Bortezomib Mediates Antiangiogenesis in Multiple Myeloma via Direct and Indirect Effects on Endothelial Cells

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Abstract
Bone marrow angiogenesis plays an important role in the pathogenesis and progression in multiple myeloma. Recent studies have shown that proteasome inhibitor bortezomib (Velcade, formerly PS-341) can overcome conventional drug resistance in vitro and in vivo; however, its antiangiogenic activity in the bone marrow milieu has not yet been defined. In the present study, we examined the effects of bortezomib on the angiogenic phenotype of multiple myeloma patient–derived endothelial cells (MMEC). At clinically achievable concentrations, bortezomib inhibited the proliferation of MMECs and human umbilical vein endothelial cells in a dose-dependent and time-dependent manner. In functional assays of angiogenesis, including chemotaxis, adhesion to fibronectin, capillary formation on Matrigel, and chorioallantoic membrane assay, bortezomib induced a dose-dependent inhibition of angiogenesis. Importantly, binding of MM.1S cells to MMECs triggered multiple myeloma cell proliferation, which was also abrogated by bortezomib in a dose-dependent fashion. Bortezomib triggered a dose-dependent inhibition of vascular endothelial growth factor (VEGF) and interleukin-6 (IL-6) secretion by the MMECs, and reverse transcriptase-PCR confirmed drug-related down-regulation of VEGF, IL-6, insulin-like growth factor-I, Angiopoietin 1 (Ang1), and Ang2 transcription. These data, therefore, delineate the mechanisms of the antiangiogenic effects of bortezomib on multiple myeloma cells in the bone marrow milieu. (Cancer Res 2006; 66(1): 184-91)

Introduction
Angiogenesis represents the formation of new blood vessels from a preexisting vasculature (i.e., capillaries and post-capillary venules; ref. 1) and results from endothelial sprouting or intussusceptive (nonsprouting) microvascular growth (2, 3). It is fundamental to physiologic processes, including embryogenesis, wound repair, and menstruation; it also plays an important role in tumor growth, invasion, and metastasis (4). New vessels promote tumor cell growth by carrying oxygen and nutrients and removing catabolites, whereas endothelial cells secrete growth factors for tumor cells (5) and a variety of matrix-degrading proteinases that facilitate invasion (6). An expanding endothelial surface also gives tumor cells more opportunities to enter the circulation and metastasize (7), whereas the release of antiangiogenic factors by tumor cells may account, at least in part, for control exerted by primary tumors over metastasis (8, 9).

The role of angiogenesis in the growth, progression, and metastatic spread of solid tumors has already been well established (10). The progression of several cancers of hematopoietic lineage, including non-Hodgkin’s lymphomas, lymphoblastic leukemia, B-cell chronic lymphocytic leukemia, acute myeloid leukemia, and multiple myeloma (11), is also correlated with degree of angiogenesis. In particular, it has been shown that bone marrow angiogenesis is a hallmark of multiple myeloma progression (12), which correlates with disease activity. Indeed, the empirical use of thalidomide therapy in multiple myeloma was based on its antiangiogenic effects, coupled with increasing evidence of the role of angiogenesis in multiple myeloma progression (13). However, responses to thalidomide do not correlate with its antiangiogenic potential, and more recently, thalidomide and its potent immuno-modulatory derivatives have been shown to (a) directly induce apoptosis and G1 growth arrest in multiple myeloma cells, (b) abrogate the increased secretion of interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) triggered by binding of multiple myeloma cells to bone marrow stromal cells (BMSC); as well as (c) stimulate autologous natural killer cell-mediated anti-multiple myeloma immunity (14).

Proteasome inhibitor bortezomib, a boronic acid dipeptide, blocks activation of nuclear factor-κB, thereby inhibiting up-regulation of IL-6 induced by multiple myeloma cell-BMSC binding; it also directly induces apoptosis in multiple myeloma cells resistant to dexamethasone, alkylating agents, and anthracycline and decreases VEGF secretion and associated angiogenesis (14, 15). It has previously been shown that proteasome inhibitors exert antiangiogenic activity in animal models (16–18); however, the antiangiogenic effects of bortezomib on multiple myeloma patient–derived endothelial cells (MMEC) is undefined. The main aim of this study was to investigate the antiangiogenic activity exerted by bortezomib on patient-derived MMECs. Our data suggest that bortezomib, at clinically achievable concentrations, inhibits the proliferation of MMECs and human umbilical vein endothelial cells (HUVEC) in a dose-dependent and time-dependent manner. In functional assays of angiogenesis, including chemotaxis, adhesion to fibronectin, capillary formation on Matrigel, and chorioallantoic membrane assay, bortezomib showed a dose-dependent inhibition of angiogenesis. Importantly, binding of MM.1S cells to MMECs triggered multiple myeloma cell proliferation, which was also abrogated by bortezomib in a dose-dependent fashion. Dose-dependent down-regulation of VEGF and IL-6 secretion by MMECs was observed; furthermore, reverse

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transcriptase-PCR (RT-PCR) confirmed down-regulation of VEGF, IL-6, insulin-like growth factor-I (IGF-I), as well as angiopoietin 1 (Ang1) and Ang2 transcription in treated cells. These data show the antiangiogenic effects of bortezomib, suggesting additional mechan-isms for its activity against multiple myeloma cells in the bone marrow milieu.

Materials and Methods

Cells and cell culture. HUVECs were purchased from Clonetics BioWhittaker (Walkersville, MD; HVEC P168) and maintained in EGM-2MV media (Clonetics BioWhittaker). MM.1S cells were kindly provided by Dr. Steven Rosen (Northwestern University, Chicago, IL) and cultured in RPMI 1640 (Sigma Chemical, St. Louis, MO) containing 10% fetal bovine serum, 2 mmol/L t-glutamine (Life Technologies, Grand Island, NY), 100 units/mL penicillin, and 100 μg/mL streptomycin (Life Technologies). Endothelial cells isolated from bone marrow of patients with multiple myeloma (MMECs) were cultured in DMEM (Sigma Chemical) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, 10 μg/mL streptomycin (Life Technologies; 0.01%), and 2 mmol/L t-glutamine (1%; Life Technologies).

Proteasome inhibitor. Bortezomib [pyrazylCONH(CHPh)CONH(CH-sobutyl)B(OH)2; Millennium Pharmaceuticals, Inc., Cambridge, MA] was dissolved in DMSO and stored at −20°C until use; it was diluted in culture medium (0-20 nmol/L) immediately before use. Bortezomib and control media contained <0.1% DMSO.

Isolation of endothelial cells from bone marrow aspirates. Microvascular endothelial cells were obtained from bone marrow of patients with multiple myeloma, after appropriate consent and in accordance with the Declaration of Helsinki Protocol.

Mononuclear cells were separated from bone marrow aspirates from patients with multiple myeloma by Ficoll-Hypaque gradient centrifugation. Cells were cultured in 25-cm² flasks in complete medium (RPMI 1640 supplemented with 10% FCS and 1% glutamine) for 2 hours. To isolate endothelial cells, adherent cells were harvested in trypsin/EDTA solution (0.05/0.02% in PBS), washed twice with PBS, suspended in FCS-free medium (serum-free medium), and immunodepleted of macrophages and possible residual plasma cells by a 30-minute incubation in CD14 (a monocyte/macrophage marker) plus CD38 (a plasma cell and hematopoietic cell marker) monoclonal antibody (mAb)-coated flasks (Immunotech, Coulter, Marseilles, France). Residual cells were suspended at 0.25 to 1 x 10⁶/mL in serum-free medium and incubated at 0.15 to 0.5 x 10⁶/mL for 30 minutes at 37°C with magnetic microbeads (Dynal, Oslo, Norway) coated with Ulex Europaeus Agglutinin-I (UEA-1; Sigma Chemical), a lectin binding a specific receptor highly expressed by and restricted to endothelial cells (19, 20). Microbeads with bound cells were recovered using a side pool magnetic separation unit and transferred to 12-well plates in 3 mL complete medium per well. Fifteen to 25 days later, there were 1 to 2 x 10⁶ cells per patient. As described previously (21), the MMEC population used in this study contained >95% factor VIII–related antigen (FVIII-RA)+ and CD31+ cells, as well as CD14 and CD38 mAbs, respectively, as well as RT-PCR and Western Blotting confirmed down-regulation of VEGF, IL-6, insulin-like growth factor-I (IGF-I), as well as angiopoietin 1 (Ang1) and Ang2 transcription in treated cells. These data show the antiangiogenic effects of bortezomib, suggesting additional mechanisms for its activity against multiple myeloma cells in the bone marrow milieu.

Preparation of conditioned media and ELISA. MMECs at 90% confluence were cultured for 24 hours in 5% CO₂ humidified atmosphere at 37°C in serum-free DMEM medium (1 x 10⁶/25-cm² flask/6 mL medium) 1% glutamine, with bortezomib (0-20 nmol/L). Culture supernatants were harvested and sequentially centrifuged at 1,200 and 12,000 rpm before filtration through sterilized 0.22-mm-pore-size filters (Costar, Cambridge, MA). The filtrates were stored at −80°C as conditioned media. Cytokines (VEGF, IFN-γ) were quantified in the endothelial cell conditioned media (MMEC and HUVEC) by ELISA (Quantikine Human VEGF-A, IL-6, R&D Systems, Inc., Minneapolis, MN), according to manufacturer’s instructions.

Cell viability and proliferation assay. The inhibitory effects of bortezomib on MMEC growth was assessed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich, Inc., St. Louis, MO) at 24 and 48 hours, as described previously (22). DNA synthesis was measured by [³H]thymidine uptake at 24 hours, as previously described (23).

Chemotaxis assay. Chemotaxis assay was done in a 48-well Boyden chamber, as previously described (24).

The lower compartment was filled with 30 μL DMEM/10% FCS (positive control) or serum-free medium alone (negative control). In the upper compartment, MMEC (1.2 x 10⁵/50 μL) were seeded in the presence or absence of bortezomib (1.25-20 nmol/L) in DMEM/10% FCS. The test was done in triplicate. The compartments were separated by a polycarbonate filter 12-μm pore size (pavolinynpyrolirodone free; Neo Probe, Inc., Gaithersburg, MD), and the lower surface was coated with collagen type IV (5 μg/mL) for 1 hour at 37°C to allow cell adhesion.

After incubation for 6 hours in a humidified 5% CO₂ atmosphere at 37°C, cells on the upper side of the filter were removed by washing in PBS and scraping, whereas those that had migrated to the lower side were fixed and stained with Diff-Quik staining set (Dade Behring, Düdingen, Switzerland). Cells were counted in eight ×400 immersion fields, and mean ± SD per filter was calculated (25).

Chorioallantoic membrane assay on Matrigel. Unpolymerized Matrigel (17 mg/mL; Becton Dickinson, Mountain View, CA) was placed (50 μL/well) in a 96-well microtiter plate (0.32 cm²/well) and polymerized for 1 hour at 37°C. HUVECs and MMECs (5 x 10³ per well) in 200 μL of DMEM/10% FCS (positive control), serum-free medium (negative control), as well as in the presence or absence of bortezomib (1.25-20 nmol/L) were layered onto the Matrigel surface. After 6 hours of incubation in a 5% CO₂ humidified atmosphere at 37°C, cell growth and tridimensional organization were observed using a reverted phase-contrast light microscope (22).

Adhesion assay to fibronectin. MMECs and HUVECs in starvation medium alone (positive control) or treated with bortezomib were plated (5 x 10³ per well) in fibronectin-coated, 96-well plates in triplicate, as described (22). Cells were then fixed with 2.5% glutaraldehyde in PBS at 30 and 90 minutes and quantitated by the colorimetric assay (26). Briefly, cells were fixed for 15 minutes at room temperature with 2.5% glutaraldehyde, stained with 0.1% crystal violet in 20% methanol for 20 minutes, solubilized with 10% acetic acid, and read at 595 nm in a Microplate Reader (Molecular Devices Corp., Sunnyvale, CA). At 30 to 45 minutes, the assay measured cell attachment via αβ integrin (27); at 45 to 90 minutes, the assay measured cell spreading mediated by cytoskeleton microtubules and microfilaments (28).

Chorioallantoic membrane assay. Cultured White Leghorn chicken eggs were incubated at 37°C at constant humidity. On incubation day 3, a square window was opened in the shell, and 2 to 3 mL of albumen were removed to allow detachment of the developing chorioallantoic membrane. On day 8, 1-mm³ gelatin sponges (Gelfoam; Upjohn Co., Kalamazoo, MI) were seeded in the presence or absence of bortezomib (20 nmol/L), as well as with bortezomib 20 nmol/L alone. Sponges loaded with the RPMI 1640 alone or supplemented with basic fibroblast growth factor (bFGF; 200 μg/mL) were used as negative and positive controls, respectively. Chorioallantoic membrane were examined daily until day 12, when the angiogenic response peaks (29). On day 12, blood vessels entering the sponge within the focal plane of the chorioallantoic membrane were recognized macroscopically (at ×50), counted by two observers in a double-blind fashion (30) under a Zeiss SR microscope. To better highlight vessels, the chorioallantoic membrane portions were removed, embedded in paraffin, sectioned at 8μm along a plane parallel to the chorioallantoic membrane, fixed in Bouin’s fluid, dehydrated in graded ethanols, and rendered transparent in methylbenzoate (29). After macroscopic counting on day 12, the embryos and their membranes were fixed in ovo in Bouin’s fluid. The sponges and the underlying and immediately adjacent chorioallantoic membrane portions were removed, embedded in paraffin, sectioned at 8μm along a plane parallel to the chorioallantoic membrane surface, and stained with a 0.5% aqueous solution of toluidine blue (Merck, Darmstadt, Germany). Angiogenesis was measured by a slightly modified planimetric point count method (29): four to six ×250 fields covering every third section within 30 serial slides of each sponge per sample were

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analyzed within a superimposed 144 intersection point square reticulum of 0.125 mm². Only transversely sectioned microvessels (i.e., capillaries and small venules with or without a 3- to 10-μm lumen occupying the intersection points) were counted and calculated as the mean ± 1 SD per section, per chorioallantoic membrane, and groups of chorioallantoic membrane. The angiogenic response was calculated, with results expressed as number of eggs out of total (N = 20) with >50% inhibition in the angiogenic response compared with vehicle. Mean ± SD vessel counts were determined for each analysis.

**RT-PCR.** RT-PCR was done as previously described (31) using 2 μg total RNA extracted with Trizol reagent (Invitrogen/Life Technologies, Carlsbad, CA) and reverse transcribed by Moloney murine leukemia virus reverse transcriptase (Invitrogen, San Diego, CA). Then, 1 μg of cDNA was subjected to PCR for the selected genes. Primers (Invitrogen) used were 5'-CAGACAGTGGCTTTAAC-3' and 5'-CATGCGAGATCTCATACTC-3' for VEGF; 5'-ACATCTCCCACCTCTCTGTGTTTCTT-3' and 5'-CCCTCTCATTTGTTTCA-3' for Ang1; 5'-CTCCAGCAAATTCATCATTG-3' and 5'-GCTTCCCAGCTTCCATGT-3' for Ang2; as well as 5'-CTCTTTACAAATTCCATCATTG-3' and 5'-CGCCA-CAGTTTCCCGAGGG-3' for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as control. Primers used for IL-6 were from human IL-6 primer pair (R&D Systems). PCR profiles were VEGF: 1 cycle of denaturation at 95°C for 90 seconds, 30 cycles at 95°C for 60 seconds, 60°C [annealing temperature (Tm)] for 45 seconds, and 72°C for 60 seconds; Ang1: one cycle of denaturation at 95°C for 100 seconds, five cycles at 97°C for 30 seconds, 60°C Tm for 30 seconds, and 72°C for 30 seconds followed by 30 cycles at 95°C for 35 seconds, 60°C Tm for 30 seconds, and 72°C for 30 seconds with a final extension at 72°C for 7 minutes; IL-6: one cycle of denaturation at 94°C for 45 seconds, 30 cycles at 94°C for 45 seconds, and 72°C for 45 seconds; for Ang2: one cycle of denaturation at 95°C for 10 minutes, 35 cycles at 95°C for 30 seconds, 56°C Tm for 30 seconds, and 72°C for 30 seconds; Ang1: one cycle of denaturation at 95°C for 90 seconds, 35 cycles at 95°C for 30 seconds, 55°C Tm for 30 seconds, and 72°C for 30 seconds; Ang2: one cycle of denaturation at 95°C for 90 seconds, 35 cycles at 95°C for 30 seconds, 60°C Tm for 30 seconds, and 72°C for 30 seconds. The PCR products were separated by electrophoresis on 1.5% agarose gels and stained with ethidium bromide. The bands were detected by a trans-illuminator, and the intensity was assessed by Image J Analysis Software, which converts the band area into pixels.

**Real-time RT-PCR.** One microgram of total RNA was reverse transcribed into total cDNA. Real-time RT-PCR was done on an ABI PRISM 7000 Sequence Detector using dedicated reagents (Applied Biosystems, Foster City, CA). Each gene was analyzed in parallel with the Abelson (ABL) housekeeping gene: the absolute levels of VEGF, IL-6, Ang1, Ang2, and IGF-I were determined for each analysis.

**Statistical analysis.** Statistical significance of differences observed in drug-treated versus control cultures was determined using the Wilcoxon rank signed tests. The minimal level of significance was P < 0.05.

**Results**

**Bortezomib exerts an antiproliferative activity in MM.1S cells triggered by binding to MMECs.** Because we have shown that BMSCs promote multiple myeloma cell growth, survival, and drug resistance (34), we first similarly examined whether MMECs similarly promoted growth of MM.1S cells. Binding to MMECs up-regulated (3.5-fold) [3H]thymidine uptake in MM.1S cells, which was completely abrogated by bortezomib in a dose-dependent fashion (Fig. 1A). Importantly, bortezomib did not trigger cytotoxicity in MMECs at 24 hours, whereas a dose-dependent cytotoxicity was observed after 48 hours of exposure to increasing concentrations of the drug.

**Figure 1.** Bortezomib inhibits proliferation of MM.1S cells triggered by binding to MMECs, without affecting the viability of MMECs at 24 hours. MMECs were cultured with MM.1S cells in the presence of increasing concentrations of bortezomib (0-20 nmol/L). DNA synthesis was assessed by the addition of tritiated thymidine during the last 8 hours of 24-hour cultures. Bortezomib abrogated proliferative activity in MM.1S cells triggered by binding to MMECs in a dose-dependent manner (A). MMECs were cultured in the presence of increasing concentrations of bortezomib (0-20 nmol/L) for 24 hours (B) and 48 hours (C), and viability was then assessed by MTT assays. Bortezomib does not induce cytotoxicity in MMECs at 24 hours, whereas a dose-dependent cytotoxicity was observed after 48 hours of exposure to increasing concentrations of the drug.

**Bortezomib inhibits MMEC functions relevant to angiogenesis.** We next investigated whether bortezomib could directly inhibit in vitro chemotaxis, adhesion to fibronectin, capillary formation on Matrigel, as well as angiogenesis in the chorioallantoic membrane assay in vivo. We first examined the effect of bortezomib on MMEC migration. As shown in Fig. 2A, serum-induced migration of MMECs was significantly inhibited by...
bortezomib at IC_{50} of 10 nmol/L. Similar results were observed with HUVECs (data not shown).

We next examined the capacity of MMECs to attach to and spread on fibronectin. Bortezomib did not affect MMEC adhesion to fibronectin at 30 minutes; in contrast, cell spreading evaluated at 90 minutes was significantly inhibited by bortezomib (1.25-20 nmol/L) in a dose-dependent fashion (Fig. 2B).

The effect of bortezomib on vessel morphogenesis by MMECs was also investigated. Specifically, the in vitro ability of MMECs to form capillaries was studied by the Matrigel assay. After 6 hours, MMECs spread throughout the Matrigel surface and aligned to form branching, anastomosing, and thick tubes with multicentric junctions, which gave rise to a closely knit network of capillary-like structures (Fig. 2C, i). Bortezomib triggered a dose-dependent inhibition of capillary formation by MMECs. For example, 2.5 nmol/L bortezomib partially inhibited capillary-like tubes (Fig. 2C, ii), with nearly complete inhibition at 7.5 nmol/L, as evidenced by either isolated or aggregated spherical cells in small clumps, with few elongated cells and no anastomosed tubes (Fig. 2C, iv). Similar effects were obtained with HUVECs (data not shown).

**Bortezomib inhibits angiogenesis in vivo.** We further examined the antiangiogenic activity of bortezomib in vivo using the chorioallantoic membrane assay (Fig. 3). Bortezomib (Fig. 3C) caused a significant inhibition of basal angiogenesis in the chick embryo induced by sponges loaded with RPMI 1640 alone (Fig. 3A). Moreover, chorioallantoic membrane implanted with FGF-2 (positive control; Fig. 3B) or with MMEC conditioned media (Fig. 3D) triggered increased macroscopic vessels (34 ± 5 and 32 ± 6, respectively). Importantly, bortezomib significantly inhibited MMEC conditioned medium–induced angiogenesis (Fig. 3E), evidenced by vessel counts (10 ± 3 versus 32 ± 6) and by the number of eggs (20 of 20) with >50% inhibition in the angiogenic response compared with vehicle (15 of 20).

**Bortezomib down-regulates the expression of genes required for the angiogenic phenotype and inhibits IL-6 and VEGF secretion by patient-derived MMECs.** We investigated the effect of bortezomib on the expression of VEGF, IL-6, IGF-I, Ang1, and Ang2 genes that are primarily involved in the angiogenic cascade. RT-PCR analysis showed that bortezomib induced significant dose-dependent down-regulation of these genes with the exception of Ang1. Ang1 expression was reduced only using the highest dose of bortezomib (i.e., 7.5 nmol/L; Fig. 4).

A more precise quantitation of the bortezomib induced modulation of VEGF, IL-6, Ang1, and Ang2 genes was expressed as percentages of the baseline value, using Real-time RT-PCR. A pronounced dose-dependent down-regulation was observed: average VEGF = −20% and −30%; IL-6 = −26% and −59%; Ang2 = −31% and −63%; IGF-I = −60% and −73% at 5 and 7.5 nmol/L, respectively. Down-regulation of Ang1 was observed only at the highest tested dose: +6% and −25% at 5 and 7.5 nmol/L, respectively (Fig. 5).

Because we have previously shown that bortezomib inhibits cytokine secretion from BMSCs (35, 36), we next similarly examined whether bortezomib also blocks secretion of IL-6 and VEGF from MMECs. We found a concentration-dependent reduction of both IL-6 (Fig. 6A) and VEGF (Fig. 6B) production in the presence of bortezomib. Importantly, bortezomib did not induce significant cytotoxicity in MMECs (Fig. 1B).

**Discussion**

The 26S proteasome is a multicatalytic enzyme complex expressed in the nucleus and cytoplasm of all eukaryotic cells...
It is an essential enzyme complex for the nonlysosomal, ATP-dependent proteolytic pathway, catalyzing the rapid degradation of intracellular proteins regulating cell cycle, apoptosis, cell adhesion, transcription, antigen presentation by MHC class I molecules, and angiogenesis (37). The coordinate balance between the expression and the degradation of these proteins is required for the homeostasis of cellular functions. It plays a crucial role in the up-regulation or down-regulation of growth signaling pathways by targeting proteins for degradation. Tumorigenesis is directly dependent on signaling pathways that increase growth,
chemotaxis, production of antiapoptotic factors, and angiogenesis. Together, these factors contribute to tumor cell growth, spread, and survival; therefore, inhibition of the proteasome is a promising novel therapeutic approach to cancer.

Bortezomib (Velcade, formerly PS-341) is the first proteasome inhibitor to have shown anticancer activity in both solid tumors and hematologic malignancies. It inhibits the growth of lung cancer cell lines \textit{in vitro} and \textit{in vivo} in athymic nude mouse xenografts (38). Specifically, it produces additive growth delays against Lewis lung carcinoma when coupled with 5-fluorouracil, cisplatin, taxol, and doxorubicin (39). In murine models, it also induces marked \textit{in vivo} antitumor activity against human prostate cancer, Burkitt's lymphoma, and adult T-cell leukemia (40–42). In addition, its antiangiogenic activity has been shown in an orthotopic pancreatic cancer model (43). Multiple myeloma is the prototype cancer where bortezomib has shown marked \textit{in vitro} activity, which was followed by rapid translation to phase I, II, and III clinical trials, and resulted in accelerated approval by the Food and Drug Administration for the treatment of patients with relapsed refractory disease.

Here, we studied the \textit{in vitro} and \textit{in vivo} antiangiogenic potential of bortezomib against multiple myeloma cells in the bone marrow milieu (44, 45). Proteasome inhibitors have shown a greater activity against dividing endothelial cells than against quiescent cells (16), suggesting that they target the aberrant blood vessel development associated with tumor growth. Lactacystin, a specific proteasome inhibitor (17), inhibits angiogenesis \textit{in vitro} using the chick embryo chorioallantoic membrane. We have previously shown that bortezomib shows antiangiogenic activity in the tumor-host environment and also in a human plasmacytoma model in severe combined immunodeficient mouse, as evidenced by decreased microvascular density (18). However, the exact mechanism for this decreased microvascular density was undefined.

In this study, we showed the direct effects of bortezomib on multiple myeloma patient derived endothelial cells and associated functional sequelae to characterize the antiangiogenic potential of bortezomib.

We first examined whether bortezomib targets angiogenesis in multiple myeloma by a direct effect on MMEC functions \textit{associated with angiogenesis \textit{in vitro}}: proliferation, chemotaxis, adhesion molecule profile, and capillary formation on Matrigel. We examined the effect of bortezomib on neovascularization in the developing chick embryo chorioallantoic membrane, an \textit{in vivo} model of angiogenesis. We also investigated whether bortezomib affects the expression of genes involved in the angiogenic cascade (VEGF, IL-6, Ang1, Ang2, and IGF-I), or the secretion of important angiogenic cytokines (VEGF and IL-6), to determine if bortezomib acts indirectly against MMECs via modulation of these cytokines. We showed drug-induced inhibition of all these functions in a dose-dependent manner, including inhibition of angiogenesis \textit{in vivo} in the chorioallantoic membrane model, where bortezomib caused a significant inhibition of both basal angiogenesis and that induced by bFGF or by MMEC conditioned media.

It has been shown (46) that VEGF triggers blood vessel formation and plasma cell growth within the bone marrow of multiple myeloma. VEGF-A is primarily produced by plasma cells and stimulates proliferation and chemotaxis of endothelial cells (both necessary for angiogenesis) via VEGF receptor-2 (VEGFR-2) and of residual stromal cells via VEGFR-1. Activation of these cells results in VEGF-C and VEGF-D production, which stimulates multiple myeloma cell growth via VEGFR-3. Presently, we showed a concentration-dependent reduction of VEGF and IL-6 by MMECs after treatment with bortezomib.
We studied whether VEGF, IL-6, IGF-I, Ang1, and Ang2 genes in MMECs are modulated by bortezomib (47). Their expression in endothelial cells increases with the angiogenic switch and persists throughout angiogenesis, except during vessel stabilization, when inhibitors prevail (48). IGF-I is an angiogenic factor that acts both directly through activation of the relative receptor IGF receptor-1 and indirectly through up-regulation of VEGF gene expression (49). Ang1 and Ang2 are mandatory for vessel sprouting and remodeling (47), as well as survival factors that inhibit apoptosis. Ang1 promotes sprouting in the presence of VEGF, induces branching networks with the typical organization of mature vessels, and stabilizes perivascular endothelial cell interactions. Ang2 exerts a vessel destabilizing effect, which allows VEGF-mediated vascular reorganization. Here, we showed that both MMECs and HUVECs express VEGF, IL-6, IGF-I, Ang1, and Ang2; and that exposure to bortezomib induced a significant down-regulation of all these genes except Ang1 in a dose-dependent fashion. These data support the view that bortezomib exerts an indirect antiangiogenic activity on human MMECs through the down-regulation of genes required for autocrine and paracrine growth of endothelial cells isolated from the bone marrow of patients with multiple myeloma. In summary, this study shows that bortezomib, a potent and selective inhibitor of the proteasome, targets angiogenesis in multiple myeloma by a direct effect on MLEC functions associated with angiogenesis both in vitro in proliferation, chemotaxis, adhesion on fibronectin, capillary formation on Matrigel assay, and in vivo in the chorioallantoic membrane assay. Drug-related down-regulation of genes mandatory for autocrine and paracrine growth of endothelial cells, including VEGF, IL-6, IGF-I, Ang1, and Ang2, was observed. In addition, these translated proteins decreased in MMEC conditioned media. These data therefore show that bortezomib acts both directly and indirectly against MMECs, defining another mechanism which may contribute to its anti-multiple myeloma activity.

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