

# Stathmin is key in reversion of doxorubicin resistance by arsenic trioxide in osteosarcoma cells

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**Abstract.** Osteosarcoma is the most common type of malignant bone tumor in children and adolescents. Numerous patients are unable to be cured due to the development of resistance of the osteosarcoma cells to chemotherapeutic drugs. Therefore, reversion of drug resistance is urgently required for the treatment of osteosarcoma. Arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) is an active ingredient in Traditional Chinese Medicine, but the therapeutic potential of As<sub>2</sub>O<sub>3</sub> in osteosarcoma remains largely unexplored. The current study investigated the effects of As<sub>2</sub>O<sub>3</sub> on MG63 osteosarcoma cells using a cell proliferation assay, flow cytometric analysis of the cell cycle and cell apoptosis, reverse transcription polymerase chain reaction to detect stathmin mRNA expression levels and western blot analysis to detect the stathmin protein expression levels. As<sub>2</sub>O<sub>3</sub> and doxorubicin (ADM) combination treatment markedly inhibited cell proliferation in ADM-resistant MG63 (MG63/dox) osteosarcoma cells, clearly induced G2/M phase cell cycle arrest and increased the number of apoptotic MG63/dox cells. Furthermore, stathmin expression was found to be downregulated in MG63/dox cells and was sensitive to ADM treatment. Additional investigation revealed that the downregulation of stathmin expression in MG63/dox cells by stathmin small interfering RNA significantly enhanced the reversion of ADM resistance in MG63/dox by As<sub>2</sub>O<sub>3</sub>. The data indicated that As<sub>2</sub>O<sub>3</sub> reversed ADM resistance in MG63/dox cells through downregulation of stathmin and may be a potential drug for the treatment of ADM-resistant osteosarcoma.

## Introduction

Osteosarcoma is an aggressive type of cancer commonly observed in adolescents and children. With the introduction of

intensive chemotherapy, the cure rate of patients with localized osteosarcoma has improved from 15 to 20%, and achieved to ~70% with surgery alone (1). Nonetheless, approximately one-third of osteosarcoma patients experience recurrent or progressive disease, of which the majority of cases are due to the development of resistance to chemotherapeutic drugs by the osteosarcoma cells (2). Therefore, drug resistance has been a hindrance in achieving improved cure rates.

Arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) compounds have been used in Traditional Chinese Medicine for thousands of years. The therapeutic use of these compounds in acute promyelocytic leukemia (APL) was described in the 1970s. In addition to APL, As<sub>2</sub>O<sub>3</sub> has been effective in the treatment of certain solid tumors, such as gastric cancer, lung cancer, breast cancer and hepatocellular carcinoma (3,4). Studies have demonstrated that As<sub>2</sub>O<sub>3</sub> induced cytotoxic effects in these tumors in a dose- and time-dependent manner (5). However, the detailed mechanisms of As<sub>2</sub>O<sub>3</sub> cytotoxicity remain to be further elucidated.

Stathmin is a microtubule regulatory phosphoprotein that is important in the assembly of the mitotic spindle. Stathmin is a key regulator of the microtubule network and provides an attractive therapeutic target in cancer treatment (6,7). Previous studies have demonstrated that small interfering (si)RNA or antisense-mediated downregulation of stathmin expression may result in proliferation inhibition and chemosensitivity enhancement in human osteosarcoma cells (8,9).

In the present study, the effect of As<sub>2</sub>O<sub>3</sub> on MG63 human doxorubicin-resistant osteosarcoma cells was examined, along with the molecular mechanisms of the effects. The present study was conducted by analyzing the effects of As<sub>2</sub>O<sub>3</sub> on cell proliferation, cell apoptosis, and stathmin mRNA and protein expression levels in MG63 and MG63/dox human osteosarcoma cells.

## Materials and methods

**Reagents.** As<sub>2</sub>O<sub>3</sub> was obtained from ShuangLu Pharmaceutical Co., Ltd. (Beijing, China), doxorubicin (ADM) was provided by Pfizer Pharmaceuticals Corporation (New York, NY, USA), propidium iodide (PI) was purchased from Sigma-Aldrich (St. Louis, MO, USA), Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Laboratories (Kumamoto, Japan), rabbit monoclonal antibody against stathmin was purchased from Abgent (1:5,000, San Diego, CA, USA) and antibody

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against  $\beta$ -actin was purchased from CoWin Bioscience Co., Ltd. (Beijing, China).

**Cell lines.** The MG63 human osteosarcoma parental cell line was obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The MG63/dox human multidrug-resistant (MDR) osteosarcoma cell line with P-glycoprotein overexpression, which was selected in a step-wise manner through exposing drug-sensitive MG63 cells to increasing doses of ADM, was provided by Dr Yoshio Oda (Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan). These cell lines were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, Utah, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone), 100 units/ml penicillin and 100 g/ml streptomycin (Gibco-BRL, Carlsbad, CA, USA) at 37°C in a humidified 5%  $\text{CO}_2$  atmosphere.

**Cell proliferation assay.** The cells were diluted with standard culture medium to a seeding density of  $5 \times 10^3$  cells/well, suspended in 96-well plates (100  $\mu\text{l}$ /well) and incubated at 37°C for 6 h. Subsequently, the cells were incubated for 24 or 48 h in the absence or presence of various concentrations of ADM and  $\text{As}_2\text{O}_3$ . Cells without any anticancer agent treatment served as a negative control. Cell proliferation was evaluated by CCK-8 assay. Briefly, 10  $\mu\text{l}$  CCK-8 solution was added to each well containing 100  $\mu\text{l}$  DMEM. Following incubation at 37°C for 4 h, the plates were analyzed on a Multiskan MK3 ELISA reader (Thermo Scientific, Madison, WI, USA) at 450 nm.

**Cell cycle analysis.** The distribution of cells in the different phases of the cell cycle was analyzed by PI staining of fixed whole cells. The cells were incubated for 48 h with  $\text{As}_2\text{O}_3$  (2  $\mu\text{M}$ ) and ADM (200 ng/ml) and as indicated. A total of  $\sim 1 \times 10^6$  cells were harvested, washed and fixed in 70% cold alcohol overnight at -20°C. The fixed cells were washed in phosphate-buffered saline (PBS) and resuspended in 1 ml PI solution (PBS containing 0.05 mg/ml PI and 1 mg/ml RNase). The cells were incubated for 30 min at 37°C. DNA content was analyzed within 2 h using a FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) at 488 nm single laser excitation. The cell-cycle distribution was analyzed using Lysis II software (Becton-Dickinson).

**Cell apoptosis assay.** Apoptotic cells were detected by flow cytometry with Annexin V-fluorescein isothiocyanate (FITC)/PI dual staining (Invitrogen Life Technologies, Carlsbad, CA, USA). The assay was performed following the manufacturer's instructions. The emitted green Annexin V fluorescence and red PI fluorescence were detected by the flow cytometer with an excitation wavelength of 488 nm and emission wavelengths of 525 and 575 nm, respectively. For each sample, 10,000 events were recorded. The quantities of cells in early apoptosis, late apoptosis and necrosis were determined as the percentages of Annexin V+/PI-, Annexin V+/PI+ and Annexin V-/PI+ cells, respectively.

**Reverse transcription polymerase chain reaction (RT-PCR).** The harvested cells were washed with PBS and total RNA was extracted from cells using TRIzol reagent (Invitrogen

Life Technologies). RNA purity ( $\text{A}_{260}/\text{A}_{280} > 1.8$ ) was verified using a spectrophotometer, and RNA integrity was confirmed by visualization of 28 S and 18 S bands (2:1) on a 1% agarose gel. RNA (1  $\mu\text{g}$ ) was used to synthesize cDNA using Superscript First-Strand Synthesis kit (Fermentas, Waltham, MA, USA) following the manufacturer's instructions. The stathmin mRNA expression levels were detected by PCR using the following specific primers: Stathmin sense, 5'-TCC AAT CTG CAT TGA TTA CCTG-3'; and antisense, 5'-CTT CCT TCC TAA GGT CCC ACTT-3'. Human  $\beta$ -actin served as an internal loading control; the primers used were as follows:  $\beta$ -actin sense, 5'-CCA GCC GAG CCA CAT CGC TC-3'; and  $\beta$ -actin antisense, 5'-ATG AGC CCC AGC CTT CTC CAT-3'.

**Quantitative (q)PCR.** Total RNA was extracted using TRIzol and quantified by spectrophotometry. Following the reverse transcription reaction, 1  $\mu\text{l}$  cDNA served as a template.

qPCR was then performed using Platinum SYBR Green qPCR SuperMix (Takara, Dalian, China) and the ABI Prism 7500 Sequence Detection system. The primer sequences used were as follows:  $\beta$ -actin sense, 5'-GGC GGC ACC ACC ATG TAC CCT-3' and antisense, 5'-AGG GGC CGG ACT CGT CAT ACT-3'. The stathmin primer (P162495) was purchased from Shanghai Bioneer Company (Shanghai, China). Amplification conditions were as follows: 95°C for 10 min, and then 40 cycles at 95°C for 15 sec, 60°C for 60 sec and 72°C for 60 sec. The specificity of detected signals was confirmed by a dissociation curve consisting of a single peak. All samples from each experiment were run in duplicate. The fold change of stathmin small interfering (si)RNA transcript levels between the indicated group and the control equals  $2^{-\Delta\Delta\text{Ct}}$ , where  $\Delta\text{Ct}$  threshold ( $\text{Ct}$ ) =  $\text{Ct siRNA} - \text{Ct Actin}$  and  $\Delta\Delta\text{Ct} = \Delta\text{Ct indicated group} - \Delta\text{Ct control}$ .

**Western blot analysis.** The harvested cells were washed with PBS twice and lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China) on ice. The cell extracts were clarified by centrifugation and protein concentrations were determined using an Evolution 60S UV-Visible spectrophotometer (Thermo Scientific). Each protein extract (30  $\mu\text{g}$ ) was purified by 12% SDS-PAGE followed by western blot analysis using the antibodies against stathmin and  $\beta$ -actin, respectively.

**Indirect immunofluorescence assay.** Slide-cultured MG63/dox cells were incubated with ADM and  $\text{As}_2\text{O}_3$  as indicated. At 48 h after incubation, the cells were fixed in 4% paraformaldehyde at room temperature for 30 min. The slides were washed in PBS and then permeabilized by 0.1% Triton X-100 at 4°C for 20 min. Following incubation with 2% FBS in PBS for 1 h, the cells were stained with rabbit monoclonal antibody against alpha-tubulin (1:300; Epitomics, Burlingame, CA, USA) at 4°C for 12 h. Subsequent to washing with PBS three times, the slides were incubated with FITC-conjugated anti-rabbit immunoglobulin (Ig)G (Proteintech group, Inc., Chicago, IL, USA) at room temperature for 1 h. DNA was stained with DAPI at room temperature for 5 min and slides were analyzed by fluorescence microscopy using an Olympus CKX41 inverted microscope (Olympus, Tokyo, Japan).

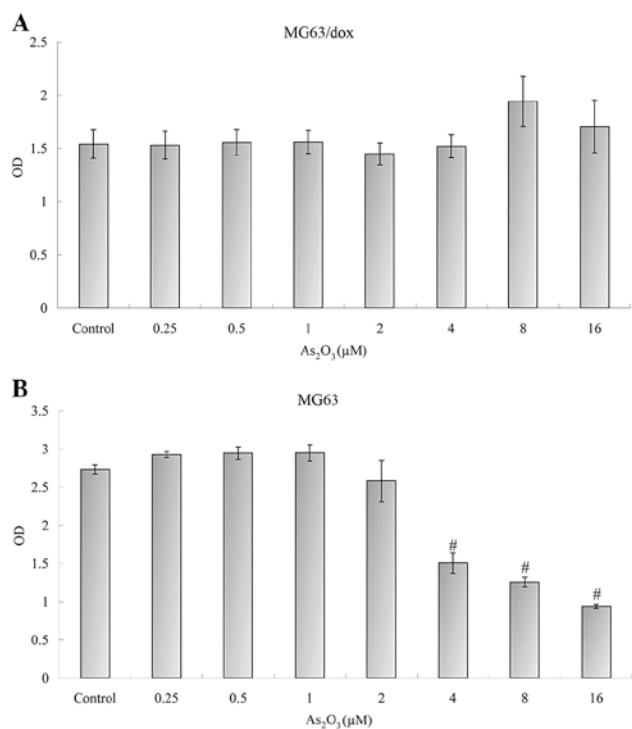


Figure 1. Effect of As<sub>2</sub>O<sub>3</sub> on (A) MG63 and (B) MG63/dox human osteosarcoma cell proliferation. Cells were treated with various concentrations of As<sub>2</sub>O<sub>3</sub> and incubated for 48 h. Cell growth was markedly inhibited at 4, 8 and 16 μM doses (<sup>#</sup>P<0.01 vs. control group). Values are expressed as the mean ± standard deviation. OD, optical density.

**siRNA transient transfection.** The cells were plated in six-well plates at 3x10<sup>5</sup> cells per well and cultured overnight to achieve 50-70% confluence prior to transfection. The respective siRNA molecules, namely stathmin sense, 5'-GUGUUGGUCUUUCUAAUGU-3'; negative control sense, 5'-CCUACGCCACCAAUUCGU-3'; and positive control GAPDH sense, 5'-GUGUGAACCAUGAGAAGUA-3' were transfected into the cells with Lipofectamine 2000 transfection reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. The cells were harvested at 48 h post transfection for PCR and western blot analysis.

**Statistical analysis.** Values are representative of triplicate determinations in two or more experiments and statistical analyses were performed with SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). The results are presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Effect of As<sub>2</sub>O<sub>3</sub> and ADM on parental MG63 and MG63/dox cell proliferation.** To confirm that As<sub>2</sub>O<sub>3</sub> and ADM combination treatment inhibited cell growth, the parental MG63 cells and the resistant MG63/dox cells were quantified using the CCK-8 assay. As<sub>2</sub>O<sub>3</sub> alone did not inhibit MG63 or MG63/dox cell proliferation effectively at any of the various doses (Fig. 1A), but As<sub>2</sub>O<sub>3</sub> was effective in significantly inhibiting the growth of MG63 cells at 4, 8 and 16 μM concentrations (P<0.01, Fig. 1B).

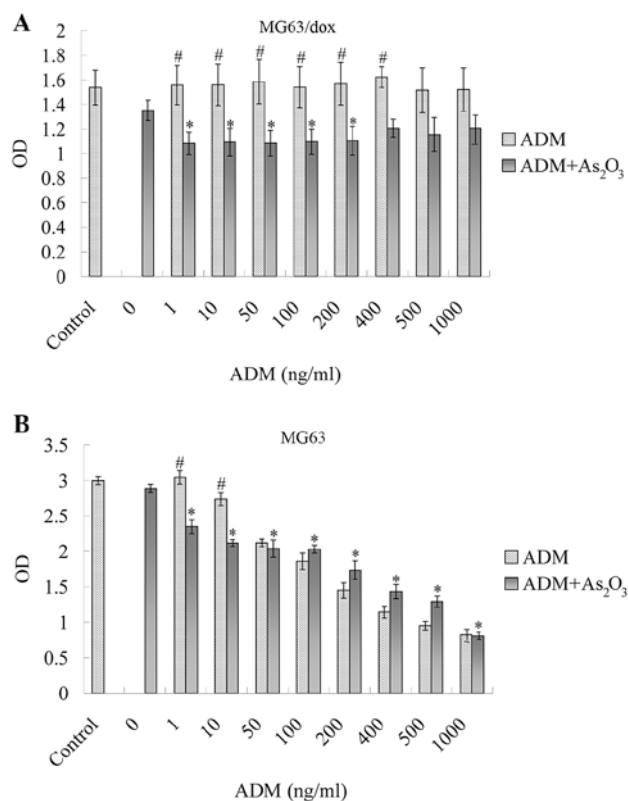


Figure 2. Effect of As<sub>2</sub>O<sub>3</sub> and ADM combination treatment on (A) MG63 and (B) MG63/dox human osteosarcoma cells. Cells were treated with 2 μM As<sub>2</sub>O<sub>3</sub> and various concentrations of ADM, and incubated for 48 h. (A) Combination treatment inhibited cell proliferation significantly compared with the control at 1, 10, 50, 100 and 200 ng/ml ADM (<sup>\*</sup>P<0.05). In contrast to ADM alone, combination treatment inhibited cell proliferation distinctly at 1, 10, 50, 100, 200 and 400 ng/ml concentrations (<sup>#</sup>P<0.05). (B) Combination treatment at various concentrations inhibited cell proliferation significantly compared with the control (<sup>\*</sup>P<0.05), and reduced cell growth at 1 and 10 ng/ml doses compared with ADM alone (<sup>\*</sup>P<0.05). The combined inhibitory effect declined at high doses of ADM. ADM, doxorubicin; OD, optical density.

As a cytotoxic drug, As<sub>2</sub>O<sub>3</sub> may produce severe side effects in patients at increasing dosages or extended treatment durations. The practical dose of As<sub>2</sub>O<sub>3</sub> in clinical therapy converted to the laboratory research should be 0.5-2 μM, and therefore, 2 μM was selected as the dose of As<sub>2</sub>O<sub>3</sub> in the follow-up experiments (10).

In contrast to the various concentrations of ADM alone, the combination of 2 μM As<sub>2</sub>O<sub>3</sub> with varying doses of ADM between 1 and 1,000 ng/ml resulted in a significant reduction in the growth of MG63/dox cells (P<0.05, Fig. 2A). Although combination treatment also significantly restrained the growth of MG63 cells compared with that of the control group (P<0.05), the addition of As<sub>2</sub>O<sub>3</sub> reduced the inhibitory effect of ADM at 50, 100, 200, 400, 500 and 1,000 ng/ml doses (Fig. 2B). The results demonstrated that combination treatment with As<sub>2</sub>O<sub>3</sub> and ADM was effective in inhibiting the growth of MDR osteosarcoma cells.

**Effect of As<sub>2</sub>O<sub>3</sub> and ADM on MG63/dox cell cycle.** Analysis of the cell-cycle phase distribution was conducted to investigate the antiproliferative mechanism of As<sub>2</sub>O<sub>3</sub> and ADM on MG63/dox cells. The results revealed significant differences

Table I. Effect of As<sub>2</sub>O<sub>3</sub> and ADM on MG63/dox cell cycle distribution.

Treatment	G0/G1 phase (%)	S phase (%)	G2/M phase (%)
Control	67.3±0.19	21.2±0.08	11.6±0.23
ADM (200 ng/ml)	68.2±0.12	19.2±0.33	12.6±0.45
As <sub>2</sub> O <sub>3</sub> (2 $\mu$ M)	53.9±0.15	24.7±0.18	21.4±0.32
As <sub>2</sub> O <sub>3</sub> and ADM	20.7±0.19 <sup>a</sup>	24.1±0.09 <sup>a</sup>	55.2±0.26 <sup>a</sup>

Values are presented as the mean  $\pm$  standard deviation of three experiments. <sup>a</sup>P<0.01 as compared with the control group. ADM, doxorubicin.

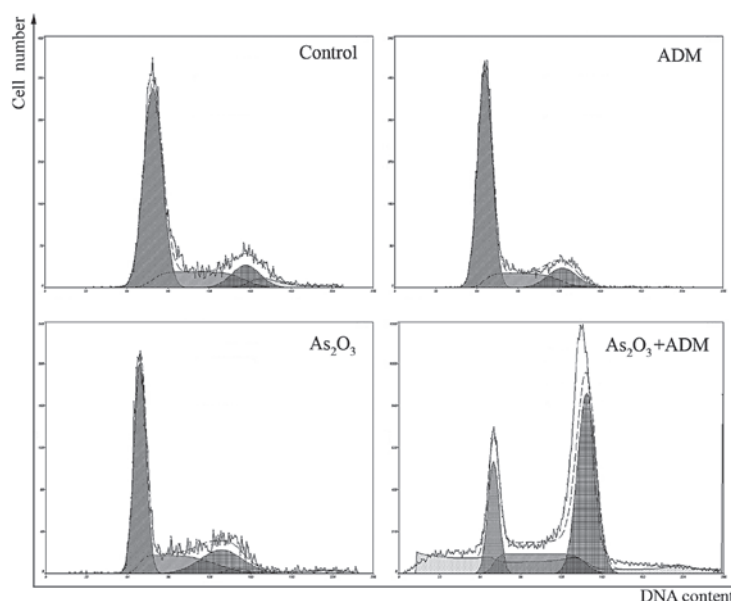


Figure 3. Effect of 48 h As<sub>2</sub>O<sub>3</sub> (2  $\mu$ M) and ADM (200 ng/ml) treatment on the cell cycle of MG63/dox human osteosarcoma cells. Increases in the fraction of cells in the G2/M phase following As<sub>2</sub>O<sub>3</sub> and ADM combination treatment were detected, with a concurrent reduction in the numbers of cells in the G0/G1 phase. ADM, doxorubicin.

in the percentages of combination treatment cells in each cell cycle phase compared with the control cells ( $P<0.01$ ; Fig. 3 and Table I). Increases in the fraction of cells in the G2/M phase were detected following As<sub>2</sub>O<sub>3</sub> and ADM treatment, and a concurrent reduction of the cell proportion in G0/G1 phase was observed. The results demonstrated that As<sub>2</sub>O<sub>3</sub> and ADM combination treatment inhibited the proliferation of MG63/dox cells through cell cycle arrest at the G2/M phase.

**Induction of apoptosis in MG63/dox cells by ADM and As<sub>2</sub>O<sub>3</sub>.** As<sub>2</sub>O<sub>3</sub> has been reported to induce apoptosis in various human cancer cells (10). To determine whether As<sub>2</sub>O<sub>3</sub> induced apoptosis in MG63/dox cells, the cells were treated with As<sub>2</sub>O<sub>3</sub> and ADM for 48 h. The results of flow cytometric analysis with Annexin V-PI staining revealed that ADM or As<sub>2</sub>O<sub>3</sub> treatment alone did not induce apoptosis; however, ADM and As<sub>2</sub>O<sub>3</sub> combination treatment increased the percentage of apoptotic cells significantly (Fig. 4).

**Effect of As<sub>2</sub>O<sub>3</sub> and ADM combination treatment on stathmin expression levels.** Stathmin, a signal transduction regulatory factor, is crucial in cell division and malignant development.

Zhang *et al* (8) observed that the stathmin gene was expressed at high levels in osteosarcoma and may become a novel target in osteosarcoma treatment. In order to determine whether stathmin was involved in As<sub>2</sub>O<sub>3</sub> and ADM-induced apoptosis, MG63/dox cells were incubated with As<sub>2</sub>O<sub>3</sub> and ADM for 48 h, and stathmin expression levels were analyzed using PCR and western blotting. The results demonstrated that incubation with As<sub>2</sub>O<sub>3</sub> and ADM resulted in significant downregulation of stathmin expression in MG63/dox cells. Treatment of the MDR cell line with As<sub>2</sub>O<sub>3</sub> or ADM alone did not induce distinctly reduced stathmin expression levels (Fig. 5).

**Effect of As<sub>2</sub>O<sub>3</sub> and ADM combination treatment on the cytoskeleton and morphology of MG63/dox cells.** Stathmin knockdown has been reported to alter the phenotype of microtubules. In order to validate the above finding that As<sub>2</sub>O<sub>3</sub> and ADM combined treatment downregulated stathmin expression, the cell microtubule network was examined by immunofluorescent imaging of cells treated with As<sub>2</sub>O<sub>3</sub> and ADM (Fig. 6). Distinguished cell morphological changes were observed using staining conditions and counting all cells within multiple images. In particular, the cells treated with ADM



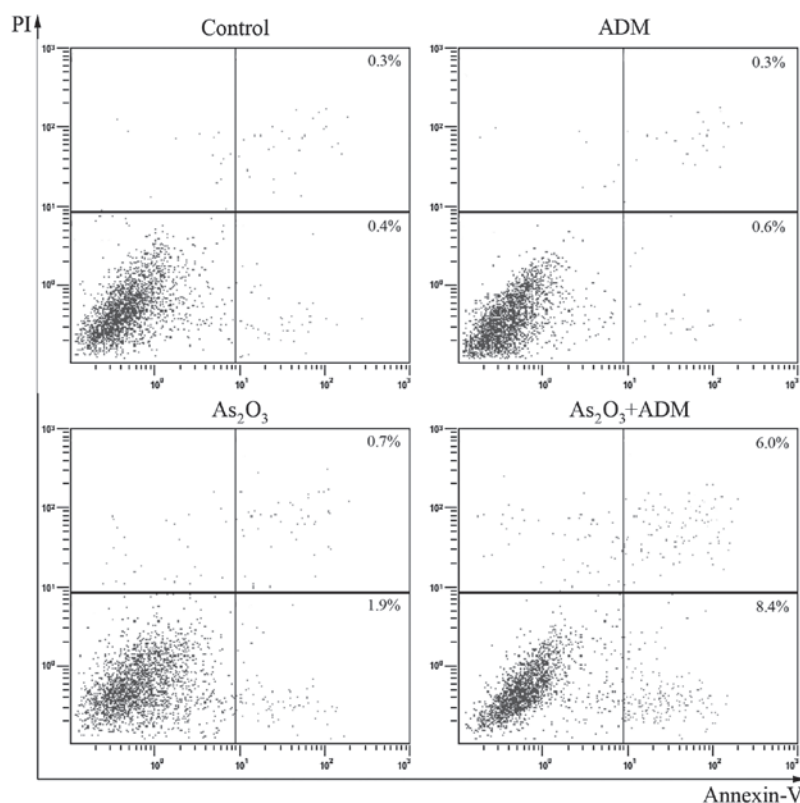


Figure 4.  $\text{As}_2\text{O}_3$  and ADM induced apoptosis in MG63/dox human osteosarcoma cells. The cells were treated with  $\text{As}_2\text{O}_3$  and ADM for 48 h. Cell apoptosis was detected by flow cytometry with Annexin V-fluorescein isothiocyanate/PI dual staining. Results are expressed as the percentage of apoptotic cells in early (bottom right) and late (top right) apoptosis.  $\text{As}_2\text{O}_3$ , arsenic trioxide; ADM, doxorubicin; PI, propidium iodide.

and  $\text{As}_2\text{O}_3$  exhibited increased numbers of short neurite-like extensions compared with the controls. Treatment of the cells with  $\text{As}_2\text{O}_3$  or ADM alone induced marginal changes in the cytoskeleton and morphology. These data indicated that  $\text{As}_2\text{O}_3$  and ADM markedly altered the organization of microtubule networks through stathmin downregulation.

**Effect of RNA interference targeting stathmin on stathmin expression levels, cell proliferation and apoptosis.** To further investigate the role of stathmin in  $\text{As}_2\text{O}_3$  and ADM-induced apoptosis, siRNA targeting stathmin was used to analyze the potential of these novel therapeutic targets in the treatment of human osteosarcoma. PCR and western blot analysis were used to determine the effect of siRNA treatment on stathmin mRNA and protein expression levels in MG63/dox cells. As shown in Fig. 7, western blotting indicated that the expression levels of stathmin protein in MG63/dox cells were significantly reduced by stathmin-siRNA. The fold change in stathmin siRNA transcript levels between the cells transfected with stathmin-siRNA and the control cells was calculated using the  $2^{-\Delta\Delta\text{Ct}}$  method (where  $\Delta$  cycle threshold (Ct) =  $\text{Ct}_{\text{siRNA}} - \text{Ct}_{\text{Actin}}$  and  $\Delta\Delta\text{Ct} = \Delta\text{Ct}_{\text{indicated group}} - \Delta\text{Ct}_{\text{control}}$ ), and ~85.5% stathmin gene expression was found to be inhibited following stathmin-siRNA transfection (Table II). These results demonstrated that siRNA sequences targeting the stathmin gene were effective in knocking down stathmin gene expression.

To analyze whether  $\text{As}_2\text{O}_3$  reversed drug resistance through stathmin inhibition, stathmin-siRNA-transfected MG63/dox

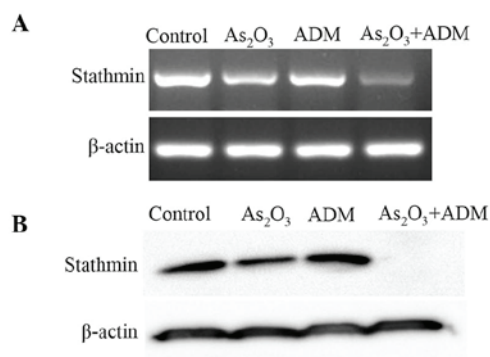


Figure 5. Exposure to  $\text{As}_2\text{O}_3$  and ADM for 48 h reduced stathmin expression levels in MG63/dox human osteosarcoma cells. (A) Stathmin gene expression levels were determined by polymerase chain reaction. (B) Western blotting was employed to detect stathmin protein expression levels. The blot was also probed with an anti- $\beta$ -actin monoclonal antibody to assess the relative protein levels in the sample lanes. ADM, doxorubicin.

cells were incubated with 2  $\mu\text{M}$   $\text{As}_2\text{O}_3$  and various concentrations of ADM for 48 h, and quantified by CCK-8 assay. Compared with the control cells, stathmin-siRNA-transfected cells exhibited significantly enhanced chemosensitivity, increasing the inhibitory rate of  $\text{As}_2\text{O}_3$  and ADM treatment by 12.77% (at 200 ng/ml ADM) to 18.91% (at 1,000 ng/ml ADM; Fig. 8). The effect of  $\text{As}_2\text{O}_3$  and ADM administration on inducing apoptosis was also detected in the stathmin-siRNA-transfected MG63/dox cells. As shown in Fig. 9, a higher apoptotic rate was demonstrated in stathmin-siRNA-transfected cells compared

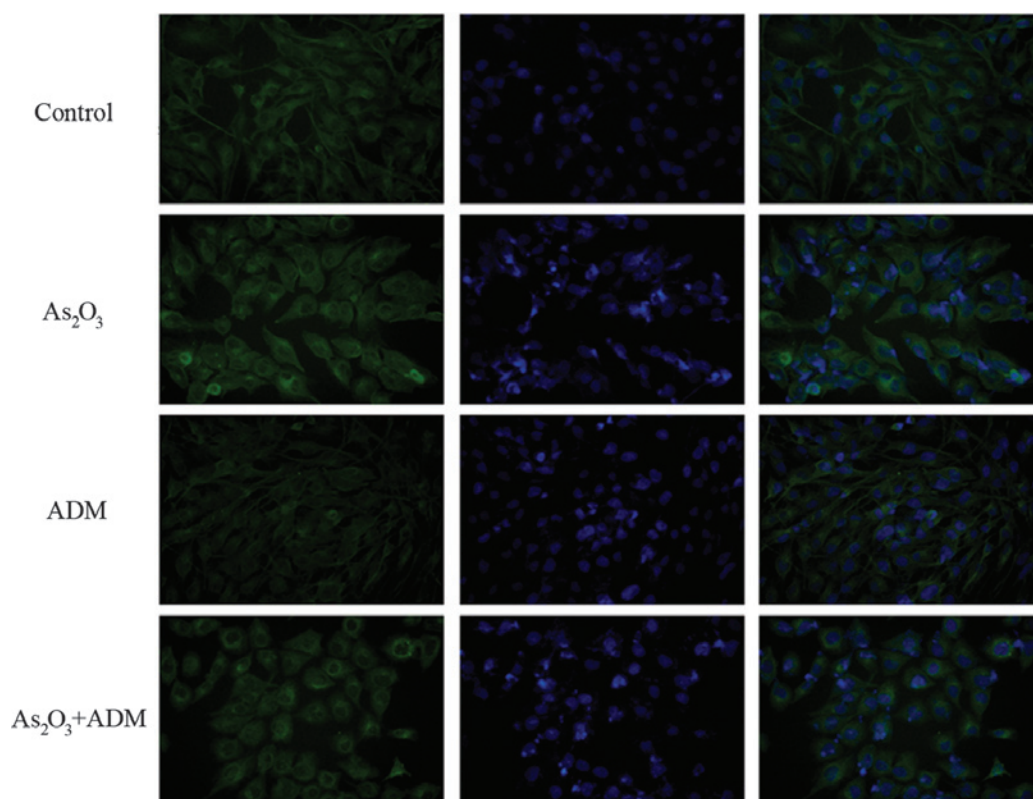


Figure 6. Effect of  $\text{As}_2\text{O}_3$  and ADM on the microtubule network of MG63/dox human osteosarcoma cells. Slide-cultured MG63/dox cells were incubated with 200 ng/ml ADM and/or 2  $\mu\text{M}$   $\text{As}_2\text{O}_3$ . At 48 h after incubation, cells were fixed in 4% paraformaldehyde and stained with anti- $\alpha$ -tubulin antibody and DAPI. The images show microtubules in green (left row), nuclei in blue (middle row) and the merged images (right row). ADM, doxorubicin.

Table II. Stathmin and  $\beta$ -actin Ct values in the control and stathmin-siRNA transfected cells.

Sample	Ct stathmin	Ct $\beta$ -actin
Control	18.93604	14.72279
Stathmin-siRNA	21.95575	14.95309

Ct, cycle threshold; siRNA, small interfering RNA. The fold change of stathmin siRNA transcript levels between stathmin-siRNA group and the control equals 85.5% (via  $2^{-\Delta\Delta\text{Ct}}$ ).

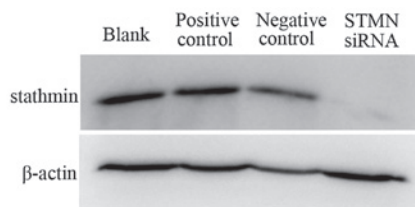


Figure 7. Expression levels of STMN protein in transfected MG63/dox human osteosarcoma cells detected by Western blot analysis. STMN immunoreactive bands were normalized to  $\beta$ -actin bands on the same membrane. STMN, stathmin; siRNA, small interfering RNA.

with MG63/dox cells following  $\text{As}_2\text{O}_3$  and ADM treatment. The above results indicated that  $\text{As}_2\text{O}_3$  and ADM suppressed cell proliferation and induced apoptosis, possibly through inhibiting stathmin expression.

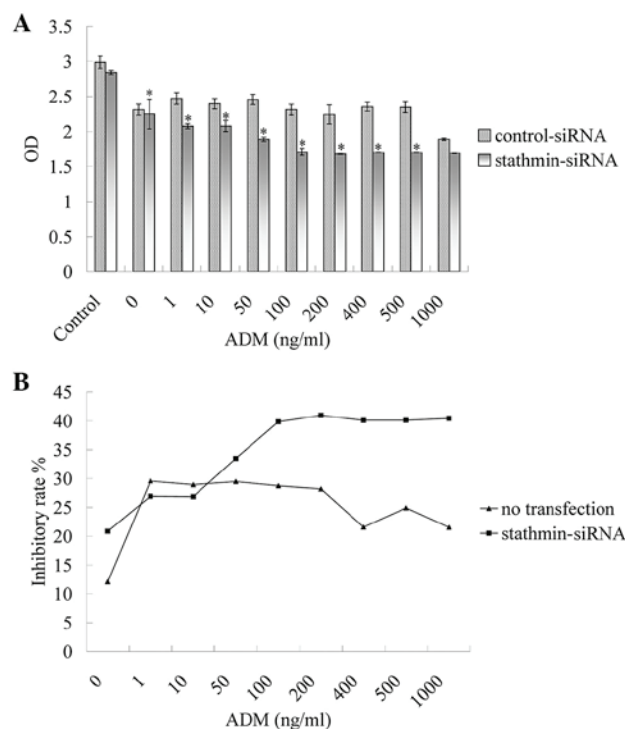


Figure 8. Effect of  $\text{As}_2\text{O}_3$  and ADM combination treatment on stathmin-siRNA-transfected MG63/dox human osteosarcoma cells. (A) Stathmin-siRNA and control-siRNA-transfected MG63/dox cells were treated with 2  $\mu\text{M}$   $\text{As}_2\text{O}_3$  and various concentrations of ADM. After 48 h incubation, cell growth was evaluated by the cell counting kit-8 assay (\* $P < 0.05$  vs. control group). (B) Stathmin-siRNA enhanced the cytotoxic effect of  $\text{As}_2\text{O}_3$  and ADM. ADM, doxorubicin; siRNA, small interfering RNA; OD, optical density.

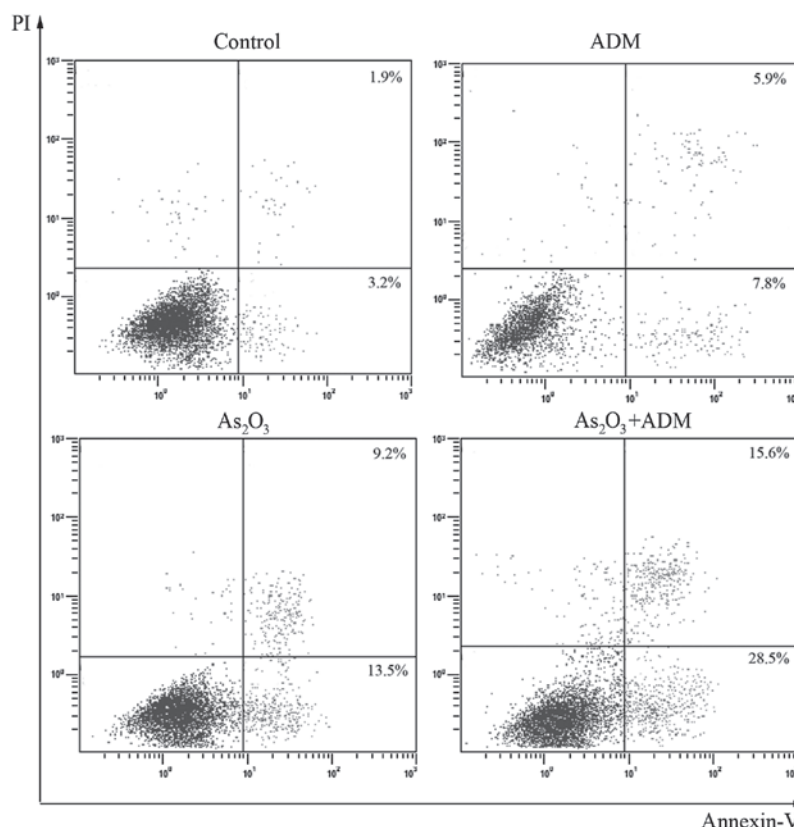


Figure 9. As<sub>2</sub>O<sub>3</sub> and ADM induce apoptosis in stathmin-siRNA transfected MG63/dox human osteosarcoma cells. The transfected MG63/dox cells were treated with 2  $\mu$ M As<sub>2</sub>O<sub>3</sub> and 200 ng/ml ADM for 48 h. Cell apoptosis was detected by flow cytometry with Annexin V-fluorescein isothiocyanate/PI dual staining. Results are expressed as percentage of apoptotic cells in early and late apoptosis. ADM, doxorubicin; PI, propidium iodide; siRNA, small interfering RNA.

## Discussion

Osteosarcoma is the most common type of primary malignant bone tumor in children and adolescents; ~60% of primary malignant bone tumors are diagnosed in the first two decades of life (11). With the introduction of intensive chemotherapeutics, including cisplatin, doxorubicin, ifosfamide and high dose methotrexate, >60% of the patients are cured (1). However, 30-40% patients with localized osteosarcoma experience recurrent or progressive disease. The majority of these patients are considered to have MDR osteosarcoma cancer cells, resistant to one or more chemotherapeutic agents (12). Resistance to these agents remains a challenge in the treatment of osteosarcoma and an obstacle to achieving improved cure rates. Thus, novel drugs and therapeutic regimens are required for more effective treatment of aggressive and recurrent MDR osteosarcoma.

As<sub>2</sub>O<sub>3</sub>-based compounds are the most widely used and analyzed arsenic-based cancer drugs. Results of *in vitro* studies and clinical trials have revealed that As<sub>2</sub>O<sub>3</sub> is effective in inhibiting the growth of APL cells (3). Recent studies have found that As<sub>2</sub>O<sub>3</sub> also exhibits an anticancer effect in particular types of non-APL cancer, including myeloid leukemia, gastric cancer, prostate and ovarian carcinomas, and breast cancer (5). However, studies regarding the effect of As<sub>2</sub>O<sub>3</sub> on osteosarcoma are rare. According to a study by Guo *et al* (13), As<sub>2</sub>O<sub>3</sub> combined with VP-16 and paclitaxel was an effective remedy in the treatment of stage III osteosarcoma. A previous study also revealed that As<sub>2</sub>O<sub>3</sub> induced apoptosis in MG63 human osteosarcoma cells (14). These findings prompted the

investigation of the effect of As<sub>2</sub>O<sub>3</sub> on MDR human osteosarcoma cells in the present study. As<sub>2</sub>O<sub>3</sub> has been reported to downregulate P-glycoprotein expression in human leukemia cells (15). In the present study, the MG63/dox MDR osteosarcoma cell line, which is characterized by upregulation of the MDR1 gene and overexpression of P-glycoprotein, was used.

The results revealed that combination treatment with As<sub>2</sub>O<sub>3</sub> and ADM was effective in inhibiting MG63 and MG63/dox cell proliferation. The MG63/dox drug-resistant cells exhibited particular sensitivity to the combination treatment. Furthermore, the combination treatment induced MG63/dox apoptosis and cell-cycle arrest. This finding is of great importance for evaluating the potential use of As<sub>2</sub>O<sub>3</sub> in treating patients with MDR.

A growing number of studies have reported that stathmin was expressed at high levels in a wide variety of human malignancies, including osteosarcoma. Stathmin is a key regulator of the microtubule network and provides an attractive therapeutic target in cancer treatment (6). High levels of stathmin expression in cancer cells were observed to correlate with cell proliferative potential and appear to be required for the maintenance of the malignant phenotype (16).

The present study provided evidence that stathmin may be involved in As<sub>2</sub>O<sub>3</sub>-induced MDR cell apoptosis. As<sub>2</sub>O<sub>3</sub> and ADM treatment not only inhibited MG63/dox cell proliferation and induced apoptosis, but also resulted in reduced stathmin expression levels, indicating that reduced stathmin expression levels are associated with As<sub>2</sub>O<sub>3</sub> and ADM-induced apoptosis. Furthermore, treatment with As<sub>2</sub>O<sub>3</sub> and ADM altered the

cytoskeleton of MG63/dox cells, while stathmin knockdown has been reported to alter the phenotype of microtubules. These results demonstrated that stathmin may be crucial in the reversion of drug resistance by  $\text{As}_2\text{O}_3$ .

To demonstrate the possible role of stathmin in the  $\text{As}_2\text{O}_3$ -induced apoptotic pathway, siRNA targeting stathmin was transfected into MG63/dox cells. The CCK-8 assay demonstrated that the combination treatment inhibited the transfected MG63/dox cell proliferation markedly compared with the non-transfection group. Flow cytometric analysis revealed that the treatment with  $\text{As}_2\text{O}_3$  and ADM induced marked apoptosis. Stathmin is a ubiquitous cytoplasmic phosphoprotein that regulates microtubule dynamics. Inhibiting stathmin expression may influence the functions of either centrosomes or G2/M checkpoint proteins at the centrosome, which may result in a G2/M block and reduce the rate of cell proliferation (17). In the present study, stathmin was demonstrated to be involved in  $\text{As}_2\text{O}_3$ -induced apoptosis in human osteosarcoma cells and this finding may support the use of stathmin as a therapeutic target in human osteosarcomas. The exact molecular mechanism that accounts for the observed interaction between stathmin inhibition and  $\text{As}_2\text{O}_3$  treatment appears complex and requires further clarification.

In conclusion, the administration of  $\text{As}_2\text{O}_3$  together with ADM may be a useful novel anticancer chemotherapy, particularly in MDR cases, as  $\text{As}_2\text{O}_3$  reversed ADM resistance in MG63/dox cells through downregulation of stathmin. The results indicated that  $\text{As}_2\text{O}_3$  is a promising chemotherapeutic agent for patients with drug-resistant osteosarcoma.

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