

Original Article

Diallyl trisulfide inhibits proliferation and promotes apoptosis of side population cells in multiple myeloma cells

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Abstract: Multiple myeloma (MM) is an incurable cancer, in which the main factors leading to death are relapse and the development of resistance to drugs. Both of these are associated with the existence of side population (SP) cells, which have features similar to those of tumor stem cells. The development of new safe agents, possibly from natural products, is necessary to more efficiently treat SP cells involved in MM. Diallyl trisulfide (DATS), a natural organosulfur compound isolated from garlic, has been shown to inhibit the occurrence and development of tumors and cancer stem cells and enhance chemosensitivity. However, the effect of DATS on MM proliferation and the biological behavior of SP cells remain unclear. Therefore, in this study, human MM RPMI-8226 and NCI-H929 cell lines were treated with different concentrations of DATS (2, 5, 10, 20, 40, or 80 μ M) for 1, 2, or 3 days and the half-maximal inhibitory concentration (IC_{50}) was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In addition, SP RPMI-8226 and NCI-H929 cells were isolated and analyzed using flow cytometry. The effects of dimethyl sulfoxide (DMSO), DATS, and bortezomib on the percentage of MM SP cells, survival rate, colony formation, cell cycle, and cell apoptosis were analyzed using an MTT assay, a colony assay, and flow cytometry analysis. DATS displayed a dose- and time-dependent effect on the survival rate of RPMI-8226 and NCI-H929 cells. DATS and bortezomib treatment reduced the percentage of MM SP cells and the SP cell survival rate and inhibited colony formation and the cell cycle. In addition, DATS and bortezomib treatment promoted SP cell apoptosis. In conclusion, DATS inhibited the proliferation and promoted the apoptosis of SP MM cells.

Keywords: Multiple myeloma, diallyl trisulfide, bortezomib, side population cells

Introduction

Multiple myeloma (MM), a type of cancer that affects the plasma cells in the bone marrow, is the second most common hematological cancer worldwide [1]. The number of new MM cases is 6.3 per 100000 persons per year and the mortality rate is 3.3 deaths per 100000 persons per year [2]. Currently, MM is treated with chemotherapy and radiation, which have improved the survival rate of patients with MM. However, the disease remains incurable and has a 5-year survival rate of approximately 47% [3]. Tumor relapse and drug resistance are the main factors leading to death in patients with MM. In addition, tumor relapse

and drug resistance are associated with the existence of tumor stem cells. Side population (SP) cells have features similar to those of cancer stem cells and have been identified in MM using flow cytometry along with Hoechst-33342 staining [4-6]. In MM, SP cells have important pathophysiological and clinical implications. Therefore, inhibiting the proliferation and promoting the apoptosis of SP cells could reduce drug resistance and cancer recurrence, thereby prolonging the tumor-free interval in patients and improving survival rates [7-10]. Bortezomib, a proteasome inhibitor, reduced SP cell numbers by suppressing proliferation through inhibition of the cell cycle. However, the drug has obvious toxic and side effects

[11]. Therefore, the development of new safer agents, possibly from natural products, is necessary for the efficient treatment of SP cells in MM.

Diallyl trisulfide (DATS), a natural organosulfur compound isolated from garlic, has been widely used in China for more than a thousand years [12]. Recorded history attests that garlic was used to stimulate the immune system, prevent or treat various diseases, and promote health [13, 14]. In numerous cancer types, DATS inhibited the occurrence and development of tumors by inhibiting cell proliferation, migration, and invasion, arresting the cell cycle, and promoting apoptosis [15, 16]. DATS also inhibited cancer stem cells and enhanced chemosensitivity [17, 18]. However, the effect of DATS on MM proliferation and the biological behavior of SP cells are still unclear.

Therefore, in this study, MM cells were treated with DATS to determine its effect on the survival rate of MM cells. In addition, SP cells were sorted from MM cells and treated with DATS to determine the effect of DATS on the biological behavior of SP cells. The results indicated that DATS inhibited the survival of MM cells and decreased the survival rate, inhibited colony formation, arrested the cell cycle, and promoted the apoptosis of SP cells.

Materials and methods

Cell culture

Human MM Roswell Park Memorial Institute (RPMI)-8226 and NCI-H929 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mmol/l L-glutamine (Gibco, Carlsbad, CA, USA). All cells were maintained in a humidified incubator in a 5% CO₂ atmosphere at 37°C.

Cell viability assay

Cell viability was detected using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's protocol (Promega, Madison, WI, USA). Briefly, RPMI-8226 and NCI-H929 cells (5×10³ cells/ml) were treated with various concentrations of DATS (Grade: reagent grade (RG); purity 98%;

Chromadex, Irvine, CA, USA) and incubated in a 96-well plate in a final volume of 0.1 ml at 37°C for 0, 24, 48, and 72 h. Thereafter, 10 µl of MTT reagent was added to each well and the plate was incubated for 4 h at 37°C. The absorbance was measured using a Vmax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 570 nm. Each sample was assayed in triplicate.

Groups and treatment

SP cells were treated with four drugs: DATS, bortezomib (10 nm/L, Selleck Chemicals LLC, Houston, TX, USA) as a positive control, and 0.1% dimethyl sulfoxide (DMSO) as a negative control. Cells were harvested after treatment following each assay.

Hoechst33342 fluorescent staining

SP cells were sorted from the MM RPMI-8226 and NCI-H929 cell lines using the Hoechst-33342 fluorescent staining method with flow cytometry. The RPMI-8226 and NCI-H929 cells were seeded into six-well plates, cultured for 48 h, and 10 µg/ml Hoechst33342 stain (Sigma-Aldrich, St. Louis, MO, USA) was added. The cells were further cultured for 20 min. After the culture medium containing Hoechst33342 was removed, the plates were washed with the medium thrice and sorted using flow cytometry.

Cell cycle analysis

For the cell cycle analysis, each group of cells was harvested, digested with trypsin, washed twice with PBS, and then centrifuged at 2000 rpm for 5 min to collect the cells. Thereafter, the cells were fixed with 70% precooled ethanol at 4°C overnight, digested with 200 µg/ml ribonuclease A at 37°C for 30 min, then 100 µl propidium iodide (PI) was added at 4°C in the dark for 30 min. The samples were analyzed using flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) and the data were analyzed using ModFit software.

Colony formation assay

A colony formation assay was performed as previously described [19]. Briefly, 2×10⁴ RPMI-8226 and NCI-H929 cell suspensions were resuspended in 0.8 ml growth medium containing 0.3% low-melting-temperature agarose

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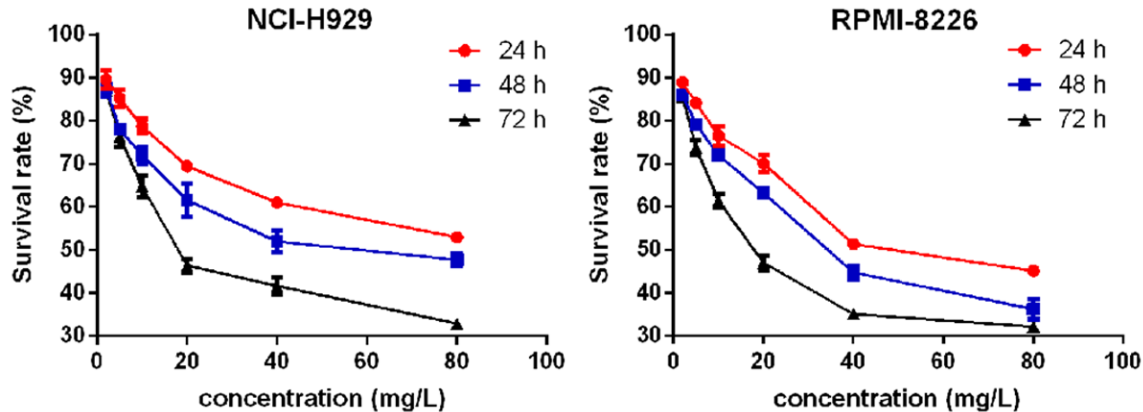


Figure 1. DATS reduced the survival rate of NCI-H929 and RPMI-8226 cells.

(Promega, Madison, WI, USA) and treated with the drugs according to their grouping. The cells were then incubated in 24-well plates in triplicate over a base layer of 0.8 ml growth medium containing 0.6% low-melting-temperature agarose. The plates were incubated for two weeks until colonies formed. The colonies were visualized and their number calculated using light microscopy (10×). The colony-forming efficiency (CFE) percentage was calculated using the following formula:

$$\text{CFE} = \frac{\text{colony number}}{\text{inoculation cell number}} \times 100\%$$

Apoptosis assay

Apoptosis was detected using an apoptosis detection kit (KeyGene, Nanjing, China). RPMI-8226 and NCI-H929 cells were collected after centrifugation at 2000 rpm for 5 min. The precipitates were digested with trypsin and washed twice with PBS. The cells were then centrifuged at 2000 rpm for 5 min and 5×10^5 cells were suspended in 500 μl of binding buffer from the detection kit. Thereafter, 5 μl each of Annexin V-fluorescein isothiocyanate (FITC) and PI were added and the cells were incubated for 15 min in the dark. The cells were then analyzed immediately using flow cytometry.

Statistical analysis

All statistical analyses were performed using the statistical package for the social sciences (SPSS) version 19.0 software (IBM Inc., USA). Continuous variables are presented as mean \pm standard deviation (SD). The differences between multiple groups were analyzed using a

one-way analysis of variance (ANOVA) followed by a post-hoc LSD test. An independent *t*-test was used to compare the differences between groups. *P*-values < 0.05 were considered statistically significant.

Results

DATS inhibited MM cell survival

The effect of DATS on the survival rate of MM cells was evaluated using an MTT assay. Treatment of RPMI-8226 and NCI-H929 cells for 1 to 3 days with 2, 5, 10, 20, 40, or 80 μM of DATS reduced the cell survival rate in a dose- and time-dependent manner (**Figure 1**). The IC_{50} values of DATS were 67.56, 44.77, and 23.35 mg/L in NCI-H929 cells at 24, 48, and 72 h, respectively. The IC_{50} values of DATS were 47.38, 36.54, and 17.92 mg/L in RPMI-8226 cells at 24, 48, and 72 h, respectively. A DATS concentration of 10 mg/L (approximate IC_{25}) was used for subsequent experiments.

Proportion of SP cells in MM cell lines

SP cells are known to promote tumor relapse and drug resistance in MM [20]. In this study, SP cells were analyzed and isolated using flow cytometry and were detected among RPMI-8226 and NCI-H929 cells at a proportion of $2.08 \pm 0.438\%$ and $1.26 \pm 0.352\%$, respectively (**Figure 2**).

DATS reduced the percentage of MM SP cells

To understand the effect of DATS on the survival rate of SP cells, the cells were analyzed

DATS inhibits proliferation of side population cells in MM cells

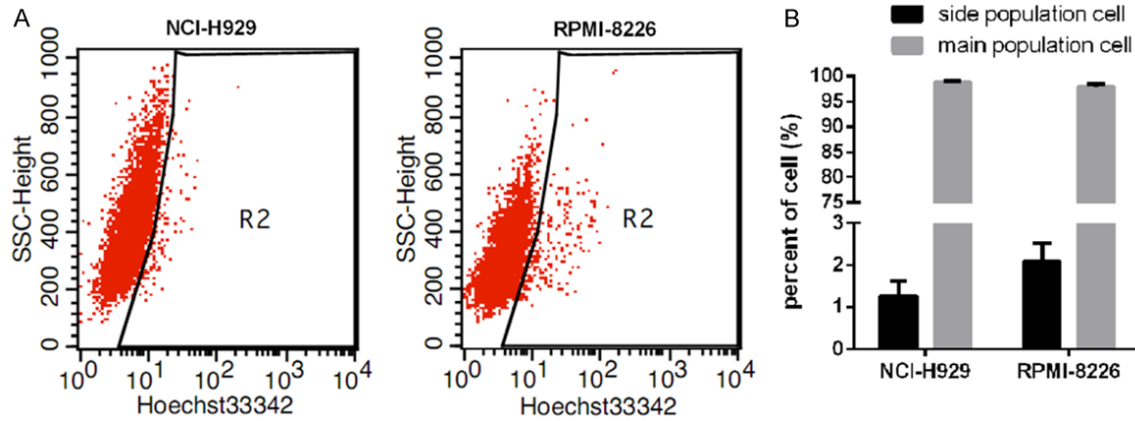


Figure 2. Proportion of SP cells in MM cell lines. A: Representative images of SP cells detected. B: Percentage of SP cells and main population cells. Data are presented as mean \pm SD.

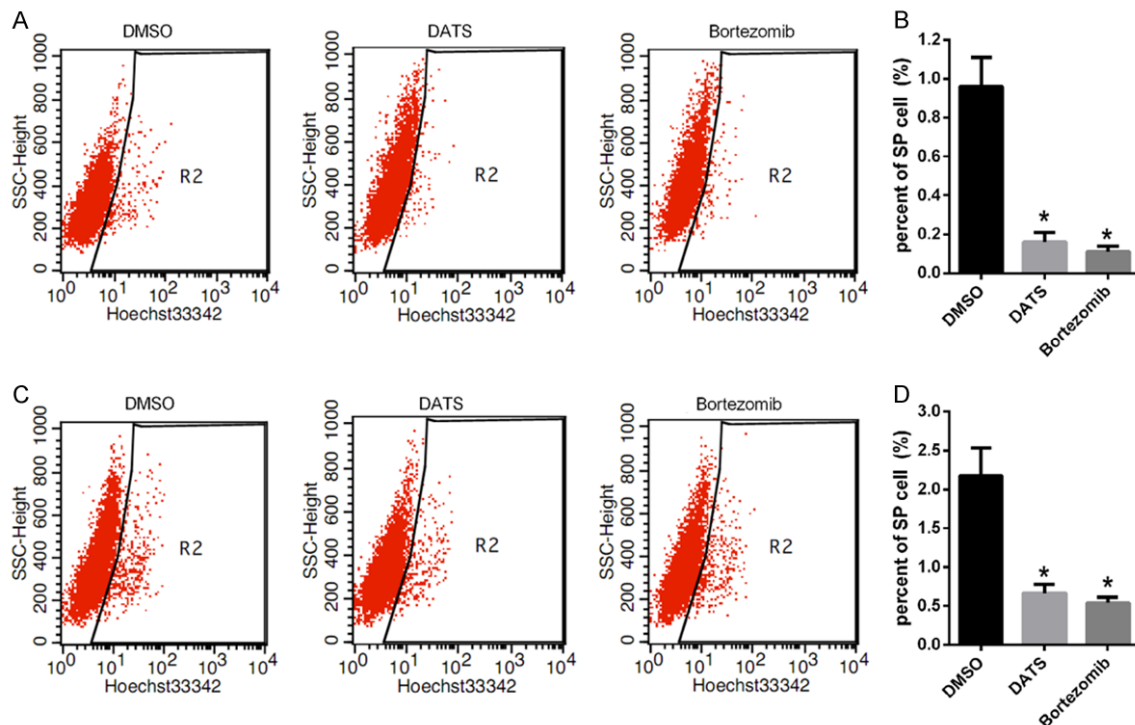


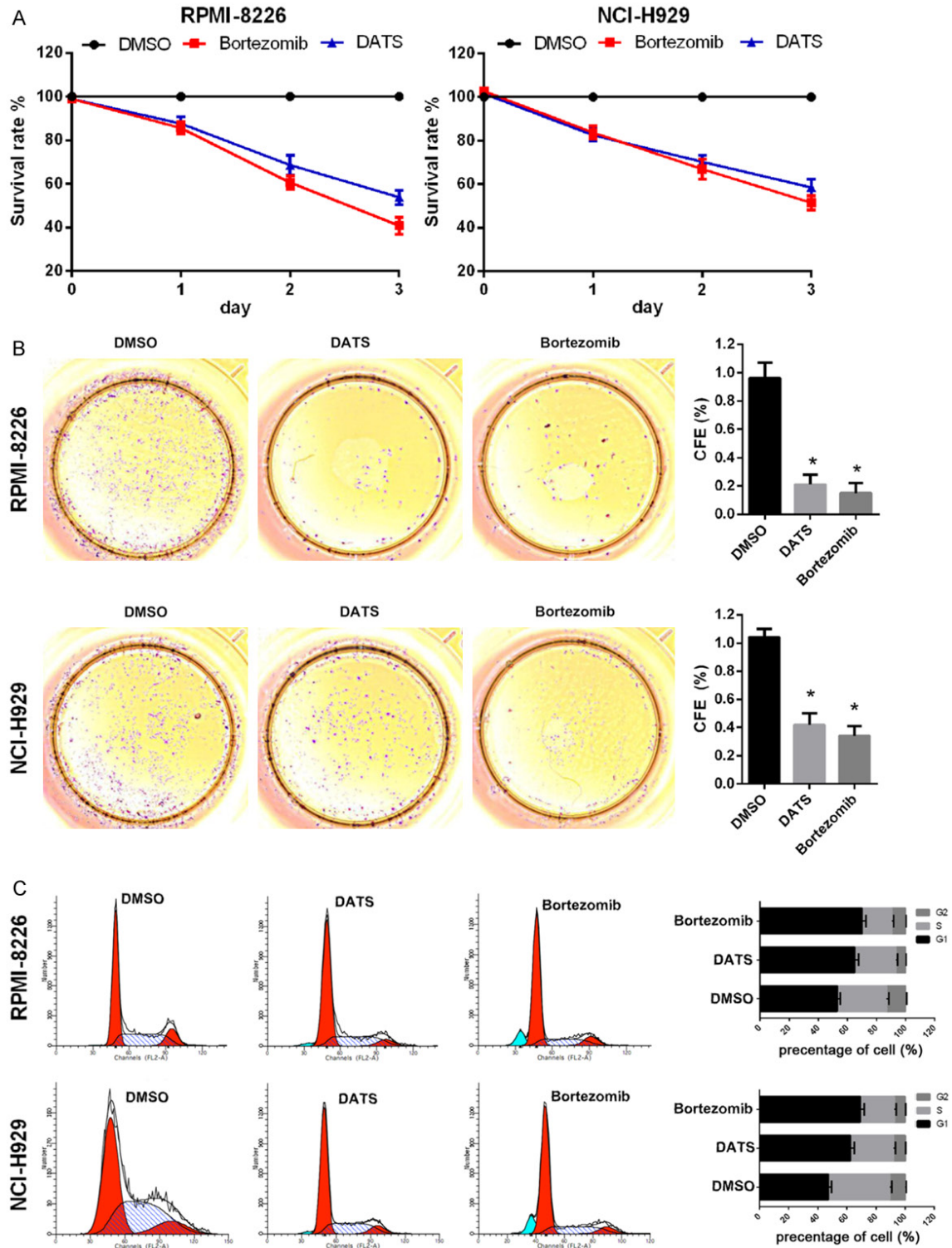
Figure 3. DATS reduced the percentage of MM SP cells. A: Representative image of SP cells detected among NCI-H929 cells. B: Percentage of SP cells among NCI-H929 cells; data are mean \pm SD, * P <0.05 vs. DMSO. C: Representative image of SP cells detected among RPMI-8226 cells. D: Percentage of SP cells among RPMI-8226 cells; data are presented as mean \pm SD, * P <0.05 vs. DMSO.

using flow cytometry after drug treatment (**Figure 3**). The results indicated that the percentage of SP cells more significantly decreased after treatment with DATS and bortezomib than it did after treatment with dimethyl sulfoxide (DMSO) (P <0.05). The effect of bortezomib treatment was not significantly different from that of DATS treatment (P >0.05).

DATS reduced the SP cell survival rate and inhibited colony formation and the cell cycle

To understand the effect of DATS on SP cells, SP cells were separated from MM cells using flow cytometry. Survival rate, cell cycle, and colony formation were evaluated after treatment with DMSO, DATS, and bortezomib. The

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MTT assay indicated that the survival rate of the SP cells more significantly decreased after

DATS and bortezomib treatment than it did after DMSO treatment (P <0.05). Furthermore,

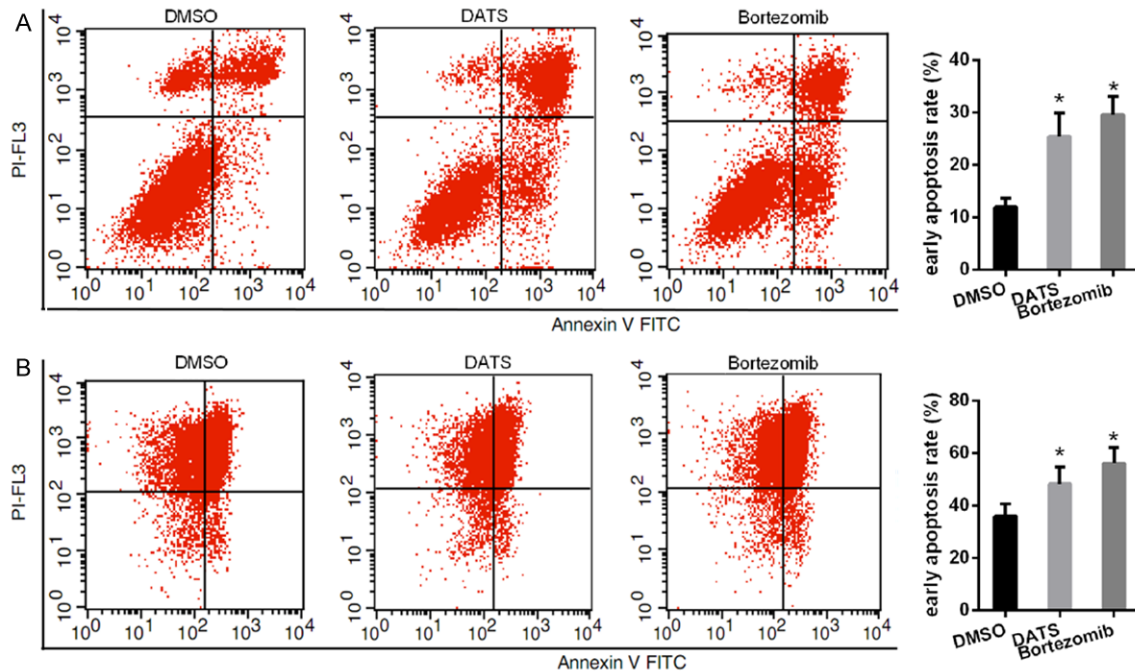


Figure 5. DATS promoted the apoptosis of MM SP cells. A: DATS promoted the apoptosis of SP cells in NCI-H929 cells. B: DATS promoted the apoptosis of SP cells in RPMI-8226 cells. Bars indicate the apoptosis rate of MM SP cells. Data are presented as mean \pm SD, * $P < 0.05$ vs. DMSO.

this effect was found to be dose- and time-dependent in RPMI-8226 and NCI-H929 cells (**Figure 4A**). In addition, DATS and bortezomib treatment inhibited colony formation more significantly than DMSO treatment ($P < 0.05$) (**Figure 4B**). The cell cycle was analyzed using flow cytometry (**Figure 4C**). Compared to DMSO treatment, DATS and bortezomib treatment significantly increased the percentage of cells in the G1-phase, which prevented their transition from the G1- to the S-phase ($P < 0.05$). The results also indicated that there was no significant difference between bortezomib treatment and DATS treatment in regards to effects on survival rate, cell cycle, and colony formation ($P > 0.05$).

DATS promoted SP cell apoptosis

SP cell apoptosis was analyzed using flow cytometry (**Figure 5**). Apoptosis was significantly greater in bortezomib- and DATS-treated SP cells compared to that in DMSO-treated cells ($P < 0.05$). The results also indicated that apoptosis was not significantly different between cells treated with bortezomib and cells treated with DATS ($P > 0.05$).

Discussion

DATS is a natural drug widely used in China to stimulate the immune system and promote health [12, 13]. Previous studies indicated that treatment with DATS suppressed the proliferation of human breast cancer cells by inhibiting estrogen receptor- α activity [21]. Furthermore, treatment with DATS inhibited the proliferation and induced the apoptosis of bladder cancer cells by mediating a caspase-dependent signaling pathway and regulating the phosphoinositide3-kinase (PI3K)/Akt and JNK pathways [22]. In this study, we found that treatment with DATS reduced the survival rate of MM cells in a dose- and time-dependent manner. The results indicated that DATS may be useful as a new agent for the treatment of MM.

MM remains incurable, with a 5-year survival rate of approximately 47%. The primary causes of MM-related death are tumor relapse and drug resistance [4]. These effects are associated with the existence of SP cells, which have features similar to those of tumor stem cells [5, 6]. The results of this study indicated that the proportion of SP cells was $2.08 \pm 0.438\%$ and $1.26 \pm 0.352\%$ among RPMI-8226 and NCI-

H929 cells, respectively. SP cells are known to promote tumor relapse and drug resistance in MM [10, 20]. DATS inhibited the proliferation and promoted the apoptosis of SP cells, an effect that would be expected to prolong the tumor-free interval and improve survival rates [7-10].

In this study, we found that bortezomib treatment significantly reduced the survival rate and colony formation, arrested the cell cycle, and promoted the apoptosis of SP cells. Previous research studies indicated that bortezomib significantly reduced SP cell numbers by suppressing proliferation through the inhibition of the cell cycle in the treatment of relapsed MM [11]. Similarly, in this study, we found that DATS reduced the survival rate and colony formation, arrested the cell cycle, and promoted the apoptosis of SP cells. DATS and bortezomib treatment displayed a comparable efficacy in inhibiting the proliferation and promoting the apoptosis of MM SP cells.

In conclusion, DATS inhibited the proliferation and promoted the apoptosis of SP cells and is a new potential agent for preventing MM progression and recurrence. However, further research is needed to verify our findings in animal models and clinical trials. In addition, we examined the toxicity and side effects of DATS to demonstrate its potential usefulness and safety for development as a therapeutic drug.

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Disclosure of conflict of interest

None.

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