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The role of proteasome activity for activating and inhibitory signalling in human platelets

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ABSTRACT

Platelets express key proteins of the proteasome system, but its functional role in the regulation of platelet integrity, however, is not fully understood yet. Therefore, this study evaluated activating and inhibitory platelet signalling pathways using the potent and selective proteasome inhibitor bortezomib.

In washed platelets, the effect of bortezomib on viability and on aggregation was assessed. In addition, fibrinogen binding and CD62P expression were determined. The influence on activating and inhibitory signalling was detected by phosphorylation levels of essential messenger molecules.

Platelet viability was maintained after incubation with 0.01 μM to 1 μM bortezomib, but tampered with 100 μM bortezomib. Agonist-induced aggregation was only reduced under 100 μM bortezomib and with weak induction by 10 μM adenosine diphosphate. Similarly, phosphorylated kinase levels of the activating signalling pathways were not affected by 0.01 μM to 1 μM bortezomib. In contrast, proteasome inhibition resulted in the reduction of inhibitor-induced vasodilator-stimulated phosphoprotein phosphorylation, accompanied with the partial decrease of induced inhibition of fibrinogen binding and CD62P expression.

In conclusion, platelet activation and aggregation are not dependent on proteasome activity. Instead, inhibitory signalling is partially attenuated under proteasome inhibition. Supramaximal inhibitory concentrations of bortezomib (above 1 μM) lead to heterogeneous effects on activating or inhibitory systems, probably caused by decreasing platelet viability.

1. Introduction

Platelets are crucial for sufficient hemostasis and involved in inflammation and immune processes [1,2]. Although being anucleated cell fragments derived from megakaryocytes, platelets contain a protein degradation apparatus with a 20S proteasome subunit and a functional protein ubiquitination system [3–5]. However, the functional role and the interaction of the proteasome system with other platelet functions are still subject of research.

Recently, we could show that proteasome blockade mediated by bortezomib, frequently used in research [6], is able to specifically suppress the basal or agonist-induced proteasome activity and polyubiquitination in platelets in a dose-dependent manner [7]. A near-total inhibition of proteasome activity occurs at concentrations of approximately 10 nM and above. Using collagen or adenosine diphosphate (ADP) as agonists, induced platelet aggregation, measured in platelet-rich-plasma (PRP), remained unaffected by inhibitory bortezomib concentrations [7]. Since proteasome activity may mediate functional effects independently from the initiation of aggregation, it is essential to investigate further processes involved in platelet activation or inhibition and its associated signalling.

In this study, therefore, we analysed dose-dependent effects of bortezomib on phosphorylated kinase levels involved in platelet adhesion, secretion or calcium mobilization including the p38 mitogen-activated protein (p38 MAP) kinase, the extracellular signal-regulated kinases 1 and 2 (ERK1/2) or Akt/PKB [8–10].

Platelet integrity is also conditioned by the inhibitory regulation of activating signalling cascades mediated by cyclic adenosine monophosphate (cAMP)- and cyclic guanosine monophosphate (cGMP)-dependent phosphorylation of effector proteins. In this context, we

Abbreviations: DEA/NO, diethylamine diazenium diolate/nitric oxide; PGE1, prostaglandin E1; VASP, vasodilator-stimulated phosphoprotein

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determined the phosphorylation at Ser^{157} or Ser^{239} of the vasodilator-stimulated phosphoprotein (VASP), an actin-binding protein and the main substrate for both cAMP- and cGMP-dependent protein kinases [11,12]. For experimentation, the nitric oxide (NO) donor diethylamine diazenium diolate (DEA/NO) was used to stimulate the cGMP-dependent pathway [13,14] and prostaglandin E1 (PGE1) to enhance cAMP synthesis and cAMP-dependent phosphorylation via PKA [15].

2. Material and methods

2.1. Materials

Bortezomib was from Selleckchem (Munich, Germany). Thrombin Receptor Activator Peptide 6 (TRAP-6) was from BACHEM (Bubendorf, Switzerland). Collagen reagent was from Takeda Pharma Ges.m.b.H (Wien, Austria). ADP was from Haemochrom Diagnostika GmbH (Essen, Germany). Prostaglandin E1 (PGE1), apyrase and Tyrode's salt solution were from Merck KGgA (Darmstadt, Germany). DEA NONOate (DEA/NO) was from Enzo Life Sciences GmbH (Loerrach, Germany). Rabbit monoclonal antibodies against phospho-ERK1/2 (catalog-number 4370S), phospho-Akt/PKB (4060S), pan-actin (8456S) and rabbit polyclonal antibody against phospho-p38 (9211L) were from New England Biolabs GmbH (Frankfurt am Main, Germany). Mouse monoclonal antibodies directed to phospho-VASP Ser^{157} (0085–100) or phospho-VASP Ser^{239} (0047–100) and FITC-conjugated mouse monoclonal antibody directed to phospho-VASP Ser^{239} (0047-100F) were from Nano Tools GmbH (Teningen, Germany). FITC-conjugated mouse monoclonal anti-CD62P antibody (SM1150F) was from OriGene Technologies GmbH (Herford, Germany), FITC-conjugated mouse monoclonal antibody against human bound fibrinogen was from STAGO (Duesseldorf, Germany). Horseradish peroxidase- and starbright 700-conjugated goat anti-mouse (1706516; 12004158) and anti-rabbit antibodies (1706515; 12004161) were from Bio-Rad Laboratories Inc. (Muenchen, Germany).

2.2. Blood collection

Venous whole blood samples (WB) were obtained from informed healthy voluntary donors without any medication intake. Peripheral blood was collected in polypropylene tubes containing 3.2% citrate buffer (106 mM trisodium citrate, Sarstedt, Nuembrecht, Germany).

Our studies with human platelets and the consent procedure were approved by our local ethics committee of the University of Wuerzburg (approval number 101/15). The participants provided their written informed consent to participate in this study. The study was performed according to our institutional guidelines and to the Declaration of Helsinki.

2.3. Preparation and stimulation of washed platelets

Washed platelets were prepared as described [11]. 3 mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) was added to WB to prevent platelet activation. PRP was obtained by centrifugation of WB at 330 × *g* for 5 min (min). Subsequently, samples of PRP were centrifuged at 430 × *g* for 10 min. Then, pelleted platelets were washed once in CGS buffer (120 mM sodium chloride, 30 mM D-glucose, 12.9 mM trisodium citrate, pH 6.5) and resuspended in Tyrode's salt solution ($CaCl_2$ x $2H_2O$ 2.65 g/L, $MgCl_2$ x $6H_2O$ 0.214 g/L, KCl 0.2 g/L, $NaHCO_3$ 1 g/L, NaCl 8.0 g/L, NaH_2PO_4 0.05 g/L, D-Glucose 1 g/L, pH 7.2–7.6) to the final appropriate concentration. For experiments with ADP, the washing buffers, except for the final Tyrode's salt solution, were supplemented with 0.3 U/mL apyrase to preserve ADP responsiveness. In addition, bortezomib was added to whole blood and to all washing buffers to avoid additional incubation times after preparation of washed platelets.

The platelet suspension was aliquoted in Eppendorf tubes (100 µL per tube) and incubated with buffer or indicated bortezomib concentrations at room temperature (RT).

After that, 1 mM $CaCl_2$ was added to washed platelets and stimulated with buffer, 5 µg/mL collagen, 10 µM TRAP-6 or 10 µM ADP for 2 min according to experiments.

2.4. Cell viability

Cell viability was measured fluorometrically by means of the VisionBlue Quick Cell Viability Kit from BioVision, Inc. (Milpitas, USA). Viable, metabolically active cells convert the cell-permeable redox dye resazurin of the VisionBlue Reagent to the pink fluorescent product resorufin. The degree of fluorescence represents an indicator of the activity of cell metabolism [16]. Aliquots with 100 µL of washed platelets suspension (5×10^8 platelets/mL) were pre-incubated with buffer, 10 µM Ca^{2+} ionophore A23187 or indicated bortezomib concentrations, followed by addition of 10 µL of redox dye resazurin for 60 min. After that fluorescence values of resorufin were measured with a 560 nm excitation/ 590 nm emission filter according to the manufacturer's instructions.

2.5. Platelet aggregation

Platelet aggregation was measured using an APACT 4004 aggregometer (LabiTec, Ahrensburg, Germany). 1 mM $CaCl_2$ was added in each cuvette with 200 µL of washed platelets (3×10^8/mL) followed by an immediate stimulation with 5 µg/mL collagen or 10 µM TRAP-6. For stimulation with 10 µM ADP, the platelet suspension was supplemented with 1 mM $CaCl_2$ and 100 µg/mL fibrinogen. Aggregation was measured for 5 min under continuous stirring at 1000 rpm and 37 °C. The maximal aggregation value was used for analysis.

2.6. Western blot analysis

Washed platelets were lysed and proteins were separated by SDS-PAGE and then transferred onto nitrocellulose membranes. The membranes were incubated with appropriate primary antibodies overnight at 4 °C. For visualisation of the signal, goat anti-rabbit or anti-mouse IgGs conjugated with starbright 700 or horseradish peroxidase were used as secondary antibodies, and if necessary, followed by detection with the ECL detection kit (GE Healthcare, Piscataway, NJ, USA). Blots were analysed densitometrically using Image Lab 6.0 Software from Bio-Rad Laboratories Inc. (Muenchen, Germany) and NIH Image J software for uncalibrated optical density.

2.7. Flow cytometric analysis of platelet VASP phosphorylation

PRP was pre-incubated with buffer, 0.01 µM, 1 µM or 100 µM bortezomib for 45 min at RT followed by 15 min incubation at 37 °C. After that, 30 µL of pre-incubated PRP was stimulated with buffer, 1 µM PGE1 or 1 µM freshly prepared DEA/NO for 5 min at 37 °C, fixed with 2.5% formaldehyde for 10 min at RT and pelleted at 21,380 × g for 1 min. The platelet pellet was re-suspended in 50 µL of 0.2% Triton-X 100 in PBS/BSA/Glc (Dulbecco's PBS (Ca^{2+}, Mg^{2+} free), 0.5% BSA, 5.5 mM D-Glucose) and stained with 10 µg/mL of FITC-conjugated anti-phospho-VASP Ser^{239} antibody for 30 min at RT. The antibody pre-incubated with an appropriate phospho-peptide was used as isotype control. Then, samples were diluted with 500 µL of PBS/BSA/Glc and analysed with the FACS Calibur flow cytometer from Becton Dickinson (Franklin Lakes, NJ, USA) using CELLQuest software, version 6.0. The platelet population was identified by its forward and side scatter distribution (Supplementary Fig. S1) and 20,000 events were analysed for mean fluorescence.

2.8. Flow cytometric analysis of platelet fibrinogen binding and CD62P expression

PRP was pre-incubated with buffer, 0.01 μM, 1 μM or 100 μM bortezomib for 45 min at RT followed by 15 min incubation at 37 °C in the presence of 150 μg/mL FITC-conjugated anti-fibrinogen antibody or 10 μg/mL FITC-conjugated anti-CD62P antibody. After that, 30 μL of pre-incubated PRP were stimulated with buffer, PGE1 (1 μM for fibrinogen binding, 0.5 μM for CD62P expression) or 1 μM freshly prepared DEA/NO for 5 min at 37 °C, followed by 2 min stimulation with 10 μM TRAP-6. The reaction was stopped by addition of 1% formaldehyde. The samples were fixed for 10 min at RT, diluted with 300 μL of PBS/BSA/Glc and analysed by flow cytometry as described above.

2.9. Statistical analysis

The n-values refer to the number of experiments, each made with different blood donors. Descriptive data were calculated with the MedCalc statistic program (MedCalc Software bvba, Mariakerke, Belgium) and GraphPad PRISM 7 (GraphPad Software, San Diego, CA, USA). Data distribution analysis was performed with Shapiro-Wilk test indicating normal distribution of data. Differences of variances between groups were analysed by one-way analysis of variance (ANOVA) followed by post-hoc Tukey-Kramer-Test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Platelet viability is affected only by high bortezomib concentrations

The suspension of freshly prepared washed platelets evoked the generation of 115.0 ± 4.7 R.F.U. (Fig. 1). Incubation of washed platelets with buffer for 60 min did not change the metabolic activity with 113.9 ± 3.6 R.F.U. However, pre-incubation of platelets with 10 μM calcium ionophore A23187, a well-characterized agent decreasing the mitochondrial potential and inducing cell death, resulted in a significant decrease of resorufin fluorescence by approximately 74%. 0.01 μM and 1 μM bortezomib did not have influence on fluorescence levels, whereas 100 μM led to a reduction of approximately 18.5% (Fig. 1).

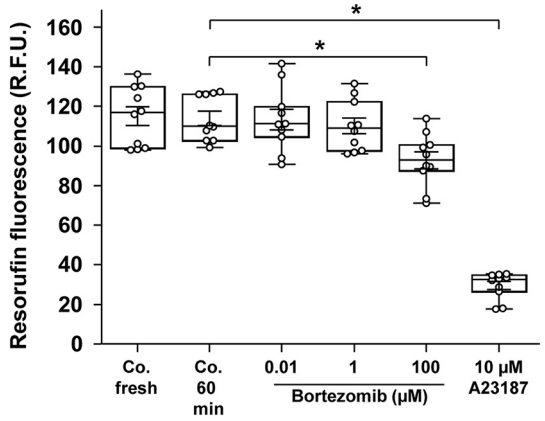

Fig. 1. Platelet viability is decreased by high bortezomib concentrations. Washed human platelets (5×10^8 platelets/mL) were pre-incubated for 60 min with buffer, indicated bortezomib concentrations or 10 μM Ca^{2+} ionophore A23187, followed by resazurin. Fluorescence of resorufin converted by viable platelets was measured. Data are presented as mean ± SEM in arbitrary resorufin fluorescence units (R.F.U); n = 10; *: $p < 0.05$, compared to unstimulated platelets (Co., control 60 min).

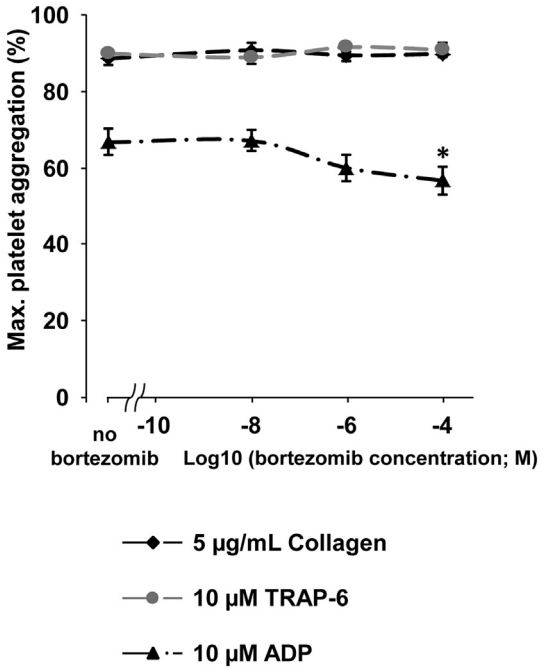

Fig. 2. Agonist-induced platelet aggregation under proteasome inhibition. Washed human platelets (3×10^8 platelets/mL) were pre-incubated with buffer or indicated bortezomib concentrations for 60 min. After stimulation with 5 μg/mL collagen, 10 μM TRAP-6 or 10 μM ADP, light transmission aggregometry was determined. Data are presented as mean ± SEM of maximal aggregation; n = 5.

3.2. High bortezomib concentrations decrease ADP induced aggregation

Platelet aggregation was measured in washed platelets pre-incubated with buffer or bortezomib concentrations of 0.01 μM–100 μM (Fig. 2). 5 μg/mL collagen and 10 μM TRAP-6 induced maximal aggregation values of 89.9% ± 1.7% and 91.2% ± 1.2%. Pre-incubation of washed platelets with all tested bortezomib concentrations did not show any effect on aggregation results (Fig. 2). In contrast, maximal ADP induced platelet aggregation was lower, reaching only 68.1% ± 3.4%. Incubation with bortezomib attenuated ADP induced aggregation, significantly for 100 μM with 58.1% ± 3.6% (Fig. 2).

3.3. Bortezomib does not have influence on agonist-induced phosphorylation of p38 MAP kinase

The activation of p38 MAP kinase is mediated by dual phosphorylation of Thr^{180} and Tyr^{182} [17]. Western Blot analysis of platelets stimulated with collagen (Supplementary Fig. S2), TRAP-6 and ADP revealed a significant increase of p38 phosphorylation from 0.49 ± 0.02 AU to 0.63 ± 0.01 AU, 0.62 ± 0.02 AU and 0.71 ± 0.08 AU, respectively (Fig. 3A–C). The proteasome inhibitor bortezomib did not change the basal level of p38 phosphorylation (Table 1). Pre-incubation of platelets with increasing concentrations of bortezomib from 0.01 μM to 100 μM followed by stimulation with collagen, TRAP-6 and ADP did not affect the level of agonist-induced p38 phosphorylation (Fig. 3A–C).

3.4. Bortezomib does not have influence on agonist-induced phosphorylation of ERK1/2 kinases

The degree of ERK1/2 activation can be assessed by the measurement of phosphorylation at Thr^{202} and Tyr^{204} [18]. Stimulation with collagen (Supplementary Fig. S2), TRAP-6 and ADP significantly increased the phosphorylation level at both sites by more than three-fold

from 0.19 ± 0.02 AU to 0.66 ± 0.02 AU, 0.63 ± 0.02 AU and 0.84 ± 0.09 AU (Fig. 4A–C). Bortezomib did not significantly affect the basal (Table 1) or the agonist-induced phosphorylation level of ERK1/2 kinases (Fig. 4A–C).

3.5. High bortezomib concentration inhibits phosphorylation of Akt/PKB

The activation and function of Akt/PKB is mediated via phosphorylation at Ser^{473} [19,20]. Stimulation of platelets with collagen (Supplementary Fig. S2) increased phosphorylation by more than two-fold from 0.28 ± 0.03 AU to 0.69 ± 0.01 AU, with TRAP-6 from 0.28 ± 0.03 AU to 0.66 ± 0.01 AU and with ADP from 0.28 ± 0.02 AU to 0.44 ± 0.02 AU (Fig. 5A–C). Incubation of quiescent platelets with bortezomib did not change the basal level of Akt/PKB phosphorylation (Table 1). The lower bortezomib concentrations of 0.01 μM and 1 μM did not change agonist-induced Akt/PKB phosphorylation, whereas 100 μM bortezomib decreased stimulated phosphorylation significantly to 0.62 ± 0.01 AU for collagen (Fig. 5A) and to 0.62 ± 0.01 AU for TRAP-6 (Fig. 5B). ADP-stimulated phosphorylation was only slightly attenuated with 0.40 ± 0.02 AU (Fig. 5C).

3.6. Proteasome inhibition by bortezomib decreases PGE1- and DEA/NO-induced VASP phosphorylation in platelets

PGE1- and DEA/NO-induced platelet VASP phosphorylation was measured by flow cytometry in PRP after pre-incubation with bortezomib or with buffer. 0.01 μM bortezomib led to the reduction of both, PGE1- and DEA/NO- induced VASP phosphorylation, from 304.3 ± 48.6 to 273.1 ± 40.4 MFI and from 266.3 ± 25.2 to 168.9 ± 47.4 MFI, respectively (Fig. 6A and B). 1 μM bortezomib resulted in a stronger decrease with MFI values of 251.0 ± 30.6 for PGE1-incubation and 133.8 ± 44.8 for DEA/NO-induced values. Instead, 100 μM bortezomib provoked instable, heterogeneous effects on VASP phosphorylation.

For Western blot analysis, lower concentrations of 2 nM PGE1 and 2 nM DEA/NO were used, since the inhibitors induce VASP phosphorylation much more effectively in washed platelets compared with PRP in flow cytometric analysis (Fig. 7). 0.01 μM bortezomib decreased DEA/NO-, and PGE1- induced VASP phosphorylation slightly at both Ser^{239} (Fig. 7A) and Ser^{157} (Fig. 7B) by approximately 5% and 10%, respectively. 1 μM bortezomib led to a reduction of DEA/NO-induced VASP phosphorylation at Ser^{239} by 7% and at Ser^{157} by 13%.

An inhibitory effect of 1 μM bortezomib was still measurable in PGE1-stimulated platelets, however, less pronounced than in the presence of 0.01 μM bortezomib. Similar to flow cytometry, 100 μM bortezomib did not inhibit DEA/NO- and PGE1-induced VASP phosphorylation at Ser^{239} and Ser^{157}.

3.7. The inhibitory effect of PGE1 and DEA/NO on agonist-induced fibrinogen binding and CD62P expression is attenuated under proteasome inhibition

Fibrinogen binding and CD62P expression were investigated in PRP, pre-incubated with different bortezomib concentrations and PGE1 or DEA/NO, followed by 2 min stimulation with 10 μM TRAP-6. 0.01 μM and 1 μM bortezomib were able to diminish PGE1- and DEA/NO-mediated inhibition of agonist-induced fibrinogen binding by approximately 10% (Fig. 8A and C), and similarly, inhibition of agonist-induced CD62P expression (Fig. 8B and D). Incubation with 100 μM bortezomib led to heterogeneous results.

4. Discussion

The significance of the proteasome system for protein degradation in eukaryotic cells and the effects of proteasome inhibition on survival and viability of tumor cells - in vitro and in vivo - has been intensively

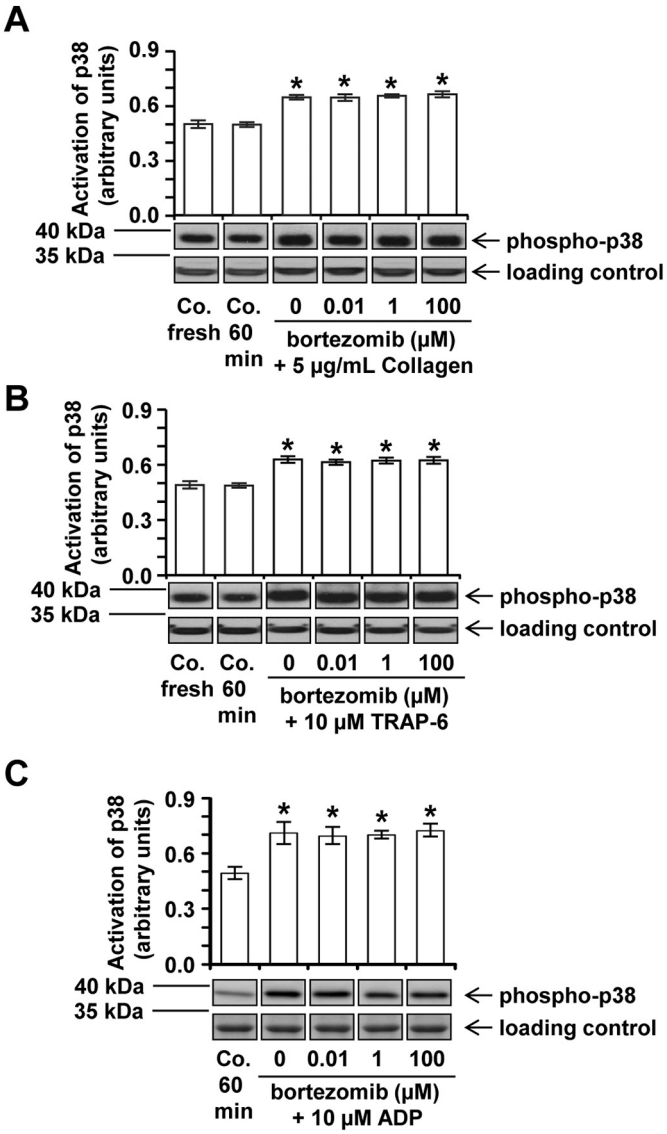

Fig. 3. Proteasome inhibition does not affect p38 MAP kinase phosphorylation. Washed human platelets (3×10^8 platelets/mL) were pre-incubated with buffer or indicated bortezomib concentrations for 60 min and stimulated as described with 5 μg/mL collagen (A), 10 μM TRAP-6 (B) or 10 μM ADP (C). The histograms show the level of phosphorylated p38 MAP kinase. Results are presented as mean ± SEM; n = 8; *: $p < 0.05$, compared to unstimulated platelets (Co., control 60 min).

investigated in several studies [21]. Instead, the role of the proteasome system in platelets has not entirely been elucidated and is still subject of research. Recently, we could show that 1 nM bortezomib completely prevented collagen-induced activation of the platelet proteasome system, whereas 1–10 nM bortezomib exerted a 4-fold to 10-fold suppression of the basal proteasome activity in a dose-dependent manner. Concomitantly, basal and collagen-induced polyubiquitination was augmented with increasing bortezomib concentrations [7].

For myeloma cells, it was demonstrated that bortezomib exerts a cytotoxic effect, with concentrations of 15 nM–30 nM inducing a decline of myeloma cell viability by > 80% measured with the resazurin indicator [22]. In human platelets, the viability is maintained after incubation with up to 1 μM bortezomib indicating a different susceptibility of platelets for bortezomib-related toxic effects compared to eukaryotic cells. Only the high 100 μM bortezomib concentration partially affected platelet resazurin-related metabolism. In consequence, and essential for experimental research, specific effects of bortezomib

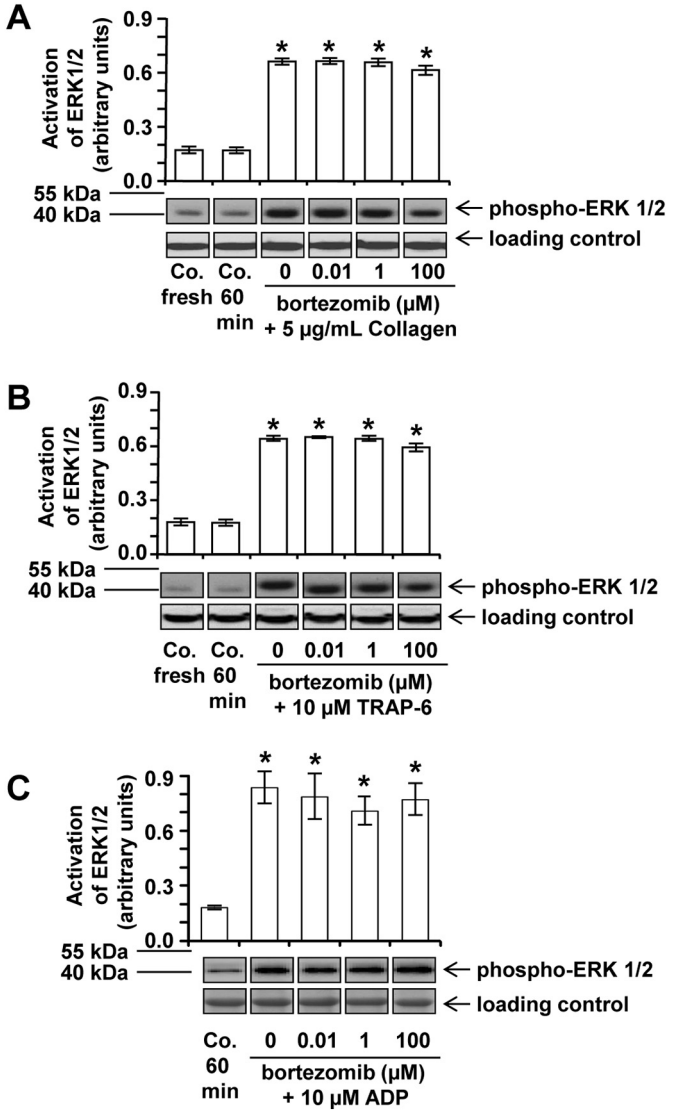

Fig. 4. Proteasome inhibition does not affect ERK1/2 phosphorylation.
Washed human platelets (3×10^8 platelets/mL) were pre-incubated with buffer or indicated bortezomib concentrations for 60 min and stimulated as described with 5 μg/mL collagen (A), 10 μM TRAP-6 (B) or 10 μM ADP (C). The histograms show the level of phosphorylated ERK1/2. Results are presented as mean ± SEM; n = 8; *: $p < 0.05$, compared to unstimulated platelets (Co., control 60 min).

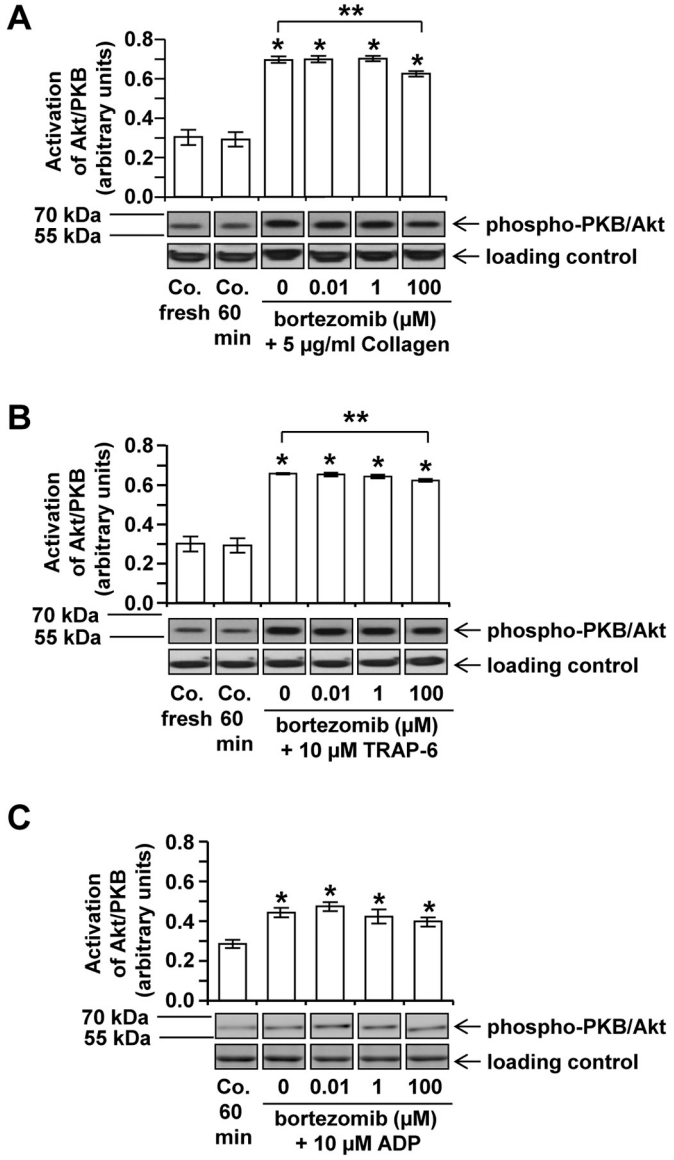

Fig. 5. Akt/PKB phosphorylation is decreased only by high bortezomib concentrations.
Washed human platelets (3×10^8 platelets/mL) were pre-incubated with buffer or indicated bortezomib concentrations for 60 min and stimulated as described with 5 μg/mL collagen (A), 10 μM TRAP-6 (B) or 10 μM ADP (C). The histograms show the level of phosphorylated Akt/PKB. Results are presented as mean ± SEM; n = 8; *: $p < 0.05$, compared to unstimulated platelets (Co., control 60 min). **: $p < 0.05$ (comparison as indicated).

are to be expected in the nM-range rather than in the μM-range.

Activating stimulation of platelets initiates shape change and, consecutively, conformational changes of fibrinogen receptors on the platelet surface, finally resulting in irreversible platelet aggregation [23]. Obviously, this process is independent from proteasome activity, since collagen- and TRAP-6-induced aggregation in bortezomib-treated washed platelets were not tampered, which is in accordance to previous aggregation studies using PRP [7]. The reported and confirmed partial reduction of aggregation induced with 10 μM ADP, as weak agonist, after incubation of platelets with bortezomib in a high concentration of 100 μM has probably been caused by toxic effects [7].

Although not essentially tampering aggregation, proteasome inhibition may interfere with activating signalling pathways. The stimulation of platelets with collagen, TRAP-6 and ADP resulted in a significant increase of phosphorylation of p38 MAP kinase at Thr^{180} and Tyr^{182}, of ERK1/2 kinase at Thr^{202} and Tyr^{204}, and of Akt/PKB at Ser^{473}, confirming the involvement of these kinases in platelet

Table 1
Influence of bortezomib on basal protein kinase phosphorylation.

Washed human platelets, either freshly prepared (control fresh) or incubated with buffer (control 60 min) or with bortezomib concentrations for 60 min as indicated, were lysed with Laemmli's SDS-gel sample buffer and analysed by Western blot using antibodies against phospho-Thr^{180} and phospho-Tyr^{182} of p38, against phospho-Thr^{202} and phospho-Tyr^{204} of ERK1/2 and against phospho-Ser^{473} of Akt/PKB. Phosphorylation intensity was normalised to actin controls. Results are presented as mean ± SEM; n = 6.

	p38	ERK1/2	Akt/PKB
Control fresh	0.49 ± 0.01	0.18 ± 0.02	0.30 ± 0.01
Control 60 min	0.49 ± 0.02	0.18 ± 0.01	0.30 ± 0.01
Bortezomib 0.01 μM	0.48 ± 0.02	0.19 ± 0.01	0.31 ± 0.01
Bortezomib 1 μM	0.48 ± 0.01	0.18 ± 0.02	0.32 ± 0.02
Bortezomib 100 μM	0.49 ± 0.01	0.18 ± 0.01	0.31 ± 0.01

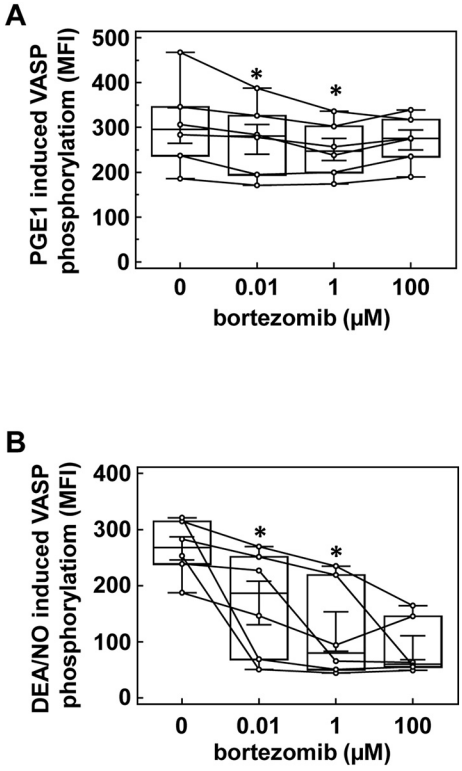

Fig. 6. Bortezomib attenuates PGE1- and DEA/NO-induced VASP phosphorylation in PRP.
PRP was pre-incubated with buffer or indicated bortezomib concentrations for 60 min followed by stimulation with 1 μM PGE1 (A) or 1 μM DEA/NO (B). VASP phosphorylation in platelets was measured by flow cytometry. Data of each experiment are presented in a box-and-whisker plot; n = 6; *: $p < 0.05$, compared to "0 μM bortezomib". Representative histograms of flow cytometry are shown above the box-and-whisker plots.

activation with different agonists [8,9,24].

In contrast, bortezomib concentrations totally blocking the proteasome activity in platelets were not able to change the basal level or the collagen- and TRAP-6 induced level of p38 MAP kinase phosphorylation, indicating that the activation of p38 is not dependent on the degree of proteasome activity in platelets. Interestingly, in some nucleated cell types, bortezomib triggered p38 MAP kinase phosphorylation, e.g. in acute myeloid leukemia cell lines or patient-derived acute myeloic leukemia cells [25], in esophageal squamous cell carcinoma cells [26] and in the human erythroleukemic cell line K562 [27].

Similar to p38 MAP kinase, bortezomib did not affect basal or agonist-induced ERK1/2 phosphorylation in platelets. Variable effects of proteasome inhibition on phospho-ERK1/2 are reported in different cell types. Bortezomib did not influence the basal level in the human erythroleukemic cell line K562 [27], whereas it enhanced phosphorylation in acute myeloid leukemia cells [25] and in cultured human Tenon's fibroblasts [28].

Only 100 μM bortezomib led to slightly lower levels of agonist-induced Akt/PKB phosphorylation in platelets. In hepatocellular carcinoma cells, bortezomib decreased basal Akt/PKB phosphorylation in a dose-dependent manner [29], and in human Tenon's capsule fibroblasts (HTF), it reduced the transforming growth factor β-induced phosphorylation of Akt/PKB [28]. Akt/PKB activity has been directly linked to processes essential for cellular metabolism and viability [30,31]. Increasing annexin V binding and the decrease of mitochondrial transmembrane potentials after incubation of platelets with 25 μM bortezomib have also implicated the induction of apoptosis in other studies [32].

The interaction of the proteasome system with inhibitory platelet pathways has been addressed in this study for the first time. Physiological inhibitors like NO and prostacyclin or pharmacological vasodilators like NO donors and PGE1 mediate their effects through cAMP- or cGMP-dependent phosphorylation of intracellular target proteins via PKA and PKG [33]. VASP is a major common substrate for both PKA and PKG in platelets and its phosphorylation directly correlates with platelet inhibition [11,33].

0.01 μM and 1 μM Bortezomib decreased both cAMP- and cGMP-mediated VASP phosphorylation in platelets. The lower levels of VASP phosphorylation manifested in an attenuated inhibition of agonist-induced fibrinogen binding and CDP62P expression. These anti-inhibitory effects were consistently detectable and may have been stronger using lower concentrations of PGE1 and DEA/NO. In contrast, 100 μM bortezomib induced heterogeneous effects on VASP phosphorylation and on associated fibrinogen binding or CD62P expression, pointing to functional toxicity of bortezomib for platelets in this concentration. In living cells, the 26S proteasome is not only important for protein degradation, but it represents a multiprotein-complex binding different regulatory proteins like proteinphosphatase 2, MLCK, F-actin or PKA. The inhibition of that complex may lead to the enhanced anchorage of PKA and PKG, becoming unavailable for signalling processes, e.g. as described for the NFkB-IkB-PKAc complex in platelets [34].

As possible limitations of the study, it should be regarded that washing procedures bear the risk of functional deterioration or preactivation of platelets. However, it was important to rule out unspecific effects of contaminating plasma on Western Blot analysis of signalling molecules. Light transmission in controls reached values as expected for normal platelet function [35] indicating reliable results for experiments with washed platelets. Since proteasome-mediated effects are presumably based on slow and time-delayed mechanisms, an incubation time of 60 min was chosen, defined as optimal according to our previous study [7]. The incubation period did not affect basal levels of kinase phosphorylation and the stimulation of phosphorylation after 60 min was comparable to freshly washed platelets pointing to intact platelet integrity. Extended incubation periods with bortezomib beyond 60 min may enhance the observed effects on inhibitory signalling, however, it would be required to perform these experiments with a different approach, e.g. by using more stable platelet-rich-plasma with platelet washing after bortezomib incubation. The bortezomib-mediated attenuation of VASP phosphorylation was only slight, but it was consistently detectable and accompanied by reduced inhibition of platelet activation. These effects may play a significant role for threshold activation of platelets, representing an issue that should be addressed in future studies with the use of agonists in lower concentrations. Since this study only used bortezomib for proteasome inhibition, it would also be of interest to prove the generalisability of the results by experimentation with other types of proteasome inhibitors, e.g. with carfilzomib, to rule out bortezomib-related effects.

5. Conclusions

In summary, it can be concluded that proteasome inhibition induced by bortezomib does not affect activating signalling pathways or the initiation of aggregation in platelets. However, proteasome inhibition partially attenuates cAMP- and cGMP-mediated-inhibition of agonist-stimulated platelets. The use of bortezomib in high concentrations (above 1 μM) may lead to impaired platelet responsiveness due to decreasing cell viability.

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The funding source had no involvement in the collection, analysis and interpretation of data, in the writing of the report, and in the decision to submit the article for publication.

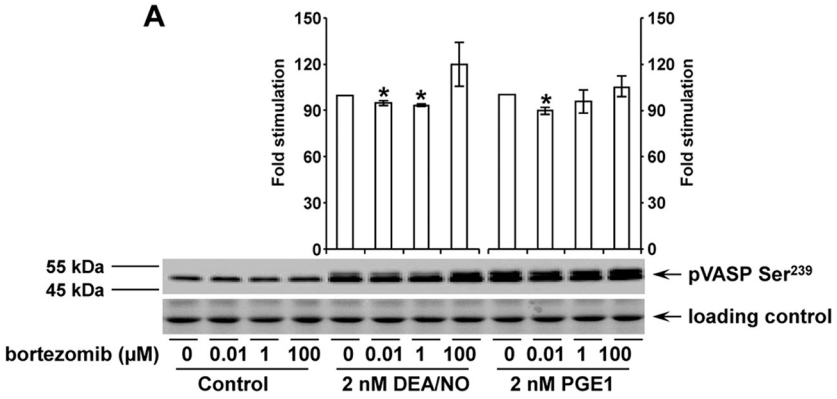
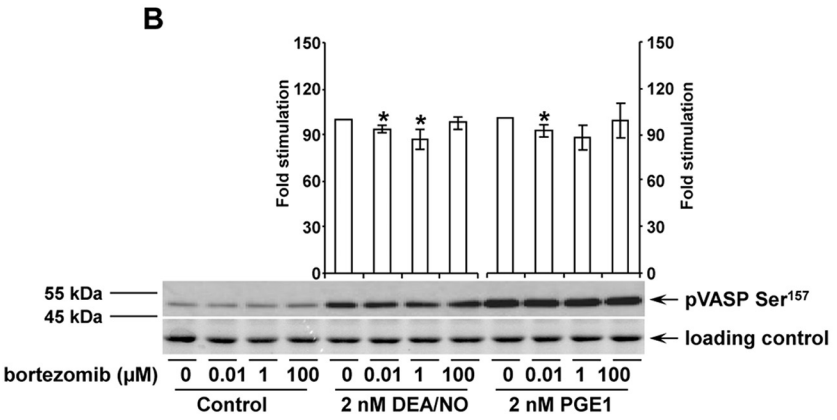


Fig. 7. Bortezomib attenuates PGE1- and DEA/NO-induced VASP phosphorylation in washed platelets.
Washed human platelets were pre-incubated with buffer or with indicated bortezomib concentrations for 60 min, followed by stimulation with 2 nM DEA/NO or 2 nM PGE1. VASP phosphorylation at Ser^{239} (A) and at Ser^{157} (B) was detected by Western blot analysis. The histograms show the changes of VASP phosphorylation compared with the corresponding control (PGE1 and DEA/NO-stimulated platelets without bortezomib). Results are presented as mean ± SEM; n = 4. $p < 0.05$, compared to "0 μM bortezomib".



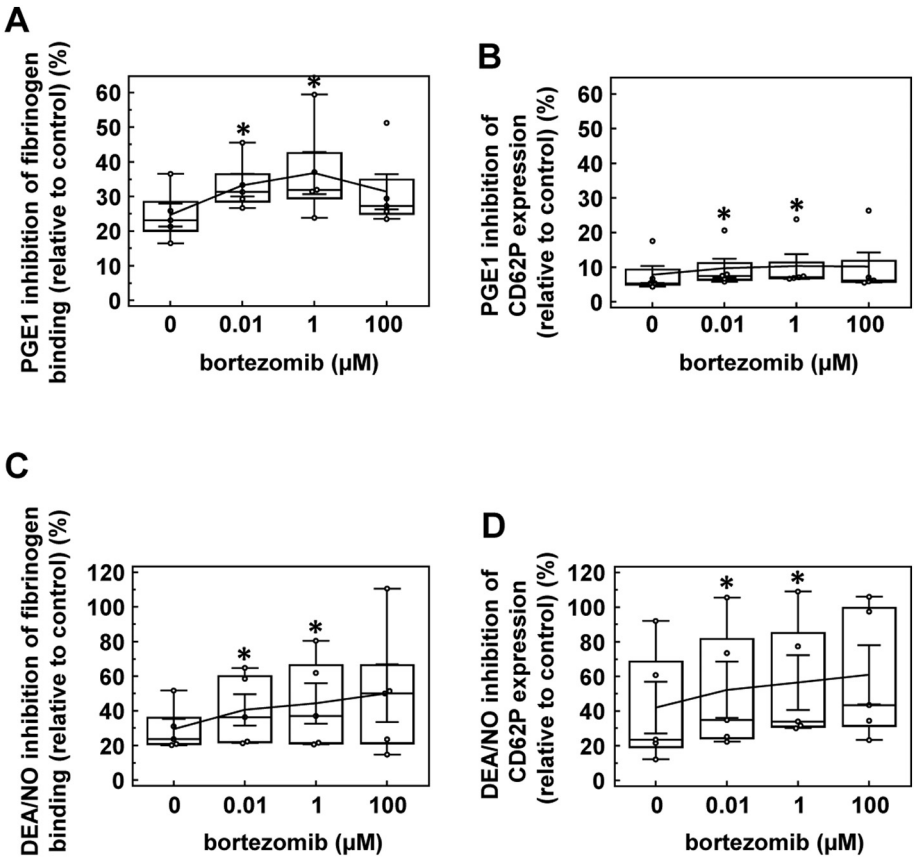


Fig. 8. Bortezomib attenuates inhibition of fibrinogen binding and CD62P expression in agonist-stimulated platelets.
The PGE1-mediated (A and B) and DEA/NO-mediated (C and D) relative inhibition of fibrinogen binding and of CD62P expression in bortezomib-treated platelets are shown. PRP was pre-incubated with buffer or indicated bortezomib concentrations for 60 min. 1 μM PGE1 (for fibrinogen binding), 0.5 μM PGE1 (for CD62P expression) or 1 μM DEA/NO were added, followed by 2 min stimulation with 10 μM TRAP-6. Fibrinogen binding and CD62P expression were measured by flow cytometry. The extent of platelet inhibition is given as percent of maximal fibrinogen binding (or CD62P expression) in platelets pre-incubated with buffer (without PGE1 or DEA/NO). Data are presented in a box-and-whisker plot; n = 5; *: $p < 0.05$, compared to "0 μM bortezomib".

Declaration of Competing Interest

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The authors have no further conflicts of interest to declare.

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