

Downregulation of P-gp, Ras and p-ERK1/2 contributes to the arsenic trioxide-induced reduction in drug resistance towards doxorubicin in gastric cancer cell lines

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Abstract. Multidrug resistance (MDR) to doxorubicin (DOX) limits its effectiveness against tumor cells. Arsenic trioxide (As_2O_3) has been reported to reduce MDR in various types of cancer, but the mechanisms involving Ras and p-glycoprotein (P-gp) remain to be fully elucidated. The objectives of the present study were to evaluate As_2O_3 in reversing MDR to DOX, and to identify the association in antitumor activities between the effectiveness of DOX and Ras/phosphorylated (p-) extracellular signal-regulated kinase (ERK)1/2 signaling in SGC7901/ADM and SGC7901/S human gastric cancer cell lines. Cytotoxicity and sensitivity towards As_2O_3 were assessed using non-toxic and mildly-toxic concentrations (0.1 and 0.5 μM , respectively). The reversing effect of As_2O_3 on MDR was investigated prior to and following treatment with a cytokine activation of the recombinant human granulocyte colony stimulating factor ERK pathway. The SGC7901/ADM and SGC7901/S cells had the same sensitivity to As_2O_3 . The SGC7901/ADM cells were resistant to DOX and As_2O_3 treatment reduced the level of resistance to DOX ($P < 0.01$). The expression of P-glycoprotein (P-gp) in the SGC7901/ADM cells was higher than in the SGC7901/S cells ($P < 0.001$). As_2O_3 treatment decreased the levels of P-gp in a time- and dose-dependent manner ($P < 0.01$). The expression of Ras was higher in the SGC7901/ADM cells than in the SGC7901/S cells, while the expression of p-ERK1/2 remained the same. As_2O_3 decreased the levels of Ras and p-ERK1/2 ($P < 0.01$). Following pretreatment with rhG-CSF, the levels of Ras and p-ERK1/2 were further decreased ($P < 0.01$). Drug-resistant gastric cancer cells had higher expression levels

of P-gp and Ras, but not of p-ERK1/2. Non- and mildly-toxic doses of As_2O_3 reduced MDR to DOX through Ras/p-ERK1/2 signaling.

Introduction

In 2008, there were a 989,600 new cases of gastric cancer (GC) and a GC-associated mortality rate of 738,000 worldwide (1). Although the rate of GC-associated mortality has declined in the last 50 years (2), it remains the second leading cause of cancer-associated mortality worldwide, with an incidence rate ranking fourth following lung, breast and colorectal cancer (3). Systemic chemotherapy remains critical in the treatment of GC.

DOX is a well-known chemotherapeutic treatment used in Hodgkin's lymphoma, non-Hodgkin's lymphoma, multiple myeloma, acute leukemia, Kaposi's sarcoma, Ewing's sarcoma, Wilms' tumor and in solid tumors of the breast, adrenal cortex, prostate, thyroid, head and neck, endometrium, lung and ovary (4). DOX is widely used to treat GC in combination regimens with etoposide, cisplatin and 5-fluorouracil. However, drug resistance to DOX significantly limits its efficacy. Resistance to DOX is often associated with the expression of p-glycoprotein (P-gp) (4).

Multidrug resistance (MDR) is a complex process due to several mechanisms. Numerous resistance-associated proteins, including P-gp, have been found in different cancer cells, and overexpression of this transmembrane glycoprotein causes accelerated efflux of several lipid-soluble chemotherapeutic agents from the cells, decreasing their efficacy and leading to treatment failure (5). The difficulty of reversing this mechanism underlies its complexity.

Arsenic trioxide (As_2O_3) appears to achieve complete remission in 70% of patients with acute promyelocytic leukemia (APL) by inducing cancer cell apoptosis (6). Additional studies have confirmed that low doses of As_2O_3 induces complete remission in 90% of relapsed patients with APL (7-9). To date, a number of studies have provided novel insights into the pathogenesis of this agent and indicate that As_2O_3 may be useful in treating other types of cancer (10,11). It has become evident that the effects of As_2O_3 are not restricted to APL cells, but that As_2O_3 may also be effective in other malignant cells in

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several of types of cancer, including chronic myeloid leukemia, multiple myeloma, and solid prostate, esophageal and ovarian tumors (12-15). A number of mechanisms are associated with the effects of As₂O₃, including the induction of apoptosis, inhibition of growth, inhibition of differentiation and angiogenesis, and inhibition of mitochondrial respiration (16-18). In addition, several signaling pathways are involved in the antitumoral activity of As₂O₃, including inactivation of the Notch, c-Jun NH₂-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) signaling pathways (19-21). Human GC cells have been found to be sensitive to As₂O₃ (22).

Few studies have investigated the involvement of Ras and P-gp in MDR in GC cancer. The present study was designed to assess As₂O₃ in reversing DIX-induced MDR in GC cancer cell lines. The association between the antitumor effects of As₂O₃ and the Ras/phosphorylated (p-)ERK1/2 signaling pathway was also examined. The results of the present study aimed to provide novel insights into the treatment of gastric cancer.

Materials and methods

Chemicals and reagents. In the present study, As₂O₃ and 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cyclosporin A (CsA) was purchased from Novartis (Bale, Switzerland). RPMI 1640, fetal bovine serum (FBS), and dimethyl sulfoxide (DMSO) were purchased from Thermo Fisher Scientific (Hyclone, Waltham, MA, USA). DOX was purchased from Zhejiang Haizheng Pharmaceutical Co. (Taizhou, China). The recombinant human granulocyte colony stimulating factor (rhG-CSF) was purchased from Qilu Pharmaceutical (Jinan, China). The mouse anti-P-gp (P-170), Ras (cat. no. ZA-0343), p-ERK1/2 (cat. no. sc-7383), horseradish peroxidase (HRP)-conjugated goat-anti-mouse secondary antibody and the corresponding immunohistochemistry kits were purchased from Beijing Golden Bridge Biotechnology Co. (Beijing, China).

Cell culture. The parental SGC7901/S human GC cell line and the DOX-resistant SGC7901/ADM cell line were obtained from the Key Laboratory of Cancer Biology and Xijing Hospital of Digestive Diseases, Fourth Military Medical University (Xi'an, China). The SGC7901/ADM cells were obtained from the SGC7901/S cells, based on a previously described method (23). In brief, the SGC7901/ADM cells were induced *in vitro* by continuous exposure of SGC7901 parent cells to adriamycin (ADM) at concentrations of 0.25 µg/l to 25 µg/l. Cell lines capable of sustained growth in medium containing 25 µg/l ADM were considered to be resistant after 3 months. The SGC7901/S cells were cultured in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% (v/v) heat-inactivated FBS. The SGC7901/ADM cells were cultured in the same medium with 1 µg/ml DOX, and were grown without DOX for 2 weeks prior to the experiments. All the cells were cultured at 37°C in a 5% CO₂ humidified atmosphere and analyses were performed in the logarithmic growth phase.

Cytotoxicity and sensitivity of SGC7901/S and SGC7901/ADM cells to As₂O₃. The SGC7901/S and SGC7901/ADM cells were

trypsinized, centrifuged at 1,000 x g for 5 min, harvested and seeded into 96-well plates, in triplicate, at a density of 1x10⁴ cells/well (100 µl) at 37°C. Following overnight incubation, the cells became adherent. The culture medium was removed and As₂O₃ was added at 12 concentrations (0.01, 0.05, 0.10, 0.25, 0.50, 0.75, 1.0, 2.5, 5.0, 10.0, 15.0 or 20.0 mM). The cells (1x10⁴ cells/well) were incubated for 24 h, 48 h and 72 h, and were further incubated for an additional 4 h in the presence of 10 µl/well of MTT reagent, containing 5.00 mg/ml in phosphate-buffered saline (PBS). Following aspiration of the medium, the resulting formazan was dissolved with DMSO (100 µl/cell). The plates were agitated mechanically for 60 sec, and the optical density (OD) or absorbance was measured immediately at 490 nm on a microplate reader (AR2010; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The cell survival rate was calculated as: (OD of the assessment group / OD of the control group) x 100%. The inhibition rate and half maximal inhibitory concentration of the reagent (IC₅₀) were calculated based on the survival rate (SR) (24,25). The dose of As₂O₃, which resulted in a SR of >95% was considered non-toxic, and the dose resulting in a SR >85% was considered mildly-toxic.

Resistance of SGC7901/ADM cells and the resistance-reversing effect of As₂O₃. The present study included five groups, which were as follows: SGC7901/ADM, SGC7901/S, SGC7901/ADM+As₂O₃ (0.10 µM), SGC7901/ADM+As₂O₃ (0.50 µM) and SGC7901/ADM+CsA (4.00 µg/ml). These two concentrations of As₂O₃ were selected as the non-toxic and the mildly-toxic doses in the experiment described above. To investigate the drug resistance-reversing effect of As₂O₃, DOX (0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0 and 30.0 µg/ml) was added to the cell culture (1x10⁴ cells/well), rather than As₂O₃. The cells were then incubated for 24 h and 48 h at 37°C. The drug resistance ratio was calculated as the IC₅₀ of the drug-resistant cells divided by the IC₅₀ of the parental cells. The reversal ratio was calculated as the IC₅₀ of the drug-resistant cells divided by the IC₅₀ of the drug-resistant cells treated with reversal agents,

Reversing effect of As₂O₃ following treatment with rhG-CSF. The SGC7901/ADM, SGC7901/S, SGC7901/ADM+0.10µMAS₂O₃, SGC7901/ADM+0.50µMAS₂O₃, and SGC7901/ADM+4.00 µg/ml CsA cells (1x10⁴ cells/well) were incubated with rhG-CSF (1.50 µg/ml) for 3 days at 37°C, and then cultured with DOX at different concentrations (0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 30.0, 40.0 and 50.0 µM) for a further 2 days at 37°C, following which the reversing effect of As₂O₃ was assessed. The blank control contained no cells in the culture medium, while the negative control contained medium with no reagent.

Analyzing the expression of P-gp using western blotting. The SGC7901/ADM and SGC7901/S cells (1x10⁴ cells/well) were grouped, as described above, and were pretreated with non- and mildly-toxic concentrations of As₂O₃ for 24, 48 and 72 h (18). The cells were collected by centrifugation at 1,000 x g for 20 min at 4°C and washed with ice-cold PBS. The cell pellets were then resuspended in lysis buffer (Invitrogen Life Technologies, Carlsbad, CA, USA), containing 50 mM Tris-chloride (pH 7.4), 150 mM sodium chloride, 0.1% sodium dodecyl sulfate, 1% Triton-100 and 1 mM ethyl-

enediaminetetraacetic acid (pH 8.0). Following incubation at 4°C for 40 min, the samples were centrifuged at 15,400 x g for 20 min. The supernatants containing cytosolic proteins were separated by polyacrylamide gel (Bio-Rad Laboratories, Inc.) electrophoresis at 200 V for 1-2 h, and were transferred onto a nitrocellulose membrane (EMD Millipore, Billerica, MA, USA) at 120 V for 3 h. The membrane was blocked with 5% skim milk powder at room temperature for 2 h, followed by incubation with primary monoclonal mouse anti-human antibody (P-gp, 1:50) for 2 h at room temperature. Following washing with blocking buffer, the membranes were incubated with HRP-conjugated goat-anti-mouse secondary antibody (1:1,000) for 2 h at room temperature, followed by chemiluminescence detection (Pierce Biotechnology, Inc., Rockford, IL, USA). Western blot analysis was performed using an image analysis system (VIDAS System; Biomérieux, Marcy l'Étoile, France).

Immunocytochemical assays

Expression levels of Ras and p-ERK1/2 in cells treated with As₂O₃. A total of six treatment groups were used to assess the levels of Ras and p-ERK1/2: SGC7901/ADM+As₂O₃ (0.10, 0.50, and 0.75 μM), SGC7901/ADM+CsA (4.00 μg/ml), SGC7901/ADM and SGC7901/S. The cells (1x10⁴ cells/well) were treated, as described above, and incubated for 24, 48, and 72 h. As previously described (13), the cells were fixed in ice-cold acetone for 15 min. Following incubating with 3% hydrogen peroxide-carbinol for 10 min, the cells were incubated at 37°C for 2 h with the following primary antibodies: Mouse-anti-human monoclonal Ras or p-ERK1/2. Following incubation with Poly Helper (50 μl; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 37°C for 30 min, the HRP-conjugated goat-anti-mouse IgG antibody solution was added and incubated again at 37°C for 30 min. Finally, staining with 3,3'-diaminobenzidine (Sigma-Aldrich) and hematoxylin (Sigma-Aldrich) were performed. The labeled cells were analyzed using an image analysis system (VIDAS, Option Company).

Expression levels of Ras and p-ERK1/2 following treatment with an ERK activator. rhG-CSF is a cytokine, which activates a number of cellular pathways, including the ERK pathway (26,27). Therefore, the SGC7901/ADM and SGC7901/S cells (1x10⁴ cells/well) were treated with rhG-CSF (1.50 μg/ml) for 3 days at 37°C, and then divided into the five treatment groups, described above. The cells were then incubated with the primary antibody. The experiments were repeated three times.

Statistical analysis. Data are presented as the mean ± standard deviation of three independent experiments. Student's t-test and analysis of variance were used to analyze the differences between groups, with a Student-Newman-Keuls (q-test) post hoc test. P<0.05 was considered to indicate a statistically significant difference. All data were analyzed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Sensitivity of SGC7901/ADM and SGC7901/S cells to As₂O₃. In the present study, SGC7901/ADM and SGC7901/S cells

were treated with different concentrations of As₂O₃ for 24, 48 and 72 h, following which the OD at 490 nm was measured and the IC₅₀ and survival rates were calculated (Table I). The IC₅₀ values of the two cell types to As₂O₃ decreased gradually with increasing treatment duration. However, no difference was observed between the two cell lines at any time-point (all P>0.05). Doses of 0.1 and 0.5 mM were considered non-toxic and mildly-toxic doses, respectively (Table I), and subsequent experiments were performed using these doses. These results revealed that the SGC7901/ADM and SGC7901/S cells had the same sensitivity to As₂O₃, which occurred in a time- and dose-dependent manner.

As₂O₃ reduces drug resistance of SGC7901/ADM cells to DOX, possibly through the p-ERK1/2 pathway. The IC₅₀ to DOX was assessed in the five groups of cells incubated for 24 and 48 h. The experimental groups were: SGC7901/ADM+0.10 μM As₂O₃ and SGC7901/ADM+0.50 μM As₂O₃. The positive control group was SGC7901/ADM+4.00 μg/ml CsA. The negative control groups were SGC7901/ADM and SGC7901/S. The results revealed that the level of drug resistance between the resistant and parental cells were 6.63- and 6.85-fold at 24 h and 48 h, respectively (Table II). The IC₅₀ values to DOX in the experimental groups were significantly lower than that in the SGC7901/ADM group when treated with 0.10 μM at 24 and 48 h (P<0.01) and with 0.50 μM at 24 and 48 h (P<0.01). The reversal in the CsA group was ~4.5-fold at different time points, while in the experimental groups, the effect was time-dependent (Table II). The IC₅₀ of As₂O₃ at 0.50 μM was markedly lower than that at As₂O₃ at 0.10 μM, particularly at 48 h. The value was close to that in the SGC7901/S group and lower than that in the CsA group, but without statistical significance (P>0.05).

Following treatment with rhG-CSF, a cytokine that activates the ERK pathway, the IC₅₀ at 48 h was assessed again (Table II). Compared with the cells without rhG-CSF treatment, the IC₅₀ to DOX of As₂O₃ (0.50 μM) was significantly higher (P<0.05), suggesting that the ERK activator had the ability to partially reduce the drug resistance-reversing effect of As₂O₃.

These findings suggested that the SGC7901/ADM cells were resistant to DOX, and that As₂O₃ had a drug resistance-reversing effect, in a time- and dose-dependent manner. In addition, p-ERK1/2 may be involved in this mechanism, which requires further investigation.

Reduction in the expression of P-gp. The expression of P-gp was detected using western blotting (Fig. 1). The expression of P-gp was more marked in the SGC7901/ADM cells than in the SGC7901/S cells (0.38±0.025 vs. 0.19±0.012, respectively; P<0.001), without significant variation in with time (Table III). In the SGC7901/ADM cells, As₂O₃ at the non-toxic concentration (0.10 μM) at 24 h did not decrease the protein expression of P-gp, however, the increase in duration significantly decreased the levels of P-gp (P<0.01). Unlike the non-toxic concentration, the mildly-toxic concentration (0.50 μM) reduced the levels of P-gp even at 24 h (P<0.01). In the positive control cells, the levels of P-gp were close to that in the SGC7901/S cells, without significant changes with time (Table III). These results revealed that the expression of P-gp in the drug-resistant cells was higher than in the parental cells. CsA appeared to reduce the protein expression of P-gp, and As₂O₃ decreased the levels

Table I. IC₅₀ volume of non- and mildly-toxic doses of arsenic trioxide at different time-points.

Cell line	IC ₅₀ μM ^a			95% >SR>85% ^b			SR>95% ^c		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
SGC7901/ADM	5.57±0.36	2.63±0.18	1.42±0.09	0.10-0.08	0.10-0.75	0.05-0.25	0.01-0.01	0.01-0.01	0.01-0.05
SGC7901/S	5.94±0.54	3.06±0.28	1.59±0.12	0.25-1.30	0.07-0.08	0.04-0.25	0.01-0.25	0.01-0.07	0.01-0.04

^aData are presented as the mean ± standard deviation of three experiments (n=3); ^bSR>95% was considered the non-toxic dose; ^c95% >SR>85% was considered a mildly-toxic dose. SR, survival rate; IC₅₀, half maximal inhibitory concentration.

Table II. Comparison of the IC₅₀ values of DOX and drug-reversing ratio of As₂O₃ at different time-points.

Factor	Treatment	-rhG-CSF		+rhG-CSF
		24 h	48 h	48 h
IC ₅₀ to DOX ^a	As ₂ O ₃ (0.1 μM)	23.46±3.30	14.35±1.84	19.28±2.16
	As ₂ O ₃ (0.5 μM)	9.52±1.02	4.28±0.55	6.85±1.31
	CsA (4 μg/ml)	7.62±0.83	5.98±0.71	7.73±2.24
	SGC7901/ADM	34.31±4.38	26.94±3.67	32.39±4.52
	SGC7901/S	5.17±0.46	3.93±0.42	5.49±0.68
Drug-resistance ratio ^b		6.63	6.85	5.90
Drug-reversal ratio ^c	As ₂ O ₃ (0.1 μM)	1.46	1.87	1.68
	As ₂ O ₃ (0.5 μM)	3.60	6.29	4.72
	CsA (4 μg/ml)	4.50	4.51	4.19

^aData are presented as the mean ± standard deviation. ^bDrug resistance ratio was calculated as the IC₅₀ of resistant cells to that of SGC7901/S cells. ^cReversal ratio was calculated as the IC₅₀ of resistant cells to the reversal-agent treated cells. As₂O₃, arsenic trioxide; CsA, cyclosporin A; IC₅₀, half maximal inhibitory concentration; DOX, doxorubicin; rhG-CSF, recombinant human granulocyte colony stimulating factor.

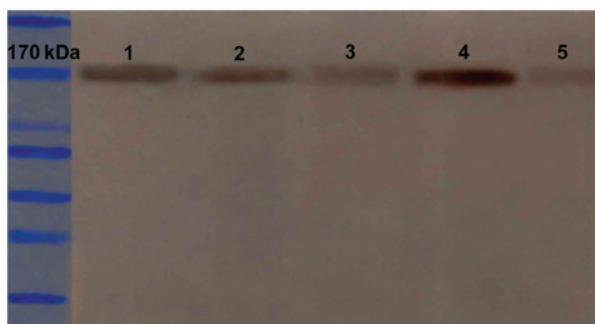


Figure 1. Protein expression levels of P-gp with or without arsenic trioxide treatment for 48 h, determined using western blotting. 1, protein expression of P-gp in SGC7901/ADM cells treated with 0.1 μM As₂O₃ for 48 h; 2, protein expression of P-gp in SGC7901/ADM cells treated with 0.5 μM As₂O₃ for 48 h; 3, protein expression of P-gp in SGC7901/ADM cells treated with 4 μg/ml CsA for 48 h; 4, protein expression of P-gp in SGC7901/ADM cells without As₂O₃; 5, protein expression of P-gp in SGC7901/S without As₂O₃. P-gp, p-glycoprotein; CsA, cyclosporin A; As₂O₃, arsenic trioxide.

of P-gp, even at non- or mildly-toxic concentrations, in a time- and dose-dependent manner.

Expression of Ras and p-ERK1/2 expression, determined using immunocytochemistry. Ras protein was predominantly

localized in the membrane and partially translocated in the cytoplasm close to the membrane (Fig. 2A). The expression of Ras was higher in the SGC7901/ADM cells than in the SGC7901/S cells (P<0.01). Following treatment with As₂O₃, the levels of Ras were reduced in a time- and dose-dependent manner (Table III; Fig. 2B). At 24 h, only the 0.75 μM group exhibited lower expression than the SGC7901/ADM cells (P<0.01). At 48 and 72 h, the cell groups treated with As₂O₃ had significantly lower levels of Ras (P<0.05). The expression of Ras in the three control groups remained stable over time. These results suggested that drug-resistant cells may have a higher expression levels of Ras expression, and that As₂O₃ decreases these levels.

Expression of p-ERK1/2 expression was detected in the cytoplasm surrounding the nucleus of cells (Fig. 3A and B). No significant differences were identified in the levels of p-ERK1/2 between the SGC7901/ADM, SGC7901/S and CsA-treated cells. Following treatment with As₂O₃, the protein expression of p-ERK1/2 decreased (Table III). At 24 h, the levels of p-ERK1/2 were decreased at all As₂O₃ concentrations, without statistical significance (P>0.05). However, in the 0.50 and 0.75 μM As₂O₃ groups, the expression of p-ERK1/2 was decreased, particularly in the As₂O₃ 0.75 μM group at 72 h (P<0.01). These results suggested that the Ras/p-ERK1/2 signaling pathway may be involved in the mechanism of reducing drug resistance to DOX by As₂O₃ in GC cells.

Table III. Comparison of the OD values of P-gp, measured using western blot analysis, and the expression levels of Ras and p-ERK1/2, measured using immunocytochemistry, at different time-points.

Protein	Treatment	OD value		
		24 h	48 h	72 h
P-gp	As ₂ O ₃ (0.1 μM)	0.349±0.001	0.32±0.003	0.29±0.002
	As ₂ O ₃ (0.5 μM)	0.30±0.002	0.28±0.002	0.16±0.001
	CsA (4 μg/ml)	0.16±0.004	0.16±0.001	0.15±0.002
	SGC7901/ADM	0.32±0.061	0.38±0.010	0.38±0.005
	SGC7901/S	0.14±0.003	0.14±0.003	0.14±0.002
Ras	As ₂ O ₃ (0.1 μM)	0.091±0.004	0.084±0.001	0.080±0.002
	As ₂ O ₃ (0.5 μM)	0.089±0.003	0.078±0.003	0.076±0.003
	As ₂ O ₃ (0.75 μM)	0.081±0.004	0.073±0.002	0.073±0.002
	CsA (4 μg/ml)	0.091±0.003	0.093±0.003	0.084±0.005
	SGC7901/ADM	0.093±0.003	0.090±0.003	0.091±0.004
	SGC7901/S	0.068±0.003	0.071±0.005	0.066±0.008
p-ERK1/2	As ₂ O ₃ (0.1 μM)	0.099±0.009	0.086±0.002	0.083±0.004
	As ₂ O ₃ (0.5 μM)	0.086±0.007	0.080±0.002	0.070±0.001
	As ₂ O ₃ (0.75 μM)	0.08±0.004	0.078±0.005	0.067±0.002
	CsA (4 μg/ml)	0.098±0.009	0.11±0.018	0.11±0.025
	SGC7901/ADM	0.11±0.008	0.11±0.012	0.11±0.014
	SGC7901/S	0.10±0.016	0.090±0.010	0.10±0.027

Data are presented as the mean ± standard deviation of three experiments. As₂O₃, arsenic trioxide; CsA, cyclosporin A; P-gp, P-glycoprotein; p-ERK, phosphorylated extracellular signal-regulated kinase; OD, optical density.

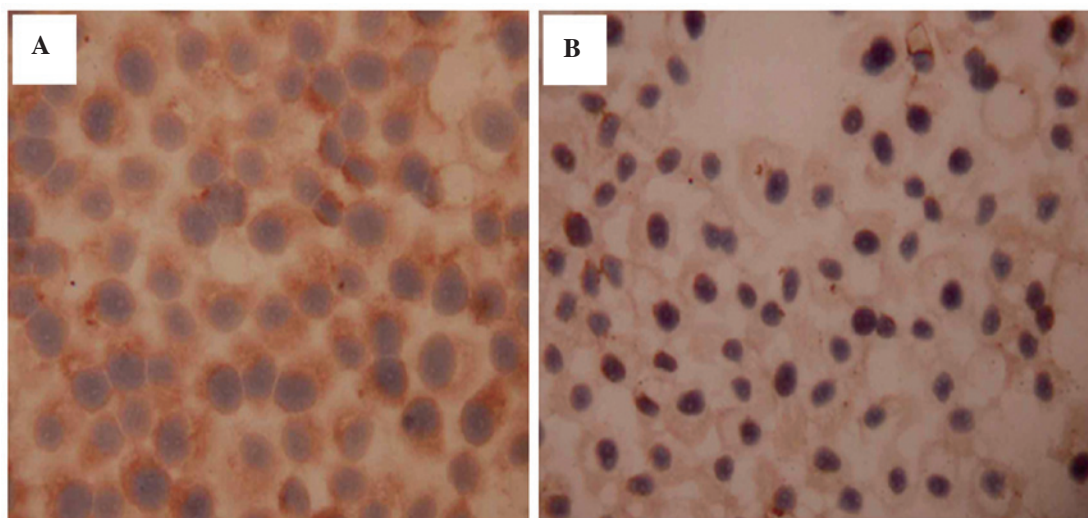


Figure 2. Immunohistochemistry of Ras protein in (A) SGC7901/ADM cells (Ras predominantly localized in the membrane and partially translocated in the cytoplasm) and (B) SGC7901/ADM cells treated with 0.5 μM As₂O₃ for 48 h (Ras protein expression observed to be reduced) (magnification, x400). As₂O₃, arsenic trioxide.

Ras/p-ERK1/2 signaling pathway is involved in reducing drug resistance. The cells were incubated with non- and mildly-toxic concentrations of As₂O₃ for 48 h and, following pretreatment with rhG-CSF, cytokines activating the ERK pathway, Ras and p-ERK1/2 were assessed using immunocytochemistry.

Following rhG-CSF treatment, the expression of Ras was similar in all groups (P>0.05). No difference was identified in

the levels of Ras between the SGC7901/ADM and CsA-treated cells (P>0.05; Table IV; Fig. 4A and B). Compared with the cells without rhG-CSF treatment, the levels of Ras were not different (P=0.12) in the 0.1 μM As₂O₃-treated groups, but were higher in the As₂O₃ 0.5 μM As₂O₃-treated groups (P<0.01).

The levels of p-ERK1/2 in all the cells increased following rhG-CSF treatment (Table IV; P<0.01). Treatment with 0.10 and

Table IV. Protein expression levels of Ras and p-ERK1/2 with or without treatment of rhG-CSF, determined using immunocytochemistry (optical density).

Protein	Treatment	rhG-CSF/-	rhG-CSF/+
Ras b	As ₂ O ₃ (0.1 μM) ^b	0.084±0.001	0.093±0.008
	As ₂ O ₃ (0.5 μM)	0.078±0.003	0.089±0.001
	CsA (4 μg/ml)	0.093±0.003	0.099±0.005
	SGC7901/ADM	0.094±0.006	0.10±0.008
p-ERK1/2 ^a	As ₂ O ₃ (0.1 μM)	0.086±0.002	0.093±0.002
	As ₂ O ₃ (0.5 μM)	0.080±0.002	0.091±0.001
	CsA (4 μg/ml)	0.11±0.018	0.12±0.019
	SGC7901/ADM ^c	0.10±0.010	0.14±0.003

Data are presented as the mean ± standard deviation of three experiments. ^aCells were incubated with non- and mildly-toxic concentrations of As₂O₃. ^bCompared with cells without rhG-CSF, the levels of Ras levels not significantly different (P=0.12) in the As₂O₃ (0.1 μM) groups, but were higher in the As₂O₃ (0.5 μM) groups (P<0.01). ^cExpression of p-ERK1/2 in the SGC7901/ADM cells increased following rhG-CSF treatment (P<0.01). As₂O₃, arsenic trioxide; rhG-CSF, recombinant human granulocyte colony stimulating factor; p-ERK, phosphorylated extracellular signal-regulated kinase; CsA, cyclosporin A.

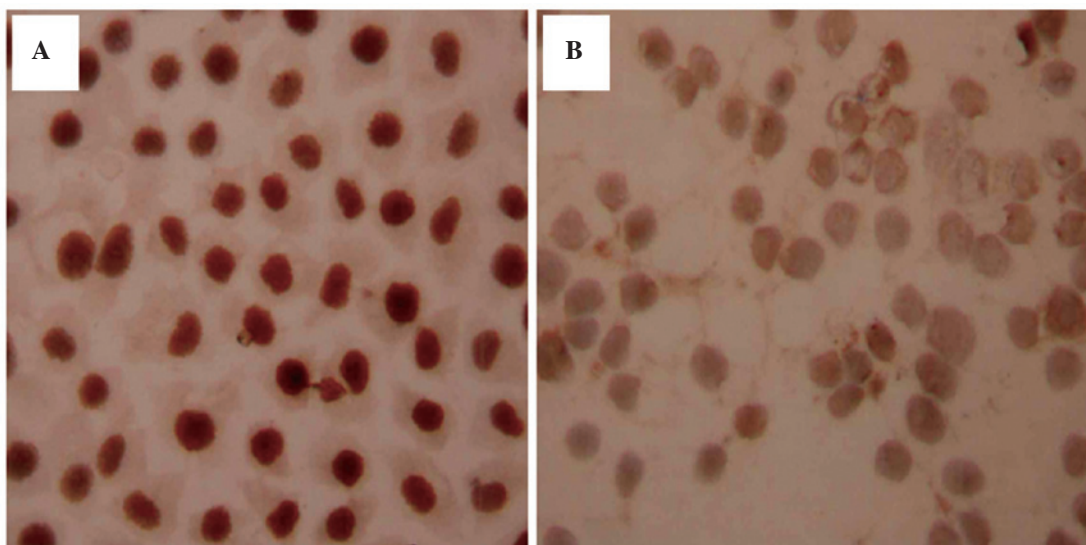


Figure 3. Immunohistochemistry of (A) p-ERK1/2 protein in the SGC7901/ADM cells and (B) SGC7901/ADM cells treated with 0.5 μM As₂O₃ for 48 h (magnification, x400). Expression of p-ERK1/2 was detected in the cytoplasm surrounding the nucleus of the cells. p-ERK, phosphorylated-extracellular signal-regulated kinase; As₂O₃, arsenic trioxide.

0.50 μM As₂O₃ reduced the levels of p-ERK1/2, compared with the negative control group (P<0.05; Fig. 5A and B), whereas the levels of p-ERK1/2 increased significantly at the same doses of As₂O₃ following rhF-CSF treatment (P<0.01). Similar to Ras, these results suggested that the expression of p-ERK1/2 decreased following treatment with the ERK activator.

Discussion

GC is one of the most common types of cancer, and generally has a poor prognosis. DOX is used as one of the key components in a number of chemotherapeutic regimens against GC, however, MDR eventually limits its use. Several types of cancer cell, including non-acute myeloid leukemia, multiple myeloma, breast cancer and APL, have been found

to be sensitive to As₂O₃ through cell growth inhibition and apoptosis (12,20,28,29). However, few studies have examined the effects of As₂O₃ in GC cells, and these studies reported a number of different mechanisms (30-32). In the present study, the human GC cell lines, SGC7901/S and SGC7901/ADM, were investigated. The latter was induced by long-term continuous exposure to DOX in a step-wise increment of various concentrations. The high IC₅₀ of SGC7901/ADM, but not of SGC7901/S, confirmed the resistance to DOX. The doses of 0.10 and 0.50 μM of As₂O₃ were defined as non- and mildly-toxic concentrations, respectively. This was different from the study by Zhao *et al* (22), which reported that 2 μM was the non-toxic concentration. In the MTT assay for the detection of cytotoxicity and sensitivity to As₂O₃, cell growth was inhibited without any treatment at 72 h. This was possibly

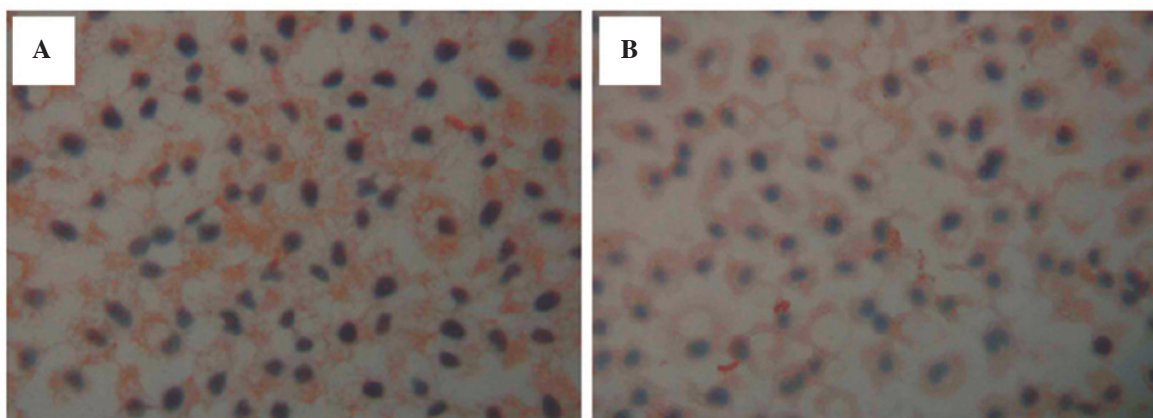


Figure 4. Immunohistochemistry of (A) Ras protein in the SGC7901/ADM cells following treatment with $0.1 \mu\text{M}$ As_2O_3 and rhG-CSF for 48 h. (B) Ras protein in the SGC7901/ADM cells following treatment with $0.5 \mu\text{M}$ As_2O_3 and rhG-CSF for 48 h (magnification, x400). No differences were identified in the levels of Ras between the SGC7901/ADM- and CsA-treated cells. Compared with the cells without rhG-CSF treatment, the levels of Ras were not significantly different ($P=0.12$) in the $0.1 \mu\text{M}$ As_2O_3 -treated groups, however were greater in the $0.5 \mu\text{M}$ As_2O_3 -treated groups ($P<0.01$). rhG-CSF, recombinant human granulocyte colony stimulating factor; As_2O_3 , arsenic trioxide.

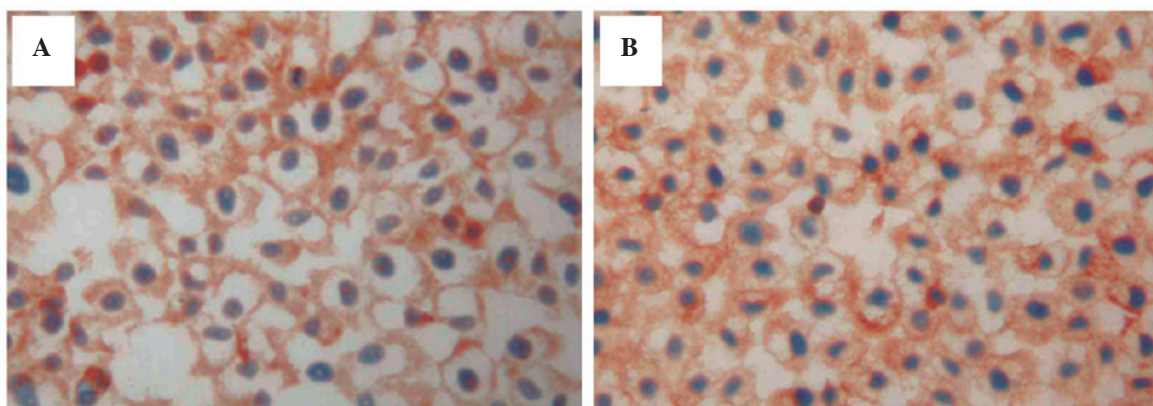


Figure 5. Immunohistochemistry of (A) p-ERK1/2 protein in the SGC7901/ADM cells following treatment with As_2O_3 ($0.1 \mu\text{M}$) and rhG-CSF for 48 h; (B) p-ERK1/2 protein in the SGC7901/ADM cells following treatment with $0.5 \mu\text{M}$ As_2O_3 and rhG-CSF for 48 h (magnification, x400). Treatment with 0.10 and $0.50 \mu\text{M}$ As_2O_3 reduced the levels of p-ERK1/2, compared with the negative control group. p-ERK, phosphorylated extracellular signal-regulated kinase; rhG-CSF, recombinant human granulocyte colony stimulating factor; As_2O_3 , arsenic trioxide.

the result of the toxic metabolic accumulation and nutrient deficiency. Previously, it was found that As_2O_3 induced APL cells to enter apoptosis (6). The present study demonstrated that As_2O_3 partially reduced *in vitro* drug-resistance, even at a non-toxic dose ($0.10 \mu\text{M}$), in a time- and dose-dependent manner.

A number of mechanisms are involved in MDR, and the overexpression of P-gp is one of the key mechanisms. Agents to reduce MDR have been investigated for a number of years (33,34). A novel inhibitor of P-gp-mediated MDR, OC144-093, was reported in 2000 (28). In the present study, the expression of P-gp in SGC7901/ADM was higher than in the parental cells and, following As_2O_3 treatment, the levels of P-gp decreased significantly. The non- and mildly-toxic doses of As_2O_3 were selected with the purpose of using them in clinical practice without serious side effects.

Several studies have reported the *in vitro* effects of As_2O_3 in the inhibition of different types of solid tumor, through a number of different mechanisms. Eguchi *et al* (19) demonstrated that apoptosis induced in human mesothelioma cells is accompanied by the activation of JNK1/2 and ERK1/2 (19). In

breast cancer, As_2O_3 inhibits cell growth through the inactivation of the Notch signaling pathway (20). To investigate the role of the Ras/p-ERK1/2 signaling pathway in the mechanism of As_2O_3 , the rhG-CSF cytokine, which activates the ERK pathway was used to treat the cells.

Ras is a monomeric GTP-coupled protein encoded by the Ras gene, which is important in cell growth regulation (35). It was reported in 1993 that Ras has direct interactions with the RAF1 serine/threonine kinase, which was the first mammalian Ras effector to be identified (36). ERK1 and ERK2, also termed mitogen-activated protein kinase (MAPK)1 and MAPK2 are MAPK isomers, which are widely expressed in eukaryotic cells. In addition, the identification of B-Raf mutations in cancer emphasize the importance for aberrant Raf-MEK-ERK signaling in oncogenesis (36). In the present study, the levels of Ras and p-ERK were determined using immunocytochemistry. The levels of Ras were higher in the SGC7901/ADM cells than in the SGC7901/S cells, however, no significant difference was observed in the levels of p-ERK1/2. The results suggested that p-ERK1/2 was not involved in the MDR mechanisms in these cells, but indicated that Ras was

involved. Following treatment with As₂O₃, the levels of Ras levels reduced, and the protein levels of p-ERK1/2 decreased until intervention with mildly-toxic concentrations of As₂O₃ (0.50 and 0.75 μM).

Following pretreatment with rhG-CSF, no change was observed in the levels of Ras in cells from the control groups, however, levels increased in the 0.50 μM As₂O₃ treatment group. The expression of p-ERK1/2 increased in all cells, suggesting that rhG-CSF activated p-ERK1/2. As₂O₃ reduced the levels of p-ERK1/2, compared with the negative control group. Thus, a p-ERK activator may partially inhibit the drug resistance-reversing effects of As₂O₃. A phase II trial revealed that As₂O₃ is inactive in patients with pancreatic cancer, who develop a progressive disease following gemcitabine treatment (37). This failure may be due to the lack of co-treatment with other chemotherapeutics. As₂O₃ has been demonstrated to be effective in treating APL without significant side effects (8,9).

The present study involved the examination of MDR induced by DOX. There are several other chemotherapeutic agents, which are actively used in the treatment of GC in a number of combination regimens (38). Further investigations are required to examine the effect of As₂O₃ in reversing the MDR induced by these other agents. In addition, it is important to further investigate the effects of As₂O₃ in GC, compared with other types of cancer. Although the present study clearly suggested the involvement of Ras in the MDR of GC, further investigations are required to clarify whether mutations in Raf are involved in drug-resistant cells. Finally, the rhG-CSF cytokine, used in the present study to activate the ERK pathway, also activates a number of other cell mechanisms, which require further investigations, including the PI3K/AKT pathway (39,40).

In conclusion, the results of the present study revealed that As₂O₃ had the ability to reverse MDR in human GC cells. This mechanism may be relevant to reduce the expression of P-gp. Drug-resistant cells may have higher expression levels of Ras, and As₂O₃ may decrease these levels. The Ras/p-ERK1/2 signal transduction pathway may be involved in this mechanism. Further investigations, involving a combination of chemotherapeutics and arsenic trioxide, are essential.

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