezomib in these models and, in the future, may help predict conditions required for efficacy with second-generation proteasome inhibitors.

Materials and Methods

Sample Preparation for Proteasome Enzymatic Assays

Blood samples were prepared with 10 μL of whole blood added to 300 μL cold lysis buffer (5 mmol/L EDTA), mixed, and incubated on ice for 15 to 20 min. Samples were centrifuged at 6,600 × g, 4°C for 20 min. The resulting supernatant (200 μL) was added to 200 μL buffer [40 mmol/L HEPES (pH 8.0)], 1 mmol/L EDTA, and 20% glycerol, mixed, and assayed immediately or stored at -80°C. Tumor and brain samples were prepared as follows: Samples (100–300 mg) were frozen and mechanically disrupted in a Covaris E-series instrument (Covaris, Inc.) in 50 mmol/L HEPES (pH 8.0), and 1 mmol/L DTT. Samples were centrifuged for 10 min at 14,000 rpm at 4°C. Supernatants were mixed with equal volumes of stabilizing buffer and assayed immediately or stored at -80°C.

20S Proteasome Assays

For the 20S β5 proteasome assay, lysates prepared from cells, blood, or tissues were thawed at room temperature and diluted 1:5 with proteasome assay buffer (20 mmol/L HEPES, 0.5 mmol/L EDTA, pH 7.4). Five microliters of lysate were added to wells of a 96-well plate followed by 100 μL of proteasome assay buffer at 37°C containing 40 μmol/L Suc-LLVY-AMC (7-amino-4-methylcoumarin) and 12 mmol/L recombinant human PA28α. The progress curves were monitored in a plate reader for 1 h at 37°C. The slopes of the progress curves were converted to substrate turnover by use of an AMC calibration standard. The 20S β1 and β2 proteasome assays were identical to the β5 proteasome assay except that substrates Z-LLE-AMC and Z-VLR-AMC were used respectively.

Cell Culture

Human cell lines 22RV1 and H460 were obtained from the American Type Culture Collection. Cells were maintained in DMEM (Invitrogen) containing high glucose, L-glutamine (Invitrogen), 110 mg/L sodium pyruvate and pyridoxine hydrochloride (Invitrogen), and supplemented with 10% fetal bovine serum (Hyclone). Cells were cultured at 37°C, 5% CO2 atmosphere, 97% relative humidity and routinely passaged by trypsin-EDTA (GIBCO) treatment.

Viability Assays

One day prior to the intended start of the assay, the 22Rv1 and H460 cells were plated in 96-well microtiter plates in a final volume of 75 μL at 1,500 cells/well. Cells were placed in a tissue culture incubator overnight (37°C, 5% CO2 and 21% O2, 97% relative humidity) to allow the cells to equilibrate. Bortezomib (25 μL) was then systematically added to the microtiter plates containing cells to a final volume of 100 μL and placed back into the tissue culture incubator. Cell viability, based on mitochondrial function, was measured 48 h later using 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium (WST) assays as measured by liquid chromatography with tandem mass spectrometry. The blood sampling. The software used was WinNonlin, Version 5.2 (Pharsight Corp.).

Quantitation of Bortezomib and Pharmacokinetic Analysis

The concentrations of bortezomib in blood and tumor tissue were determined by a method based on liquid chromatography with tandem mass spectrometry. The blood samples (50 μL) were extracted by acetonitrile containing 0.1% formic acid and an internal standard (13C-bortezomib). An aliquot of tumor tissue homogenate (2 parts water to 1 part tissue) was extracted as for a blood sample. The transition ions of 367.13/266.07 m/z were monitored by liquid chromatography with tandem mass spectrometry for quantification of bortezomib.

Blood and tumor samples were collected from the mouse at each time point (n = 3) under an anesthesia condition before sacrifice. Pharmacokinetic parameters were calculated using a noncompartmental model with sparse sampling. The software used was WinNonlin, Version 5.2 (Pharsight Corp.).

Establishment and Treatment of the CWR22 Model

Donor mice bearing CWR22 tumors (volume of approximately 500–2,000 mm3) were euthanized by CO2 asphyxiation and tumors were dissected using sterile technique. Viable tumor fragments measuring ~2 × 1.5 × 1.5 mm were implanted s.c. into the right flank of male 6- to 8-week-old
CB-17 severe combined immunodeficient mice (SCID) mice (Charles River Laboratories) using a 13-gauge trocar. Tumor volume was monitored twice weekly with caliper measurements. The mean tumor volume (MTV) was calculated using the formula Volume = (Width^2 × Length)/2. When the MTV reached approximately 200 mm^3, the mice were randomized into treatment groups containing 10 animals per group. The animals were dosed i.v. as described in text on a 2/wk schedule for a maximum period of 3 to 4 wk, or until the humane end point was reached in >50% of the mice in that group (when tumors in vehicle arm achieved 10% of animal body weight). All studies were done in accordance with standards of ethical treatment approved by the Institutional Animal Care and Use Committee (IACUC) and Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). Tumor growth inhibition (TGI) was calculated at the end of study as follows: TGI = [(control average tumor volume – treated average tumor volume) × 100/control average tumor volume]. Statistical comparisons between study groups were made with Student’s t test.

Establishment and Treatment of the H460 Model

Female Ncr nude mice (Charles River Laboratories), approximately 6 to 8 weeks old, were inoculated s.c. with 2.5 × 10^6 freshly dissociated H460 tumor cells (American Type Culture Collection). Tumor volume was monitored twice weekly with caliper measurements. The MTV was calculated using the formula Volume = (Width^2 × Length)/2. When the MTV reached approximately 200 mm^3, the mice were randomized into treatment groups containing 10 animals per group. Animals were dosed i.v. as described in text on a 2/wk schedule for a maximum period of 3 to 4 wk, or until the humane end point was reached in >50% of the mice in that group (when tumors in vehicle arm achieved 10% of animal body weight). All studies were done in accordance with IACUC- and AAALAC-approved standards of ethical animal treatment. TGI was calculated at the end of study as follows: TGI = [(control average tumor volume – treated average tumor volume) × 100/control average tumor volume].

DCE-MRI

MRI Procedures. MRI was done on a 7T (300 MHz) 210-mm horizontal bore Varian INOVA spectrometer (Varian), with 120-mm gradient insert set with 40 G/cm capabilities. A whole body 63-mm quadrature coil was used for image acquisition.

The animals were warmed by heat lamp for several minutes and were immediately anesthetized by 4% isoflurane. Following induction, the animals were transferred to the animal bed and nose cone, which maintained 2% isoflurane, and position supine for scanning. The tail vein was cannulated using a 29-g catheter (ReCathCo) and secured using medical tape. To minimize motion due to respiration, the midsection of the animal was gently secured using foam.

Following a coronal scout image, a single 2-mm-thick axial slice was selected through the middle of the flank tumor for DCE imaging. A gradient echo imaging sequence was selected to monitor the signal change associated with contrast passage into the tumor tissue, using the following parameters: repetition time = 11 ms, echo time = 2.75 ms, pulse angle = 10°, field of view = 3.5 mm × 2.5 mm, data matrix = 128 × 128, number of excitations = 4, spectral width = 100 kHz. Each image took 5.6 s to acquire, and a series of 100 images was queued for a monitoring for 9.3 min. After the 8th scan, 100 μL of 0.5 mmol/kg Gd-DPTA (Magnevist, Bayer HealthCare Pharmaceuticals, Inc.) were injected via the catheter. Following the gradient echo sequence, a multislice fast spin echo scan of the tumor was collected, using the following parameters: repetition time = 500 ms, 2 echoes, echo spacing = 12 ms, field of view = 5 mm × 5 mm, data matrix = 256 × 256, number of excitations = 4.

Data Analysis. Images were analyzed using custom software written in IDL (ITT Visual Information Solutions). Prior to modeling the signal enhancement curves, filtering and region-of-interest operations were implemented. First, the images were filtered with a standard 3 × 3 smoothing kernel. Next, the region of interest of the whole tumor was hand-drawn, and a binary mask file was created to delineate tumor and nontumor regions. The inner and outer regions (core and rim, respectively) of the tumor were segmented for independent analysis. To account for varying tumor sizes, we chose to define the rim as the outer 50% of the tumor by area. This balanced model accurately reflected the heterogeneous nature of the xenografts, while removing the bias and difficulty of manual segmentation.

The DCE-MRI data herein are presented as the signal uptake curves and the integrated area under the signal intensity curve for 240 s following contrast injection (IAUC_{240}). Data were acquired and analyzed for the entire tumor as well as for rim and core regions separately; however, only the core IAUC_{240} data are presented.

MicroCT Vascular Casting

In a subset of animals, vascular casts of the animal vessels were created to visualize the architecture of tumor blood supply and illustrate the regional heterogeneity of the xenograft models employed in this study. Animals bearing tumors of similar size (~300 mm³) were asphyxiated by CO₂, and the thoracic cage was opened to expose the heart. A 21-gauge cannula attached to polyethylene tubing was inserted into the left ventricle. The right ventricle was nicked to allow passage and drainage of the perfusate, which began with 25°C 8 mL of heparinized saline (25,000 units/L) followed by 8 mL of 4% paraformaldehyde in PBS, after which an infusion of Microfil MV-122 (Flow Tech, Inc.) was initiated. Microfil is a radiopaque silicon that can be cured in situ, providing casts of the vasculature. Microfil was prepared at a half dilution relative to the manufacturer’s recommendations, which was not appreciable, which was infused by a pump at a rate of 0.5 mL/h until it began to flow out of the right ventricle.

Twenty-four hours after infusion, the tumors were removed and placed in 10% formalin. MicroCT imaging was done on a Scanco μCT-40 (Scanco USA) at 16 μm isotropic resolution with the following parameters: 70 kVp, 114 μA, 1,024 × 1,024 data matrix, 1,000 projections (0.36° step per projection). To match the DCE-MRI data, volume-of-interest analysis was done on the 3D reconstructed microCT data.
The volume of blood vessels in a 300 × 300 × 100 volume (36.86 mm³) in the center of the tumor was measured.

**Results**

**Effect of Bortezomib on CWR22RV1 and H460 Cell Viability and Proteasome Inhibition**

Bortezomib is a modified dipeptidyl boronic acid derived from leucine and phenylalanine and is a potent, reversible, and specific inhibitor of the chymotrypsin-like activity (β5 site) of the 20S proteasome (Fig. 1A). Bortezomib strongly inhibits cell viability in many human tumor cell lines, showing an average 50% inhibition of cell growth (GI50) of 3.8 nmol/L across the entire National Cancer Institute (NCI) cell line panel (31). Prior to beginning in vivo studies we wanted to specifically evaluate the potency of bortezomib in both the CWR22 and H460 cell lines, so we carried out cell viability experiments. However, CWR22 cells can only be passaged in vivo, so we were constrained to use a derivative cell line, CWR22RV1, in our studies. The

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**Figure 1.** A, chemical structure of bortezomib. B, in vitro effects of bortezomib on mammalian cell viability. WST assays were done after incubation of 22RV1 human prostate cells (white circles) and H460 human lung cells (black cells) with indicated concentrations of bortezomib for 48 h. Data represent mean ± SE (error bars) from three independent experiments. C, in vitro effects of bortezomib on 20S proteasome activity in H460 cells. The data represent percent inhibition of β5 specific activity compared with vehicle control-treated animals (n = 2). D, in vitro effects of bortezomib on 20S proteasome activity in CWR22RV1 cells. The data represent percent inhibition of β5 specific activity compared with vehicle control-treated animals (n = 2).
CWR22RV1 cell line was serially propagated \textit{ex vivo} from a xenograft (CWR22) in nude mice after castration-induced regression and relapse of the androgen-dependent, parental CWR22 xenograft (32). Incubation of either H460 or CWR22RV1 cells with various doses of bortezomib for 48 hours resulted in a range of cell viability and allowed us to calculate an EC\textsubscript{50} value. As depicted in Fig. 1B, similar EC\textsubscript{50} values were observed in CWR22RV1 and H460 cells, 4 nmol/L and 7 nmol/L, respectively. These results are consistent with our observed EC\textsubscript{50} of <10 nmol/L for bortezomib in the majority of cell lines we have tested.\textsuperscript{1} In addition to the cell viability studies described above, we also wanted to show that bortezomib potently inhibited proteasome activity in both CWR22RV1 and H460 cells \textit{in vitro}. As expected, bortezomib treatment resulted in significant 20S proteasome inhibition (as assessed by inhibition in β5 specific activity) in both CWR22RV1 and H460 cells (Fig. 1C and D).

Figure 2. Bortezomib acute dose pharmacokinetic/pharmacodynamic studies. A, whole blood and tumor exposures in CWR22 and H460 tumor-bearing mice following an acute dose of bortezomib at 0.8 mg/kg. Data represent mean ± SD (n = 3). Pharmacodynamic responses in tumor were determined by measuring 20S proteasome β5 enzyme inhibition in tumor at different time points. Tumor samples were collected and tested for β5 proteasome activity in CWR22 tumor-bearing mice (B) or H460 tumor-bearing mice (C). The data represent percent inhibition of β5 specific activity compared with vehicle control-treated animals (n = 3).

Pharmacokinetic/Pharmacodynamic Assessment of Bortezomib in the CWR22 and H460 Xenograft Models

\textit{In vivo}, bortezomib has shown single-agent activity in lung (33), multiple myeloma (34), prostate (35), pancreatic (36), and T-cell leukemia (37) xenograft models. In these studies, treatment with bortezomib led to a reduction of tumor size, slowing of tumor growth, inhibition of tumor angiogenesis, a decrease in metastasis, and an increase in overall survival (38). Nevertheless, there are many additional s.c. xenograft models that remain resistant to bortezomib activity (39). The CWR22 prostate xenograft is routinely used as an \textit{in vivo} model and has been utilized to evaluate the antitumor activity of many well-known drugs such as doxorubicin and paclitaxel (40), geldanamycin (41), trastuzumab (42), and gefitinib (43). The H460 lung xenograft model is also well established and has been used extensively in preclinical studies to evaluate the activity of drugs such as sorafenib.
(44), oblimersen (45), and paclitaxel (46). Internally, the CWR22 model has proven to be a reliable and consistent bortezomib-sensitive in vivo preclinical xenograft model, whereas the H460 model has been bortezomib resistant.

Acute i.v. pharmacokinetic/pharmacodynamic studies were done with bortezomib in s.c. CWR22 tumor-bearing CB17 SCID mice and H460 tumor-bearing NCR-nude mice. The animals were dosed at the twice weekly i.v. maximum tolerated dose for bortezomib, which is 0.8 mg/kg, and monitored for 48 hours. Bortezomib concentrations and 20S proteasome activity in tumor were assessed at various time points (1, 8, 24, and 48 hours) throughout the study in both models to provide tumor pharmacodynamic readouts to correlate with exposures. As indicated in Fig. 2A, whole blood bortezomib levels peaked at 216 ng/mL (Cmax) in the CWR22 model and 233 ng/mL in the H460 tumor-bearing mice. Whole blood bortezomib levels from both CWR22 and H460 tumor-bearing animals closely mimicked each other, generating very similar area under the plasma concentration-time curve over 48 hour (AUC0-48 h) values (7,550 h.ng/mL and 7,670 h.ng/mL for the CWR22 and H460 tumor-bearing mice, respectively). In contrast, levels of bortezomib found in CWR22 and H460 tumors diverged from one another, resulting in differences in the observed tumor Cmax and AUC0-48 h values from the two xenograft models. The Cmax in the CWR22 model was 523 ng/mL, compared with only 108 ng/mL observed in H460 tumors, a difference of almost 5-fold. The bortezomib tumor AUC0-48 h in CWR22s was 14,600 h.ng/mL, whereas in H460s the bortezomib tumor AUC0-48 h was only 4650 h.ng/mL, or approximately 3-fold less. As a result of these findings, the calculated tumor-to-blood AUC ratios for CWR22s was 1.9 compared with only 0.61 for H460, reflecting differences in tumor penetration despite similar whole blood bortezomib levels. Caco-2 assay results show that bortezomib has good cell permeability (Papp value of approximately 10 × 10-6 cm/s) with an efflux ratio <2 (Papp B-A/Papp A-B).

To confirm that there was a correlation between tumor drug exposure and tumor pharmacodynamic response, we examined levels of 20S proteasome inhibition in tumors from both CWR22 and H460s following an acute i.v. dose of bortezomib (0.8 mg/kg). Tumor proteasome inhibition, as measured by 20S β5 specific activity, was significantly inhibited in CWR22 tumors (Fig. 2B). Maximum proteasome inhibition seen in this study was approximately 40% of vehicle control values (4 hours postdose), and by 24 hours proteasome activity had recovered to 80%. In pharmacodynamic studies done in H460 tumor-bearing mice, no significant tumor proteasome inhibition was seen over the entire time course of the study (Fig. 2C). These data suggest that the greater drug exposure seen in CWR22 tumors resulted in both higher maximum levels and sustained duration of proteasome inhibition. In contrast, bortezomib had low tumor exposures in H460s and could not achieve the concentration threshold necessary to result in any significant tumor proteasome inhibition (47).

These results make it more likely that the differences in the tumor environment of these two xenograft models may account for the different bortezomib tumor exposures in CWR22 and H460s.

**In vivo Efficacy Activity of Bortezomib in the CWR22 and H460 Models**

Efficacy studies were done in CB17 SCID male mice bearing CWR22 s.c. tumors and NCR-nude mice bearing s.c. H460 tumors using Millennium IACUC-approved research protocols. In each study a direct comparison was made between vehicle and 0.8 mg/kg bortezomib dosed i.v. on a biweekly schedule for 21 days. Efficacy was determined based
on treated/control (T/C) tumor weight ratio guidelines defined by National Cancer Institute studies, where moderate efficacy is a T/C of <0.4; TGI of ≥60% (48).

As seen in Fig. 3A, tumor growth inhibition consistent with cytostatic activity was shown by bortezomib in the CWR22 model (T/C = 0.3, P < 0.05). In contrast, bortezomib treatment showed no antitumor activity in the H460 tumor model (T/C = 1; Fig. 3B). All administered doses of bortezomib were well tolerated, without any significant (≤10%) weight loss (data not shown). These observations suggest that to achieve optimal bortezomib efficacy in these model systems it is necessary to achieve a minimum level of proteasome inhibition. In the case of CWR22s, we observed at least 40% tumor proteasome inhibition that was sustained for at least 8 hours. In contrast, in H460s, no significant tumor proteasome inhibition was seen at the obtained exposures and as a consequence, no antitumor activity was seen in the efficacy study.

**DCE-MRI and MicroCT Vascular Casting Assessment of the CWR22 and H460 Models**

To further understand why efficacious proteasome inhibition levels were achieved in CWR22 but not H460 xenografts,

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**Figure 4.** The signal intensity changes in CWR22 and H460 tumor rim and core following contrast agent injection using DCE-MRI (A), and the quantification of the area under the curve of the signal intensity graph for the core regions of the tumors (B). The uptake curve shows that although the outer, highly perfused rim is similar in both tumors, the central regions of the tumors are different, and the H460 is 56% reduced in vascular perfusion and permeability as compared with the CWR22.
we investigated if architecture in naïve tumors may be a concomitant factor using DCE-MRI and microCT vascular casting. As shown in Fig. 4, the perfusion and permeability of the tumor as represented by the integrated signal area, IAUC$_{240}$, of core region of the H460 xenograft was 53% less than the CWR22 model ($P < 0.0001$). Conversely, the outer rims of the tumors were comparable by IAUC$_{240}$ and were highly enhanced by MRI. Representative animals (tumors) were cast by Microfil and scanned by microCT, and the relative vessel density could be visualized in 3D. Figure 5 shows an H460 (A) and CWR22 (B) tumor scanned at 16 μm resolution and rendered in 3D for display. Both tumors have extensive and large vessels on the outer edge (rim) of the tumor, whereas only the CWR22 tumor appears well vascularized in the central core region. Quantitatively, we measured the vessel density of the CWR22 core to be on average nine times the density of an equivalent-size H460 tumor. In these studies, untreated tumor architecture and perfusion were investigated; and, in future work, it would be interesting to explore the effect (if any) of the proteasome inhibitor on tumor vessels.

Figure 5. MicroCT vascular casting of the H460 (A) and CWR22 (B) tumors allows us to visualize the heterogeneous vessel distribution. The perfused contrast agent enhances the vessels during high-resolution imaging (16 μm) and provides corroborating evidence that the outer region of the tumors is serviced by large vessels in both cell lines; however, the CWR22 is more densely packed with small vessels than the H460 in the central core of the tumor.

Discussion
Characterizing the pharmacokinetic profile and pharmacodynamic responses that reflect appropriate target inhibition is critical to understanding the factors that drive efficacy in preclinical models. Here we did an analysis of the activity of bortezomib in two xenograft models, one bortezomib-responsive and the other bortezomib-resistant, to better understand the relationship that exists between pharmacokinetic/pharmacodynamic response and efficacy. In CWR22 xenografts, we see drug exposures in tumor that are higher than those seen in H460 xenografts. However, blood exposures across the two models are the same, showing that there are some differences between the two tumor models that must account for the differences in drug exposure. The observed differences in tumor pharmacokinetics were further correlated with differences seen in the tumor pharmacodynamic response in the two tumor models. Tracking with pharmacokinetics, we observed a significant and sustained tumor proteasome response in the CWR22 model (both in vitro and in vivo), but saw no significant tumor proteasome inhibition in H460s (although bortezomib treatment showed significant proteasome inhibition in H460s in vitro, as shown in Fig. 1C). Consistent with these pharmacokinetic/pharmacodynamic studies we observed a strong antitumor effect of bortezomib in the CWR22 model, but did not observe any antitumor activity in the H460 model, after dosing bortezomib twice per week i.v. at maximum tolerated dose (0.8 mg/kg) for 3 to 4 weeks.

We investigated the differences in tumor vessel function and architecture in the two models. DCE-MRI is an established method for noninvasive assessment of vascular function based on rapid scanning following the injection of a low molecular weight $T_1$ contrast agent. The dynamic signal changes in tissue contrast are proportional to the concentration of the contrast agent in the tissue and related to the functionality of the tumor vessels including permeability, capillary surface area, extravascular extracellular volume, and vascular transfer constant (49–51). Several studies have shown the use of these methods for preclinical (22, 23, 52) and clinical (53–56) evaluation of cancer therapies with an antivascular mechanism of action. In this study, it isn’t the mechanism of drug efficacy that we are exploring but the impact of vascular function in tumor on pharmacokinetics, pharmacodynamics, and efficacy in two different cancer models.

A measure of perfusion and permeability of the tumor can be obtained by calculating the integrated signal change in the tumor during the period following the i.v. injection of the MRI contrast agent. Due to the relatively low uptake of contrast into the central (core) region of the H460 tumor, we used a 240-second integration time to improve signal-to-noise. The signal uptake curves (Fig. 4A) and the quantification of the signal increase, IAUC$_{240}$ (Fig. 4B), show that there is a marked perfusion difference in the two experimental models. It is important to consider, however, that the MRI readout is a measure of the biodistribution of the contrast agent and not bortezomib. Thereby we cannot with certainty say by the MRI data

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that the bortezomib distribution is limited to the core of
the tumor; however, the low-molecular-weight contrast
agent does inform us that the degree of perfusion and
permeability is drastically divergent between these two
tumor models and could contribute to differences in phar-
macokinetics.

To confirm the DCE MRI finding, high-resolution mi-
croCT images of tumor vascular casts were created by the
perfusion of the mouse with a silicon agent, which cures
forming a permanent model of the vessels. The agent uti-
lized in these studies was doped with lead chromate, en-
hancing its contrast in CT imaging studies. Published
reports match internal validation done on the agent, sug-
gest that vessels >18 μ can be resolved utilizing this tech-
nique (25). Representative images of the vascular structure
are shown in Fig. 5A and B. The outer rim of the tumors
appear similarly vascularized with large vessels. Referring
to Fig. 4A, the MRI-measured perfusion of this region of
both tumors is similar and may be related to the comparable
density and permeability of the blood vessels. In contrast,
the inner core of the H640 tumor (Fig. 5A) is relatively
devoid of vessels in comparison with the CWR22 tumor
(Fig. 5B). These tumors are similarly sized so the variation
is not due to a size discrepancy. Rather, it seems that the
heterogeneity in vessel development is a characteristic of
the cell line. We quantified a central region of the tumor
measuring approximately 37 mm3 and found the CWR22
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Tumor Architecture and Bortezomib Efficacy

The objective of these studies was to better understand
how differences in tumor architecture and tumor vasculature
potentially affected the relationship between pharmacokinetics/
pharmacodynamics and efficacy in preclinical models of can-
cer. Here we used DCE MRI and high-resolution CT imaging
to characterize the vasculature of CWR22 and H460 xenograft
tumors and were able to identify prominent differences in
vessel perfusion, permeability, and architecture that we be-
lieve resulted in variations in bortezomib tumor exposure.
These differences in exposure altered tumor pharmacody-
namic responses, and ultimately resulted in significant dif-
ferences in efficacy. Presumably, differences in tumor
vasculature should not only affect bortezomib, but could po-
tentially alter the pharmacokinetics of other cytotoxic drugs
as well. A study examining the relationship between plasma
and tumor pharmacokinetics in cancer patients found tumor
pharmacokinetics to be more influenced by tumor perfusion
than plasma drug exposures (57). In addition, numerous
companies have attempted to exploit the leaky vasculature
found in tumors by developing drug delivery systems (e.g.,
nanoparticles, liposomes) that would allow compounds to
preferentially accumulate in cancer tissue (58–60). These
studies suggest that the combination of the physicochemical
properties of bortezomib and the specific vascular architec-
ture of various tumors may limit the clinical indications
where we can expect bortezomib to show strong efficacy.
Thus, a number of groups are focused on developing sec-
ond-generation small molecule proteasome inhibitors with
different physicochemical properties that may allow im-
proved tissue distribution and ultimately improved efficacy
in solid tumor indications.

Disclosure of Potential Conflicts of Interest

M.J. Williamson, M.D. Silva, J. Terkelsen, R. Robertson, L. Yu, C. Xia, B.
Bannerman, Y. Cao, and E. Kupperman are employees of Millennium: The
Takeda Oncology Company.

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