Arsenic trioxide inhibits Ewing's sarcoma cell invasiveness by targeting p38^{MAPK} and c-Jun N-terminal kinase

Shuai Zhang, Wei Guo, Ting-Ting Ren, Xin-Chang Lu, Guo-Qing Tang and Fu-Long Zhao

Ewing's sarcoma is the second most frequent primary malignant bone tumor, mainly affecting children and young adults. The notorious metastatic capability of this tumor aggravates patient mortality and remains a problem to be overcome. We investigated the effect of arsenic trioxide (As_2O_3) on the metastasis capability of Ewing's sarcoma cells. We performed 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-2*H*-tetrazolium bromide assays to choose appropriate concentrations of As_2O_3 for the experiments. Migration, invasion, and adhesion assays were performed to assess the effect of As_2O_3 on the metastasis of Ewing's sarcoma. Immunofluorescent staining was used to observe cytoskeleton reorganization in Ewing's sarcoma cells treated with As_2O_3. Changes in matrix metalloproteinase-9 expression and the mitogen-activated protein kinase (MAPK) pathway were investigated using western blot. Inhibitors of p38^{MAPK} (sb202190) and c-Jun NH_2-terminal kinase (JNK, sp600125) were used in invasion assays to determine the effect of p38^{MAPK} and JNK. We found that As_2O_3 may markedly inhibit the migration and invasion capacity of Ewing's sarcoma cells with structural rearrangements of the actin cytoskeleton. The expressions of matrix metalloproteinase-9, phosphor-p38^{MAPK}, and phosphor-JNK were suppressed by As_2O_3 treatment in a dose-dependent manner. The inhibitors of p38^{MAPK} (sb202190) and JNK (sp600125) enhanced the inhibition induced by As_2O_3, which was counteracted by anisomycin, an activating agent of p38^{MAPK} and JNK. Taken together, our results demonstrate that As_2O_3 can inhibit the metastasis capability of RD-ES and A-673 cells and may have new therapeutic value for Ewing's sarcoma.

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Keywords: arsenic trioxide, Ewing's sarcoma, invasion, migration, mitogen-activated protein kinase

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Introduction

Ewing's sarcoma is one of the most common bone malignancies with a relatively poor long-term outcome for children and young adults [1]. Recently, combined therapy with surgery, radiation, and chemotherapy was used to overcome Ewing's sarcoma and improve the 5-year survival rate to approximately 50% [2–4]. Nevertheless, although considerable progress has been made in the treatment of Ewing's sarcoma using multimodal therapy, patients with metastatic disease at presentation have significantly worse outcomes [5].

Arsenic trioxide (As_2O_3), an active ingredient in traditional Chinese medicine, has been used successfully in the treatment of acute promyelocyte leukemia [6,7]. Numerous studies have uncovered antitumor effects at clinically tolerable concentrations for some solid tumors, including for some musculoskeletal tumors, because of its ability to promote cell differentiation, induce apoptosis, and inhibit growth and angiogenesis [8,9]. We previously reported the positive effect of As_2O_3 combined with VP-16 and paclitaxel in the treatment of osteosarcoma and Ewing's sarcoma [10]. Subsequently, we found that As_2O_3 can inhibit osteosarcoma cell invasion *in vitro* through the suppression of MEK1/2 and extracellular signal-regulated kinase (ERK)1/2 phosphorylation[11]. In this study, we further investigated whether low doses ($< 2\,\mu mol/l$) of As_2O_3 have an inhibitive effect on the adhesion, migration, and invasion capacity of Ewing's sarcoma cells. The low drug concentrations in serum have been shown to be physiologically achievable with only mild-to-moderate side effects [6,12].

Mitogen-activated protein kinase (MAPK) is a member of the serine–threonine kinase family. The MAPK pathway has been shown to exist in most mammalian cells and plays a vital role in the transmission of extracellular signals to their intracellular targets and to have subsequent effects on proliferation, apoptosis, adhesion, and differentiation [13]. Three classic MAPK pathways have been identified in mammalian cells: ERK1/2, c-Jun NH_2-terminal kinase/ (JNK), and p38^{MAPK}. These parallel pathways may be activated by various types of stimuli and contribute to respective cellular influences, which may be independent or overlapping [14]. MAPKs have also been reported to be involved in the cytological effects induced by As_2O_3, such as apoptosis and differentiation [8,15,16].

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Here, we examine the influence of As_2O_3 on the metastasis of Ewing's sarcoma cells and show that As_2O_3 can inhibit the mobility, migration, and invasive capacity of two Ewing's sarcoma cell lines, affecting their cytoskeleton arrangement. We also focused our analysis on the different roles of MAPK pathways in the inhibition induced by As_2O_3, finding that the inaction of JNK1/2 and $p38^{MAPK}$ may be involved. Thus, this study may highlight some clinical implications for the design of treatment strategies in human Ewing's sarcoma.

Materials and methods

Cell culture and reagents

Cell lines were obtained from American Type Culture Collection (Manassas, Virginia, USA). Human Ewing's sarcoma cell line A-673 was cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, Utah, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, New York, USA), and RD-ES was cultured in Roswell Park Memorial Institute 1640 medium (Hyclone) supplemented with 15% fetal calf serum (Gibco). The cell lines were maintained in a humidified chamber with 5% CO_2 at 37°C. Antibodies to ERK1/2, p-ERK1/2, JNK1/2/3, p-JNK1/2/3, p38, and p-p38 were purchased from Bioworld (St. Louis Park, Minneapolis, USA). The antibody to β-actin, Fli-1(C-19), and horseradish peroxidase-conjugated goat anti-mouse or rabbit antibodies were purchased from Santa Cruz (California, USA). Antibody to matrix metalloproteinase-9 (MMP-9) was purchased from Cell Signaling Test (Danvers, Massachusetts, USA). Transwell chambers (24-well) with 8.0 μm-pore membranes were purchased from Corning (New York, USA). Growth factor-reduced Matrigel was purchased from BD Biosciences (Franklin Lakes, New Jersey, USA). Tetramethylrhodamine B isothiocyanate (TRITC)-labeled phalloidin (P1951) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). The inhibitors of p38 (sb202190; s7067) and JNK (sp600125; s5567) and anisomycin (A9789) were also purchased from Sigma-Aldrich.

Cytotoxicity assays

The in-vitro cytotoxic effect of As_2O_3 on Ewing's sarcoma tumor cells was determined by using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2 *H*-tetrazolium bromide (MTT) assay as described before [17]. In brief, cells growing in plates were dispersed in 0.05% trypsin solution and resuspended in DMEM or Roswell Park Memorial Institute 1640 medium containing FBS. Approximately 5000 cells were added to each well of a 96-well plate and incubated for 24 h. Various concentrations of As_2O_3 were added to each well after changing the media with or without respective concentration serum. Thereafter, the cells were cultured at 37°C for another 48 h, and cell cytotoxicity was estimated by measuring absorbance at 492 nm using an enzyme-linked immunosorbent assay microplate reader following the supplier's instructions.

Cell migration assays

Cell migration was assessed using tissue culture-treated 6.5-mm modified Boyden transwell chambers (8-μm pore size; Costar 3422, Corning Inc., New York, USA). A-673 and RD-ES cells were treated with various concentrations of As_2O_3. After incubation for 24 h, 4×10^4 cells were resuspended in 130 μl of serum-free medium and then seeded carefully into the upper part of each chamber. A volume of 800 μl of DMEM or RMPI 1640 supplemented with appropriate FBS was added to the lower chamber. After incubation for 24 h at 37°C in 5% CO_2 in an incubator, the cells remaining in the top chamber were removed and the top surface of each membrane was cleared with a cotton swab. Cells on the lower surface of the transwell chamber membrane were then fixed in methanol, stained with crystal violet staining solution, and counted. The number of migrated cells was determined by counting cells in five high-powered fields per well.

For the wound-healing assay, cells were grown to a confluent monolayer in six-well plates for 24 h. Thereafter, the monolayers were wounded by scraping with a 200 μl of sterile pipette tip and washed twice with phosphate-buffered solution (PBS) to remove any cellular debris. The wounded monolayers were then incubated in medium with various concentrations of As_2O_3 for another 24 h. After incubation, the monolayers were observed and corresponding images were taken at 0 and 24 h.

Cell invasion assay

The general procedure of the invasion assay is similar to that of the migration assay described above. In brief, the membranes in chambers were coated with an equal thickness of 100 mg/ml of Matrigel (BD Biosciences, 356234) before cells were seeded into the upper chamber. After incubation for 36 h (A-673) or 48 h (RD-ES) at 37°C, the invaded cells on the lower surface of the membrane were analyzed in the same way as in the migration assay.

Immunofluorescent staining assay

The A-673 and RD-ES cells were treated with 0 μmol/l or 2 μmol/l of As_2O_3 for 24 h after being attached to the surface of coverslips completely. After the treatment, the cells were washed with PBS three times and then fixed in 4% paraformaldehyde for 20 min. Thereafter, cells were blocked in PBS containing 0.5% bovine serum albumin for 10 min and permeabilized in 0.1% Triton X-100 for 5 min. The cells were then incubated for 30 min with TRITC-labeled phalloidin (Sigma, St. Louis, Missouri, USA, P1951) at 37°C, after which 1 μg/ml of 4′,6-diamidino-2-phenylindole in PBS was used to stain the cell nucleus for 30 s. After three additional washes, the coverslips were examined and photographed by immunofluorescence microscopy.

Adhesion assay

The adhesion assay was performed using the MTT assay. After incubation with various concentrations of As_2O_3 for

24 h, the Ewing sarcoma cells (1×10^5) were resuspended in 200 µl of medium without serum and planted into the Matrigel-precoated (100 µg/ml) 96-well plate. Then, groups of cells were washed at 30 and 60 min to remove nonadherent cells. After the wash, an MTT assay was carried out to measure the adhered cells at 492-nm wavelength.

Western blot assay and densitometric analyses

The procedure of western blotting analysis is briefly described below. After treating with different concentrations of As_2O_3 for 24 h, Ewing's sarcoma cells were washed twice with ice-cold PBS and lysed in radioimmunoprecipitation assay lysis buffer (20 mmol/l of Tris–HCl, 150 mmol/l of NaCl, 1%NP-40, 5 mmol/l of EDTA, and 1 mmol/l of Na_3VO_4, pH 7.5) containing protease inhibitor cocktail (Sigma-Aldrich) and then incubated for 25 min and centrifuged for 20 min at 13 000 x*g* at 4°C. Thereafter, the supernatant was recovered and quantified using the Pierce bicinchoninic acid protein quantization assay (Pierce, Rockford, Illinois, USA). An aliquot (50–100 µg of protein per lane) of the total protein was loaded onto SDS-polyacrylamide gel electrophoresis gels for electrophoresis and then blotted to polyvinylidene fluoride membranes (BioRad, Richmond, California, USA). The membranes were probed with appropriate primary and secondary antibodies. After western blot, densitometric assays were performed to determine the change of some protein band intensities using the Image J computer software (Wayne Rasband, Washington D.C., USA).

Statistical analysis

All statistical analyses were carried out with the SPSS statistical software package 13.0 (SPSS, Inc., Chicago, Illinois, USA). The data, shown as mean ± standard deviation, were analyzed by the analysis of variance, and *P* values less than 0.05 were considered statistically significant ($*P < 0.05$, $**P < 0.01$).

Results

Effect of arsenic trioxide on the cytotoxicity of Ewing's sarcoma cells

As shown in Fig. 1, the treatment of RD-ES cells with 0, 5, or 10 µmol/l of As_2O_3 led to a dose-dependent decrease in cell viability, regardless of whether serum was present. However, no significant reduction was observed with 0–2 µmol/l of As_2O_3. Interestingly, a more prominent decrease in A-673 cell viability was observed after treatment with 2 µmol/l of As_2O_3. No significant reduction of A-673 cell viability was observed at low concentrations (< 0.5 µmol/l) of As_2O_3. Consequently, the respective noncytotoxic concentrations of As_2O_3 were used in the following in-vitro experiments.

Arsenic trioxide inhibits Ewing's sarcoma cell motility, migration, and invasion *in vitro*

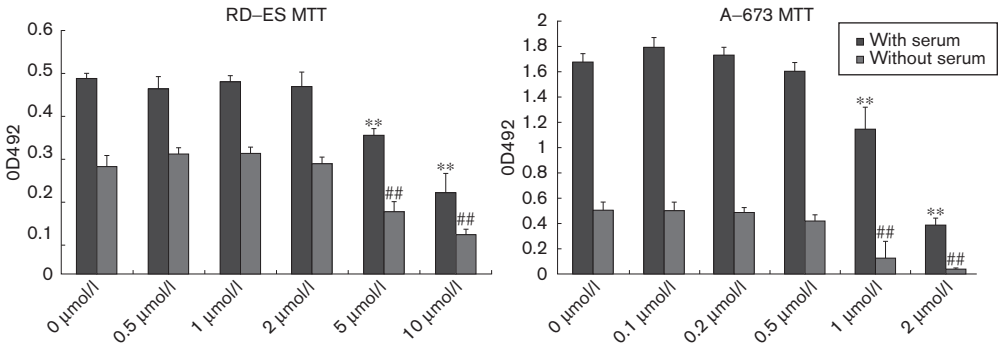
To study the motility of Ewing's sarcoma cells under the influence of As_2O_3, wound-healing assays were performed. Images were taken at 0 and 24 h after cells were wounded with sterile pipette tips. The cell-free 'scratch' healing conditions are shown in Fig. 2, and these suggested that the motility of Ewing's sarcoma cells was impaired by As_2O_3 in a dose-dependent manner.

Migration and invasion are indispensable for metastasis *in vivo*. Inhibition of the migration and invasion capacity of Ewing's sarcoma cells may decrease the potential for metastasis. Thus, we analyzed the effect of As_2O_3 on the migratory and invasive properties of Ewing's sarcoma cells using the Boyden transwell chambers assay. As shown in Fig. 3, the ability of Ewing's sarcoma cells to migrate and invade was inhibited in a dose-dependent manner, confirming the results of the wound-healing assay.

Effect of arsenic trioxide on Ewing's sarcoma cell adhesion

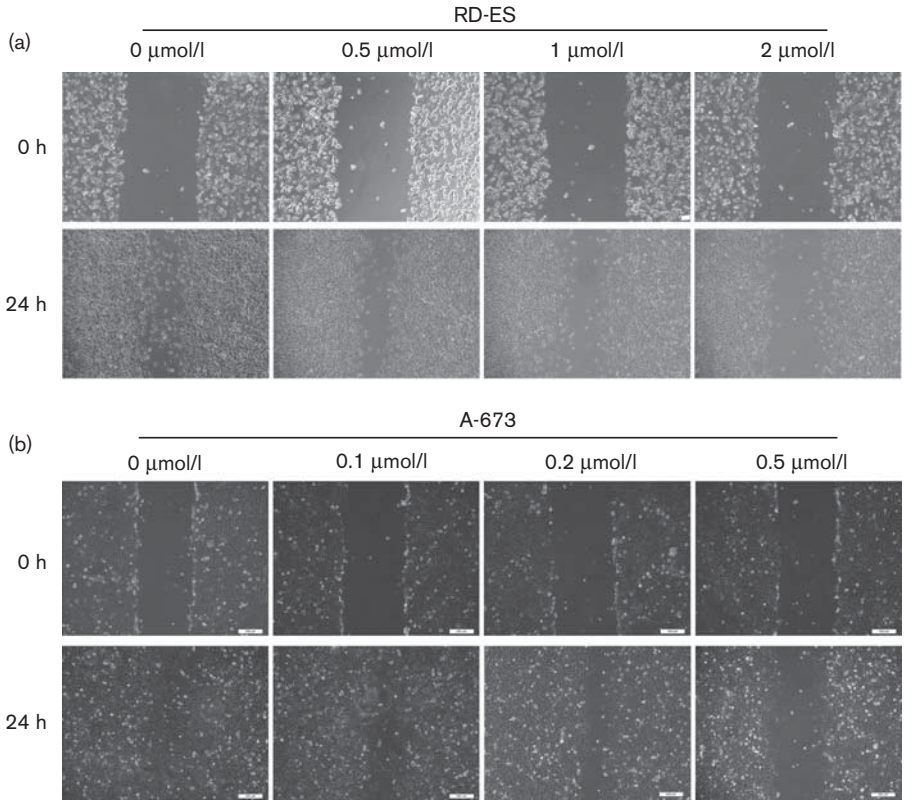
The adhesion of cancer cells to the extracellular matrix plays a significant role in the metastatic process after

Fig. 1



The cytotoxic effect of arsenic trioxide (As_2O_3) on Ewing's sarcoma cells. During the logarithmic phase, RD-ES and A-673 cells were treated with the indicated concentrations of As_2O_3 for 48 h, with or without serum. 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-2*H*-tetrazolium bromide-based assays were used to determine cytotoxicity. $**P<0.01$ compared with the untreated group with serum, $\#\#P<0.01$ compared with the untreated group without serum.

Fig. 2



Effect of arsenic trioxide (As_2O_3) on the motility of Ewing's sarcoma cells. RD-ES (a) and A-673 (b) cells were wounded after monolayer formation. The cell monolayers were incubated with the indicated concentrations of As_2O_3 in medium for 24 h. Images were taken under a microscope 0 and 24 h after wounding.

disaggregation from the primary tumor. Therefore, an inhibition of adhesion may contribute to a reduction in metastatic potential. We found that As_2O_3 reduced the adhesion of A-673 cells, but RD-ES cells were unaffected even at a concentration of 2 μmol/l of As_2O_3 (Fig. 4).

Arsenic trioxide affects the cytoskeleton of Ewing's sarcoma cells

Rearrangement of the cytoskeleton is an important characteristic of cancer cell metastasis during migration to other tissues and organs [18]. Polymerization of actin filaments at the leading edge and pseudopod formation in malignant cancer cells were observed upon cell spreading and were associated with a wealth of cell migration and invasion capability. As shown in Fig. 5, more cytoskeleton changes resulting in actin depolarization at the edges of the cell were visualized in Ewing's sarcoma cells treated with 2 μmol/l (RD-ES) or 0.5 μmol/l (A-673) of As_2O_3 compared with the untreated group. This result revealed that As_2O_3 may affect the structural rearrangements in the actin cytoskeleton of Ewing's sarcoma cells.

Arsenic trioxide inhibits the expression of phosphorylated $p38^{MAPK}$ and JNK in Ewing's sarcoma cells

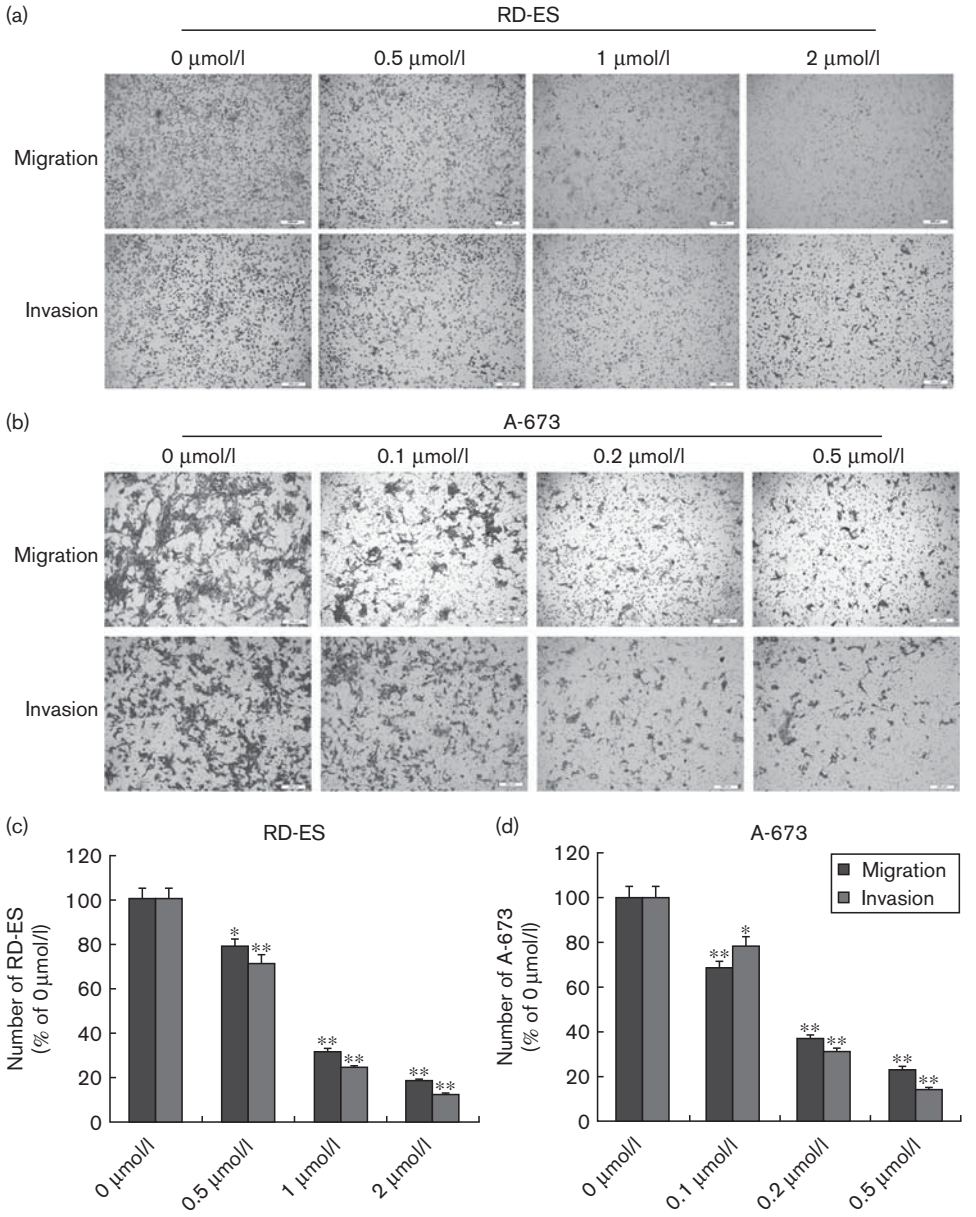
To investigate whether the MAPK pathway is involved in the decreased metastasis of Ewing's sarcoma cells induced by As_2O_3, we assessed the expression of total and phosphorylated ERK1/2, $p38^{MAPK}$, and JNK. As shown in Fig. 6, phosphorylation of $p38^{MAPK}$ and JNK was repressed by As_2O_3 in a dose-dependent manner. Unexpectedly, the expression of p-ERK1/2 was barely affected by As_2O_3. We also evaluated the expression of EWS/Fli-1, which is the critical oncogene responsible for the transformation of Ewing's sarcoma through the activation or repression of specific target genes and found that it was not affected by As_2O_3 treatment.

MMPs can degrade major components of the extracellular matrix and play critical roles in tumor invasion and metastasis [19,20]. MMP-9 expression decreased 24 h after As_2O_3 treatment.

JNK and $p38^{MAPK}$ inhibitors enhanced the inhibition of cell invasion induced by As_2O_3

To further confirm the influence of JNK and $p38^{MAPK}$ on the inhibition of cell invasion induced by As_2O_3,

Fig. 3



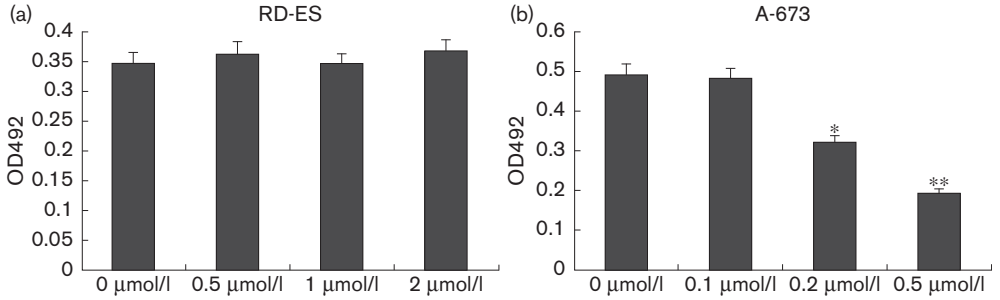
Arsenic trioxide (As_2O_3) inhibits Ewing's sarcoma cell migration and invasion *in vitro*. After treatment with the indicated concentrations of As_2O_3, RD-ES (a) and A-673 (b) cells that migrated or invaded under the membrane were observed under a microscope. Magnification: × 200. The migration and invasion were quantified by counting the number of RD-ES (c) and A-673 (d) cells that migrated into the inner membrane. Untreated cells were used as a control. Data are presented as mean ± standard deviation of three separate experiments and calculated as the percentage of control. $^*P<0.05$, $^{**}P<0.01$ compared with the control group.

sb202190 and sp600125, which specifically inhibit p38 and JNK, respectively, were used in invasion assays using A-673 and RD-ES cells. The results shown in Fig. 7 indicate that the use of inhibitors alone may reduce invasion, and its combination with As_2O_3 may further inhibit cell invasion. We then determined the action of inhibitors by the western blot assay. As shown in Fig. 8, they were found to inhibit the expression of phosphorylated $p38^{MAPK}$ and JNK.

Anisomycin counteracted As_2O_3-induced effects on migration, invasion, cytoskeleton rearrangement, and MMP-9 inhibition

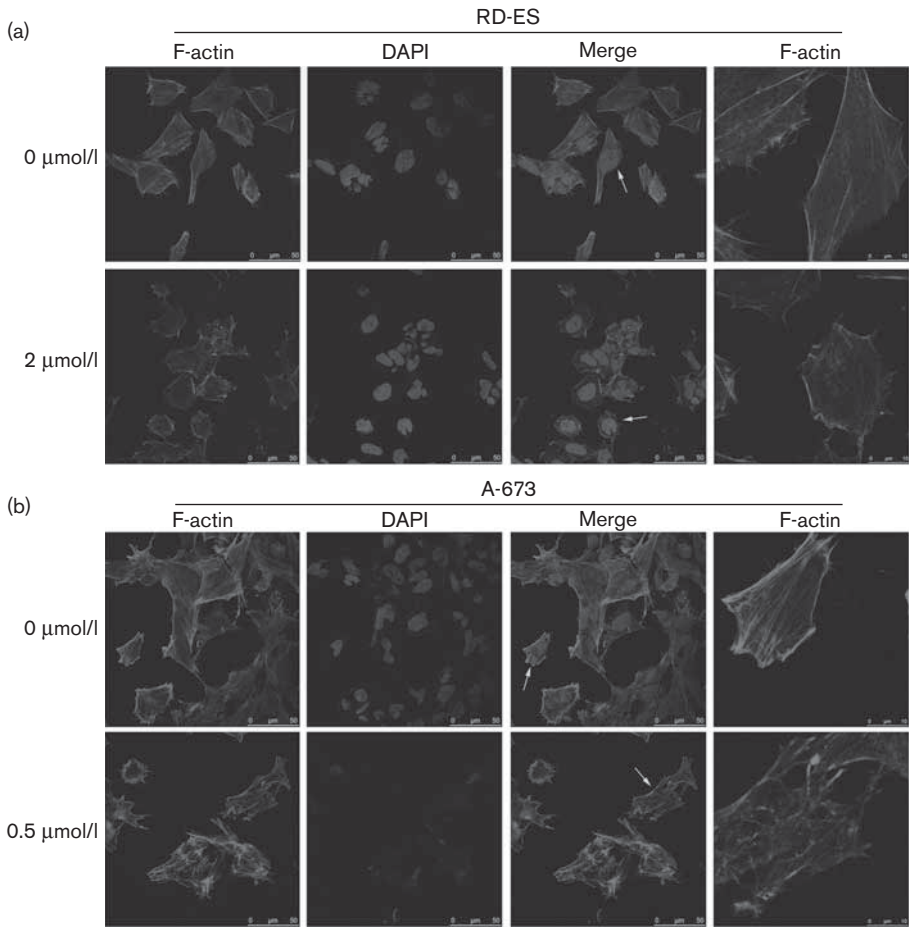
We used anisomycin, an activating agent of JNK and $p38^{MAPK}$, to confirm the action of JNK and $p38^{MAPK}$ in As_2O_3-induced effect on Ewing's sarcoma. As shown in Fig. 9, anisomycin counteracts the reduction of migration and invasion induced by As_2O_3 in Ewing's sarcoma cells. Anisomycin also restored the F-actin rearrangement caused

Fig. 4



Effect of arsenic trioxide (As_2O_3) on the adhesion of Ewing's sarcoma cells. RD-ES (a) and A-673 (b) cells incubated for 24 h with the indicated concentrations of As_2O_3 were seeded onto 96-well plates coated with Matrigel. After 60 min, the remaining absorbency of the attached cells at 492 nm in each well was measured. Data are given as the mean ± standard deviation of three independent experiments. $^*P<0.05$ and $^{**}P<0.01$ compared with the control group.

Fig. 5



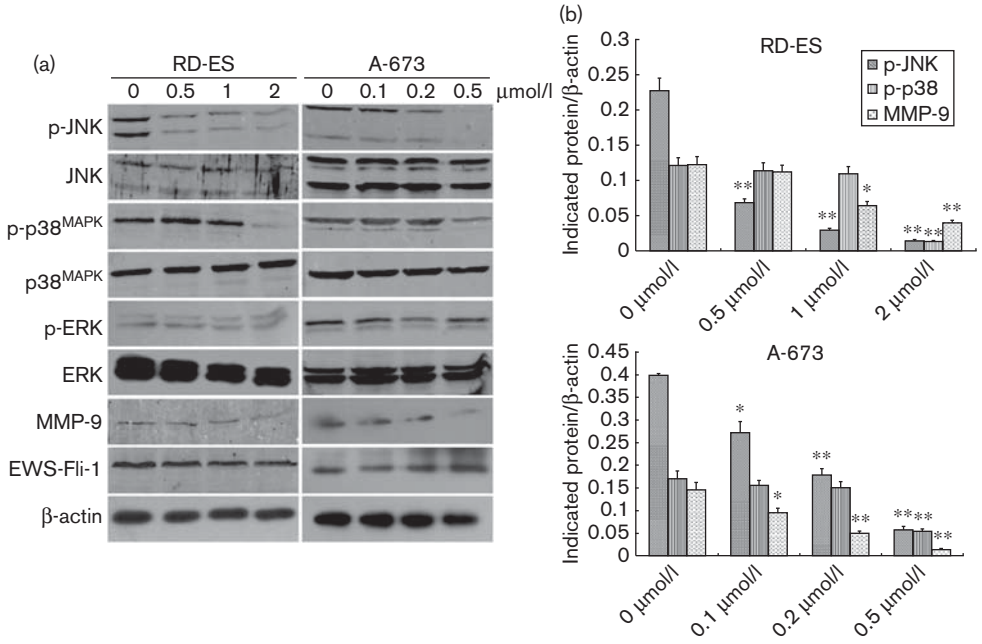
Actin rearrangements in Ewing's sarcoma cells treated with arsenic trioxide (As_2O_3). Cytoskeleton assays of RD-ES (a) and A-673 (b) cells were performed using confocal microscopy after 24 h incubation with As_2O_3. Actin rearrangements in Ewing's sarcoma cells were visualized by immunolocalization with TRITC-phalloidin (red). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). Treatment with As_2O_3 caused F-actin rearrangement and actin impolarization at the edges of the cells. Scale bar = 50 μm.

by As_2O_3, at least partially. Inhibition of MMP-9 expression induced in Ewing's sarcoma by As_2O_3 was also eliminated by anisomycin practically.

Discussion

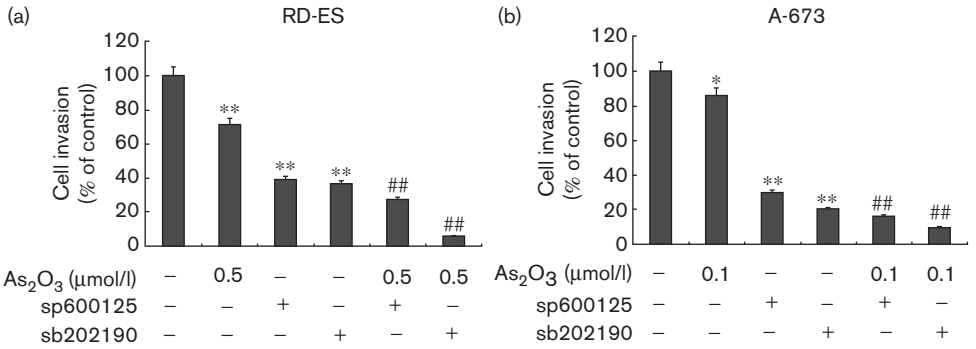
As the most widely studied and used arsenic-based drug, As_2O_3 has been proposed as an alternative for

Fig. 6



Effect of arsenic trioxide (As_2O_3) on mitogen-activated protein kinase (MAPK) expression. Western blot analysis was performed using cell lysates prepared from tumor cells treated with various concentrations of As_2O_3 and the indicated antibodies. (a) The expression levels of total and phosphorylated MAPKs, matrix metalloproteinase-9 (MMP-9), and EWS/Fli-1 in RD-ES and A-673 cells were evaluated as described in 'Materials and methods'. (b) Densitometric assays of phosphorylated JNK, $p38^{MAPK}$, and MMP-9 were performed and data were presented as mean ± standard deviation of three independent experiments. *$P<0.05$, **$P<0.01$ compared with control.

Fig. 7



Effects of arsenic trioxide (As_2O_3) and inhibitors on the invasion capacity of Ewing's sarcoma cells. After incubation with the indicated concentrations of As_2O_3, RD-ES and A-673 cells were pretreated with sp600125 (20 μmol/l) or sb202190 (20 μmol/l) for 1 h. Thereafter, a Matrigel invasion assay was performed to determine the invasion capability of the cells. Untreated cells were used as control. Data are presented as a percentage of control and expressed as mean ± standard deviation of three independent experiments. *$P<0.05$, **$P<0.01$ compared with no treatment. ##$P<0.01$ compared with treatment with 0.5 μmol/l or 0.1 μmol/l of As_2O_3.

therapy-resistant cancers, particularly acute promyelocyte leukemia. Manifold tumor-derived cells have shown susceptibility to As_2O_3 *in vitro* [8,21–23]. Although the drug's mechanism of action and application in many solid tumors has been increasingly known, studies on its antimetastatic effects are rare [24,25]. Ewing's sarcoma has a grievous impact on health because it commonly occurs in children and young adults. Because of the absence of effective therapies, prognosis is still dismal (approximately 20% overall survival) for patients presenting with clinically detectable metastases [26,27]. We recently investigated the effect of As_2O_3 on musculoskeletal tumors and found that it can induce apoptosis among Ewing's sarcoma cells [10] and inhibit osteosarcoma invasion [11]. In this

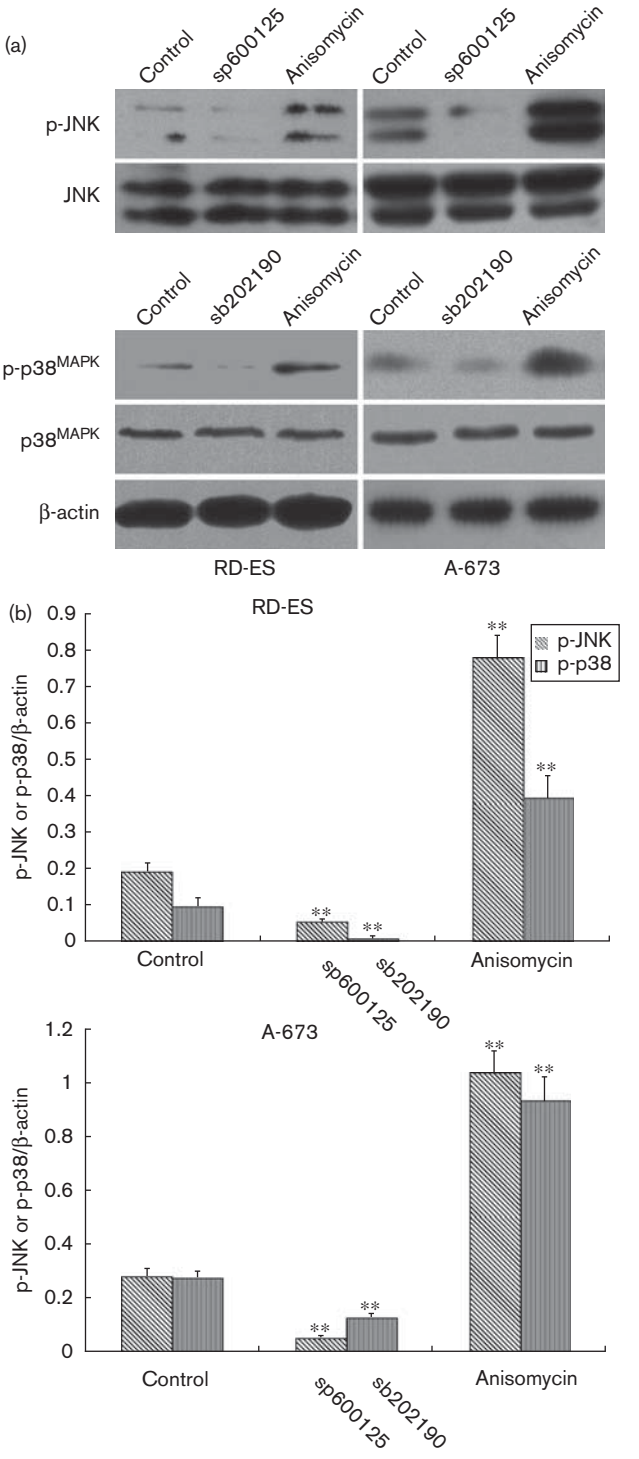
study, we presented the effect of As_2O_3 on the metastatic capability of Ewing's sarcoma cells.

To eliminate the inference of As_2O_3-induced apoptosis, we carried out cytotoxicity assays to determine the concentrations of As_2O_3 that have no effect on the proliferation of Ewing's sarcoma cells. The cell line A-673 was more sensitive to As_2O_3 than was RD-ES. Our results are similar to those of Beauchamp *et al.* [28]. The different cell lines had different sensitivities to As_2O_3, but the concentration ranges for both cell lines were much lower than clinically tolerable doses (< 5 μmol/l).

The metastatic spread of cancer remains one of the greatest barriers to curing the disease. Several complicated processes contribute to the metastasis of solid tumors, and the migration and invasion of tumor cells through the basement membrane are very important. We showed that As_2O_3 can significantly inhibit the migration and invasion capacity of Ewing's sarcoma cells in a concentration-dependent manner, and it has an effect on cytoskeleton rearrangement. We also found that As_2O_3 can decrease the mobility of Ewing's sarcoma cells on culture plates, which is an important factor influencing migration. The effect of As_2O_3 on Ewing's sarcoma mentioned above is similar to previously reported effects on other kinds of solid tumor cells [11,24,29]; thus, it is suggested that As_2O_3 may inhibit the migration of a wide range of cancer cells. We also investigated the effect of As_2O_3 on the adhesion of Ewing's sarcoma cells and found different influences on RD-ES and A-673 cells. As_2O_3 can decrease the attachment of A-673 cells to Matrigel, but it has no such effect on RD-ES cells. It may be because RD-ES has a mixed-growth property of adherent growth and clusters in suspension according to American Type Culture Collection description, wheres A-673 is adherent. Taking into account the inconsistent effect of As_2O_3 treatment on adhesion in other research, the results may be due to cell specificity [11,24]. Macroscopically, tumor cell invasion involves adhesion to and proteolysis of the basement membrane before migration through it. Among several kinds of proteins relative to cell invasion, MMPs are prominent components of tumor cells, degrading their ECM proteins [30]. On the basis of the dose-dependent depression of MMP-9 after treatment with As_2O_3, we infer that reduced MMP-9 expression may be a factor involved in the As_2O_3-induced inhibition of invasion in RD-ES and A-673 cells.

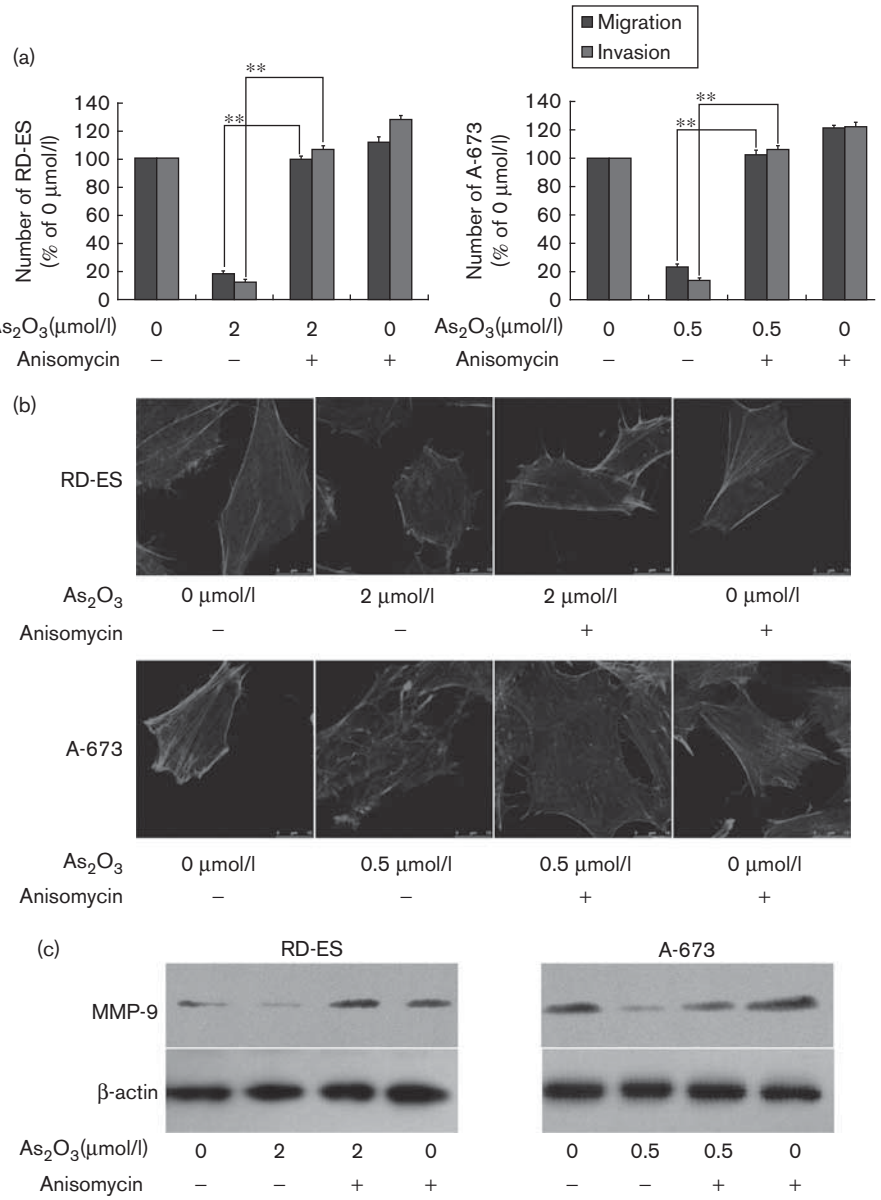
The expression levels of phosphorylated JNK and $p38^{MAPK}$, but not of ERK1/2, were suppressed after As_2O_3 treatment, whereas phosphorylated $p38^{MAPK}$ was inhibited only at the highest concentration of As_2O_3 in Ewing's sarcoma cells. The findings suggest that JNK may be an important modulator in the process induced by As_2O_3, whereas $p38^{MAPK}$ may be involved partially. Previous research revealed that As_2O_3 induced apoptosis of multiple kinds of cancer cells by activating JNK and

Fig. 8



Effect of inhibitors (sp600125, sb202190) and anisomycin on expression of phosphorylated c-Jun NH_2-terminal kinase and phosphorylated $p38^{MAPK}$. RD-ES and A-673 cells were pretreated with sp600125 (20 μmol/l) or sb202190 (20 μmol/l) for 1 h or with anisomycin (20μg/ml) for 30 min. (a) Western blot assay was performed to detect the expression of indicated protein. Beta-actin expression was used to assess equal lane loading. (b) Densitometric assays were performed and data are presented as mean ± standard deviation of three independent experiments. $^{**}P<0.01$ compared with control.

Fig. 9



Effect of anisomycin on reversing the arsenic trioxide (As_2O_3)-induced effects of migration/invasion, cytoskeleton rearrangement, and matrix metalloproteinase-9 (MMP-9) inhibition. (a) Anisomycin reverses the inhibition by As_2O_3 on the migration and invasion of Ewing's sarcoma cells. Data are presented as mean ± standard deviation of three independent experiments. $^{**}P < 0.01$ compared with treatment with 2 μmol/l or 0.5 μmol/l of As_2O_3 only. (b) Anisomycin partially reverses the F-actin disorganization induced by As_2O_3. (c) Anisomycin nearly eliminated the reduction of MMP-9 expression caused by As_2O_3.

$p38^{MAPK}$ [22,31]. Our results presented the reverse effect of low-concentration As_2O_3 on JNK and $p38^{MAPK}$. Bing Liu *et al.* [32] reported the opposing effects of low-dose As_2O_3 on hepatocellular carcinomas cells. Huei-Sheng Huang *et al.* [33] also reported that treatment with JNK inhibitor sp600125 could enhance apoptosis of HaCaT keratinocytes induced by As_2O_3. Further investigations are still warranted for the lack of relative research on low-concentration As_2O_3 and the controversy over its reversed effect.

MAPKs play an important role in cancer invasion with the capability of regulating the proteolytic enzymes that can degrade the basement membrane [34,35]. JNK and $p38^{MAPK}$ were reported to be involved in modulations of migration, invasion, and actin cytoskeleton rearrangement of various cancer cells [36–38]. Lu *et al.* [39] reported that the inactivation of JNK may be one reason for the decreased expression of MMP-2/9 by α-solanine and may inhibit the migration and invasion of human melanoma cells. Kumar *et al.* [40] found that $p38^{MAPK}$

regulates MMP-2/9 mRNA and MMP-2/9 activity relative to the invasive capacity of bladder cancer cells. Lee *et al.* [37] also reported that the NF-κB, JNK, and $p38^{MAPK}$ pathways are MMP-9 mediators induced by TLR2, TLR3, or TLR5 activation of human epidermal keratinocytes. The results for phosphorylated JNK, phosphorylated $p38^{MAPK}$, and MMP-9 levels in the research mentioned above are consistent with our results. The consistency implies that the inactivation of JNK and $p38^{MAPK}$ may be associated with the reduction in MMP-9 and may be involved in the inhibition of invasion and migration. Anisomycin, which is a protein activator of JNK and $p38^{MAPK}$ [41], was used to confirm the action of JNK and $p38^{MPAK}$ in the effect induced by As_2O_3. According to our results, anisomycin counteracted the inhibition of migration, invasion, cytoskeleton rearrangement, and MMP-9 expression induced by As_2O_3. Previous research has shown that AP-1, which is the downstream element of MAPKs, may be one of the transcriptional factors that induce expression of MMPs [34,42]. It has been suggested that JNK and $p38^{MAPK}$, which can activate AP-1 components c-jun and c-fos, respectively [43,44], may be involved in inducing MMPs by activating AP-1. Although ERK1/2 can also induce the activity of AP-1, it is not involved according to our results. However, the detailed mechanism of phosphorylated JNK and $p38^{MAPK}$ modulation in As_2O_3-induced invasion inhibition in Ewing's sarcoma remains unknown, and further investigations are needed.

In conclusion, our findings indicate that As_2O_3 may inhibit the migration and invasion of Ewing's sarcoma cells *in vitro* in a dose-dependent manner. Inactivation of JNK and $p38^{MAPK}$ is involved in the inhibitory effects, accompanying a reduction in MMP-9 and rearrangement of the cytoskeleton. Our results show an additional insight into the antitumor effect of As_2O_3 and provide a theoretical basis of using As_2O_3 as a possible therapeutic candidate aimed at controlling the invasion of Ewing's sarcoma.

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Conflicts of interest

There are no conflicts of interest.

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