

## RESEARCH ARTICLE

# Effects of Arsenic Trioxide Alone and in Combination with Bortezomib in Multiple Myeloma RPMI 8266 Cells

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### Abstract

The aim of this study was to detect the efficiency of arsenic trioxide (ATO) alone or together with bortezomib to inhibit proliferation and induce apoptosis in a multiple myeloma (MM) RPMI 8266 cells. Mechanisms of action were also investigated. RPMI 8266 cells were treated with ATO alone and in combination with bortezomib for 24 hours, and cell viability was assessed by modified MTT. Annexin V-FITC and PI staining was used to detect the apoptosis rate and cell cycling was investigated by flow cytometry, along with expression of cell surface death receptor-4 (DR4) and death receptor-5 (DR5). Western blotting was applied to detect the expression of bcl-2, caspase-3, caspase-8, and caspase-9. As a result, the ATO combined with bortezomib group showed more inhibition of RPMI 8266 cell viability than the ATO group. Expression of DR4 and DR5 on the cell surfaces, and the apoptosis rate were increased after treatment by ATO alone or combined with bortezomib. The cells appeared to arrest in G2/M phase after treatment. Expression of bcl-2 was more significantly decreased in the combination group, and that of caspase-3, caspase-8 and caspase-9 was significantly increased as well. Therefore, bortezomib can enhance ATO actions to induce apoptosis in RPMI 8266 cells, with decrease in expression of bcl-2 and increase of caspase-3, caspase-8 and caspase-9 proteins.

**Keywords:** Arsenic trioxide - bortezomib - multiple myeloma - apoptosis

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### Introduction

Multiple myeloma (MM) is hematological malignant disorder characterized by proliferation and accumulation of clonal abnormal plasma cells in the bone marrow. It accounts for 1% of neoplastic diseases and 13% of hematologic cancers (Mousa et al., 2007; Palumbo and Anderson, 2011; Sanaat et al., 2011). MM is an incurable plasma cell malignancy by far. Drug resistance and relapse is the main reason for its refractory feature (Badros et al., 2005; Hou et al., 2005; Morales et al., 2008).

Arsenic trioxide (ATO) is an effective chemotherapeutic agent in treating some kind of tumors including MM (Wang et al., 2008; Yedjou et al., 2010). The mechanisms of action derivatives in this disease and other malignancies include induction of apoptosis, inhibition of proliferation and inhibition of angiogenesis (Mousa et al., 2007). Moreover, ATO as a single agent induced clinical responses in 21-33% of patients with relapsed/refractory MM. Bortezomib, a highly selective, reversible inhibitor of the 26S subunit of the proteasome complex, offers 50% of responses in refractory/relapsed patients affected by MM (Canestraro et al., 2010).

Although the two drugs showed effective in MM, there

is a high percentage of patients resistant to each drug. ATO and bortezomib have different mechanism in induction of apoptosis and inhibition of proliferation. Therefore, with the purpose of evaluating whether the combination of ATO and bortezomib would achieve attractive perspective results, the MM cell line, in this study, RPMI 8266 cells were treated with ATO alone and in combination with bortezomib for evaluating a possible synergistic effect and its mechanism.

### Materials and Methods

#### *Cell culture and drug preparation*

MM cell line RPMI 8266 cells were cultured in 25 cm<sup>2</sup> tissue culture flask under standard culture medium RPMI-1640 (GiBco, USA) supplemented with 10% fetal calf serum (FCS) (Beijing Ding States Biological Technology Co., Ltd, China). The medium was changed twice a week. ATO (Harbin Yida Pharmaceutical Co., Ltd, China), packaged and stored at room temperature before being used to the required concentration, was diluted with normal saline to 1000 µmol/L solution. Bortezomib (Millennium, USA) stored at -20°C before being used to the required concentration was diluted with saline to 1000 nmol/L.

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### Cell viability assay

The effect of the drugs on RPMI 8266 cell viability was assessed by modified MTT assay (purchased from Beijing Ding States Biological Technology Co., Ltd. China). The cells were seeded into three 96-well tissue culture plates at a density of  $5 \times 10^3$  cells/well, and treated with ATO alone (0.1, 1, 5, 10, 50  $\mu\text{mol/L}$ ), bortezomib alone (0.1, 1, 5, 10, 50  $\text{nmol/L}$ ) and combination of the two drugs, 10  $\text{nmol/L}$  of bortezomib was added to each concentration of ATO and each group in a plate should be in a final volume of 100  $\mu\text{l}$  culture medium, and incubated for 24 hours. For MTT assay, 10  $\mu\text{l}$  (5  $\text{mg/ml}$ ) MTT solution was added to each well and incubated for 4 hours. 100  $\mu\text{l}$  of the solution was added into each well and incubated overnight at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ . The absorbance was measured in microplate reader (BIO-TEK Inc) at wave length 570  $\text{nm}$ . Each experiment was repeated in triplicate format, and results were expressed as mean  $\pm$ SD.

### Apoptosis detection by annexin V-FITC/PI

Apoptosis was also evaluated by Annexin V-FITC and propidium iodide (PI) kit (purchased from Beyotime Institute of Biotechnology - China). Briefly,  $7 \times 10^5$  cells/well were seeded in 6-well plates and treated with ATO alone (1, 10, 50  $\mu\text{mol/L}$ ), bortezomib alone (1, 10, 50  $\text{nmol/L}$ ) and combination of the two drugs, 10  $\text{nmol/L}$  of bortezomib was added to each concentration of ATO and incubated at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  in humidified incubator for 24 hours. The cell was centrifuged for 5 minutes, supernatant was discarded and the cells was washed with PBS one time. 195  $\mu\text{l}$  Annexin V-FITC liquid and 5  $\mu\text{l}$  Annexin V-FITC were added gently to the suspended cells, mixed gently and incubated in room temperature (20-25  $^\circ\text{C}$ ) at dark for 10 minutes (Aluminum foil can be used to avoid light). Centrifugation for 5 minutes, supernatant was discarded. 190  $\mu\text{l}$  Annexin V-FITC liquid was added gently to the suspended cells. 10  $\mu\text{l}$  of propidium iodide staining solution was added, gently mixed and incubated in ice bath at dark. Samples were analyzed by flow cytometry to determine the percentage of cells displaying annexin V positive/PI-negative (early apoptosis) or annexin V positive/PI-positive staining (late apoptosis and cell death).

### Cell cycle detection

Cell cycle distribution was determined by staining DNA with PI using Cell cycle and apoptosis detection kit (purchased from Beyotime Institute of Biotechnology, China). The RPMI 8266 cells were seeded into 6-well tissue culture plates at a density of  $7 \times 10^5$  cells/well and divided into three group, ATO group (final concentration was 5.0  $\mu\text{mol/L}$ ), bortezomib group (final concentration was 10  $\text{nmol/L}$ ) and Combination group (final concentration was 5  $\mu\text{mol/L}$ +10  $\text{nmol/L}$ ). All groups were incubated at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  in humidified incubator for 24 hours. The cells were centrifuged for 5 minutes. Supernatant was discarded, and the cells were washed with pre-cooled PBS one time and transferred into 1.5 ml centrifuge tubes and fixed by 1ml ice-bathed pre-cooled 70% ethanol, mixed gently and incubated at  $4^\circ\text{C}$  for 12 hours, then the tubes were centrifuged for 5

minutes in 1000g, the supernatant discarded carefully and the cells were washed with pre-cooled PBS. 0.5 ml of propidium iodide staining solution was added to each tube of cell samples and incubated in dark at  $37^\circ\text{C}$  for 30 minutes. Flow cytometry was used to detect the red fluorescence, excitation wavelength at 488  $\text{nm}$  and simultaneous detection of light scattering to analysis the cell cycle.

### Death receptor detection by flow cytometry

Cell surface expression of DR4 and DR5 on RPMI 8266 cell was measured by flow cytometry.  $1 \times 10^6$  cells/well was seeded in 6-well plates, was divided into ATO group (final concentration for 5.0  $\mu\text{mol/L}$ ), bortezomib group (final concentration as 10  $\text{nmol/L}$ ), combination group (final concentration as 5  $\mu\text{mol/L}$ +10  $\text{nmol/L}$ ) and the control group (add the same volume of PBS) and incubated for 24 h. Washed twice with ice cold PBS and incubated for 30 min at  $4^\circ\text{C}$  with primary anti-human-DR4 (mouse IgG1) or anti-human-DR5 (mouse IgG1) monoclonal antibodies (Santa Cruz Company, USA), cells were washed with ice-cold PBS and incubated with FITC-labeled goat anti-mouse secondary antibodies for 30 min at  $4^\circ\text{C}$  in darkness. Sample was analyzed with a FACScan flow cytometer and CellQuest software (Beckton Dickinson, USA). Each experiment was repeated three times.

### Western blot analysis

$1 \times 10^7$  of RPMI 8266 cells were treated with ATO (5.0  $\mu\text{mol/L}$ ), bortezomib (10  $\text{nmol/L}$ ) and combination (5  $\mu\text{mol/L}$ +10  $\text{nmol/L}$ ) for 24 hours. Treated cells were lysed in buffer composed of RIPA (50  $\text{mM}$  Tris-pH 7.4, 150  $\text{mM}$  NaCl, 1% NP-40, 0.5 sodium deoxycholate, 0.1% SDS) and PMSF in ratio of (PMSF: RIPA 1:100) (Beyotime Institute of Biotechnology, China). Total protein concentration in the supernatant was determined using a bicinchoninic acid assay (Beyotime Biotechnology, China). Proteins were normalized for all concentrations to 50  $\mu\text{g}$  per lane and was electrophoresed in 12.5% polyacrylamide gels, and transferred onto PVDF membrane (Beijing Ding States Biological Technology Co., Ltd. China). The membrane with transferred proteins was blocked for 1 h by incubation in 5% dry skimmed milk in TBST (0.1% Tween-20 in Tris buffered saline). The membranes were incubated with the indicated primary antibody Bcl-2 monoclonal antibody, Caspase-3, Caspase-8, Caspase-9 and  $\beta$ -actin (Beyotime Institute of Biotechnology, China) overnight at  $4^\circ\text{C}$ , washed with TBS (pH 6.8) and Tween 20 (TBS-T) three times, incubated with the secondary antibody (Beyotime Institute of Biotechnology, China) for 1 hour at room temperature, and washed with TBS-T three times. The immunocomplex was visualized by enhanced chemiluminescence western blotting detection reagents.

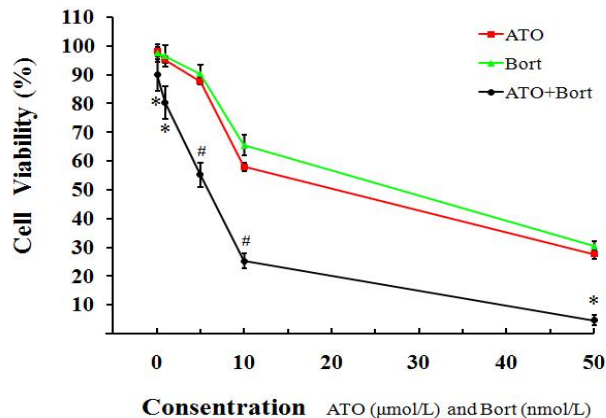
### Statistical analysis

Each experimental value was expressed as mean  $\pm$  standard deviation (SD), analyzed by using Origin7.0 software. The significance of differences between groups was considered as  $P \leq 0.05$ . All data represented the mean of triplicates.

## Results

### Cell viability was decreased by treatment with ATO or ATO combined with bortezomib

MTT assay was performed to determine whether bortezomib enhanced ATO to inhibit the proliferation of RPMI 8266 cell. The result showed that the cell survival decreased gradually with the increase of drug concentration, in ATO group the cell viability was decreased to (98.2±1.58)%, (95.3±1.24)%, (87.8±1.10)%, (58.0±1.43)% and (27.7±1.71)%, due to the increase of drug concentration at 0.1, 1, 5, 10 and 50 µmol/L, respectively and the half maximal inhibitory concentration

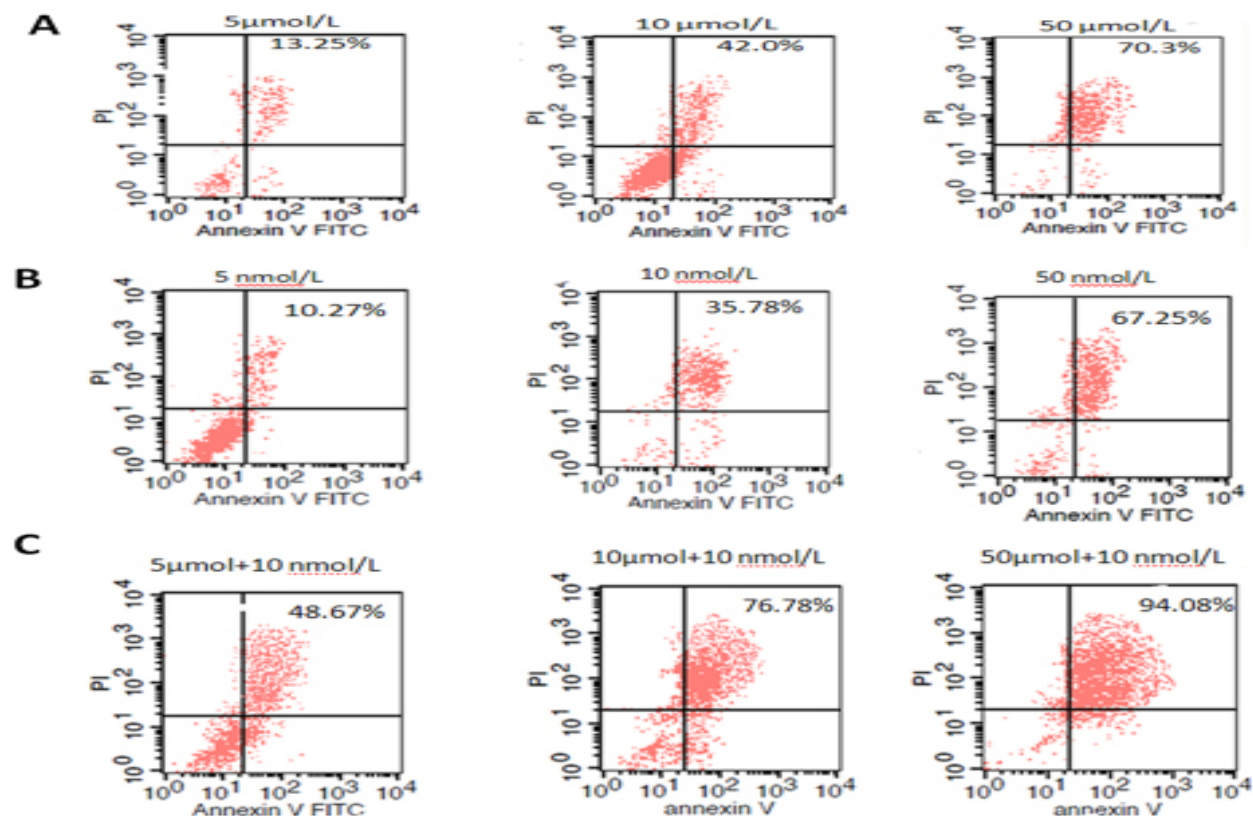


**Figure 1. Cell Viability in ATO, Bortezomib and Combined Group.** The result showed that the cell survival decreased gradually with the increase of drug concentration, in ATO, bortezomib and combined group (\* $P < 0.05$ , # $P < 0.001$ )

(IC50) for ATO was 18.6 µmol/L. In bortezomib group, the cell viability was also decreased to (97.6±2.94)%, (96.6±3.85)%, (90.4±3.12)%, (65.6±3.50)% and (30.5±1.56)% with increase of drug concentration at 0.1, 1, 5, 10 and 50 nmol/L, respectively and IC50 for bortezomib was 28 nmol/L and in combination group the cell viability was decreased to (90.1±5.56)%, (80.3±5.71)%, (55.3±4.24)%, (25.3±2.66)% and (4.7±1.88)% with increase of drug concentration, respectively, as shown in Figure 1.

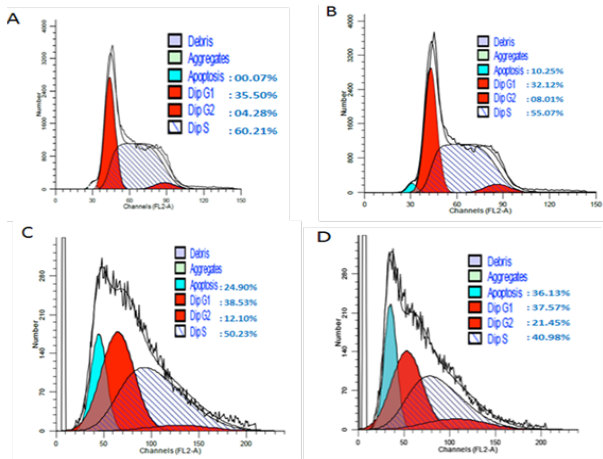
### Cell apoptosis was increased by treatment with ATO or ATO combined with bortezomib

The cells undergoing apoptosis were detected by Flow cytometry analysis using Annexin V and PI staining. Compared to the negative control, the apoptosis rates of ATO group (5, 10, 50 µmol/L) and bortezomib group (5, 10, 50 nmol/L) were increased gradually according to the drug concentrations. Figure 2.A showed the results of ATO group at different concentration, (13.25±1.60)%, (42.0±3.24)% and (70.3±4.21)% VS control (2.48±0.47)%. Figure 2B showed the results of bortezomib group at different concentration, (10.27±2.14)%, (35.78±3.68)%, and (67.25±3.20)% VS control (2.48±0.47)%. And in combination group (10 nmol/L of bortezomib was added to each concentration of ATO), the apoptosis rates of each concentration in the combination group were relatively higher than single treatment group (48.67±3.89, 76.78±4.65 and 94.08±4.32%, respectively) as shown in Figure 2C. This indicated that the combination can further increase the apoptosis induced by ATO and bortezomib.

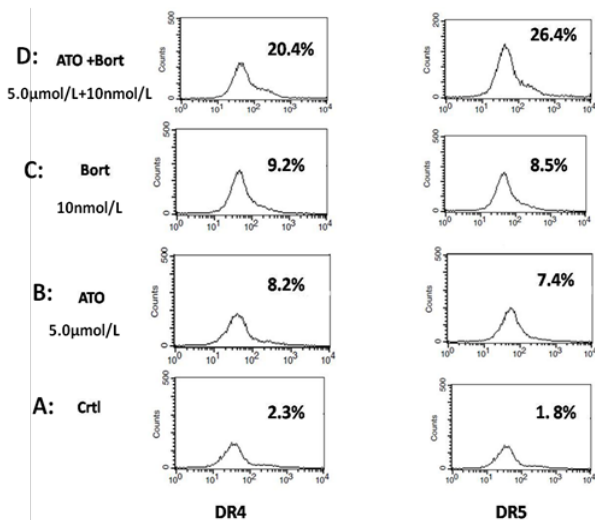


**Figure 2. The Apoptosis of RPMI 8266 Cells in Different treated Groups.** A: ATO group, B: Bortezomib group, C: ATO+Bortezomib group. Cells in the lower right quadrant (LR) represented early apoptosis and in the upper right quadrant (UR) represented the late apoptosis





**Figure 3. The Effect of ATO, Bortezomib and Combination of the Two Drugs on Cell Cycle Distribution of RPMI 8266 Cells by Flow Cytometry.** A: control, B: ATO group, C: bortezomib group, D: combination group. The values indicated the percentage of cells in the indicated phases of the cell cycle



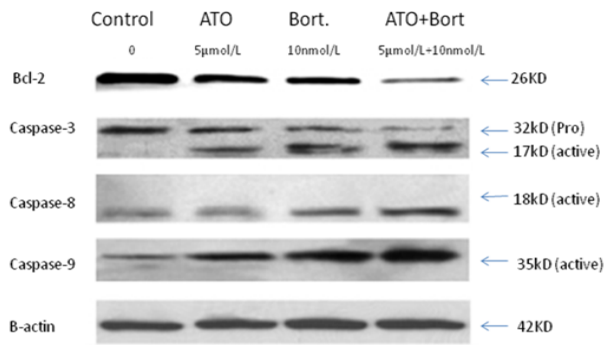
**Figure 4. The Effect of ATO, Bortezomib and Combination of the Combined Drugs on Expression of DR4 and DR5 on RPMI 8266 Cells.** A: Control without treatment, B: ATO group, C: bortezomib group, D: combination group

*Cell cycle was arrested at G2/M phase in treated groups*

Compared to control group and the treated groups of ATO and bortezomib showed increased number of cells at the G2/M phase, but induced a significant decrease in the number of cells at S phase. And in combination of ATO and bortezomib, the ratio of apoptosis and G2/M phase was relatively higher than single treatment group. It indicated that the RPMI 8266 cells were arrested further at G2/M phase in the combination group as shown in Figure 3.

*The expression of death receptor increased in treated groups*

Cell surface expression of DR4 and DR5 on RPMI 8266 cell was measured by flow cytometry. RPMI 8266 cells were treated with single-agent ATO (5.0 μmol/L), bortezomib (10 nmol/L), and combination of the two drugs (5 μmol/L ATO+10 nmol/L bortezomib). Figure 4



**Figure 5. The Expression of bcl-2, Caspase-3, Caspase-8 and Caspase-9 Proteins in Different Groups by Western Blot.** Control group without treatment, ATO group treated by 5.0 μmol/L ATO, bortezomib group treated by 10 nmol/L bortezomib, combined group treated by 5.0 μmol/L ATO and 10 nmol/L bortezomib. β-actin was used as loading control

showed the changes in the expression of DR4 and DR5. Compared with the control group, the change in the combination group increased more significantly than in the single group ( $P < 0.01$ ).

*The expression of Bcl-2, caspase-3, caspase-8 and caspase-9 in treated groups*

The result showed that both ATO and bortezomib induced down-regulation of Bcl-2 expression and up-regulation of caspase-3, caspase-8 and caspase-9 activation. More increased expression of caspase-3, caspase-8 and caspase-9 proteins and more decreased expression of Bcl-2 protein in combination group as shown in Figure 5.

**Discussion**

ATO as a single agent or bortezomib offers response in refractory/relapsed patients with MM (Canestraro et al., 2010). However, resistance to each drug is still a big issue. We postulated that ATO and bortezomib have different mechanisms, and the combination of the two drugs will have synergistic effects.

In this study, the results showed that ATO and bortezomib have direct effects on MM cell line RPMI 8266 cells. It revealed that ATO and bortezomib inhibited the proliferation, promoted apoptosis of cells and that the combination group showed synergistic effects. Moreover, flow cytometric analysis showed that as compared with the control group, arrested cell cycle were arrested at G2/M phase in all the treated groups, and the ratio of G2/M phase increased gradually and reduced S-phase cells with the increase of the drug concentration. And the combination of the two drugs enhanced these effects.

The mechanism involved in the apoptosis was also investigated in this study. The apoptosis was induced mainly through the two major pathways, intrinsic (mitochondrial) pathway and the (death-receptor) extrinsic pathway. Where the death receptor was initiated by ligation of transmembrane death receptors including tumor necrosis factor (TNF) family receptors and their ligands, and activated caspase-8. Intrinsic pathway included the activation of the release of mitochondrial constituents and

activation of caspase-9. Then, the two pathways activated caspase-3 and induced apoptosis (Johnstone et al., 2002; Tardy et al., 2006).

The results of this study showed that, the ATO and bortezomib induced DR4, DR5 up-regulation on RPMI 8266 cells, and the synergy raised to increase TRAIL receptor binding probability, in turn increase the activity of the TRAIL to promote apoptosis.

For the intrinsic pathway, we focused on detection of Bcl-2, active caspase-3 and 9 proteins, because Bcl-2 family proteins regulate mitochondria dependent apoptosis with the balance of the anti- and proapoptotic members (Canestraro et al., 2010). The release of cytochrome c and AIF is controlled by Bcl-2 family proteins, Bcl-2 and Bcl-xL inhibit the release of cytochrome c and AIF, whereas the release of cytochrome c and AIF enhanced by Bax (Gupta et al., 2003). It was shown that both ATO and bortezomib can induce apoptosis. They induced apoptosis by down-regulation of Bcl-2 gene expression and up-regulation of caspase-9 and caspase-3 as shown in figure 5.

In conclusion, ATO and bortezomib induced apoptosis in RPMI 8266 cells and the mechanism related to decrease of bcl-2 protein (endogenous apoptotic signaling pathway) and they also induced apoptosis through the mechanism related to the change of DR4 and DR5 (extrinsic apoptotic pathway). ATO and bortezomib have synergistic inhibition and apoptosis in RPMI 8266 cells through the two pathways. The combination of ATO and bortezomib provided a novel strategy for the treatment of MM. And it is worthwhile to investigate in the clinical settings.

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