

# shRNA-mediated Bmi-1 silencing sensitizes multiple myeloma cells to bortezomib

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**Abstract.** The introduction of bortezomib has resulted in a paradigm shift in the treatment of multiple myeloma (MM) and has contributed to the improved survival of patients with MM. Inevitably, resistance to therapy develops, and thus the clinical efficacy of bortezomib is hampered by drug resistance. The oncogene B-cell-specific Moloney murine leukemia virus insertion site-1 (Bmi-1) has been implicated in the pathogenesis of various human malignancies. Furthermore, RNA interference (RNAi)-mediated Bmi-1 silencing has been shown to sensitize tumor cells to chemotherapy and radiation. The role of Bmi-1 in influencing the response to bortezomib therapy has not been investigated to date. In the present study, Bmi-1 was silenced in two MM cell lines (U266 and RPMI8226) using short hairpin RNA (shRNA) targeting Bmi-1 (shBmi-1). A cell counting kit-8 (CCK-8) assay was performed to analyze cell proliferation and evaluate the 50% inhibitory concentration (IC<sub>50</sub>) values of bortezomib. Cell cycle progression and apoptosis were analyzed by flow cytometry (FCM), and the mRNA and protein expression of associated genes (Bmi-1, p14, p21, Bcl-2 and Bax) was quantified by RT-qPCR and western blot analysis, respectively. The IC<sub>50</sub> values significantly decreased in the cells transfected with shBmi-1 ( $p < 0.05$ ). The depletion of Bmi-1 sensitized the MM cells to bortezomib, which increased the G<sub>1</sub> phase duration and enhanced bortezomib-induced apoptosis ( $p < 0.05$ ). The expression of p21 and Bax (apoptosis inducer) was upregulated, whereas that of the anti-apoptotic protein, Bcl-2, was downregulated in the Bmi-1-silenced cells ( $p < 0.05$ ). The depletion of Bmi-1 enhanced the sensitivity of MM cells to bortezomib by inhibiting cell proliferation and inducing cell cycle arrest and apoptosis.

Our data suggest that Bmi-1 may serve as an important novel therapeutic target in MM.

## Introduction

Multiple myeloma (MM) is a type of cancer that arises from the neoplastic proliferation of plasma cells and is characterized by the proliferation of malignant plasma cells into the bone marrow (BM) and the excessive secretion of monoclonal immunoglobulin or Bence Jones proteins. The major clinical manifestations include extensive bone destruction, anemia, hypercalcemia, hyperviscosity and renal dysfunction (1). Of note, the incidence of MM in Western countries is higher than that in Asian countries (2). Despite advances in the understanding of the molecular pathogenesis of MM and promising new therapies, only 25-35% of patients respond to therapy in the relapsed and refractory setting (3,4).

The boronic dipeptide, bortezomib, a reversible proteasome inhibitor, has shown marked anticancer activity in various cancer cell types, including MM cells, which are resistant to conventional therapies (5,6). Despite the promising clinical activity, the efficacy of bortezomib may differ among tumor types, and some patients with MM fail to respond to bortezomib therapy (7). Moreover, almost one-third of patients with MM never respond to treatment with bortezomib, depending on the clinical situation (7). Whether these observations are related to the common mechanisms of drug resistance frequently observed for anticancer drugs remains unclear. However, the understanding of the characteristics of drug resistance and the enhancement of the sensitivity to bortezomib may help to overcome drug resistance. In addition, recent evidence has demonstrated that silencing the expression of oncogenes, such as myeloid cell leukemia sequence-1 (Mcl-1) and melanoma antigen gene (MAGE)-C1/CT7, can sensitize MM cells to bortezomib (8,9), suggesting that RNA interference (RNAi) may be an effective method for enhancing the sensitivity of MM cells to bortezomib or even reversing resistance.

B-cell-specific Moloney murine leukemia virus insertion site-1 (Bmi-1), a member of the polycomb family, was initially identified as an oncogene that cooperates with c-Myc in the initiation of lymphoma in murine models (10,11). Over the years, Bmi-1 has been reported to be involved in axial patterning (12), hematopoiesis (13), the regulation of proliferation and senescence (14). It has also been found to be essential for the

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self-renewal of normal and malignant stem cells (15). Several lines of evidence have also indicated that Bmi-1 is extensively upregulated in a variety of malignancies, including non-small cell lung cancer (16), leukemia (17), as well as breast (18), colorectal (19), pancreatic (20) and prostate cancer (21). Moreover, it has been demonstrated that Bmi-1 is overexpressed in MM and can regulate the growth and clonogenic capacity of MM cells both *in vitro* and *in vivo* (22). However, whether Bmi-1 can affect the sensitivity of MM cells to bortezomib is largely unknown. Furthermore, it has been reported that the downregulation of Bmi-1 can result in cancer cell apoptosis and that the RNAi-mediated depletion of Bmi-1 can sensitize tumor cells to chemotherapy and radiotherapy (23-25). Thus, we hypothesized that the abrogation of Bmi-1 expression may be an effective strategy for sensitizing MM cells to bortezomib.

In this study, we demonstrate that Bmi-1 plays an important role in the sensitization of MM cells to bortezomib. To determine the combined effect of RNAi and bortezomib treatment on MM cells *in vitro*, we introduced a lentiviral interference vector expressing short hairpin RNA (shRNA) to silence Bmi-1 in MM cells. Our results demonstrate that Bmi-1 silencing sensitizes MM cells to bortezomib by inhibiting cell proliferation and inducing cell cycle arrest and apoptosis, suggesting that Bmi-1 may serve as a potential and specific novel therapeutic target in MM.

## Materials and methods

**Cell culture.** HEK-293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. U266 and RPMI8226 cells were cultured in RPMI-1640 containing 10% fetal bovine serum, 50 U/ml penicillin and 50 µg/ml streptomycin.

**Lentivirus production and infection.** Bmi-1 shRNA was designed and cloned into the pLVTHM lentiviral vector. The shRNA sequence for Bmi-1 was as follows: 5'-GAGATAATAA GCTTGCTA-3' (26). A shRNA targeting a scrambled sequence (general sequence, 5'-TTCTCCGAACGTGTACGT-3') (27) served as the negative control. The clone identity was verified by restriction digestion analysis and plasmid DNA sequencing. The pWPXL vectors were transfected into the HEK-293T cells with the packaging plasmid, psPAX2, and the VSV-G envelope plasmid, pMD2.G (a gift from Dr Didier Trono, School of Life Sciences, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The viral particles were harvested 48 h following transfection. The U266 and RPMI8226 cells ( $1 \times 10^5$ ) were infected with  $1 \times 10^6$  recombinant lentivirus-transducing units plus 6 µg/ml of polybrene (Sigma, Natick, MA, USA).

**RNA extraction and quantitative reverse transcription polymerase chain reaction (RT-qPCR).** Total RNA was extracted from the U266 and RPMI8226 cells using TRIzol reagent (Invitrogen) and reverse transcribed to generate cDNA. cDNA was synthesized using the PrimeScript RT Reagent kit (Takara, Tokyo, Japan). qPCR analyses were performed using SYBR Premix Ex Taq (Takara). The primers used for the PCR amplification were as follows: Bmi-1 forward, 5'-AAATCA GGGGTTGAAAAAT CT-3' and reverse, 5'-GCTAACCA

CCAATCTTCCTTTG-3'; p14 forward, 5'-GCTACTGAGGAG CCAGCGTCTA-3' and reverse, 5'-AGCACCACCAGCGTGT CCAG-3'; and β-actin forward, 5'-GCTACTGAGGAGCCAG CGTCTA-3' and reverse, 5'-AGCACCACCAGCGTGTCCAG-3'.

**Western blot analysis.** The proteins were separated on a 12% SDS-PAGE gels and then transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked using 5% non-fat milk and incubated with a mouse anti-Bmi-1, anti-p21 or anti-Bcl-2 monoclonal antibody (mAb) (Cell Signaling Technology, Inc., Beverly, MA, USA) or a mouse anti-β-actin mAb (Sigma). The proteins were visualized and quantified using ECL reagents (Pierce Biotechnology, Inc., Rockford, IL, USA).

**Cell proliferation.** Cell proliferation was measured using the cell counting kit-8 (CCK-8) assay kit (Dojindo Corp., Kumamoto, Japan). A total of 4,000 cells were plated into each well of a 96-well plate, wherein 10 µl of the CCK-8 solution was added to 90 µl of the culture medium. The cells were subsequently incubated for 2 h at 37°C, and the optical density was measured at 450 and 650 nm. Three independent experiments were performed.

**FACS analysis for determination of cell cycle progression and apoptosis.** The U266 and RPMI8226 cells were collected and fixed in ice-cold 70% ethanol overnight. The fixed cells were washed with phosphate-buffered saline and stained with a freshly prepared solution containing 25 µg/ml propidium iodide (PI; Sigma), 10 µg/ml RNase A, 0.05 mM ethylene diamine and 0.2% Triton X-100 tetra-acetic acid in phosphate-buffered saline for 30 min in the absence of light. For each sample, at least 20,000 cells were analyzed using a flow cytometer (Beckman Coulter EPICS Altra Cell Sorter; Beckman Coulter Inc., Miami, FL, USA) and Multicycle AV software for Windows 5.0 (Phoenix Flow Systems, San Diego, CA, USA).

Cells ( $2 \times 10^5$ ) from the control group and the interference group were collected, stained according to the instructions provided with the Annexin V-PE/7-AAD Apoptosis Detection kit (Sigma), and subjected to flow cytometry (FCM) using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) for the detection of apoptosis.

**Statistical analysis.** The results in this study are presented as the means ± SEM. The data were analyzed using the Student's t-test (two-tailed) and a value of  $p < 0.05$  was considered to indicate a statistically significant difference, unless otherwise specified.

## Results

**Establishment of cell lines stably transfected with shRNA targeting Bmi-1 (shBmi-1).** To determine the biological role of Bmi-1 in the survival of MM cells, two cell lines stably transfected with shBmi-1 were established. The results from RT-qPCR demonstrated that Bmi-1 mRNA expression in the shBmi-1-transfected cells was significantly lower than that in the control cells ( $p < 0.05$ ; Fig. 1A and B). Additionally, western blot analysis revealed that the Bmi-1 protein levels were markedly reduced in the shBmi-1-transfected cells (Fig. 1C and D), demonstrating that the constructed lentivirus-mediated RNAi

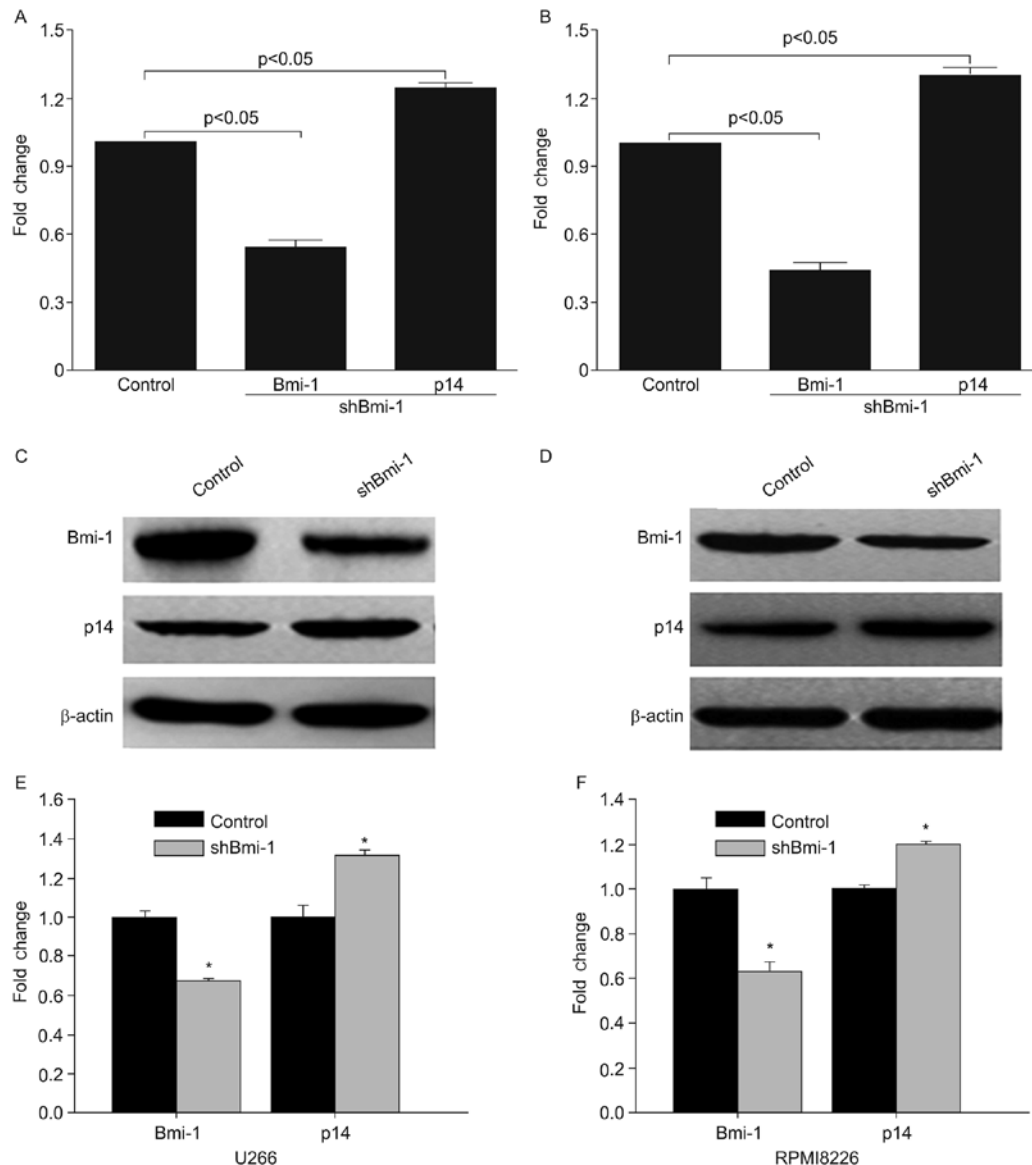


Figure 1. Effect of short hairpin RNA (shRNA) targeting B-cell-specific Moloney murine leukemia virus insertion site-1 (Bmi-1; shBmi-1) on the mRNA and protein expression of Bmi-1 and p14. (A, C and E) U266 cells; (B, D and F) RPMI8226 cells. (A and B) mRNA expression levels of Bmi-1 and p14 determined by RNA extraction and RT-qPCR following lentivirus transfection.  $\beta$ -actin was used for normalization, and the control values were normalized to 1 for comparative analysis. (C and D) Western blot analysis of Bmi-1 and p14 protein levels following lentivirus transfection. (E and F) Densitometric analysis.  $\beta$ -actin served as the internal reference. Triplicate experiments showed consistent results. \* $p < 0.05$ , vs. control.

expression vector expressing Bmi-1 (shBmi-1) inhibited the expression of Bmi-1. Bmi-1 has been shown to inhibit the INK4a/ARF locus, which exerts a significant negative effect on p14ARF transcriptional regulation (10). By detecting p14 gene expression, we established that the reduced expression of Bmi-1 resulted in an increased p14 mRNA and protein expression (Fig. 1). These results demonstrate that shRNA technology can be used to effectively inhibit Bmi-1 mRNA and protein expression in myeloma cells, indicating that myeloma cell lines in which the expression of Bmi-1 was effectively silenced by RNAi were created successfully.

*Bmi-1 knockdown sensitizes U266 and RPMI8226 cells to bortezomib.* To examine the effects of bortezomib on the survival of cells in which Bmi-1 was knocked down, a CCK-8

assay was used to detect the proliferation of U266-shBmi-1, RPMI8226-shBmi-1, U266-control and RPMI8226-control cells. The results indicated that the silencing of the Bmi-1 gene gradually enhanced the inhibition of MM cell proliferation in a time-dependent manner compared to the control (Fig. 2A and B). Subsequently, the stably transfected cell lines were treated with various concentrations (0, 10, 20, 40 and 80 nM) of bortezomib for 72 h, and a CCK-8 assay was performed in order to detect the proliferative ability of the cells. The results indicated a marked inhibition of cell proliferation in the shBmi-1-transfected cells compared with the control group (Fig. 2C and D). The 50% inhibitory concentration (IC<sub>50</sub>) values of bortezomib in the U266-control and U266-shBmi-1 cells were  $24.73 \pm 0.375$  nM and  $18.59 \pm 0.286$  nM ( $p < 0.05$ ), respectively. The IC<sub>50</sub> values of bortezomib in the RPMI8226-control and RPMI8226-shBmi-1

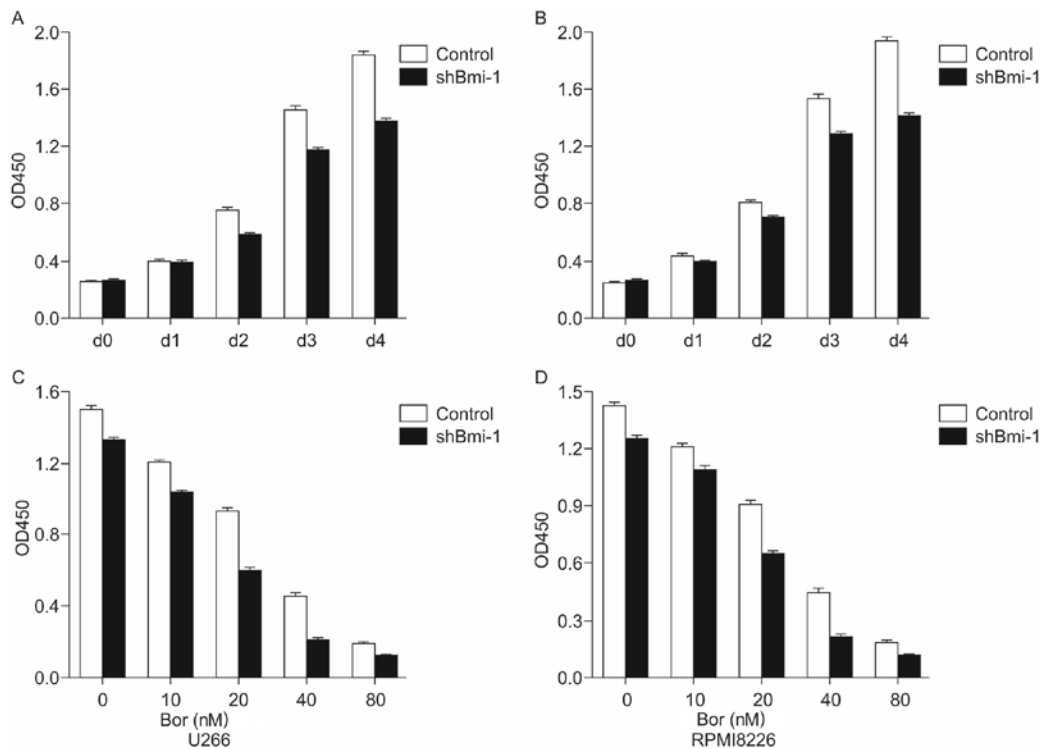


Figure 2. Inhibitory effect of short hairpin RNA (shRNA) targeting B-cell-specific Moloney murine leukemia virus insertion site-1 (Bmi-1; shBmi-1) combined with or without bortezomib treatment on multiple myeloma (MM) cells. (A and B) A CCK8 cell proliferation assay of U266-control, U266-shBmi-1, RPMI8226-control and RPMI8226-shBmi-1 cells was performed for 2 h after 0, 24, 48, 72 and 96 h. (C and D) U266-control, U266-shBmi-1, RPMI8226-control, and RPMI8226-shBmi-1 cells were exposed to bortezomib for 72 h, and a CCK8 assay was used to evaluate cell viability. The data are presented as the means  $\pm$  SD of three independent experiments. Bor, bortezomib.

cells were  $32.99 \pm 0.458$  nM and  $21.56 \pm 0.526$  nM ( $p < 0.05$ ), respectively. These results indicated that the knockdown of Bmi-1 sensitized the U266 and RPMI8226 cells to bortezomib.

*Bmi-1 silencing induces myeloma cell cycle arrest in the G<sub>1</sub> phase.* FCM was used to detect the cell cycle progression of the U266 and RPMI8226 cells which were stably transfected with shBmi-1 followed by treatment with or without 20 or 30 nM of bortezomib. The G<sub>1</sub> distribution rates of the U266-control and U266-shBmi-1 cells were  $39.91 \pm 0.26\%$  and  $45.65 \pm 0.68\%$  ( $p < 0.05$ ), respectively. The G<sub>1</sub> distribution rates of the RPMI8226-control and RPMI8226-shBmi-1 cells were  $42.30 \pm 0.47\%$  and  $50.86 \pm 0.38\%$  ( $p < 0.05$ ), respectively. Furthermore, in combination with bortezomib treatment for 48 h, the silencing of Bmi-1 induced a significant increase in the number of myeloma cells arrested in the G<sub>1</sub> phase ( $p < 0.05$ ; Fig. 3). The G<sub>1</sub> distribution rates of the U266-control and U266-shBmi-1 cells were  $45.35 \pm 0.77\%$  and  $56.81 \pm 0.50\%$  ( $p < 0.05$ ), respectively. The G<sub>1</sub> distribution rates of the RPMI8226-control and RPMI8226-shBmi-1 cells were  $49.27 \pm 0.90\%$  and  $56.94 \pm 0.61\%$  ( $p < 0.05$ ), respectively. These data demonstrate that the silencing of Bmi-1 enhances the effects of bortezomib on cell cycle arrest.

*Bmi-1 silencing enhances the apoptotic effects of bortezomib.* Annexin V-PE/7-AAD staining was utilized to evaluate the effects of Bmi-1 knockdown on MM cell apoptosis. The results indicated that the percentage of U266-control and

U266-shBmi-1 apoptotic cells was 9.8 and 12.12% ( $p < 0.05$ ), respectively, and the percentage of RPMI8226-control and RPMI8226-shBmi-1 apoptotic cells was 9.6 and 15.69% ( $p < 0.05$ ), respectively (Fig. 4). Furthermore, we examined the apoptotic rate of the U266-shBmi-1 and U266-control cells as well as that of the RPMI8226-shBmi-1 and RPMI8226-control cells following treatment with 20 or 30 nM bortezomib for 72 h. The FCM data demonstrated that the apoptotic rate of the U266-shBmi-1 and RPMI8226-shBmi-1 cells was 24.55 and 49.14%, respectively, compared with 20.83 and 22.50% of the U266-control and RPMI8226-control cells, respectively ( $p < 0.05$ ; Fig. 4). These observations indicated that the silencing of Bmi-1 enhanced the ability of bortezomib to induce MM cell apoptosis.

*Bmi-1 silencing combined with bortezomib regulates the expression levels of p21, Bax and Bcl-2.* To further explore the mechanisms underlying the enhancement of bortezomib-induced apoptosis in Bmi-1-silenced cells, we examined the expression levels of p21, Bax and Bcl-2 in the U266-shBmi-1 and U266-control cells (treated with or without 20 nM bortezomib) and in the RPMI8226-shBmi-1 and RPMI8226-control cells (treated with or without 30 nM bortezomib). Compared with the control group, the protein expression levels of p21 and Bax in the shBmi-1 cells were significantly increased, while the expression of the anti-apoptotic protein, Bcl-2, was significantly reduced ( $p < 0.05$ ; Fig. 5). Furthermore, when combined with the results obtained at 48 h

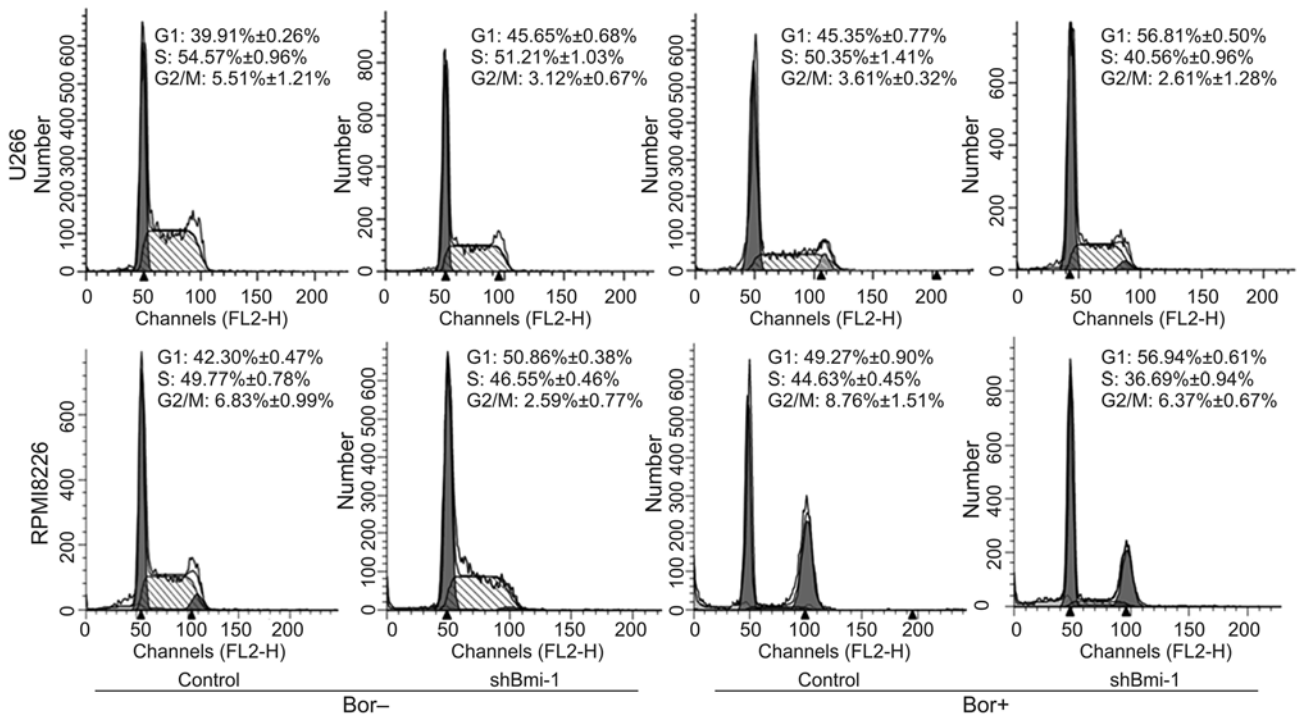


Figure 3. FACS analysis of cell cycle distribution. U266-control, U266-shBmi-1, RPMI8226-control, and RPMI8226-shBmi-1 cells were treated with (Bor+) or without 20 or 30 nM bortezomib (Bor-) for 48 h, and the cells were harvested for propidium iodide (PI) staining followed by flow cytometry (FCM). The percentage of cells displaying G<sub>1</sub>, S, and G<sub>2</sub>/M phase content are provided on each cell cycle figure. The data are presented as the means ± SD of three independent experiments. shBmi-1, small interfering RNA targeting B-cell-specific Moloney murine leukemia virus insertion site-1.

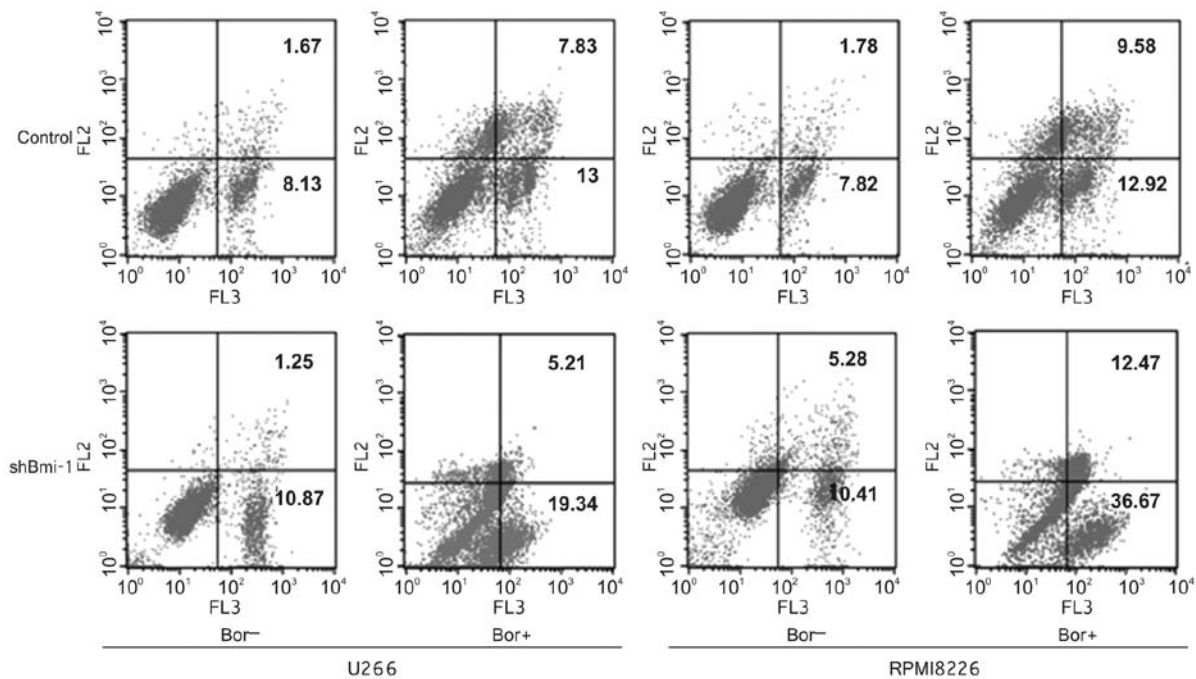


Figure 4. Silencing of B-cell-specific Moloney murine leukemia virus insertion site 1 (Bmi-1) combined with or without bortezomib treatment affects cell apoptosis of multiple myeloma (MM) cells. U266-control, U266-shBmi-1, RPMI8226-control and RPMI8226-shBmi-1 cells were treated with (Bor+) or without (Bor-) 20 or 30 nM bortezomib for 48 h, and the cells were harvested for Annexin V-PE/7-AAD staining followed by flow cytometry (FCM). The values shown in the lower-right quadrant and upper-right quadrant represent the percentage of early and late-apoptotic cells, respectively.

of bortezomib treatment, the difference was even more evident. These results suggest that the robust increase in p21 and Bax

expression and the prominent decrease in Bcl-2 expression enhanced the sensitivity of MM cells to bortezomib.

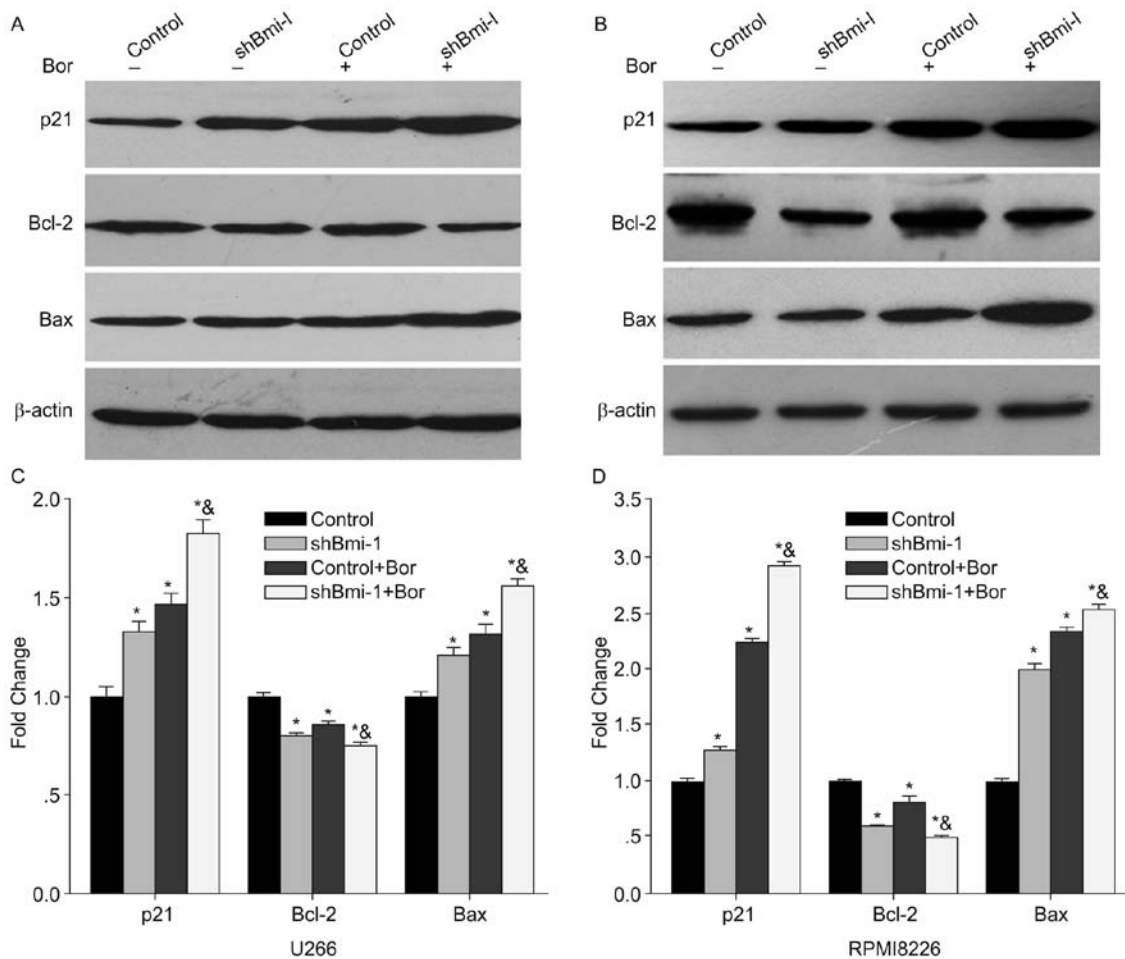


Figure 5. Effect of short hairpin RNA (shRNA) targeting B-cell-specific Moloney murine leukemia virus insertion site 1 (Bmi-1; shBmi-1) combined with bortezomib treatment on the protein expression of p21, Bcl-2 and Bax in multiple myeloma (MM) cells. (A and C) U266 cells; (B and D) RPMI8226 cells. (A and B) U266-control, U266-shBmi-1, RPMI8226-control and RPMI8226-shBmi-1 cells were incubated for 48 h in the presence (+) or absence (-) of 20 or 30 nM bortezomib (Bor). Total protein was electrophoresed and separated on a 12% SDS-polyacrylamide gels. The protein expression levels of p21, Bcl-2 and Bax were determined by western blot analysis. (C and D) Densitometric analysis. Triplicate experiments demonstrated consistent results. \* $p < 0.05$ , vs. control. & $p < 0.05$ , compared to control + Bor.

## Discussion

MM is a malignant mature B cell neoplasm characterized by the clonal proliferation of plasma cells in BM, which is characterized by a profound genomic instability involving both numerical and structural chromosomal aberrations of potential prognostic relevance (28). As an incurable cancer, MM can occur *de novo* or proceed from a monoclonal gammopathy of undetermined significance (MGUS; ~1% per year) (4). Multilevel molecular changes, such as the activation of oncogenes, inactivation of tumor suppressor genes, and alteration of the BM microenvironment, are involved in the pathogenesis and progression of MM. Due to comprehensive advances in the understanding of the molecular pathogenesis of MM, some therapeutic medicines, such as protease inhibitors, lenalidomide and farnesyl transferase inhibitors, that target signaling pathways or the BM microenvironment have been introduced to clinical practice, and have significantly improved the survival and prognosis of patients with MM (2). Among the therapeutic drugs, bortezomib, which is a proteasome inhibitor, has been shown to specifically inhibit the 26S proteasome subunit and I- $\kappa$ B degradation, prevent NF- $\kappa$ B activation,

and induce apoptosis in MM. It has also been shown to have marked clinical activity in the relapsed or refractory MM setting (5). In the present study, we also demonstrated that bortezomib induced a dose- and time-dependent increase in cell toxicity and a decrease in the viability of MM cells. Despite a promising clinical activity, some patients with MM have failed to respond to bortezomib therapy. Moreover, only 25-35% of patients respond to bortezomib treatment in the relapsed and refractory setting (3,4). Therefore, to improve the outcome of this incurable disease, further research is required to overcome resistance to bortezomib in MM. Studies have demonstrated that the administration of bortezomib in combination with other chemical medications can sensitize cancer cells to bortezomib or even reverse resistance (29,30). Furthermore, it has been reported that the RNAi-mediated silencing of the oncogenes, Mcl-1 and MAGE-C1/CT7, can also sensitize MM cells to bortezomib, suggesting that RNAi may be a potential method for enhancing the sensitivity of MM cells to bortezomib.

Bmi-1, the first polycomb-group gene to be identified, was originally isolated as an oncogenic partner of c-Myc in the initiation of lymphoma in murine models. There is accumulating evidence confirming that Bmi-1 plays a crucial role in diverse

biological and pathological processes, such as axial patterning, hematopoiesis, the regulation of proliferation, senescence and the maintenance of cancer stem cell self-renewal. Consistent with its role in inhibiting the p16ink4a locus, Bmi-1 is upregulated extensively in a variety of malignancies, including MM, and can regulate cell proliferation and carcinogenesis. Previous studies have also suggested that Bmi-1 is associated with the protection of tumor cells from apoptosis (31,32). Moreover, the increased expression of Bmi-1 in certain tumors correlates with a poor prognosis (20,32,33). Additionally, increasing evidence indicates that the RNAi-mediated depletion of Bmi-1 can sensitize tumor cells to chemotherapy or radiotherapy. In an attempt to enhance sensitivity to bortezomib, these observations prompted us to investigate the possibility of combining Bmi-1 silencing and bortezomib treatment as a clinical therapeutic strategy for MM.

In order to evaluate the possibility of developing Bmi-1 into a novel therapeutic agent for the treatment of MM, two MM cell lines (U266 and RPMI8226), which overexpress Bmi-1, were employed in the present study. A lentiviral interference vector expressing shRNA was introduced into the U266 and RPMI8226 cells, effectively silencing Bmi-1. We established that the proliferation of U266 and RPMI8226 cells was markedly inhibited after the silencing of Bmi-1, and the depletion of Bmi-1 resulted in an enhanced sensitivity of these cells to bortezomib. FACS observations revealed that silencing Bmi-1 expression in combination with bortezomib treatment prompted cell cycle arrest in the G<sub>1</sub>-S phase and enhanced bortezomib-induced apoptosis. These results indicate that the silencing of Bmi-1 sensitizes MM cells to bortezomib by inhibiting cell proliferation and inducing cell cycle arrest and apoptosis.

Bortezomib induces apoptosis by stabilizing p53 and activating p53 downstream target genes, such as p21. However, bortezomib can induce apoptosis in cells that do not contain wild-type p53 and the effect of bortezomib on p21 may actually limit its direct cytotoxic activities (34). Furthermore, bortezomib may selectively induce apoptosis in SV40-transformed, but not in normal, fibroblasts, although the accumulation of p21 occurs in both cell types (35). Therefore, the accumulation of p21 may be involved in, but is not absolutely required for, the induction of apoptosis. However, p21, a cyclin-dependent kinase inhibitor, can induce growth arrest by inhibiting cyclin-dependent kinases that drive cell cycle progression, sensitizing cancer cells to chemotherapy (36). The Bcl-2 family is comprised of structurally related proteins that can either inhibit (i.e., Bcl-2) or promote (i.e., Bax) cell death (37). The ratio of Bcl-2 to Bax is a key regulator for determining apoptosis following damage to DNA. Proteasome inhibitors can induce apoptosis by inducing an accumulation of pro-apoptotic Bcl-2 family members. In addition, evidence indicates that bortezomib overcomes the protective effects of Bcl-2 overexpression by inhibiting the degradation of Bax (38,39). However, alterations in the balance between Bcl-2 and Bax may be involved in resistance to bortezomib (40). Therefore, in this study, we focused on the expression levels of p21, Bcl-2 and Bax. The results indicated that the knockdown Bmi-1 resulted in the upregulation of p21, the downregulation of Bcl-2 and the accumulation of Bax in the cells treated with bortezomib. These findings suggest that p21 is involved in the Bmi-1-driven cell proliferation inhibition and

G<sub>1</sub> phase arrest in MM cells. Furthermore, the results indicated that Bmi-1 downregulated the Bcl-2/Bax ratio and enhanced bortezomib-induced apoptosis.

In conclusion, to the best of our knowledge, this is the first report of the anticancer potential of bortezomib treatment combined with the depletion of Bmi-1 in MM. The present study demonstrates that the knockdown of Bmi-1 sensitizes MM cells to bortezomib treatment and that the silencing of Bmi-1 enhances bortezomib-induced apoptosis. Furthermore, we demonstrate that the knockdown of Bmi-1 upregulates the expression of p21 and Bcl-2 and decreases the expression of Bax. The alteration in the Bcl-2/Bax ratio prompted bortezomib-induced apoptosis in Bmi-1-silenced MM cells. Taken together, our data suggest that bortezomib treatment combined with Bmi-1 knockdown may be a potential therapeutic strategy for MM.

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