Proteasome Inhibitors Enhance Endothelial Thrombomodulin Expression via Induction of Krüppel-Like Transcription Factors

Toyoko Hiroi, Clayton B. Deming, Haige Zhao, Baranda S. Hansen, Elisabeth K. Arkenbout, Thomas J. Myers, Michael A. McDevitt, Jeffrey J. Rade

Objective—Impairment of the thrombomodulin–protein C anticoagulant pathway has been implicated in pathological thrombosis associated with malignancy. Patients who receive proteasome inhibitors as part of their chemotherapeutic regimen appear to be at decreased risk for thromboembolic events. We investigated the effects of proteasome inhibitors on endothelial thrombomodulin expression and function.

Methods and Results—Proteasome inhibitors as a class markedly induced the expression thrombomodulin and enhanced the protein C activating capacity of endothelial cells. Thrombomodulin upregulation was independent of NF-κB signaling, a principal target of proteasome inhibitors, but was instead a direct consequence of increased expression of the Krüppel-like transcription factors, KLF2 and KLF4. These effects were confirmed in vivo, where systemic administration of a proteasome inhibitor enhanced thrombomodulin expression that was paralleled by changes in the expression of KLF2 and KLF4.

Conclusions—These findings identify a novel mechanism of action of proteasome inhibitors that may help to explain their clinically observed thromboprotective effects. (Arterioscler Thromb Vasc Biol. 2009;29:00-00.)

Key Words: ●●●
We hypothesize that some of the clinically-observed thromboprotective effects of proteasome inhibitors in myeloma patients may be attributable to modulation of the TM–protein C anticoagulant pathway. The goal of the present study was to investigate the effect of proteasome inhibitors on the expression and function of TM in endothelial cells.

Methods

Cell Culture and Reagents

Human umbilical vein endothelial cells (HUVECs; American Type Culture Collection CRL-1730) were maintained in EGM-2 media (Lonza) under 5% CO₂ at 37°C. Cells of passage 2 to 5 were used for all experiments. Bortezomib was provided by Millennium Pharmaceuticals. All other chemicals were purchased from Sigma–Aldrich unless otherwise indicated.

Animal Studies

Animal protocols were approved by the Johns Hopkins Animal Care and Use Committee. C3H/HeN male mice weighting 19 to 21 g (Charles River Laboratories) were given intraperitoneal injections of bortezomib (0, 0.4 or 0.8 mg/kg) once daily for 7 days. One hour after last injection, mice were anesthetized with isoflurane and organs harvested for mRNA and protein expression analysis.

Real-Time Quantitative PCR

Total RNA was extracted from HUVECs using RNeasy Mini kit (Qiagen) or from tissue using TRIZOL Reagent (Invitrogen). After treatment with DNase, samples were subjected to reverse transcription and standard multiplex real-time PCR in duplicate using TaqMan Universal PCR Master Mix Reagents on a 7900HT Sequence Detection System (Applied Biosystems). Levels of mRNA were measured by the standard curve methods using pooled total RNA extracted from control cells or tissue. Target mRNA levels were normalized to 18S ribosomal RNA (rRNA). The following primers/labeled probes were purchased from Applied Biosystems: human KLF2 (Hs00360439_g1), mouse KLF2 (Mm00437014_s1), and 18S ribosomal RNA control (4308329). Primers/labeled probes were purchased from Applied Biosystems: human KLF2 (Hs00360439_g1), mouse KLF2 (Hs00360439_g1), mouse KLF4 (Mm00516105_g1), human KLF4 (Hs00358836_m1), mouse KLF4 (Mm00516105_g1), human TM (Hs00151358_m1), mouse TM (Mm00437014_s1), and 18S ribosomal RNA control (4308329). Primers for human TM were custom made from the following sequences: forward primer-CCCCAACACCCAGGCCTAGCT, reverse primer-CGTCGATGTCCGTGCAGAT; probe-TGCCCTGAAGGCTACATCC-5’.

Quantification of NF-κB Activation

NF-κB activation was measured by an ELISA-based method (Trans-AM NF-κB p65; Active Motif) as previously described.5

Western Blot and Immunohistochemical Analysis

Western blotting was performed using primary antibodies to human TM (#2375; American Diagnostica), mouse TM (#sc-7097; Santa Cruz Biotechnology), and β-actin (#A5441; Sigma–Aldrich) as previously described.5,11 Immunohistochemical staining was performed on formalin-fixed and paraffin-embedded sections of mouse liver as previously described.10 Immunofluorescence staining was performed using primary antibodies to mouse TM (#sc-7097; Santa Cruz Biotechnology) and mouse CD31 (#ab28364; Abcam) with fluorescent-labeled secondary antibodies (#A21206; Alexa Fluor 488-labeled anti-Rabbit IgG and #A11058; Alexa Fluor 594-labeled anti-goat IgG, Invitrogen).

In Situ Protein C Activation Assay

The capacity to generate activated protein C (APC) in situ was measured on HUVECs as previously described.5

siRNA-Mediated Gene Knockdown

HUVECs (50% to 60% confluent) were transfected with 100 nmol/L of siRNA reagents specific for the human KLF2, KLF4, KLF6, and RelA genes (siGENOME ON-TARGETplus SMARTpool duplex) using DharmaFECT siRNA transfection reagent #1, according to the manufacturer’s instructions (Dharmacon). Control cells were incubated with vehicle (DharmaFECT siRNA transfection reagents) alone or with siGENOME nontargeting siRNA pools. Proteasome inhibitor was added to the medium 48 hours after transfection and incubated for an additional 20 hours.

Nuclear Run-On Assay

A nuclear run-on assay was performed on HUVECs treated with bortezomib or vehicle for 20 hours as previously described.15 mRNA was reverse transcribed then subjected to quantitative PCR analysis as described above.

Statistical Analysis

All data are presented as the mean±SEM. Comparison between 2 groups is by 2-tailed t tests and between multiple groups is by 1 factor ANOVA followed by a Tukey multiple comparison test for intergroup comparisons. Only probability values <0.05, considered to be statistically significant, are shown.

Results

Effect of Proteasome Inhibition on TM Expression and Function

We first characterized the ability of bortezomib, a dipeptide boronic acid derivative that is the only proteasome inhibitor approved for human use, to inhibit 20S proteasome activity and NF-κB activation in HUVECs (please see supplemental materials, available online at http://atvb.ahajournals.org). Proteasome and NF-κB activity were suppressed in a dose-dependent manner, with >70% of proteasome inhibition occurring within 3 hours of exposure to bortezomib (supplemental Figure I). In a preliminary experiment, we measured changes in mRNA expression of a series of coagulation-related molecules in HUVECs after a 20-hour exposure to 5 nmol/L bortezomib (supplemental Figure IIA). Compared to controls, bortezomib significantly increased mRNA levels of the anticoagulant molecules TM and eNOS (129±15% and 72±5% of control, respectively, P<0.002 for both), while suppressing levels of the procoagulant molecules protease-activated receptor-1 (PAR-1) and vWF (~50±1% and −25±2% of controls, respectively, P<0.05 for both) as well as the fibrinolytic molecules urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA; ~44±1% and −34±5%, respectively, P<0.01 for both). Bortezomib significantly suppressed cytokine-induced NF-κB activation in a dose-dependent manner, paralleling the degree of proteasome inhibition, and suppressed TNF-α-mediated induction of mRNA encoding E-selectin and vascular adhesion cell molecule-1 (VCAM-1) by >99% and intercellular adhesion molecule-1 (ICAM-1), monocytes chemotactic protein-1 (MCP-1), and tissue factor by >67% (supplemental Figure IIB).

Based on the above preliminary data, we chose to further explore the effects of proteasome inhibitors on TM expression and function. HUVECs exposed to bortezomib for 20 hours exhibited a dose-dependent increase in TM mRNA and protein expression (Figure 1A and 1B). Similar effects were observed in human aortic endothelial cells (supplemental Figure III). Consequent to increased TM expression was an increased capacity of endothelial cells to generate APC (Figure 1C). Time course studies revealed that bortezomib caused an initial fall in TM expression followed by a marked rebound that was not diminished by exposure to TNF-α (Figure 1D). TM expression was also induced in a dose-dependent
manner after exposure to the proteasome inhibitors epoxomicin, a natural peptidyl epoxyketone, and MG132, a synthetic tripeptide aldehyde, indicating a class effect (Figure 1E).

To determine whether the NF-κB inhibitory properties of proteasome inhibitors play a role in TM induction, we compared the effects of bortezomib to both chemical and molecular inhibitors of NF-κB activation. Parthenolide, a soluble sesquiterpene lactone NF-κB inhibitor without proteasome inhibitory properties, had no effect on basal TM gene expression whereas bortezomib caused a nearly 4-fold increase despite equipotent NF-κB inhibition (Figure 2A). Both agents effectively prevented TM downregulation by TNF-α. As confirmation, HUVECs were transfected with siRNA targeting the RelA (p65) subunit of NF-κB or a nontarget control. Suppression of RelA gene expression prevented cytokine-induced NF-κB activation but did not diminish TM upregulation by bortezomib (Figure 2B). These data indicate that proteasome inhibitors upregulate TM expression via a mechanism that is independent of NF-κB signaling.

Proteasome Inhibition Induces the Expression of Krüppel-Like Transcription Factors

We next investigated the molecular mechanism by which proteasome inhibitors stimulate TM expression. Pretreatment of HUVECs with cycloheximide abrogated bortezomib-induced TM upregulation, indicating that new protein synthesis is required (Figure 3A). By nuclear run-on assay, exposure to 5 and 10 nmol/L bortezomib increased levels of TM mRNA >10-fold.
(Figure 3B), indicating that proteasome inhibitors induce TM expression via transcriptional activation.

KLF transcription factors, particularly KLF2 and KLF4, have recently been recognized as important mediators of vascular endothelial cell function. Both are capable of stimulating the expression of TM and eNOS, similar to the observed effects of proteasome inhibitors. We therefore investigated whether KLF transcription factors are involved in TM regulation by proteasome inhibitors. HUVECs were first exposed to ascending doses of bortezomib for 20 hours, and changes in the expression of KLF2, KLF4, and KLF6 mRNA measured. Although bortezomib induced all 3 in a dose-dependent manner, the effect was most pronounced for KLF2 and KLF4, with 8- to 18-fold increases at doses \( \leq 5 \) nmol/L (Figure 4A). Similar effects on KLF2 and KLF4 mRNA expression were observed in cells treated with MG132 and epoxomicin (supplemental Figure IV). HUVECs were then transfected with siRNA targeting each of the KLF species or a nontarget control before a 20-hour exposure to 5 nmol/L bortezomib. siRNA treatment resulted in \( \approx 80\% \) inhibition of target gene expression after bortezomib exposure (supplemental Figure V). Blocking the induction of both the KLF2 and KLF4 genes, but not the KLF6 gene, effectively prevented the upregulation of TM by bortezomib (Figure 4B). Furthermore, combined inhibition of KLF2 and KLF4 appeared to have additive suppressive effects compared to each one alone. These data support the concept that proteasome inhibitor-induced upregulation of TM is mediated by upregulation of the KLF2 and KLF4 transcription factors.

**Systemic Proteasome Inhibition Enhances In Vivo TM Expression**

To investigate whether physiological doses of a proteasome inhibitor could alter TM expression in vivo, mice were administered ascending intraperitoneal doses of bortezomib for 7 days after which TM, KLF2, and KLF4 mRNA levels were measured in several organs (Figure 5A). The most pronounced effect was observed in the liver, where levels of TM mRNA and protein (Figure 5B) increased markedly in a dose-dependent manner (7- and 10-fold over controls at a...
The major findings of this study are: (1) proteasome inhibitors as a class markedly stimulate endothelial TM expression resulting in enhanced capacity of endothelial cells to generate APC; (2) proteasome inhibitors effectively prevent the down-regulation of TM by inflammatory cytokines; (3) the upregulation of TM by proteasome inhibitors is independent of their NF-kB inhibitory properties but rather mediated by induction of Krüppel-like transcription factors; and (4) systemic administration of a proteasome inhibitor can enhance endothelial expression of KLF2, KLF4, and TM in vivo.

In response to injury or inflammation, the normal anticoagulant properties of the endothelium become impaired and are opposed by the nascent expression of tissue factor which initiates thrombin generation and thrombosis. The critical importance of an intact TM–protein C anticoagulant pathway to maintaining endothelial thromboreistance is highlighted by the observation that mice with deletions of TM, protein C, or the endothelial protein C receptor (which facilitates APC generation by the thrombin/thrombomodulin complex) die in utero or in the perinatal period from thrombosis. The acquired loss of TM expression, with a consequent reduction in APC generating capacity, is thought to be a major contributor to the thrombotic manifestations of several inflammatory conditions, including bacterial sepsis, transplant rejection, and radiation enteropathy.

Dysfunction of the TM–protein C anticoagulant pathway has been implicated in thrombosis associated with multiple myeloma and other malignancies. Levels of soluble TM, caused by the release of membrane-bound TM from injured endothelial cells, are elevated at the time of diagnosis and fall with treatment. TM gene expression is well-recognized to be negatively regulated by inflammatory cytokines, such as TNF-α and interleukin-1β. Serum levels of both cytokines are markedly elevated in patients diagnosed with multiple myeloma and, like soluble TM, fall during treatment. Approximately 10% to 20% of patients with multiple myeloma also develop resistance to the anticoagulant actions of APC which is associated with an increased incidence of VTE. In contrast to congenital APC resistance attributable to the presence of the factor V Leiden mutation, the mechanism of acquired APC resistance associated with cancer appears related to increased levels of factors V and VIII which overwhelms the anticoagulant effects of APC. It is therefore mechanistically plausible that agents such as proteasome inhibitors, with the potential to both increase endothelial TM expression and APC-generating capacity as well as inhibit tissue factor expression, could reduce the risk of VTE in patients with multiple myeloma. Our results provide a conceptual basis for more in-depth in vivo animal and human clinical studies investigating this possibility.

Proteasome inhibition was found not only stimulate baseline TM expression but also block its downregulation by TNF-α. We previously reported that TM downregulation by cytokines and endotoxin is mediated by activation of NF-κB, which competes for limited cellular quantities of the transcriptional coactivator p300 necessary for TM gene expression. As the inhibition of NF-κB is thought to mediate the primary antitumor effects of bortezomib, it was reasonable to speculate that NF-κB signaling might be involved in modulation of TM expression by proteasome inhibitors. The fact that TM upregulation was not diminished by blocking relA expression (Figure 2B) and required new protein synthesis (Figure 3A) ruled out a role for NF-κB signaling in mediating TM upregulation by proteasome inhibitors. Rather, this effect was found to be mediated by induction of the Krüppel-like transcription factors KLF2 and KLF4. KLF2 is known to be capable of stimulating TM transcription by binding to a specific GC-rich site in the TM promotor. Overexpression of both KLF2 and KLF4 in endothelial cells recapitulates many of the phenotypic changes observed with proteasome inhibition, including a marked increases in baseline TM and.

**Figure 4.** Induction of TM by proteasome inhibitors is mediated by upregulation of Krüppel-like transcription factors. A, Changes in the levels of KLF2, KLF4, and KLF6 mRNA were measured in HUVECs treated with bortezomib for 20 hours at the indicated doses (n=3 per group of 1 of triplicate experiments; P<0.01). B, Effect of KLF knockdown on TM expression. HUVECs were transfected with 100 nmol/L siRNA targeting the KLF2, KLF4, KLF6 genes and a nontarget (Non-T) control 48 hours before the treatment with ±5 nmol/L bortezomib for 20 hours (n=3 per group of 1 of triplicate experiments).
An intriguing finding of our study was the observed differential effects of bortezomib on TM induction in various organs. TM induction was most pronounced in the liver and kidney with no significant induction observed in the heart or lung. TM upregulation was only observed in tissue where KLF2 or KLF4 were also upregulated. Susceptibility to thrombus formation in general, and TM expression in particular, is known to vary between different vascular beds. It is therefore possible that there are tissue-specific differences in the molecular pathways by which proteasome inhibitors regulate endothelial thromboresistance. These results, however, may be better explained by the pharmacokinetics of bortezomib. After intravenous injection, the drug is rapidly cleared from the blood with a large volume of distribution. Radiolabeling studies in rats revealed that the liver and kidney have the highest bortezomib uptake, with virtually no uptake in the brain. The maximum tolerated daily intraperitoneal dose (0.8 mg/kg) given to mice in our study was equivalent to approximately half the intravenous bolus dose (1.3 mg/m²) administered to myeloma patients on a biweekly basis in clinical studies. It is possible that alternate dosing schemes utilizing chronic intermittent bolus injections, such as those used in clinical studies, would achieve more widespread and consistent organ effects.
In summary, proteasome inhibitors as a class stimulate the expression of TM and enhance endothelial cell thromboreistance. These effects are mediated via induction of the Kruppel-like transcription factors, KLF2 and KLF4, and may help explain the reduction in thromboembolic events observed in patients who receive proteasome inhibitors for the treatment of plasma cell malignancies.

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Disclosures
None.

References
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SUPPLEMENTAL MATERIAL

Supplemental Methods

Quantification of 20S Proteasome Activity. 20S proteasome activity (kindly performed by Dr. William J. Riordan of Millenium Pharmaceuticals) was measured in whole cell lysates as previously described \(^1\) using a 96-well plate format with a BioTek medel synergy HT microplate reader (BioTek Instruments, Inc Winooski, VT). Proteasome inhibition was determined by calculating the percent change of the chymotryptic specific activity compared to untreated controls.

Quantification of NF-κB Activation. NF-κB activation was measured by an ELISA-based method (Trans-AM NF-κB p65; Active Motif, Carlsbad, CA) as previously described.\(^2\)

Supplemental Figures

Supplemental Figure I. Effect of bortezomib on endothelial cell proteasome and NF-κB signaling activity. A) 20S proteasome activity was measured in the cellular lysates of HUVECs treated with bortezomib at the indicated doses for 20 hours. (n=3 per group,*P <0.01, ** P <0.001 versus 0 nM bortezomib.) B) Time course of 20S proteasome inhibition in the cellular lysates of HUVECs treated with 5 nM bortezomib for up to 24 hours. (n=3 per group, *P <0.001 versus time 0 control.) C) NF-κB activity in whole cell extracts of HUVECs treated with 5 nM bortezomib for 6 hours then stimulated with vehicle or 100 ng/mL TNF-α for 3 hours. (n=3 per group, *P <0.001 versus 0 nM bortezomib + TNF-α.)
Supplemental Figure II. Effects of proteasome inhibition on endothelial expression of coagulation and inflammation-related genes. A) Changes in the expression of coagulation-related genes in HUVECs treated with 5 nM bortezomib for 20 hours. B) Changes in the induction of inflammation-related genes in HUVEC treated with 5 nM bortezomib for 3 hours then stimulation with vehicle or 100 ng/mL TNF-α for an additional 17 hours. Values shown are the mean ± SEM of n=3 samples per group in one of at least two replicated experiments. Target gene expression was normalized to rRNA expression. TM = thrombomodulin; eNOS = endothelial nitric oxide synthase; TFPI = tissue factor pathway inhibitor; HS = heparan sulfate proteoglycan; EPCR = endothelial protein C receptor; tPA = tissue-type plasminogen activator; uPA = urokinase-type plasminogen activator; vWF = vonWillebrand factor; PAR-1 = protease activated receptor-1; PAI-1 = plasminogen activator inhibitor-1; VCAM-1 = vascular cell adhesion molecule-1; ICAM-1 = intercellular adhesion molecule-1; MCP-1 = monocyte chemotactic protein-1; TF = tissue factor.

Supplemental Figure III. Effect of proteasome inhibition on TM expression in human aortic endothelial cells. Dose-related change in TM gene expression, normalized to rRNA, in human aortic endothelial cells treated with bortezomib at the indicated doses for 20 hours. * p<0.001 versus control (0 nM bortezomib). Values shown are the mean ± SEM from three independent experiments.
Supplemental Figure IV. A). Effect of proteasome inhibition on KLF2 and KLF4 expression. Dose-related change in KLF2 and KLF4 gene expression in HUVECs treated with epoxomicin (left panel) and MG132 (right panel) at the indicated doses for 20 hours. *p<0.001 versus control (0 nM proteasome inhibitors).

Supplemental Figure V. Effect of siRNA on KLF expression. HUVECs were transfected with 100 nM siRNAs targeting the KLF2, KLF4, KLF6 genes and a non-target (Non-T) control 48 hours prior to the treatment with ± 5 nM bortezomib for 20 hours. Values shown are the mean ± SEM from three independent experiments. **p<0.01, ***p<0.001 versus control (0 nM proteasome inhibitors, Non-T). †p<0.05, ††p<0.01, †††p<0.001 versus 5 nM bortezomib, Non-T.
Reference List
