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Research Article

Carfilzomib Potentiates Platelet Activation and Thrombus Growth

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ABSTRACT

Background: Multiple Myeloma (MM) patients treated with carfilzomib are at high risk of Venous Thromboembolism (VTE), however, the underlying aetiology and mechanism remains unknown. Carfilzomib is an irreversible Proteasome Inhibitor (PI) known to have cardiac toxicity, but less is known about its thrombogenic effects. Platelets play a critical role in the pathogenesis of thrombosis; however, limited studies have reported the effects of carfilzomib on platelet function.

Objectives: To characterise the effects of carfilzomib on platelet activation and thrombus formation.

Methods: Effects of carfilzomib on platelet activation, collagen adhesion and thrombus formation are characterised using *in-vitro* and *ex-vivo* thrombosis models.

Results: Carfilzomib potentiated thrombin-induced platelet activation, demonstrated by increased P-selectin expression and integrin $\alpha_{IIb}\beta_3$ activation compared to first-generation PI, Bortezomib and control that showed no effect. The addition of dexamethasone to carfilzomib further increased thrombin-induced platelet activation compared to carfilzomib alone. Carfilzomib potentiated platelet adhesion to type-1 collagen and increased thrombus formation under arterial flow compared to Bortezomib and control that showed no effect. The increased thrombus formation under arterial flow was further enhanced when carfilzomib was combined with dexamethasone.

Conclusion: These findings suggest that carfilzomib-induced thrombosis risk in patients with multiple myeloma may be the result of enhanced platelet thrombotic function.

Keywords: Multiple myeloma; Bortezomib; Carfilozomib; Proteosome inhibitor; Platelets; Thrombosis

INTRODUCTION

Patients with Multiple Myeloma (MM) are at increased risk of Venous Thromboembolism (VTE). Large population-based studies have demonstrated that patients with MM are 7 to 9-fold more likely to be diagnosed with VTE compared to the general population [1,2]. Although the underlying mechanism of VTE in MM is incompletely understood, therapy-related causes are important as most VTE incidences occur within the first 6 months from initiating MM therapy.

Proteasome Inhibitors (PI) used in combination with other drugs such as immunomodulators form the backbone of MM therapy, three PIs are currently approved for use in MM; bortezomib,

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carfilzomib, and ixazomib. Carfilzomib is a second-generation PI approved for the treatment of relapsed/refractory MM (RRMM), additionally; recent studies have shown that carfilzomib is also effective in treating newly diagnosed MM (NDMM). Cardiovascular toxicity is a known adverse effect of carfilzomib; however, VTE associated with carfilzomib is also a significant complication. Most of the evidence regarding increased VTE incidence stems from the RRMM studies containing carfilzomib-combination therapies, which report VTE rates of about 10% [3], while recent studies in NDMM using carfilzomib-combination therapies have also reported VTE rates between 5%-14% [4,5]. In contrast, bortezomib and ixazomib have not been associated with an increased thrombosis risk. Significantly, analysis of studies utilising bortezomib-based regimens have reported that bortezomib may in fact be protective against thrombosis. The mechanisms underlying the difference in thrombotic potential between carfilzomib and the other PIs are unclear, although pharmacodynamics of the PI may be an important factor, as carfilzomib is an irreversible PI whereas bortezomib and ixazomib are both reversible PIs. The mechanisms of carfilzomib-associated VTE are not known, however platelets are likely to play an important role, given their critical role in the pathogenesis of thrombosis.

Platelets contain the entire molecular machinery of the proteasome system, which is activated upon platelet stimulation by thrombin and collagen; moreover, PIs block collagen-induced platelet aggregation and thrombus formation in mice models [6-8]. These observations suggest an important role of the proteasome in platelet function and that carfilzomib could potentially modulate platelet activation as a contributing factor of carfilzomib-induced VTE.

To date, only a few studies have investigated the direct effect of proteasomal inhibitors on normal platelet function. Therefore, this study aims to investigate the direct effect of the proteasomal inhibitors, Carfilzomib and Bortezomib (CAR and BOR) on both C57BL/6 mice and healthy human platelets using on *ex vivo* and *in vitro* platelet activation studies and thrombus formation model under arterial flow.

MATERIALS AND METHODS

Drugs

DEX phosphate (DBL™) and CAR were purchased from AMGEN Inc. BOR was provided by Peter MacCallum Cancer Centre. BOR and CAR were dissolved in Phosphate Buffered Saline (PBS) pH 7.4. DEX were provided in liquid form. Vehicle control or sham control included human platelets and mice treated with PBS.

Animal ethics

The experimental protocol was reviewed and approved by the RMIT University Animal Ethics Committee (#AEC 23436). For *ex vivo* model study, cohorts of C57BL/6-mice were administered by oral gavage once (single-doses of proteasome inhibitors according to individual body weight: 40 mg per kg BOR or CAR). Control mice (sham control) were administered with PBS pH 7.4 *via* oral gavage. Blood samples were collected 2 hours after administration of drugs or PBS to enable peak drug concentration in blood samples. All mice had free access to fresh water and food supplements.

Human ethics

The collection of human blood for this research was approved by the RMIT University Human Research Ethics Committee (HREC reference number: 22442).

Platelet analysis and preparation

Haematological parameters were determined in Platelet Rich Plasma (PRP) or whole blood as described in previously. Human and murine platelets were washed and prepared. Then, platelet preparations were immediately used for flow cytometry studies.

Flow cytometry

Human washed platelets (50 µL of 100 × 10^9/L) were pre incubated with Fluorescein IsothioCyanate (FITC)-conjugated anti-human P-selectin (CD62P); monoclonal antibody (PAC-1); and Phycoerythrin (PE)-conjugated antibodies including anti-human CD41a, anti-human CD42a, anti-human GPIbα-IX-V complex (CD42b) and anti-human GPVI; anti-human integrin $\alpha_2\beta_1$ (CD49b) antibodies. In addition, murine washed platelets were preincubated with FITC-conjugated hamster anti-mouse CD61 (β_3), FITC-conjugated rat anti-mouse P-selectin (CD62P), FITC-conjugated rat anti-mouse CD9 (p24), anti-mouse GPVI, anti-mouse GPIbα (CD42b), or PE-conjugated JON/A (integrin alphaIIbbeta3)(Becton Dickinson Pty Ltd, Mulgrave, Victoria).

Flow and adhesion conditions

Glass µ-slides IV 0.1 with outer dimensions 75.5 × 25.5 mm (H × L) were coated with 500 µg/mL type I equine ligament collagen for 2 hours at 37°C. Non adherent collagen was washed off with PBS, pH 7.4. All blood samples were incubated with 0.05% (w/v) rhodamine 6G dye at 37°C for 60 minutes to allow fluorescent visualisation of platelets. Labelled platelets in blood were perfused over a matrix of type I collagen-coated µ-slide IV 0.1 micro-capillaries for 6 minutes at a shear stress rate of 1800 seconds-1 using a infuse/withdrew PHD 22/2200 syringe pump. Perfusion tubes were rinsed with PBS pH 7.4 after each perfusion at the same shear rate for 6 minutes. Thrombi formation derived from platelet adhesion to type I fibrillar collagen and subsequent platelet aggregation on the surface of the µ-slides, was visualised in real time using a Zeiss Axiovert M1 microscope (objective LD 20 × /0.4 NA) equipped with HAL 100-W bright light and HBO 100-W fluorescent light lamp sources and an AxioCam MRm camera.

Platelet thrombi formation was analysed by 3D deconvolution of rendered Z-stack slices of fluorescent images of the microchannel taken during a time-lapse recording using Zen Zeiss software. Following deconvolution, the threshold was adjusted to a lower intensity to distinguish platelets from the background, subsequently the same threshold settings were applied to all Z-stack images. Thrombus volume (µm^3) was

determined by multiplying the surface coverage area (μm^2) with the height (μm) of platelet thrombi.

Statistical analysis

Statistical analysis was conducted using Graph Pad Prism software program version 8.4.2. All results are expressed as the mean ± Standard Error of Mean (SEM). Unpaired student's t test was used to determine whether significant differences existed between a treatment and its vehicle control. A P value of ≤ 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Carfilzomib, not bortezomib, potentiates platelet thrombus formation *in vitro*

To examine whether Proteasome Inhibitors (PI) affect *in vitro* platelet thrombus formation under flow, whole blood collected from C57BL/6 mice pre-treated with Bortezomib (BOR) (40 mg/kg), Carfilzomib (CAR) (40 mg/kg), or sham control, were perfused across type I collagen-coated microcapillary perfusion chambers at high shear (1800s-1), under real-time fluorescence microscopy. Compared to sham control, platelet adhesion and thrombus growth were enhanced in CAR-treated mice from the initial time point (2 minutes) and sustained up to the latest time point (6 minutes) (Figures 1A and 1B). In contrast, BOR-treated mice showed no difference in platelet adhesion and thrombus growth compared to sham control at all-time points observed (Figures 1A and 1B).

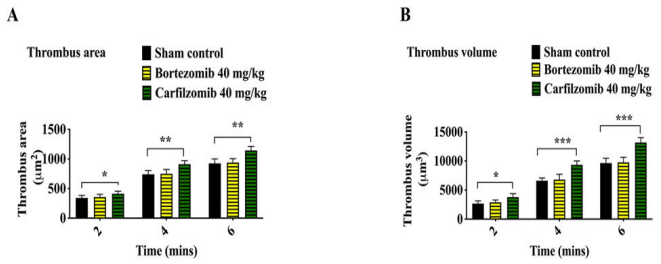

Figure 1: Effect of BOR or CAR on kinetics of mice thrombus *ex vivo* on immobilised type I collagen under arterial flow over time. Rhodamine G6-labelled whole blood from C57BL/6 mice pretreated with the sham control, 40 mg/kg bortezomib (BOR), or 40 mg/kg carfilzomib (CAR) for two hours, was perfused over immobilised type 1 collagen (500 μg/mL) under physiological arterial flow conditions at a shear rate of 1800 seconds-1. Three dimensional (3D) deconvolved reconstructions of thrombi formed were analysed for surface coverage (thrombus area)(μm^2), thrombus height (μm), and thrombus volume (μm^3). Column graphs show A. Thrombus area (μm^2) and B. Thrombus volume (μm^3) (calculated as (area × height), of blood from C57BL/6 mice pretreated with the sham control, BOR, or CAR, over a six-minute period. Data represent the mean ± SEM, of three independent experiments. (P>0.05 NS, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.005; n=4; unpaired student's t test).

The effect of PI on human thrombus formation was also investigated using whole blood from healthy donors incubated with vehicle, BOR (500 ng/ml) or CAR (1000 ng/ml) and perfused across type-1 collagen-coated microcapillary chambers at high shear (1800s-1). The chosen concentrations of BOR and CAR reflect steady-state *in vivo* levels observed in plasma (C max drug plasma concentration) [9,10]. Similar to the *ex-vivo* findings, platelet adhesion and thrombus growth were enhanced in CAR-treated blood compared to vehicle control, from 2 minutes and sustained up to the six-minute time point (Figures 2).

Furthermore, BOR-treated blood showed no difference in platelet adhesion and thrombus growth compared to vehicle control (Figures 2). Next, we examined the effect of combining Dexamethasone (DEX) with CAR or BOR to emulate clinically multiple myeloma treatment. DEX-treated blood showed no difference in initial platelet adhesion and thrombus growth compared to vehicle control (2 minute); however, thrombus growth was significantly increased at the later time points (4 and 6 minutes) (Figure 2). Significantly, CAR+DEX treated blood demonstrated enhanced initial platelet adhesion and thrombus formation at 2 minutes and propagated thrombus formation at the later time points (4 and 6 minutes), suggesting a possible synergistic affect between CAR and DEX. Conversely, at all time points, BOR+DEX-treated blood showed no difference in platelet adhesion and thrombus growth compared to DEX alone (Figure 2).

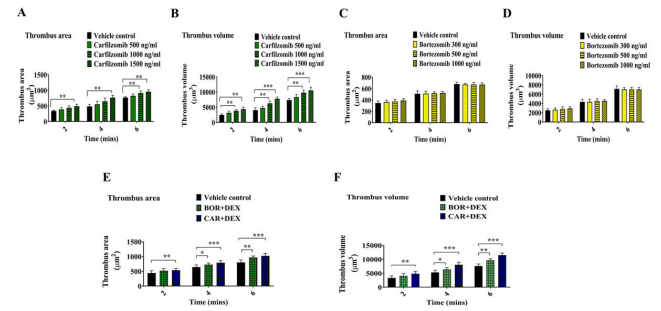

Figure 2: Effect of BOR, CAR, CAR+DEX and BOR+DEX on kinetics of human thrombus *in vitro* on immobilised type I collagen under arterial flow over time. Rhodamine G6-labelled whole blood from a healthy donor was incubated with the vehicle control or with (A-B) CAR (500, 1000, or 1500 ng/mL) or with (C-D) BOR (300, 500, or 1000 ng/mL) or with (E-F) CAR+DEX or BOR+DEX and perfused over immobilised type 1 collagen (500 μg/mL) under physiological arterial flow conditions at a shear rate of 1800 seconds-1 over a six-minute period. Three dimensional (3D) deconvolved reconstructions of thrombi were analysed for surface coverage (thrombus area)(μm^2) and thrombus volume (μm^3). Column graphs show Thrombus area and Thrombus volume (calculated as area x height). Data represent the mean ± SEM, of 4 independent experiments. (P>0.05, NS, **P ≤ 0.01, ***P ≤ 0.005; n=4; unpaired student's t test).

Carfilzomib, not bortezomib, potentiates thrombin-induced platelet activation

To investigate whether CAR potentiates thrombus formation *in vitro* by enhancing platelet activation, we performed thrombin-induced P-selectin expression and integrin $\alpha_{IIb}\beta_3$ activation studies. Platelets from wild-type C57BL/6 mice treated with sham

control, BOR (40 mg/kg), or CAR (40 mg/kg) were incubated with anti-mouse FITC-conjugated P-selectin or PE-conjugated JON/A (active integrin $\alpha_{IIb}\beta_3$) antibodies and stimulated with thrombin (0.25 U/ml). Platelets from CAR treated mice showed a significant increase in thrombin-induced P-selectin expression and JON/A binding compared to sham control (Figures 3A and 3B). Conversely, platelets from BOR treated mice showed no significant differences in both thrombin-induced P-selectin expression and JON/A binding compared to sham control (Figures 3A and 3B). Similar results were seen in thrombin-stimulated human platelets incubated with vehicle or increasing doses of CAR or BOR. Washed human platelets were incubated with FITC-conjugated P-selectin or PAC-1 (active integrin $\alpha_{IIb}\beta_3$) antibodies and stimulated with thrombin (0.25 U/ml). Platelets treated with CAR showed a significant dose-dependent increase in thrombin-induced P-selectin expression and PAC-1 binding compared to vehicle. No difference was seen between vehicle and BOR treated platelets in both thrombin-induced P-selectin expression and PAC-1 binding (Figures 3E and 3F). Platelets treated with DEX+CAR appeared to be further enhanced thrombin-induced P-selectin and PAC-1 binding but not BOR (Figures 3C-3F).

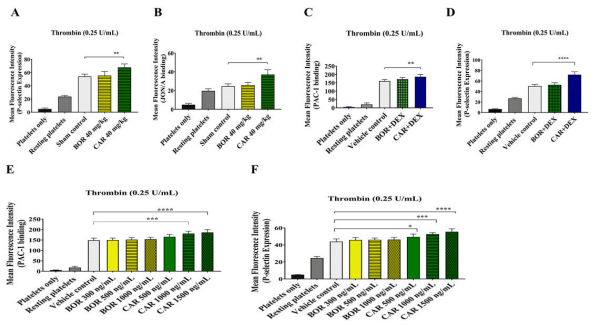

Figure 3: *Ex vivo* and *in vitro* effect of BOR, CAR, CAR+DEX and BOR+DEX on thrombin-mediated alpha granule release and active conformation of integrin $\alpha_{IIb}\beta_3$. (A-B) Washed platelets from C57BL/6 mice pretreated with sham control, 40 mg/kg (BOR), 40 mg/kg (CAR) for 2 hours were tested either resting or thrombin activated P-selectin expression or JON/A antibody binding. (C-F) Platelets (50 µL of 100 × 10^9/L) were washed before being incubated with the vehicle control or with (E-F) 300, 500, or 1000 ng/mL BOR or with 500, 1000, or 1500 ng/mL CAR or with (C-D) CAR+DEX, or with (C-D) BOR+DEX for 10 minutes at 37°C. Platelets were then activated with thrombin (0.25 IU/mL), or remained as resting platelets (not activated with thrombin) and stained with (A,D) FITC-P-selectin, or (C, E) FITC-PAC-1 monoclonal antibody.

Pre-treatment of platelets with Carfilzomib or Bortezomib does not change expression of platelet adhesion receptors

There is emerging evidence that proteasomes may regulate platelet function by influencing the platelet cytoskeleton and the signalling pathways of adhesion receptors [7]. To determine whether CAR-potentiated platelet thrombus formation and thrombin-induced platelet activation was due to an increase in platelet adhesion receptor expression, we assessed platelet surface expression of integrin $\alpha_{IIb}\beta_3$, GPIbα, CD9 and GPVI, after pre-treatment with CAR or BOR. Briefly, resting platelets from C57BL/6 mice were prepared from mice pretreated with the sham control, 40 mg/kg BOR, or 40 mg/kg CAR, and labelled with CD61 (integrin $\alpha_{IIb}\beta_3$), anti-mouse GPIbα, anti-mouse CD9, or anti-mouse GPVI-FITC conjugated antibodies. Flow cytometry was then used to measure glycoprotein expression using conjugated antibodies bound to platelet glycoprotein receptors. C57BL/6 mice treated with sham, CAR or BOR showed no difference in the level of glycoprotein surface expression (Figure 4A). This was also demonstrated in washed human platelets treated with vehicle, increasing doses of CAR or BOR and the combination treatments (Figure 4C). In addition, there was no difference in the level of glycoprotein surface expression in washed human platelets treated with BOR+DEX or CAR+DEX compared to vehicle control (Figures 4B and 4C).

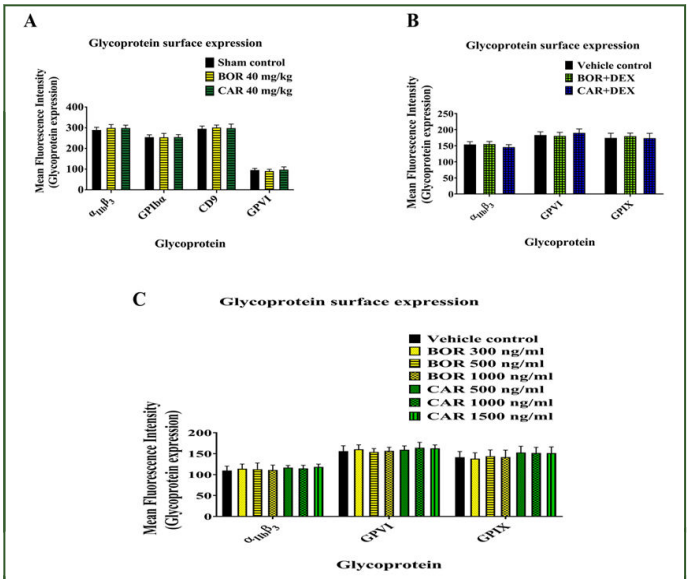

Figure 4: Effect of BOR, CAR, CAR+DEX and BOR+DEX platelet glycoprotein expression. (A) Washed platelets from C57BL/6 mice pretreated with sham control, 40 mg/kg (BOR), 40 mg/kg (CAR) for 2 hours. (B-C) Resting platelets (50 µL of 100 × 10^9/L) were incubated with the vehicle control or with (C) CAR (500, 1000, or 1500 ng/mL) or with (C) BOR (300, 500, or 1000 ng/mL) or with (B) CAR+DEX, or with (B) BOR+DEX for 45 minutes at room temperature. Resting platelets (50 µL 100 × 10^9/L) were labelled with anti-mouse CD61 (integrin $\alpha_{IIb}\beta_3$), GPIbα (CD42b), anti-mouse CD9 (p24), or anti-mouse GPVI FITC-conjugated antibodies. Platelets were then labelled with anti-human CD41a (integrin $\alpha_{IIb}\beta_3$), anti-human GPVI, or anti-human GPIX PE conjugated antibodies. Flow cytometry was used to determine P-selectin expression and measure PAC-1 binding to active integrin $\alpha_{IIb}\beta_3$ (**P 0.01, ***P 0.005; n=4; unpaired student's t-test).

The risk of VTE is high in NDMM, significantly a diagnosis of VTE within 6-12 months of initiating MM therapy is associated with increased mortality [11], therefore, understanding VTE associated with MM is a critical issue. Recent reports have shown that carfilzomib-based therapy in NDMM has a higher incidence of VTE compared to bortezomib-based therapy [4,5]. These observations further contribute to the hypothesis that

carfilzomib accentuates thrombosis whereas bortezomib has no significance or may have a protective contribution to thrombosis [12]. Using *ex vivo* and *in vitro* thrombosis models we demonstrate that carfilzomib potentiates thrombin-induced platelet activation which corresponded with increased thrombus formation under arterial flow. Moreover, our findings showed that bortezomib had no effect on platelet activation or thrombus formation (Table 1).

Table 1: Summary of the effect of single and common combined PIs-based agents.

Single agents	**Human platelets**		
	Thrombi size	**α granule exocytosis**	**integrin $\alpha_{IIb}\beta_3$**
BOR	No change	No change	No change
CAR	↑↑	↑↑	↑↑
Single agents	**Mice platelets**		
	Thrombi size	**α granule exocytosis**	**integrin $\alpha_{IIb}\beta_3$**
BOR	No change	No change	No change
CAR	↑↑	↑↑	↑↑
Combination	**Human platelets**		
	Thrombi size	**α granule exocytosis**	**integrin $\alpha_{IIb}\beta_3$**
BOR+DEX	No change	No change	No change
CAR+DEX	↑↑	↑↑	↑↑

A recent retrospective cohort study reported in NDMM patients that carfilzomib-based treatment had a greater than three-fold incidence of VTE compared to bortezomib-based therapy [13]. This observation is consistent with previous findings that bortezomib lowers VTE risk when used in combination with regimens that have thrombogenic potential whereas carfilzomib potentiates the thrombogenic potential [14]. Additionally, several studies have also suggested that carfilzomib increases cardiotoxic events such as cardiac failure, ischaemic heart disease and stroke. The proposed mechanism of carfilzomib-related cardiovascular disease is likely due to an effect on endothelial function causing impaired vascular relaxation and increased vasospasm. Moreover, the effects of carfilzomib on platelet function are yet to be determined.

Most of our understanding of the role of the proteasome in regulating platelet function stems from studies using bortezomib. Agonist-induced platelet activation demonstrates increased protein ubiquitination and enhanced proteasomal peptide hydrolysing activity, blocking this process using bortezomib results in an anti-thrombotic effect. This has been demonstrated in mice carotid artery thrombosis models, in which bortezomib significantly prolonged the time to occlusive carotid artery thrombosis [7]. Additionally, bortezomib treatment results in reduced platelet aggregation, spreading, and clot-retraction in response to thrombin, ADP or collagen stimulation [6,7]. A potential mechanism for the proteasome in regulating platelet function may involve cleavage of the cytoskeleton binding proteins Filamin A and Talin-1, PIs reduce cleavage of Filamin A and Talin-1 which translated to inhibition of ADP- and thrombin- mediated platelet aggregation [7]. Expanding on these observations, Grundler et al. showed that the proteasome may regulate collagen-induced platelet aggregation and cytoskeleton activation *via* activation of the NFkB pathway [6]. Conversely, other studies have reported that the antithrombotic effect of bortezomib is due to increased nitric oxide synthesis and thrombomodulin expression in endothelial cells *via* upregulation of the transcription factor, Kruppel-Like Factor 2 (KLF2) and not a direct antiplatelet effect. While there is some evidence suggesting that bortezomib may have an anti-thrombotic effect with supportive clinical observations in bortezomib-treated MM patients, the mechanism remains unclear.

The varying effects on thrombogenic potential between carfilzomib and bortezomib are likely due to off-target non-proteasomal activity [15]. Bortezomib inhibits several non-proteasomal serine proteases such as Cathepsin G (CatG). In contrast, carfilzomib displays minimal or no activity against serine proteases. *In vivo* studies showed that peripheral blood mononuclear cells from bortezomib-treated MM patients show a dose-dependent inhibition of CatG, whereas PBMC from carfilzomib-treated MM patients showed no detected inhibition of CatG [15]. Neutrophil derived CatG is a potent activator of the platelet PAR-4 receptor, leading to enhanced platelet activation and aggregation, this process plays a key role in platelet-neutrophil interaction and thrombus growth at sites of vessel wall injury. Another serine protease that is inhibited by bortezomib but not carfilzomib that may impact platelet function is HtrA2/Omi [15]. Htr/OMI is released from mitochondria following platelet activation with collagen and thrombin and participates in the activation of caspases 3/7 and 9 and subsequent platelet aggregation *via* exposure of phosphatidylserine on the membrane of the platelets [16]. Inhibitors of HtrA2/Omi have been reported to decrease the level of activated caspases 3/7 and caspase [16,17].

Resting platelets undergo a continuous cycle of ubiquitination and deubiquitination, and interruption of this balance towards deubiquitination promotes platelet reactivity. Moreover, increasing proteasome ubiquitination using deubiquitination inhibitors reduces integrin $\alpha_{IIb}\beta_3$ activation, and decreases platelet adhesion and thrombus formation to collagen under flow. It is likely that this effect is attributable to the inhibition of kinase phosphorylation and subsequent attenuation of platelet receptor signalling, since, previous reports have shown that deubiquitination inhibitors reduce the phosphorylation of key effector kinases in thrombin or collagen-simulated platelets. In the activated platelet, the UPS may function as a mechanism to downregulate platelet reactivity. The tyrosine kinase Syk is ubiquitylated in response to activation of GPVI by collagen and inhibition of deubiquitination results in reduced GPVI-dependent platelet aggregation [18]. In addition, platelets from

mice deficient in the ubiquitin ligase c-Cbl are hyperactivated in response to GPVI agonists correlating with loss of Syk ubiquitylation [19]. Furthermore, integrin $\alpha_{IIb}\beta_3$ activation and signalling results in numerous ubiquitination and activation of downstream proteins including C-type lectin-like receptor 2 (CLEC-2) and NF-kB proteins [8]. It is plausible that platelet activation leads to increased ubiquination of signalling proteins marking them for proteasomal degradation, however, in the presence of a deubiquitination inhibitor or a PI, this mechanism which aims to down-regulate platelet activation, is prevented [20]. Therefore, the hypothesis that carfilzomib prevents down-regulation of platelet activation by irreversibly inhibiting the platelet proteasome leading to inadvertent sustained platelet activation and promoting thrombosis in MM patients may be a key focus for future studies [21].

CONCLUSION

This study showed that carfilzomib significantly increases *in vitro* and *ex vivo* thrombus formation under arterial flow, along with platelet activation, however no effects were observed in bortezomib treated platelets. These findings suggest that carfilzomib-induced thrombosis risk in patients with multiple myeloma may be the result of enhanced platelet thrombotic function.

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CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

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