

## Original Article

# Effects and mechanisms of bortezomib combined with arsenic trioxide on multiple myeloma

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**Abstract:** Multiple myeloma (MM) is a malignant plasma cell disease belonging to the category of B-cell lymphomas, with high incidence rates. It is a common hematological malignancy. At present, main treatment methods of MM include bortezomib protease inhibitors, immunomodulatory, and traditional chemotherapy drugs. Arsenic trioxide ( $As_2O_3$ ) has shown good effects in treating tumors, including MM. However, the effects of bortezomib combined with arsenic trioxide on MM have not been elucidated. In this study, cell proliferation was detected by MTT assays. Cell apoptosis was detected by Annexin V-FITC assays. Caspase 3 activity and Interleukin-6 (IL-6) secretion was also detected and examined. Autophagy protein Beclin-1 expression was detected using Western blotting. Reactive oxygen species (ROS) were measured by  $H_2DCFDA$  probing. Bortezomib or  $As_2O_3$  treatment significantly inhibited cell proliferation, promoted cell apoptosis, and inhibited cell autophagy. The bortezomib and  $As_2O_3$  combined group showed stronger effects, inhibiting cell proliferation and producing apoptosis and autophagy ( $P < 0.05$ ). Bortezomib or  $As_2O_3$  treatment also inhibited IL-6 levels and enhanced ROS production. Bortezomib and  $As_2O_3$  combined administration showed more significant inhibition of IL-6 and enhancement of ROS levels ( $P < 0.05$ ). Current results indicate that bortezomib combined with arsenic trioxide can inhibit cell proliferation by reducing IL-6 secretion, promoting ROS production, restraining apoptosis, and inhibiting autophagy.

**Keywords:** Multiple myeloma, bortezomib, arsenic trioxide, apoptosis, proliferation, IL-6

## Introduction

Multiple myeloma (MM) originates from plasma cells in the bone marrow and belongs to B cell lymphomas [1]. Incidence of MM has continually increased, accounting for 10% of hematopoietic malignancies. Onset age can be observed in all age groups, but is more common in ages over 40 [2, 3]. Pathological manifestations of MM show abnormally plasma cells clone and proliferation. A large amount of monoclonal protein (M protein) can be found in the blood or urine. Tumor cells can infiltrate multiple tissues or organs, thereby causing various clinical symptoms [4, 5]. MM has a slow onset, without clinical symptoms in early stages. Subsequent manifestations include multiple osteolytic lesions, infections, nervous system symptoms, hypercalcemia, amyloidosis, anemia, hemorrhaging, and kidney damage [6, 7]. The etiology and pathogenesis of MM is complicated and has not been fully elucidated. It has been

suggested that the pathogenesis of MM is caused by multiple genes and multiple steps [8]. As a refractory malignant tumor, in addition to symptomatic treatment, MM is mainly treated with traditional chemotherapy drugs, hematopoietic stem cell transplantation, and immunomodulatory therapy. However, there are many related problems, including side effects of drugs and poor tolerance. Therefore, MM is still defined as a disease that cannot be completely cured [9, 10].

Recent studies have found that emerging targeted therapeutic proteasome inhibitors, such as bortezomib, for treatment of MM have become a new and important method [11, 12]. However, the mechanisms of treatment with bortezomib have not been clarified. There are still some MM patients suffering from drug resistance and recurrence [13, 14]. Arsenic trioxide (ATO), also known as  $As_2O_3$ , has shown great progress in the treatment of malignant

tumors, including leukemia [15, 16]. However, the roles of  $As_2O_3$  in MM have been rarely reported. It is very important to find an effective treatment method or combination treatment plan that improves clinical efficacy. Therefore, this study aimed to analyze the effects of bortezomib combined with  $As_2O_3$  on MM, examining the related mechanisms.

### Materials and methods

#### *Main reagents and instruments*

MM cell line OPM-2 was preserved in liquid nitrogen at the laboratory. RPMI medium, fetal bovine serum (FBS), and penicillin-streptomycin were purchased from Hyclone. Dimethyl sulfoxide (DMSO) and MTT powder were purchased from Gibco. Trypsin-EDTA was purchased from Sigma. Bortezomib and  $As_2O_3$  were purchased from Sigma. IL-6 ELISA kit was purchased from R&D. PVDF membranes were purchased from Pall Life Sciences. Western blotting related chemical reagents were purchased from Beyotime. ECL reagent was purchased from Amersham Biosciences. Rabbit anti-human Beclin-1 monoclonal antibodies and goat anti-rabbit horseradish peroxidase (HRP) labeled IgG secondary antibodies were purchased from Cell signaling. ROS activity detection kit was purchased from Shanghai Biyuntian Biotechnology Co., Ltd. The Caspase 3 active kit was purchased from Boster. LabSystem Version 1.3.1 microplate reader was purchased from Bio-Rad. YJ-A ultra-clean workbench was purchased from Suzhou Sutai Purification Equipment Engineering Co., Ltd. Finally, the DxFLEX flow cytometer was purchased from Beckman Coulter.

#### *Methods*

**OPM-2 cell grouping:** Multiple myeloma cells OPM-2, preserved in liquid nitrogen, were resuscitated. After passage, they were randomly divided into 4 groups, including the control group, bortezomib group treated with 4  $\mu$ M bortezomib for 48 hours,  $As_2O_3$  group treated with 2  $\mu$ M  $As_2O_3$  for 48 hours, and the combined group (bortezomib +  $As_2O_3$ ) treated with 4  $\mu$ M bortezomib and 2  $\mu$ M  $As_2O_3$  for 48 hours.

**MTT assays:** OPM-2 cells in the logarithmic phase were seeded in 96-well culture plates at  $5 \times 10^3$  cells/well. After 24 hours of cultivation, the supernatant was discarded and the cells

were randomly divided into 2 treatment groups, as mentioned above. After 48 hours of incubation, 20  $\mu$ l sterile MTT was added to the wells for 4 hours. DMSO (150  $\mu$ l/well) was added for 10 minutes. Absorbance (A) values were measured at a wavelength of 570 nm, calculating cell proliferation rates. The experiment was repeated at least three times.

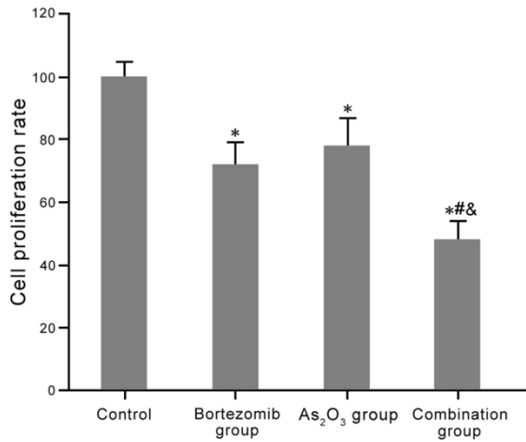
**Western blotting:** The cells were added with RIPA and lysed on ice for 30 minutes. After treatment via ultrasounds at 5 seconds for 4 times, the cells were centrifuged at 4°C and 10,000 g for 15 minutes. Isolated proteins were electrophoresed using 10% SDS-PAGE. The gel was transferred to PVDF membranes using the semi-dry transfer method at 100 mA for 1.5 hours. After blocking for 1 hour, the membranes were incubated with Notch1 primary antibody (1:2000) at 4°C overnight. After incubation with a secondary antibody (1:2000), void of light for 30 minutes, the membranes were imaged using a chemiluminescence reagent for 1 minute. They were analyzed with image processing system software and Quantity one software. The experiment was repeated four times (n=4).

**Caspase 3 activity detection:** Caspase 3 activity was examined, according to kit instructions. The cells were digested by trypsin and centrifuged at 4°C and 20,000 g for 5 minutes. Next, the cells were lysed on ice for 15 minutes and centrifuge at 5°C and 20,000 g for 5 minutes. Finally, the cells were added with 2 mM Ac-DEVD-pNA and measured at 405 nm, calculating Caspase 3 activity levels.

**Cell apoptosis assays:** The cells were digested and washed with pre-cooled PBS. They were then centrifuged at 1,000 rpm for 5 minutes and fixed with 75% pre-cooled ethanol at 4°C overnight. Next, the cells were resuspended in 800  $\mu$ l 1 $\times$ PBS and 1% BSA mixture. After incubating in 100  $\mu$ g/mL PI solution (3.8% Sodium Citrate, pH 7.0), the cells were added with 100 RNase (RnaseA, 10 mg/mL) at 37°C, void of light for 30 minutes. After being added with 300  $\mu$ l 1 $\times$  binding buffer, the cells were tested via flow cytometry.

**ELISA:** IL-6 expression in the supernatant of each group was detected using the ELISA method. The 96-well plates were added with 50  $\mu$ l sequentially diluted standard to the corresponding reaction wells. Moreover, 50  $\mu$ l sam-

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**Figure 1.** Effects of bortezomib combined with As<sub>2</sub>O<sub>3</sub> on OPM-2 cell proliferation. \*P<0.05, compared with control; #P<0.05, compared with bortezomib group; &P<0.05, compared with As<sub>2</sub>O<sub>3</sub> group.

ples were added to the reaction well. After being added with the corresponding reagent at 37°C for 10 minutes, the plates were treated with 50 µl stop solution. OD values of each well were measured using a microplate reader. A standard curve was prepared according to OD values. Corresponding sample concentrations were calculated.

**ROS detection:** For ROS detection, 2', 7'-Dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA) probes were employed. HAECs were incubated with H<sub>2</sub>DCFHDA for 30 minutes at 37°C. Fluorescence intensity levels were immediately measured using FACS Calibur (BD, USA), equipped with an argon ion laser (488 nm excitation), with 20,000 cells per sample measured.

### Statistical analysis

Measurement data were presented as mean ± standard deviation and were compared using t-tests or one-way ANOVA. All data analyses were performed with SPSS 11.5 software. Enumeration data were compared using  $\chi^2$  tests. Pearson's correlation analysis was also conducted. P<0.05 indicates statistical differences.

## Results

### Effects of bortezomib combined with As<sub>2</sub>O<sub>3</sub> on OPM-2 cell proliferation

MTT assays were adopted to test the effects of bortezomib and/or As<sub>2</sub>O<sub>3</sub> on OPM-2 cell prolifer-

ation. Bortezomib and As<sub>2</sub>O<sub>3</sub> treatment, alone, significantly inhibited cell proliferation (P<0.05). The combination of bortezomib and As<sub>2</sub>O<sub>3</sub> exhibited more obvious effects, compared with bortezomib or As<sub>2</sub>O<sub>3</sub> alone (P<0.05) (**Figure 1**).

### Influence of bortezomib combined with As<sub>2</sub>O<sub>3</sub> on OPM-2 cell apoptosis

Flow cytometry was selected to evaluate the influence of bortezomib combined with As<sub>2</sub>O<sub>3</sub> on OPM-2 cell apoptosis. Bortezomib and As<sub>2</sub>O<sub>3</sub> treatment, alone, markedly promoted cell apoptosis (P<0.05). The combination of bortezomib and As<sub>2</sub>O<sub>3</sub> exhibited more obvious effects, compared with bortezomib or As<sub>2</sub>O<sub>3</sub> alone (P<0.05) (**Figure 2**).

### Impact of bortezomib combined with As<sub>2</sub>O<sub>3</sub> on the caspase 3 activity in OPM-2 cells

Bortezomib and As<sub>2</sub>O<sub>3</sub> treatment, alone, apparently enhanced caspase 3 activity (P<0.05). The combination of bortezomib and As<sub>2</sub>O<sub>3</sub> demonstrated more significant effects, compared with bortezomib or As<sub>2</sub>O<sub>3</sub> alone (P<0.05) (**Figure 3**).

### Effects of bortezomib combined with As<sub>2</sub>O<sub>3</sub> on IL-6 secretion of OPM-2 cells

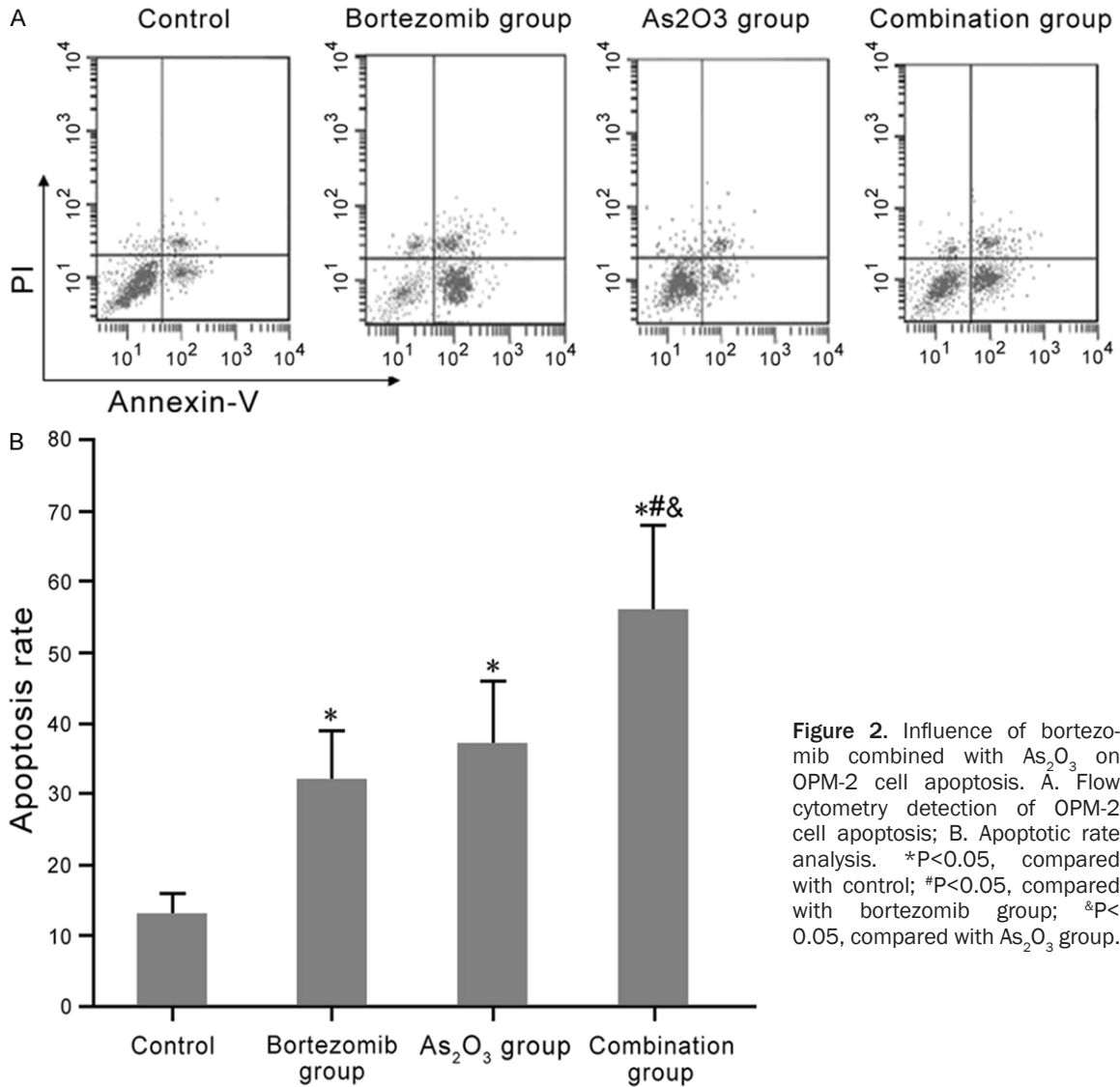
Bortezomib and As<sub>2</sub>O<sub>3</sub> treatment, alone, significantly reduced IL-6 secretion (P<0.05). The combination of bortezomib and As<sub>2</sub>O<sub>3</sub> revealed more significant effects, compared with bortezomib or As<sub>2</sub>O<sub>3</sub> alone (P<0.05) (**Figure 4**).

### Impact of bortezomib combined with As<sub>2</sub>O<sub>3</sub> on ROS production in OPM-2 cells

Bortezomib and As<sub>2</sub>O<sub>3</sub> treatment, alone, obviously enhanced ROS production (P<0.05). The combination of bortezomib and As<sub>2</sub>O<sub>3</sub> showed more significant effects, compared with bortezomib or As<sub>2</sub>O<sub>3</sub> alone (P<0.05) (**Figure 5**).

### Influence of bortezomib combined with As<sub>2</sub>O<sub>3</sub> on beclin-1 expression in OPM-2 cells

Bortezomib and As<sub>2</sub>O<sub>3</sub> treatment, alone, obviously downregulated beclin-1 expression in OPM-2 cells (P<0.05). The combination of bortezomib and As<sub>2</sub>O<sub>3</sub> exhibited more significant effects, compared with bortezomib or As<sub>2</sub>O<sub>3</sub> alone (P<0.05) (**Figure 6**).



**Figure 2.** Influence of bortezomib combined with As<sub>2</sub>O<sub>3</sub> on OPM-2 cell apoptosis. A. Flow cytometry detection of OPM-2 cell apoptosis; B. Apoptotic rate analysis. \*P<0.05, compared with control; #P<0.05, compared with bortezomib group; &P<0.05, compared with As<sub>2</sub>O<sub>3</sub> group.

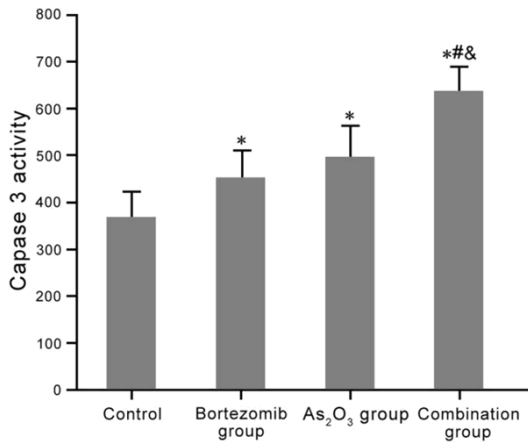
**Discussion**

Proteasome inhibitors have become a novel direction in the treatment of MM. Bortezomib acts as a reversible inhibitor with 26S proteasome protease-like activity in mammalian cells. *In vitro* experiments have demonstrated that bortezomib shows cytotoxicity in various types of leukemia and liver cancer. Thus, it can be used to treat MM that was untreated or MM that is unsuitable for high-dose chemotherapy. It was also adopted to treat relapsed MM patients [17, 18]. However, bortezomib can cause adverse effects, including heart vascular reactions, diffuse intravascular coagulation (DIC), gastrointestinal reactions, and liver and kidney damage. Moreover, it has been associ-

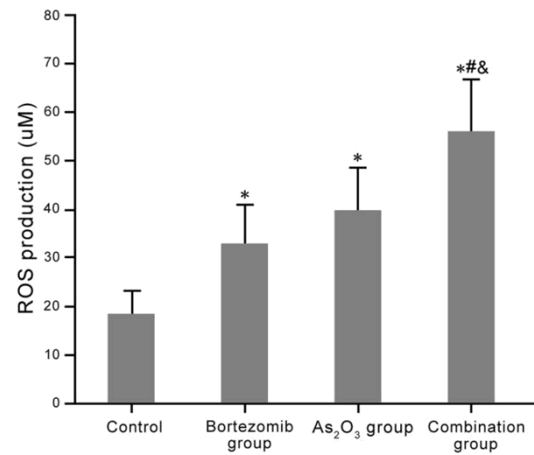
ated with MM resistance and recurrence [19]. As<sub>2</sub>O<sub>3</sub> has been proven to treat MM, but with unclear mechanisms [20]. The roles and related mechanisms of bortezomib in combination with As<sub>2</sub>O<sub>3</sub> for treatment of MM have not been elucidated. Therefore, this study first analyzed the roles of bortezomib combined with As<sub>2</sub>O<sub>3</sub> in MM tumor cells. It was observed that bortezomib and As<sub>2</sub>O<sub>3</sub> treatment, alone, significantly inhibited cell proliferation, promoted cell apoptosis, and increased caspase 3 activity. The combination of bortezomib and As<sub>2</sub>O<sub>3</sub> exhibited more significant effects, however, compared with bortezomib or As<sub>2</sub>O<sub>3</sub> alone.

Caspase 3 activity plays an important role in apoptosis by regulating DNA repair [21]. Au-

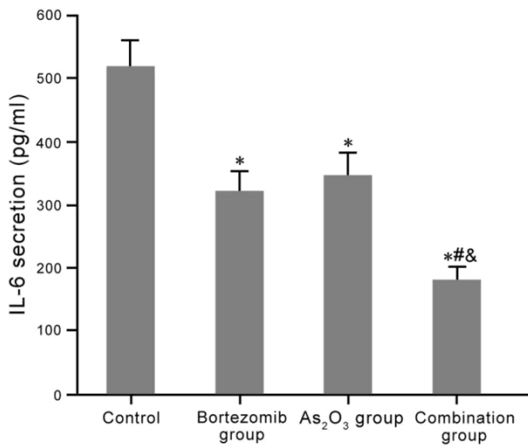
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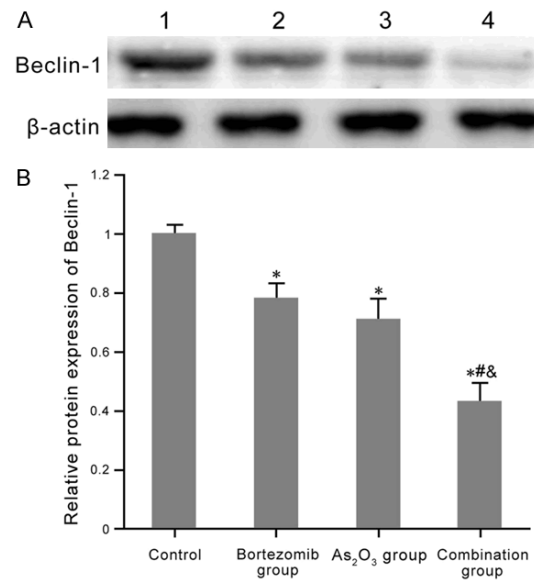
**Figure 3.** Impact of bortezomib combined with As<sub>2</sub>O<sub>3</sub> on caspase 3 activity in OPM-2 cells. \*P<0.05, compared with control; #P<0.05, compared with bortezomib group; &P<0.05, compared with As<sub>2</sub>O<sub>3</sub> group.



**Figure 5.** Impact of bortezomib combined with As<sub>2</sub>O<sub>3</sub> on ROS production in OPM-2 cells. \*P<0.05, compared with control; #P<0.05, compared with bortezomib group; &P<0.05, compared with As<sub>2</sub>O<sub>3</sub> group.



**Figure 4.** Effects of bortezomib combined with As<sub>2</sub>O<sub>3</sub> on IL-6 secretion of OPM-2 cells. \*P<0.05, compared with control; #P<0.05, compared with bortezomib group; &P<0.05, compared with As<sub>2</sub>O<sub>3</sub> group.



**Figure 6.** Influence of bortezomib combined with As<sub>2</sub>O<sub>3</sub> on beclin-1 expression in OPM-2 cells. A. Western blot detection of beclin-1 expression in OPM-2 cells. 1, control, 2, bortezomib group, 3, As<sub>2</sub>O<sub>3</sub> group, 4, combination group. B. Beclin-1 protein expression analysis. \*P<0.05, compared with control; #P<0.05, compared with bortezomib group; &P<0.05, compared with As<sub>2</sub>O<sub>3</sub> group.

tophagy is an important process of intracellular catabolism in the evolution of conserved eukaryotes. In the process of tumor formation, autophagy provides more abundant nutrition for cancer cells, promoting tumor growth. Autophagy protein beclin-1 can reflect the degree of autophagy in cells [22, 23]. The current study confirmed that bortezomib and As<sub>2</sub>O<sub>3</sub> treatment, alone, significantly downregulated beclin-1 expression. The combination of bortezomib and As<sub>2</sub>O<sub>3</sub> revealed more significant effects, compared with bortezomib or As<sub>2</sub>O<sub>3</sub> alone. A key factor in regulating MM, IL-6 can stimulate the proliferation of MM cells. Production of ROS during cell survival can enhance the toxic-

ity of As<sub>2</sub>O<sub>3</sub> to tumor cells [24, 25]. Furthermore, this study confirmed, for the first time, that bortezomib and As<sub>2</sub>O<sub>3</sub> treatment, alone, obviously reduced IL-6 secretion and enhanced ROS production. The combination of bortezomib and As<sub>2</sub>O<sub>3</sub> exhibited more significant effects, however, compared with bortezomib or



As<sub>2</sub>O<sub>3</sub> alone. Therefore, results suggest that the combination of bortezomib and As<sub>2</sub>O<sub>3</sub> can inhibit the secretion of IL-6 and promote the production of ROS, thereby inhibiting proliferation of MM tumor cells. The current study aimed to analyze the efficacy and specific mechanisms of the combination of bortezomib and arsenic trioxide through animal experimentation and clinical studies.

### Conclusion

Bortezomib combined with arsenic trioxide can inhibit cell proliferation by reducing IL-6 secretion, promoting ROS production, restraining apoptosis, and inhibiting autophagy.

### Disclosure of conflict of interest

None.

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