Bortezomib induces thrombocytopenia by the inhibition of proplatelet formation of megakaryocytes

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Abstract

Bortezomib is a potent proteasome inhibitor that has been extensively used to treat multiple myeloma. One of the most common grade 3 adverse events is cyclic thrombocytopenia. In this study, we studied the mechanism by which bortezomib induces thrombocytopenia in a mouse model. After the intravenous administration of bortezomib (2.5 mg/kg) via tail vein, platelet counts significantly decreased on days 2–4 and recovered to the normal range on day 6. Bortezomib (2.5 mg/kg) injected into mice in vivo did not affect colony-forming unit-megakaryocytes (CFU-Mk) or megakaryocytes in the bone marrow. However, proplatelet formation (PPF) significantly decreased on days 2 and 4, after bortezomib administration to mice. Meanwhile, CFU-Mk formation and the ploidy distribution of cultured megakaryocytes in vitro were not affected by bortezomib used at concentrations of ≤1 ng/mL. The PPF of megakaryocytes in vitro significantly decreased with 0.1, 1, 10, and 100 ng/mL bortezomib. Considering the bortezomib concentration in clinical studies, these data strongly suggest that decreased PPF activity induces thrombocytopenia. To elucidate the mechanism behind decreased PPF, Western blot was performed. Activated Rho expression increased after the incubation of murine platelets with bortezomib. Decreased PPF activity was eliminated by the addition of Y27632, a Rho kinase inhibitor, in vitro. Given that the Rho/Rho kinase pathway is a negative regulator of PPF, bortezomib increases activated Rho, inducing decreased PPF, which results in decreased platelet count.

Key words bortezomib; thrombocytopenia; megakaryocyte; proplatelet formation; Rho/Rho kinase pathway

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Bortezomib is a potent proteasome inhibitor that has been extensively used for refractory/relapsed or newly diagnosed multiple myeloma. It induces the apoptosis of myeloma cells by inhibiting nuclear factor (NF)-kB activity through the inhibition of proteasomes (1). Several clinical studies have demonstrated that the overall response rates upon using bortezomib alone in relapsed or refractory multiple myeloma patients were from 33% to 50% (2–5). One of the most common grade 3 adverse events is cyclic thrombocytopenia, which was reported in 20–30% of patients: 28% in the SUMMIT study and 24% in the CREST study (2, 5). However, the mechanism behind bortezomib-induced thrombocytopenia remains unknown.

Lonial et al. (6) reported that bortezomib induced thrombocytopenia with no decrease in megakaryocytes in the bone marrow in an experimental murine model, although the detailed mechanism was not described. This prompted us to study the mechanism by which bortezomib induces thrombocytopenia in a mouse model.

Recently, the Rho/Rho kinase pathway was reported to be a negative regulator in proplatelet formation (PPF) (7), although there are very limited published data on signal transduction in the regulation of PPF. Therefore, we used Western blot analysis to study the molecular signaling effects of bortezomib on Rho/Rho kinase activation using murine platelets instead of megakaryocytes.
Materials and methods

Animals and chemicals

All the animal procedures were approved by the Institutional Animal Care and Use Committee of Iwate Medical University. Male ddY mice, 6-wk old, were purchased from Japan SLC Inc. (Shizuoka, Japan). The animals were maintained under typical conditions at our animal center for 2 wks, before use. Male ddY mice, 8-wk old, were used in all the experiments.

Bortezomib (Velcade®) was purchased from Japanese Janssen Pharmaceutical Co. (Tokyo, Japan). Y27632 (Rho kinase inhibitor) was obtained from Calbiochem® (Merck Millipore, Billerica, MA, USA). RhoA pull-down activation assay biochem kits were obtained from Cytoskeleton Inc. (Denver, CO, USA). Protease inhibitors were obtained from Calbiochem Inc. (Darmstadt, Germany). All the reagents were of analytical grade. Bortezomib was dissolved in distilled water, and the other inhibitors were dissolved in DMSO. Stock solutions of bortezomib (100 mM) were stored at −20°C, until use.

In vivo experiments

Dose escalation study. The optimal dose of bortezomib to induce thrombocytopenia in mice is not yet to be identified. The mice received 0.6–3.0 mg/kg bortezomib via tail-vein injection, and blood samples were collected after 3 d. As 2.4 mg/kg bortezomib significantly induced thrombocytopenia (P < 0.01) (Fig. 1), 2.5 mg/kg bortezomib was used in all the in vivo experiments.

The time course of platelet counts, reticulated platelets, thrombopoietin (TPO) levels in plasma, and megakaryocytes in bone marrow. After the intravenous administration of bortezomib (2.5 mg/kg) via tail vein, peripheral blood was obtained by cardiac puncture. The platelet counts were measured using a Coulter T540 (Beckman Coulter, Inc., Brea, CA, USA). Reticulated platelets were measured by flow cytometry (EPICS®XL-MCL, Beckman Coulter, Inc.) (8). Plasma TPO levels were measured using an ELISA kit (Quantikine Mouse TPO Immunoassay, R&D Systems, Minneapolis, MN, USA). The megakaryocytes in bone marrow (the femora) were fixed in 10% buffered formalin, decalci-fied, and embedded in paraffin. Coded histology sections were stained with hematoxylin and eosin and mounted with cytoseal medium (Richard Allen Scientific, Kalamazoo, MI, USA). Megakaryocyte number was obtained from the mean of the megakaryocyte numbers of 10 high-power fields (HPFs, ×400) selected at random.

In vivo effects of bortezomib on colony-forming unit-megakaryocytes (CFU-Mk). Bone marrow (BM) cells obtained from femoral bone, 1 d after the intravenous administration of bortezomib (2.5 mg/kg) via tail vein, were cultured using a CFU-Mk assay kit (MegaCult-C, Stem Cell Technologies, Vancouver, BC, Canada) containing recombinant murine TPO (50 ng/mL), recombinant human IL-6 (20 ng/mL), and recombinant murine IL-3 (10 ng/mL) for 7 d at 37°C in 5% CO₂, at 100% humidity. Cultures were performed in quadruplicate for each concentration of bortezomib. The slides were fixed with acetone and stained for acetylcholine esterase (AChE) (9).

Megakaryocytic colonies were counted and categorized into 3 types based on the number of megakaryocytes of which they were composed: small (3 to 20 AChE (+) cells per colony), medium (21 to 49 AChE (+) cells per colony), and large (more than 50 AChE (+) cells per colony).

In vivo effects of bortezomib on PPF. Murine megakaryocytes were partially purified using Percoll® (10) and bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) gradient methods (11) from the femoral BM cells at 48, 96, and 192 h after the intravenous administration of bortezomib (2.5 mg/kg) via tail vein. Megakaryocytes were plated in 96-micro-well culture plates (300 megakaryocytes/well) and cultured in IMDM (Iscove’s Modified Dulbecco’s Medium) (Life Technologies, Grand Island, NY, USA) supplemented with 1% ITS-G (Life Technologies) at 37°C in 5% CO₂, at 100% humidity for 24 h. Cultures were performed in quadruplicate for each concentration of bortezomib.

The megakaryocytes in each well were fixed with 1% glutaraldehyde and stained for AChE by the method described above (9). Megakaryocytes bearing 2 or more cytoplasmic processes that were at least twice the length of the cell body diameter were considered to be proplatelet-displaying megakaryocytes (12). Percentage PPF was defined as the number of AChE-positive cells with PPF divided by the total number of AChE-positive cells.

In vitro study

In vitro effects of bortezomib on megakaryocytic colony formation. The femoral BM cells were cultured using a
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CFU-Mk assay kit (MegaCult-C) containing recombinant murine TPO (50 ng/mL), recombinant human IL-6 (20 ng/mL), recombinant murine IL-3 (10 ng/mL), and various concentrations of bortezomib (0, 0.1, 1, 10, 100 ng/mL) at 37°C for 5% CO2 at 100% humidity for 7 d. Cultures were performed in quadruplicate for each concentration of bortezomib. Megakaryocytic colonies were fixed with acetone, stained for AchE (9), and counted by the method described above.

In vitro effects of bortezomib on ploidy distribution. The femoral BM cells were cultured in IMDM supplemented with 10% fetal calf serum (Gibco®; Life Technologies) with recombinant human TPO (50 ng/mL) and murine recombinant murine SCF (20 ng/mL). Seven days later, ploidy distributions were analyzed by the method described by Tomer et al. (13).

In vitro effects of bortezomib with or without Y27632 on PPF. Murine megakaryocytes were partially purified as described above (10, 11). They were then plated and cultured in IMDM, supplemented with 1% ITS-G at 37°C in 5% CO2 at 100% humidity for 24 h, with various concentrations of bortezomib (0, 0.1, 1, 10, 100 ng/mL) and with or without Y27632, a Rho kinase inhibitor, in a 96-micro-well plate. Cultures were performed in quadruplicate for each concentration of bortezomib. Percentage PPF of megakaryocytes was calculated as described above.

Western blot analysis. To estimate the mechanism behind the effect of bortezomib on thrombocytopenia, Western blot analysis of murine platelet proteins was performed using a Rho pull-down activation assay biochem kit. Briefly, murine platelets were incubated with bortezomib for 20 h in IMDM supplemented with 10% FCS. Murine platelets were washed with cold phosphate-buffered saline (PBS) twice and lysed in 0.7 mL of lysis buffer (20 mM Tris-HCl, pH 8, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA) and protease inhibitors (cocktail Set III, EDTA-free, Calbiochem®). Rhotekin-RBD protein on colored agarose beads was added to the solution and incubated at 37°C for 30 min. After washing with cold PBS, the sample was diluted in Laemmli sample buffer, and proteins were separated on 12% SDS-polyacrylamide gels (Mini-Protean® TGX gel, Bio-Rad Lab., Hercules, CA, USA). After the transfer of proteins to nitrocellulose membrane (Bio-Rad Lab.), membranes were incubated with unlabeled anti-Rho antibody in blocking buffer (5% BSA in PBS containing 0.1% Tween 20 [TBST]) (Kanto Chem. Ltd., Tokyo, Japan). Membranes were washed with TBST twice and developed using the Vectastain Elite ABC Standard Kit (Vector Laboratories, Inc., Burlingame, CA, USA).

Statistical analysis

Statistical analysis was performed using 2-tailed Student’s t test or ANOVA test. A P value of <0.05 was considered to be statistically significant.

Results

All the data are shown as mean ± standard error of the mean (SEM).

In vivo experiments

The time course of platelet counts, reticulated platelets, TPO levels in plasma, and megakaryocytes in bone marrow. After the intravenous administration of bortezomib (2.5 mg/kg) via tail vein, platelet counts decreased on day 4 and recovered to the normal range on day 6, as shown in Fig. 2. Minimum platelet counts were 604 ± 83 × 10^9/L on day 4 (P < 0.01, n = 5). Rebound thrombocytosis was observed on day 8. Reticulated platelets (RP) were also measured. Percentage RP significantly increased on day 6 (6.2 ± 0.7, P < 0.01, n = 6), indicating that the young platelets appeared on day 6. Plasma TPO levels increased on day 2–4, and the maximum plasma TPO concentration was 703.8 ± 150.2 pg/mL on day 4 (P < 0.05, n = 4). Megakaryocytes in the femur were similar in number and morphology between bortezomib-treated and untreated mice (Fig. 3, n = 5, day 0: 12.1 ± 0.6 megakaryocytes/HPFs, day 4: 11.9 ± 1.1 megakaryocytes/HPFs, day 6: 10.4 ± 0.6 megakaryocytes/HPFs).

![Figure 2](image-url)

Figure 2 Platelet counts and percentage of reticulated platelets in bortezomib-treated mice. Bortezomib-treated mice had a significant reduction in platelet counts on days 2 and 4. The platelet counts returned to normal on day 6. The percentage of reticulated platelets on day 6 significantly increased. Solid lines: bortezomib-treated mice; dashed lines: untreated mice. Error bars represent SEM; **P < 0.01 (days 0 and 4; n = 5; days 2, 8, and 10: n = 4; day 6: n = 6);
In vivo effects of bortezomib on CFU-Mk. Bortezomib was administered to mice via the tail vein. Twenty-four hours later, BM cells were harvested and cultured for 7 d. Cultures were carried performed in quadruplicate for each concentration of bortezomib. A total of 39.2 ± 2.5 CFU-Mk were in untreated mice (n = 5), while 34.2 ± 2.2 CFU-Mk were in bortezomib-treated mice (n = 5) per 1 × 10^5 bone marrow cells. Large CFU-Mk were 1.6 ± 0.7 colonies per 1 × 10^5 bone marrow cells and 0.2 ± 0.2 colonies per 1 × 10^5 bone marrow cells in untreated and bortezomib-treated mice, respectively. Middle CFU-Mk were 15.0 ± 1.5 colonies per 1 × 10^5 bone marrow cells and 14.2 ± 0.6 colonies per 1 × 10^5 bone marrow cells in untreated and bortezomib-treated mice, respectively. Small CFU-Mk were 23.3 ± 1.7 colonies per 1 × 10^5 bone marrow cells and 20.2 ± 1.6 colonies per 1 × 10^5 bone marrow cells in untreated and bortezomib-treated mice, respectively. There were no significant differences between untreated and bortezomib-treated mice (Fig. 4).

In vivo effects of bortezomib on PPF. Proplatelet formations of megakaryocytes, obtained from bortezomib-treated mice on days 2, 4, 6, and 8, were evaluated. PPF significantly decreased only on day 2 (6.0 ± 1.3%, P < 0.01, n = 6) and day 4 (5.3 ± 1.1%, P < 0.01, n = 6) (Fig. 5).

In vitro experiments

In vitro effects of bortezomib on CFU-Mk. CFU-Mk in vitro showed no significant decrease at 0.01 to 1 ng/mL bortezomib. CFU-Mk were 39.2 ± 2.5 colonies per 1 × 10^5 bone marrow cells at 0 ng/mL (n = 5), 36.6 ± 2.6 colonies per 1 × 10^5 bone marrow cells at 0.01 ng/mL (n = 5), 37.6 ± 2.2 colonies per 1 × 10^5 bone marrow cells at
0.1 ng/mL (n = 5), and 37.0 ± 1.9 at 1 ng/mL (n = 5) per 1 × 10^5 bone marrow cells. However, CFU-Mk decreased at 10 ng/mL (12.8 ± 6.3, P < 0.01, n = 5) and were scarcely seen at 100 ng/mL. Large CFU-Mk were 1.6 ± 0.7 colonies at 0 ng/mL (n = 5), 1.0 ± 0.5 colonies at 0.01 ng/mL, 0.6 ± 0.4 colonies at 0.1 ng/mL, and 0.8 ± 0.8 colonies at 1.0 ng/mL, per 1 × 10^5 bone marrow cells. Middle CFU-Mk were 15.0 ± 1.5 colonies at 0 ng/mL, 14.6 ± 2.0 colonies at 0.01 ng/mL, 14.6 ± 1.7 colonies at 0.1 ng/mL, and 14.4 ± 1.7 colonies at 1.0 ng/mL. Small CFU-Mk were 23.2 ± 1.7 colonies at 0 ng/mL, 21.2 ± 2.2 colonies at 0.01 ng/mL, 22.8 ± 0.6 colonies at 0.1 ng/mL, and 22.2 ± 2.1 colonies at 1.0 ng/mL bortezomib. There were no significant differences between them (Fig. 6).

In vitro effects of bortezomib on ploidy distribution. Ploidy distributions of cultured megakaryocytes with or without bortezomib were determined by 2-color analysis using flow cytometry. There were no significant differences between cultured megakaryocytes with or without bortezomib (Fig. 7).

In vitro effects of bortezomib with or without Y27632 on PPF. Proplatelet formation significantly decreased at 0.1, 1, 10, and 100 ng/mL bortezomib (0 ng/mL: 26.2 ± 0.8%; 0.01 ng/mL: 23.1 ± 1.7%; 0.1 ng/mL: 18.1 ± 1.5% P < 0.01; 1 ng/mL: 12.7 ± 1.3% P < 0.01; 10 ng/mL: 9.9 ± 1.4% P < 0.01; 100 ng/mL: 2.9 ± 1.2% P < 0.01; 0 ng/mL: n = 12, 0.01 and 0.1 ng/mL: n = 8, 1 and 10 ng/mL: n = 10; and 100 ng/mL: n = 7) (Fig. 8). However, decreased PPF at 1 ng/mL of bortezomib was eliminated by the addition of 10 nM of Y27632, a Rho kinase inhibitor (0 ng/mL of bortezomib: 25.7 ± 2.0%, 1 ng/mL of bortezomib: 13.9 ± 1.3%, P < 0.01, 1 ng/mL of bortezomib + 10 nM Y27632: 28.9 ± 1.6, P < 0.01; n = 6 for each concentration point) (Fig. 9).

Discussion

Thrombocytopenia is a common adverse event during bortezomib treatment for multiple myeloma (2, 5). Its kinetic data differ from those induced by other cytotoxic agents (14, 15), and it is characterized by transient thrombocytopenia. In this study, we studied the following items, including bortezomib’s in vivo effects on CFU-Mk, CFU-Mk proliferation and maturation, and PPF of megakaryocytes in mice. At first, bortezomib was administered to mice, in which significant thrombocytopenia was induced, to evaluate the in vivo effects on CFU-Mk, megakaryocytes, and PPF. CFU-Mk, including immature to mature CFU-Mk, were not affected by the administration of bortezomib (Fig. 4). Megakaryocytes in the bone marrow, in terms of number and morphology, were also not affected (Fig. 3). Meanwhile, PPF was decreased (nificant decrease at 0.001 to 1 ng/mL of bortezomib. Significant decrease (P < 0.01) occurred at 10 and 100 ng/mL of bortezomib. CFU-Mk counts were 12.8 ± 6.3 at 10 ng/mL but were scarcely observed at 100 ng/mL. Error bars represent SEM; **P < 0.01. n = 5 for each concentration point.

Figure 6 In vitro effects of bortezomib on CFU-Mk. Cultures were performed in quadruplicate. CFU-Mk formation in vitro showed no significant decrease at 0.001 to 1 ng/mL of bortezomib. Significant decrease (P < 0.01) occurred at 10 and 100 ng/mL of bortezomib. CFU-Mk counts were 12.8 ± 6.3 at 10 ng/mL but were scarcely observed at 100 ng/mL. Error bars represent SEM; **P < 0.01. n = 5 for each concentration point.

Figure 8 In vitro effects of bortezomib on proplatelet formation. Proplatelet formation significantly decreased on using 0.1, 1, 10, and 100 ng/mL of bortezomib. Cultures were carried out in quadruplicate. Error bars represent SEM; *P < 0.05, **P < 0.01 (0 ng/mL: n = 12; 0.01 and 0.1 ng/mL: n = 8, 1 and 10 ng/mL: n = 10; 100 ng/mL: n = 7).
Significantly decreased on days 2 and 4 after bortezomib administration, resulting in thrombocytopenia.

These data led us to examine the in vitro effects on CFUMegs formation, that is, the proliferation and maturation to megakaryocytes, ploidy distribution of megakaryocytes, and PPF in the presence of bortezomib. As shown in Fig. 6, CFU-Mk formation, including immature to mature CFU-Megs, was not affected in the presence of bortezomib ≤1 ng/mL. The ploidy distribution of megakaryocytes cultured in vitro with TPO and stem cell factor revealed no difference between cultured megakaryocytes with and without bortezomib (1 ng/mL) (Fig. 7). In contrast, bortezomib ≥0.1 ng/mL induced a significant decrease in the frequency of megakaryocyte PPF (Fig. 8).

The phase 1 study of bortezomib revealed a 2-compartment pharmacokinetic model with a very short initial distribution half-life (t1/2: 0.22–0.46 h) followed by a more sustained terminal elimination half-life (t1/2: more than 10 h) (16). The plasma levels of bortezomib in patients are considered to be <5 ng/mL 1 h after injection and more than 1 ng/mL after 24 h. The data described above show that bortezomib only affects PPF, the step of platelet production, and not progenitor cells or megakaryocyte development at bortezomib concentrations shown in clinical studies.

To estimate the role of G-proteins, which are considered to be important in the reorganization of the actin and microtubule cytoskeleton (17, 18), we carried out Western blot analysis of murine platelets instead of murine megakaryocytes because we could not obtain a sufficient number of purified megakaryocytes. Chang et al. (7) reported that the Rho/Rho kinase pathway, a well-known regulator of the actin cytoskeleton, acts as a negative regulator of PPF. Therefore, we focused on the expression of activated Rho. In a Western blot of platelets to evaluate the Rho expression activated by bortezomib, bortezomib was observed to induce the increased expression of activated Rho (Fig. 10). These data indicate that bortezomib stimulated a negative regulator, the Rho/Rho kinase pathway, resulting in decreased PPF.

There are very limited published data on the regulation of production/degradation of Rho proteins. Rolli-Derkinderen et al. (19) observed that endogenous RhoA is subjected to proteasomal degradation and that RhoA degradation is a physiological process that may serve to regulate the level of RhoA and consequently RhoA-dependent signaling in living cells. They showed that a proteasome inhibitor, lactacystin, significantly induced the time-dependent accumulation of activated RhoA, indicating that proteasomal degradation of activated RhoA participates in the regulation of RhoA expression. The increased activated Rho expression by bortezomib in this study is considered to be derived from a similar mechanism. To confirm the significant role of activated Rho expression, a PPF assay was carried out. PPF decreased with only bortezomib (1 ng/mL), while the decreased PPF was eliminated in the presence of Y27632 (10 nM), a Rho kinase inhibitor (Fig. 9).

In conclusion, bortezomib modulates Rho/Rho kinase. Consequently, bortezomib decreased PPF, resulting in thrombocytopenia.

References
