The thromboprotective effect of Bortezomib is dependent on the transcription factor Kruppel-like factor 2 (KLF2)

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Key Points:

Transcription factor Kruppel-like factor 2 (KLF2) is a critical determinant of vascular thrombosis. The antithrombotic effect noted with Bortezomib is KLF2-dependent.

ABSTRACT

Multiple myeloma confers a high risk for vascular thrombosis, one that is enhanced further by treatment with immunomodulatory agents. Strikingly, inclusion of the proteasome inhibitor bortezomib reduces thrombotic risk, yet the molecular basis for this observation remains unknown. Here, we show that bortezomib prolongs thrombosis times in the carotid artery photochemical injury assay in normal mice. Cell-based studies show that bortezomib increases expression of the transcription factor Kruppel-like factor 2 (KLF2) in multiple cell types. Global postnatal overexpression of KLF2 (GL-K2-TG) increased time to thrombosis while global postnatal deletion of KLF2 (GL-K2-KO) conferred an anti-parallel effect. Finally, studies in GL-K2-KO mice showed that the thromboprotective effect of bortezemib is KLF2-dependent. These findings identify a transcriptional basis for the anti-thrombotic effects of bortezomib.
Introduction

Cancer is associated with a high risk of thrombosis\(^1\). Among hematological malignancies, multiple myeloma(MM) has an especially high rate of both arterial and venous thrombotic events (VTE)\(^2\). Further, patients treated with the immunomodulatory drugs(IMiDs) thalidomide and lenalidomide experience a substantially elevated VTE risk(15-30%)\(^3\). In sharp contrast, MM patients receiving proteasome inhibitor bortezomib(BZ) have a VTE risk that is less than 4%\(^4,5\). Consistently, BZ treatment in hypertensive rat models decreases arterial thrombosis\(^6\). Although \textit{in vitro} and \textit{ex vivo} studies show that BZ inhibits platelet aggregation, the magnitude of this effect is insufficient to explain the profound decrease in VTE noted with BZ\(^7-9\). Consequently, alternative mechanisms are likely operative and account for the anti-thrombotic effects of BZ.

\textit{KLF2} is a member of the zinc finger family of transcription factors that is highly expressed in endothelial and hematopoietic cells\(^10-13\). Studies in our laboratory identify \textit{KLF2} as a key regulator of endothelial\(^14-16\) and myeloid inflammation with favorable antithrombotic properties\(^17,18\). Further, studies conducted by us and others reveal that BZ induces KLF2 mRNA in endothelial\(^19\) and hematopoietic cells. As endothelial and hematopoietic cells are centrally involved in thrombotic events, we hypothesized that the antithrombotic effect noted with BZ may be \textit{KLF2}-dependent.

Methods

CAG-Cre-ERT\(^2\)(control) mice(Jackson Laboratory) were crossed with floxed \textit{KLF2} mice or floxed stop KLF2 mice to generate global \textit{KLF2} knockout(GL-K2-KO) or global \textit{KLF2} overexpressing(GL-K2-TG) mice(previously described\(^20\)) respectively. Indicated mice and cells were treated with BZ at the mentioned dose and schedule.
All the mice colonies were maintained in a clean animal facility, and all animal experimentation was approved by the Case Western Reserve University Institutional Animal Care and Use Committee.

Further details are provided in Supplemental Methods.

**Results and discussion:**

**Bortezomib prolongs time to occlusion in a carotid artery thrombosis assay.** To determine if BZ confers an antithrombotic effect, C57BL/6J mice treated with a non-myelosuppressive dose of BZ (0.3mg/kg intraperitoneal thrice weekly for 2 weeks) were subjected to carotid artery thrombosis using the photochemical injury model. Wild type mice treated with BZ demonstrated a significantly prolonged time to occlusive carotid artery thrombosis compared to saline treatment (40.8 ± 6 minutes vs. 29.4 ± 6 minutes, p = 0.006, n= 7 per group) (Figure 1A). There was no difference between WBC and platelet counts and RBC hemoglobin in saline and BZ-treated animals (Figure 1B). Further, the antithrombotic effect was not associated with concomitant changes in the tail-bleeding (Figure 1C) or in platelet activation assays (Figure 1D).

**Bortezomib induces KLF2.** A large body of work suggests that interaction between endothelial and hematopoietic cells (e.g. platelets and myeloid cells) regulates thrombosis. As shown in Figure 2A, BZ induced KLF2 mRNA in both endothelial (HUVEC) and hematopoietic (RAW264.7 and MEG-01) cell lines. This induction is specific to KLF2 in the myeloid and megakaryocytic cell lines. Although KLF4 is concomitantly induced in endothelial cells (HUVEC), it was not as robust as the increase in KLF2 levels (Supplemental Figure 1). Consistently, treatment of C57BL/6J mice with BZ induced KLF2 mRNA in peripheral white blood cells (Figure 2B). To further understand how KLF2 levels are induced, we examined the effect of BZ on a KLF2 promoter luciferase construct and found that BZ induced KLF2 promoter activity (Supplemental Figure 2).
Kruppel-like factor 2 (KLF2) is a critical determinant of the thrombotic phenotype. Cell-based gene expression studies in endothelial and myeloid cells by our group suggest that KLF2 likely confers an anti-thrombotic effect\textsuperscript{13,15}. However, the significance of these observations has not been studied \textit{in vivo}. We performed carotid artery thrombosis assays in mice with global postnatal KLF2 deletion (GL-K2-KO) or overexpression (GL-K2-TG). Compared to the controls, GL-K2-KO mice have a significantly shortened time to occlusive thrombosis (Figure 2C; 20.6 ± 2 minutes vs. 32.8 ± 4 minutes, p=0.0001, n= 6-7). Conversely, GL-K2-TG mice demonstrated significantly prolonged times to occlusive thrombosis (44.8 ± 1 minutes vs. 32.8 ± 4 minutes, p= 0.0003, n= 5-7). These results identify KLF2 as a critical determinant of thrombosis \textit{in vivo}.

The antithrombotic effect of BZ is KLF2-dependent. To determine if the anti-thrombotic effect of BZ is KLF2-dependent, we treated GL-K2-KO and control mice with BZ. Although occlusive carotid artery thrombosis times were significantly prolonged in the control mice treated with BZ (36 ± 5 minutes vs. 51.2 ± 8 minutes, p= 0.009, n= 5-7) (Figure 2D), no prolongation was noted in the GL-K2-KO (26.1 ± 4 minutes vs. 25.6 ± 4 minutes, p=0.8, p= 5-7) mice after BZ treatment demonstrating that the thromboprotective effect of BZ is dependent on KLF2.

KLF2 alters the expression of thrombotic targets. We analyzed primary endothelial cells and peritoneal macrophages obtained from mice with KLF2 knockout or overexpression. KLF2 overexpression was associated with a significant decrease in inducible nitric oxide synthase (iNOS) and monocyte chemotactic factor-1 (MCP-1) in peritoneal macrophages and protease-activated receptor 1 (PAR-1) and thrombomodulin (TM) in endothelial cells. Conversely, KLF2 knockout demonstrated a significant increase in iNOS levels in peritoneal macrophages and plasminogen activator inhibitor 1 (PAI-1) in endothelial cells. (Figure 2E).

Our findings are the first \textit{in vivo} studies to implicate a transcription factor, namely KLF2, as a key orchestrator of the thromboprotective effect observed with BZ. Previous \textit{in vitro} studies
demonstrate that BZ treatment is associated with increased thrombomodulin expression that was dependent on KLF2. Further, studies identify that BZ induces endothelial NOS and decreases secondary thrombotic events and inflammatory responses. Given that KLF2 is known to confer potent anti-inflammatory effects and induce eNOS, it is possible that these observations are secondary to increased KLF2 levels as suggested by our studies.

An additional mechanism implicated in the anti-thrombotic effect of BZ is inhibition of platelet aggregation. However, Avcu et al. described their observations as a mild platelet defect that was insufficient to explain the significant antithrombotic effect. Consistent with this view, we did not observe a significant effect on platelet activation following treatment with BZ. Hence, our findings that BZ's anti-thrombotic effects are abrogated in the absence of KLF2 implicates a more fundamental underlying mechanism. Interestingly, BZ treatment in hematopoietic cells (myeloid and megakaryocyte) specifically induces only one member of the KLF family, i.e. KLF2. Although both KLF2 and KLF4 are induced in the endothelium, the effect on KLF2 transcript levels is more robust. Since KLF4, a closely related member of the Kruppel family of transcription factors also has thromboprotective properties, the demonstration that the antithrombotic effect of BZ is essentially lost in the absence of KLF2 compels us to postulate that in the context of BZ treatment, KLF2 must provide a more dominant antithrombotic effect, one that cannot be compensated for by endothelial induction of KLF4 that can occur with BZ treatment. We also demonstrate that KLF2 alters key thrombotic targets such as TM, PAI-1, and PAR-1. Although there is significant overlap in the number of downstream targets altered by both KLF2 and KLF4, it is likely that genes more critical to the thrombotic process are affected by KLF2 alone and reflect the dependency of BZ on this transcription factor for the antithrombotic benefit.

In summary, this study implicates the transcription factor KLF2 as a critical determinant of thrombosis. Our study also demonstrates that BZ induces KLF2 in both hematopoietic and
endothelial cells and provides *in vivo* genetic evidence that the antithrombotic benefit noted with BZ is *KLF2*-dependent. Hence, *KLF2* may be a novel target amenable to pharmacological manipulation in the management of thrombosis. Further studies are being pursued to elucidate the relative contribution of each cell type in the thromboprotective effect and will provide useful insights in the molecular mechanisms and potential targets for amelioration of thrombosis.
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Authorship

Contribution: L.N. and H.S. performed experiments. L.N., G.B.A., and Z.L. analyzed and interpreted the data. L.N. and M.K.J. designed the research. G.B.A., Z.L. and A.H.S. provided critical advice on research design and experiments. L.N. drafted the manuscript. All authors critically reviewed and approved the final version of the manuscript.

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REFERENCES


Figures Legends

**Figure 1. Bortezomib (BZ) has an antithrombotic effect.** (A) C57BL/6J mice at 8-12 weeks of age treated with intraperitoneal BZ injections (0.3 mg/kg) thrice weekly for 2 weeks. Carotid artery thrombosis (photochemical injury model, Rose Bengal) was performed 24-30 hours after the last dose of BZ. (B) White blood cell count (WBC), Hemoglobin (HB) and Platelet counts (PLTS) in C57BL/6J mice treated with BZ. (C) C57BL/6J mice treated with BZ as described in (A) were subjected to tail bleeding assay 24 hours after last dose of BZ. (D) Platelet flow cytometry in human α-thrombin stimulated JONA/A binding Fig.D(i) or P-selectin expression Fig. D(ii), convulxin stimulated JON/A binding Fig. D(iii), and fibrinogen binding after ADP stimulation D(iv) in platelets from C57BL6J mice treated with saline versus BZ. The concentration-dependent results are the mean ±SEM of 5 individual experiments.

**Figure 2. Antithrombotic effect of BZ is KLF2-dependent.** (A) Endothelial (HUVEC), monocytic (RAW 264.7) and megakaryocyte (MEG-01) cells lines treated with BZ at 5µM for 24 hours (*P<0.05, n=3-5). Total RNA was isolated and KLF2 mRNA expression analyzed by qPCR and normalized to GAPDH. (B) C57BL/6J mice were treated with BZ and white blood cells (WBC) isolated for KLF2 mRNA expression, normalized to GAPDH (*P<0.05, n=5). (C) Carotid artery thrombosis assay (photochemical injury model) was performed in CAG-Cre-ERT² (CRE), GL-K2-KO, and GL-K2-TG mice. (D) CAG-Cre-ERT² (CRE) and GL-K2-KO mice were treated with intraperitoneal BZ (0.3 mg/kg) versus saline thrice weekly for 2 weeks followed by carotid artery thrombosis assay (photochemical injury model) that was performed 24-30 hours after the last dose of BZ. (E) Total RNA was isolated from primary KLF2 knockout and overexpressed macrophages (peritoneal macrophages) and endothelial cells (cardiac and pulmonary) and mRNA expression analyzed as indicated by qPCR and normalized to GAPDH.
MCP-1, monocyte chemotactic protein 1; iNOS, inducible nitric oxide synthase; TF, tissue factor; TM, thrombomodulin; PAI-1, plasminogen activator inhibitor-1; PAR-1, protease-activated receptor 1.
Figure 1.
Figure 2.
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