

INHIBITION OF MITOGEN-ACTIVATED PROTEIN KINASE KINASE ENHANCES APOPTOSIS INDUCED BY ARSENIC TRIOXIDE IN HUMAN BREAST CANCER MCF-7 CELLS

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SUMMARY

1. Arsenic trioxide (As_2O_3) has recently been used to treat acute promyelocytic leukaemia and has activity *in vitro* against several solid tumour cell lines where the induction of differentiation and apoptosis are the prime effects. The mechanism of As_2O_3 -induced cell death has yet to be clarified, especially in solid cancers.

2. In the present study, the human breast cancer cell line MCF-7 was examined as a cellular model for As_2O_3 treatment. The involvement of extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK) was investigated in As_2O_3 -induced cell death.

3. It was found that As_2O_3 activates the prosurvival mitogen-activated protein kinase kinase (MEK)/ERK pathway in MCF-7 cells, which, conversely, may compromise the efficacy of As_2O_3 . Hence, a combination treatment of As_2O_3 and MEK inhibitors was investigated to determine whether this treatment could lead to enhanced growth inhibition and apoptosis in MCF-7 cells.

4. Inhibition of MEK/ERK with the pharmacological inhibitors U0126 (10 $\mu\text{mol/L}$) or PD98059 (20 $\mu\text{mol/L}$) together with As_2O_3 (2 and 5 $\mu\text{mol/L}$) resulted in a significant enhancement of growth inhibition in breast cancer MCF-7 cells as determined by the 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide assay and [Methyl- ^3H]-thymidine incorporation. Furthermore, the results demonstrated that combined treatment with As_2O_3 and the MEK1/2 inhibitor U0126 could augment breast cancer MCF-7 cell apoptosis approximately twofold compared with the effects of the two drugs alone, as determined by Hoechst 33258 or annexin V/propidium iodide (PI) staining and flow cytometry.

5. In addition, As_2O_3 activated p38 in a dose-dependent manner, but had no effect on JNK1/2. Treatment with a p38 inhibitor did not prevent As_2O_3 -induced apoptosis.

6. In conclusion, the results of the present study showed that enhanced apoptosis is detected in breast cancer MCF-7 cells in

the presence of As_2O_3 and an MEK inhibitor, which may be a new promising adjuvant to current breast cancer treatments.

Key words: apoptosis, arsenic trioxide, MCF-7, mitogen-activated protein kinase.

INTRODUCTION

Arsenic trioxide (As_2O_3) has been adopted from traditional Chinese medicine and is highly effective in the treatment of patients suffering from acute promyelocytic leukaemia (APL) refractory to all-*trans*-retinoic acid; As_2O_3 has become an extremely important component in the clinical management of this leukaemia.^{1–4} Over the past 10 years, numerous studies have been performed to investigate the mechanisms of action of As_2O_3 on APL. These *in vitro* studies were performed using NB4 cells,⁵ an APL cell line with chromosome translocation t(15;17) from a relapsed APL patient. In NB4 cells, As_2O_3 was shown to exert dose-dependent dual effects. At low concentrations (0.1–0.5 $\mu\text{mol/L}$), As_2O_3 induces differentiation, whereas at high concentrations (0.5–2 $\mu\text{mol/L}$) it induces apoptosis.⁵ The therapeutic efficacy of As_2O_3 in APL patients and studies in the APL cell line NB4 have prompted an investigation of the effects of As_2O_3 in other cell lines and further elucidation of the mechanisms of actions in mediating these effects. Several studies have shown that As_2O_3 also exhibits potent growth inhibitory effects in several other cell lines of diverse malignant phenotypes, including cervical cancer, lung cancer, glioma, prostate cancer and breast cancer cells.^{6–11} Despite the well-documented clinical efficacy of arsenic in leukaemia therapy, the precise mechanisms of regulating the arsenic-dependent induction of apoptosis on neoplastic cells have not been elucidated.

Mitogen-activated protein kinases are a family of widely expressed serine-threonine kinases regulating important cellular processes. In mammals, three major MAPK family subgroups exist: extracellular signal-regulated kinase (ERK), c-Jun N-terminal of stress-activated protein kinases (JNK) and the p38 group of protein kinases.¹² The signalling cascades involving JNK and p38 are key mediators of stress signals and seem to be responsible mainly for protective responses, stress-dependent apoptosis and inflammatory responses. Conversely, the ERK pathway plays a major role in regulating cell proliferation and differentiation and provides a protective effect against apoptosis.¹²

c-Jun N-terminal kinase and p38 have been investigated in As_2O_3 -induced apoptosis. However, whether the activation of JNK

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Received 14 April 2005; revision 23 June 2005; accepted 3 July 2005.

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or p38 is involved in As₂O₃-induced apoptosis depends on the type of cell. c-Jun N-terminal kinase activation mediates As₂O₃-induced apoptosis in acute promyelocytic leukaemia cells and in an acute myeloid leukaemia (M2)-derived cell line, but not in HepG2 cells.^{13–15} Apoptosis induced by As₂O₃ in leukaemia U937 cells is dependent on activation of p38 but, in the NB4 cell line, p38 is a negative regulator of As₂O₃-induced apoptosis.^{16,17} The roles of JNK or p38 in As₂O₃-induced apoptosis in MCF-7 breast cancer cells remain to be clarified.

Combination of As₂O₃ with other drugs that activate additional apoptotic signals, or inhibit survival signals, may provide a rational molecular basis for novel chemotherapeutic strategies. A rational molecular target is the ERK MAPK. Extracellular signal-regulated kinase phosphorylates additional kinases and specific transcription factors, such as Elk-1 and c-Fos, are important in cell proliferation. However, the link between ERK activation and As₂O₃-induced cell death is not straightforward.^{16–18}

In the present study, we mainly tested the combined effects of As₂O₃ and MEK1/2 inhibitors on the induction of apoptosis of a breast cancer cell line. The human breast adenocarcinoma cell line MCF-7, an oestrogen receptor (ER)-positive breast cancer cell line, was examined as a cellular model for As₂O₃ treatment to mimic the early phase of breast cancer. The results showed that As₂O₃ increased MEK1/2 activity and combined treatment with As₂O₃ and MEK1/2 inhibition led to enhanced cell death in MCF-7 cells.

METHODS

Reagents and antibodies

A stock solution of As₂O₃ was prepared by dissolving As₂O₃ powder (Sigma, St Louis, MO, USA) in phosphate-buffered saline (PBS) to a concentration of 10 mmol/L. Primary antibodies, including polyclonal anti-phospho-p42/44 MAPK (Thr202/Tyr204), polyclonal anti-p44/42 MAPK (ERK1/2), polyclonal anti-phospho-stress-activated protein kinase (SAPK)/JNK (Thr183/Tyr185), polyclonal anti-SAPK/JNK, polyclonal anti-phospho-p38 MAPK (Thr180/Tyr182), polyclonal anti-p38 MAPK, anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (HRP) and the MEK1/2 inhibitors U0126 and PD98059 were all purchased from Cell Signalling Technology (Beverly, MA, USA).

Cell lines and tissue culture

The MCF-7 cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mmol/L L-glutamine, penicillin (100 U/mL) and streptomycin (100 mg/mL; Sigma), referred to as complete medium. Medium containing As₂O₃ was prepared by serial dilution of the 10 mmol/L As₂O₃ stock solution in RPMI 1640.

Cell viability assay

Cells were seeded in 96-well plates (5000 cells/well) and treated with indicated concentrations of As₂O₃ in the presence or absence of 10 μmol/L U0126 or 20 μmol/L PD98059 for 48 h. The 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was used to assess cell viability: MTT, 20 μL dissolved in PBS (5 mg MTT/mL PBS), was added to the cells. After 4 h incubation at 37°C, culture medium containing MTT was removed and 100 μL dimethylsulphoxide (DMSO) was added to solubilize the blue formazan formed by viable cells. Plates were read using an ELISA plate reader (EL808; Bio-Tek Instruments, Winooski, VT, USA) at 570 nm. Cell viability is expressed as a percentage ratio of exposed cells to control cells.

[Methyl-³H]-thymidine incorporation into DNA

Cells were seeded onto 96-well plates (5000 cells/well) and treated with indicated concentrations of As₂O₃ in the presence or absence of 10 μmol/L U0126 or 20 μmol/L PD98059 for 48 h. After treatment, 0.5 μL [methyl-³H]-thymidine solution (37 kBq/μL; Amersham Bio-science< Buckinghamshire, UK) was added to each well and cells were incubated at 37°C for 6 h. The cell suspension was then transferred to glass microfibre filters (Whatman, Maidstone, UK) using a cell harvester. Nucleic acids incorporating [Methyl-³H]-thymidine were trapped on the filter paper. Radioactivity was measured using a scintillation counter. The percentage of [methyl-³H]-thymidine incorporated was calculated as follows:

$$\% \text{ Incorporation} = 100 \times ((\text{c.p.m. of tested sample})/(\text{c.p.m. of control}))$$

Assays for the detection of apoptosis

Apoptosis was determined two ways. For nuclear morphology, cells that had been cultured on glass-bottom dishes were fixed, washed twice with PBS and stained with Hoechst 33258 staining solution according to the manufacturer's instructions (Beyotime, Jiangsu, China). Stained nuclei were observed under a fluorescence microscope. For flow cytometry with annexin V-propidium iodide (PI) staining, cells were seeded onto six-well plates at a density of 3×10^5 cells/well. After treatment, both floating and trypsinized adherent cells were collected by centrifugation at 250 g for 5 min. The number of cells in each sample was adjusted to 5×10^5 cells. Cells were pelleted and incubated with 100 μL annexin V incubation reagent (Beckman Coulter, Marseille, France) containing 10 μL of 10× binding buffer, 10 μL PI, 2 μL annexin V conjugate and 78 μL distilled water for 15 min in the dark at room temperature. After incubation, 400 μL of the 1× binding buffer was added to each sample. The fluorescent signals of the annexin V conjugate and PI were detected at channels of fluorescence intensity FL1 and FL2 (Cytomics FC500; Beckman Coulter).

Western blot analysis

Briefly, cells were lysed in buffer (0.5% NP-40, 10 mmol/L Tris-HCl, pH 7.2, 0.15 mol/L NaCl, 0.02% NaN₃, 1 mmol/L phenylmethylsulphonyl fluoride), kept on ice for 30 min and centrifuged at 12 879 g for 10 min at 4°C. Supernatants were collected, whereas nuclear debris were pelleted and discarded. Lysates (30 μg) were run on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in Tris-glycine buffer. Gels were transferred to polyvinylidene difluoride (PVDF) using a semidry blotting apparatus in 25 mmol/L Tris, 192 mmol/L glycine and 20% methanol. After blocking in 2% non-fat dry milk for 1 h, membranes were

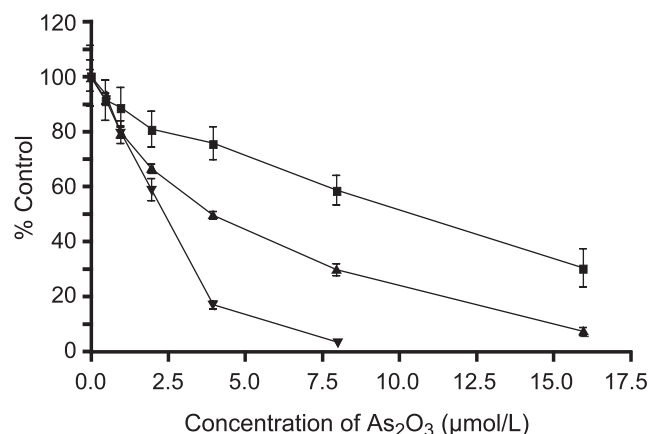


Fig. 1 Cell survival of MCF-7 cells following As₂O₃ treatment for various times. Cells were treated with different concentrations (0.5–16 μmol/L) of As₂O₃ for 24 (■), 48 (▲) and 96 h (▼). Cell growth inhibition was measured with the 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide assay, as described in the Methods. Data are the mean ± SD for six replicate measurements.

first probed with the specific primary antibody detecting the protein (according to the manufacturer's instructions (Cell Signalling Technology)). Blots were then incubated with a specific HRP-linked secondary antibody and developed using an enhanced chemiluminescence kit (Amersham Biosciences).

Statistical analysis

Data are expressed as the mean±SD. Two-way analysis of variance (ANOVA) was used to assess significant differences between treatment groups. *P* < 0.05 was considered statistically significant.

RESULTS

Effect of As₂O₃ on cell survival and apoptosis of MCF-7 cells

Figure 1 shows the dose- and time-response curves for the effects of As₂O₃ on the viability of MCF-7 cells. The MCF-7 cells were

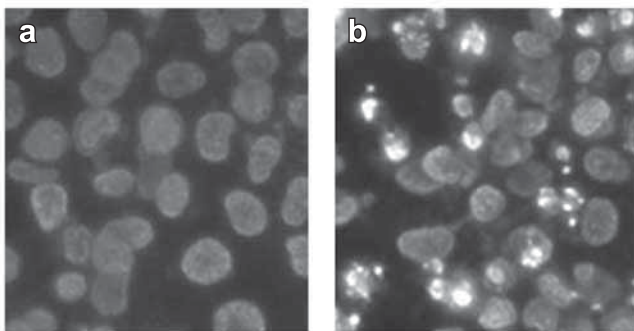


Fig. 2 Arsenic trioxide-induced morphological changes in MCF-7 cells. MCF-7 cells were either untreated (a) or were treated with 5 μmol/L As₂O₃ for 3 days (b) and were fixed and stained with Hoechst 33258. Apoptotic cells show condensed and fragmented fluorescent nuclei. Original magnification ×200.

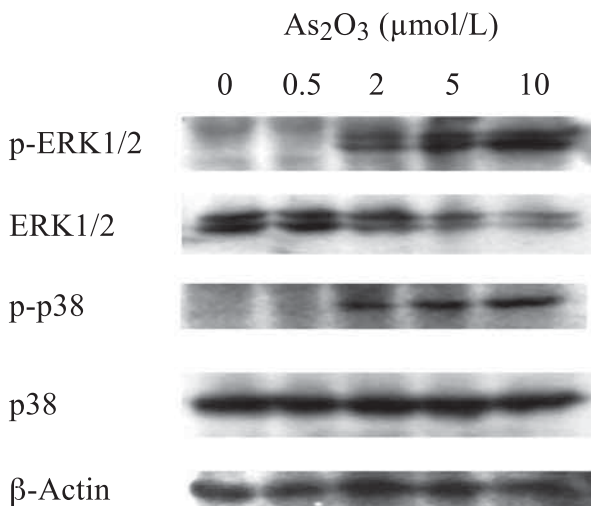


Fig. 3 Expression of mitogen-activated protein kinases (MAPK), specifically extracellular signal-regulated kinase (ERK) and p38, and phosphorylated-MAPK (p-ERK, p-p38) in As₂O₃-treated MCF-7 cells. Cells were incubated with 0.5, 2, 5, 10 μmol/L As₂O₃ for 60 min. Cell lysates were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the p-ERK, p-p38, ERK and p38 protein bands were detected by western blot analysis. Experiments repeated three times gave similar results.

incubated with 0.5–16 μmol/L As₂O₃ for 24, 48 and 96 h. The curves show the reduction in cell viability following treatment of MCF-7 cells with increasing concentrations of As₂O₃ and for

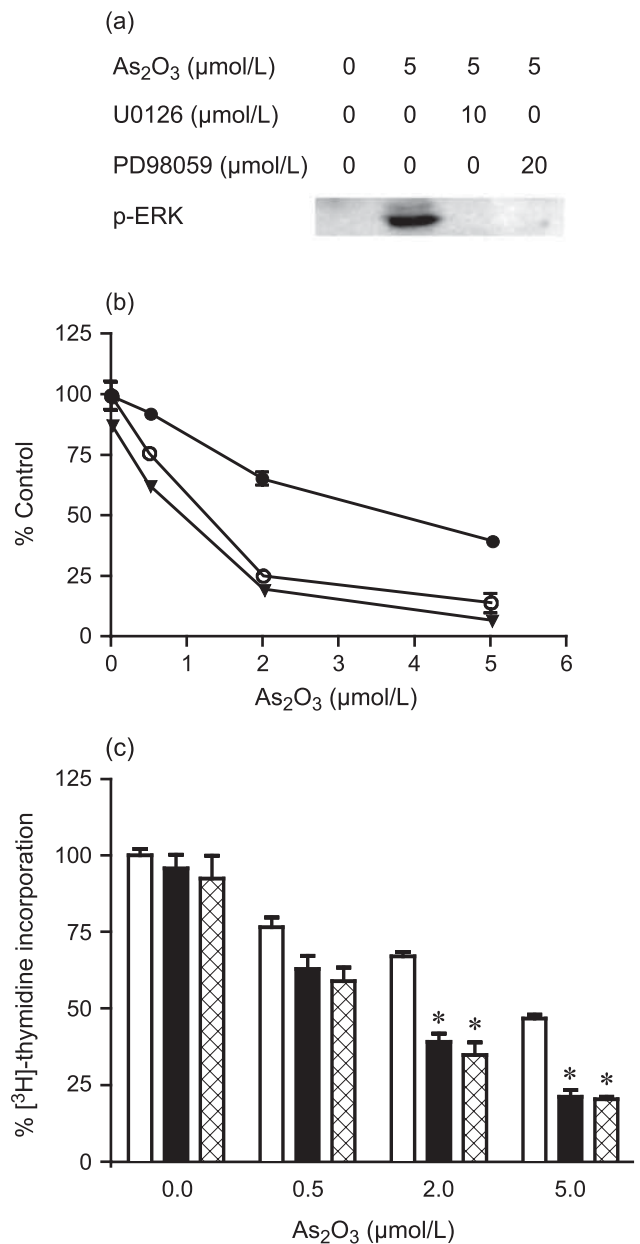


Fig. 4 Analysis of the effects of As₂O₃ and mitogen-activated protein kinase kinase (MEK) inhibitors on the inhibition of MCF-7 breast cancer cell growth and DNA synthesis. (a) The MEK inhibitors U0126 or PD98059 blocked extracellular signal-regulated kinase (ERK) activation by As₂O₃. MCF-7 cells were treated simultaneously with or without 10 μmol/L U0126 or 20 μmol/L PD98059 for 60 min. (b) MCF-7 breast cancer cells were treated with the indicated concentrations of As₂O₃ in the absence (●) or presence of 10 μmol/L U0126 (○) or 20 μmol/L PD98059 (▼) for 48 h and cell growth inhibition was measured using the 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide assay, as described in the Methods. (c) MCF-7 breast cancer cells were treated with the indicated concentrations of As₂O₃ in the absence (□) or presence of 10 μmol/L U0126 (■) or 20 μmol/L PD98059 (▨) for 48 h and [³H]-thymidine incorporation was determined as in the Methods. Data are the mean±SD for four replicate experiments. Percentage is expressed relative to untreated control, which is defined as 100%. **P* < 0.05 compared with As₂O₃-treated samples.

longer periods of time. The 50% inhibitory concentration (IC₅₀) for As₂O₃ against MCF-7 cells following treatment for 24, 48 and 96 h was 8.6, 3.3 and 1.86 $\mu\text{mol/L}$, respectively. To investigate whether induction of apoptosis was the mechanism of cell death, MCF-7 cells were treated with 5 $\mu\text{mol/L}$ As₂O₃ for 3 days and stained with Hoechst 33258. Apoptotic cells, with condensed and fragmented fluorescent nuclei, were observed in As₂O₃-treated samples, whereas untreated cells presented round, uniformly coloured nuclei (Fig. 2). Further confirmation of As₂O₃-induced apoptosis was obtained using flow cytometry with annexin V–PI staining.

Effect of As₂O₃ on MAPK

To determine the involvement of MAPK in As₂O₃-induced cell death, western blots of MCF-7 cell extracts treated with As₂O₃ were performed using antibodies specific for phosphorylated forms. The blots showed a dose-dependent activation of ERK1/2 and p38, but not JNK1/2. (Fig. 3)

Analysis of As₂O₃ and MEK inhibitors on the inhibition of cell growth breast cancer cells

In other systems, ERK generally plays a critical role in cell proliferation and growth;²² thus, it was reasoned that ERK activation by As₂O₃ may enhance cell proliferation and compromise the efficacy of this drug. A logical approach to investigate this is to use pharmacologic blockers of MEK to inhibit As₂O₃-induced ERK activation and its downstream effects. To test this hypothesis, a combination of As₂O₃ and the MEK1/2 inhibitors U0126 or PD98059 was used to treat human breast carcinoma cell lines. The results showed that a combination of As₂O₃ and U0126 or PD98059 inhibited cell proliferation (Fig. 4a,b). At low concentrations of 0.5 $\mu\text{mol/L}$, As₂O₃, U0126 or PD98059 showed a modest, albeit not significant, enhancement of the inhibition of cell growth, whereas at higher concentrations of 2 and 5 $\mu\text{mol/L}$, As₂O₃, U0126 or PD98059 were able to significantly inhibit cell proliferation compared with As₂O₃ alone in MCF-7 cells. These results are

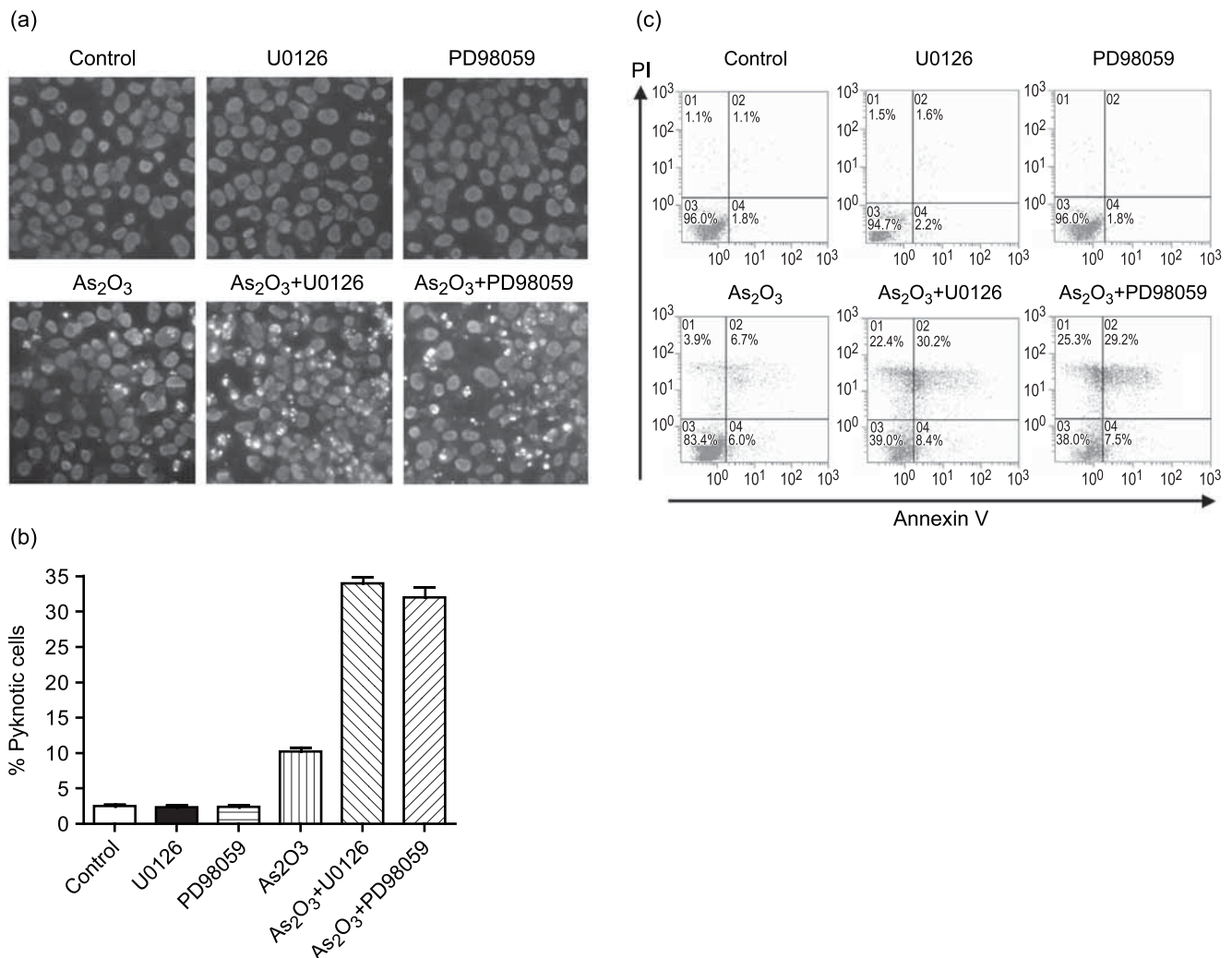


Fig. 5 Arsenic trioxide and the mitogen-activated protein kinase kinase (MEK) inhibitor U0126 cause enhanced cell apoptosis. MCF-7 cells were treated with 2 $\mu\text{mol/L}$ As₂O₃ in the absence or presence of 10 $\mu\text{mol/L}$ U0126 or 20 $\mu\text{mol/L}$ PD98059. (a) After 48 h treatment, MCF-7 cells were harvested and stained for Hoechst 33258. (b) Pyknotic cell number is expressed as a percentage of total cells. (c) After 48 h treatment, MCF-7 cells were harvested and double stained for annexin V and propidium iodide and analysed by flow cytometry. Early apoptotic cells are annexin V+/PI-, late apoptotic cells are Annexin V+/PI+, necrotic cells are Annexin V-/PI+ and healthy cells are Annexin V-/PI-. The percentage of cells of the total in each of the four populations is indicated. A representative experiment of three performed is shown.

consistent with those reported above, namely that ERK1/2 was activated when MCF-7 cells were treated with As₂O₃ at concentrations higher than 2 µmol/L.

Arsenic trioxide and the MEK inhibitors U0126 or PD98059 enhance cell apoptosis

To assess whether apoptosis was involved in the enhanced inhibition of cell growth observed above, Hoechst 33258 staining or annexin V–PI staining was performed with As₂O₃, MEK inhibitor (U0126 or PD98059) or a combination of the two. Hoechst 33258 staining of MCF-7 cells revealed that the number of cells with condensed and fragmented fluorescent nuclei increased from 10% (treated with As₂O₃ alone) to 34% (treated with As₂O₃ and U0126) or 32% (treated with As₂O₃ and PD98059; Fig. 4a,b). Similarly, As₂O₃ caused 12.7% apoptosis and U0126 caused 3.8% apoptosis, as measured by the number of annexin V-positive cells. When cells were treated with both As₂O₃ and U0126, there was a significant increase in the number of annexin V-positive cells to 38.6% (Fig. 4c). These results indicate that MEK inhibition enhances As₂O₃-induced cell apoptosis.

Arsenic trioxide-induced cell death is independent of p38 pathways

To determine whether p38 kinase is involved in As₂O₃-induced apoptosis, 10 µmol/L SB203580, a selective inhibitor of p38 kinase, was added to MCF-7 cells treated with As₂O₃. Hoechst 33258 staining of MCF-7 cells was used to evaluate apoptosis. The results showed that SB203580 did not block the apoptosis induced by As₂O₃. The addition of SB203580 to MCF-7 cells treated with As₂O₃ increased the apoptotic fraction, but this was not statistically significant (Fig. 6).

DISCUSSION

Increasingly, more studies have revealed the effectiveness of As₂O₃ in acting against solid tumours since its pilot treatment of APL patients.^{1–4} In view of the promising effectiveness of As₂O₃ as a chemotherapeutic drug, the potential application of As₂O₃ in breast cancer treatment was explored in the present study. The cytotoxicity of As₂O₃ against human breast cancer MCF-7 cells was assessed using several parameters. The results of the MTT assay showed a dose- and time-dependent inhibition of the growth of MCF-7 cells by As₂O₃. The antitumour effects of As₂O₃ against APL cell lines and other solid human tumours are as a result of the induction of apoptosis.^{19–21} In the present study, As₂O₃ was also found to induce apoptosis in MCF-7 cells. This was supported by evidence that MCF-7 cells treated with 5 µmol/L As₂O₃ for 3 days showed condensed and fragmented fluorescent nuclei following Hoechst 33258 staining. To further confirm the induction of apoptosis by As₂O₃, we examined the externalization of phosphatidylserine (PS) by flow cytometry with annexin V–PI staining. The data indicated that PS externalization and necrotic cells were evident in MCF-7 cells treated with As₂O₃. These results suggest that As₂O₃-induced apoptosis contributes to the growth inhibition of MCF-7 cells.

The As₂O₃-induced apoptosis occurs via a variety of mechanisms that include caspases, reactive oxygen species, p53, Bcl-2, protein kinase C and MAPK.^{13,14,22–28} However, which mechanism is actually critical for As₂O₃-induced apoptosis, especially in solid cancers, has yet to be determined. In the present study, we focused mainly on the role of MAPK in As₂O₃-induced apoptosis of MCF-7 cells. The MAPK signalling cascades regulate a variety of cellular activities, including cell growth, differentiation, survival and death. In mammals, the MAPK family is divided into three major groups, namely ERK, JNK and p38, based on their degree of homology, biological activities and phosphorylation motifs.

c-Jun N-terminal kinase and p38 belong to the SAPK family and have been investigated in As₂O₃-induced apoptosis. It has been reported that As₂O₃ induced JNK activation and apoptosis in APL and that a JNK inhibitor significantly protected against As₂O₃-induced apoptosis.¹³ In the acute myeloid leukaemia (M2)-derived cell line NKM-1, As₂O₃ induced sustained activation of JNK and apoptosis and addition of a JNK inhibitor reduced the percentage of apoptotic cells after As₂O₃ treatment.¹⁴ Arsenic trioxide is able to induce apoptotic activity in K562 cells and its apoptotic mechanism may be associated with the activation of p38.²⁹ Arsenic trioxide leads to the activation of p38, which plays a role in inducing apoptosis by As₂O₃ in human leukaemia U937 cells.¹⁶ In HepG2 cells, activation of p38 by As₂O₃ may not be the

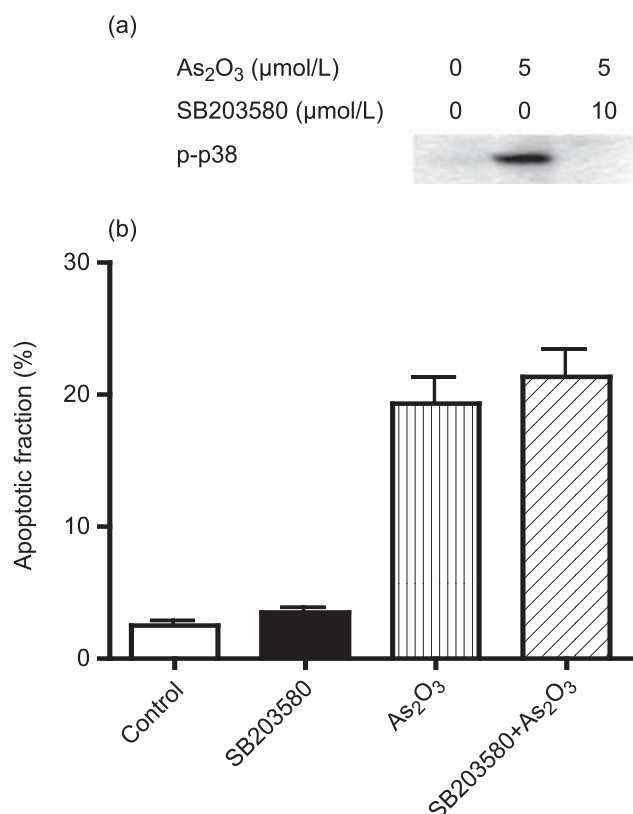


Fig. 6 No inhibition by the p38 inhibitor SB203580 of As₂O₃-induced MCF-7 cells apoptosis. (a) The p38 inhibitor SB203580 blocked p38 activation by As₂O₃. MCF-7 cells were treated simultaneously with or without 10 µmol/L SB203580 for 60 min. (b) MCF-7 cells were treated with 5 µmol/L As₂O₃ in the presence or absence of 10 µmol/L p38 inhibitor SB203580 for 48 h. The apoptotic fraction was obtained by Hoechst 33258 staining. Results are the mean ± SD of three independent experiments.

major determinant of the course of the apoptotic processes.¹⁵ Pharmacological inhibition of p38 potentiates arsenic-dependent apoptosis and suppression of growth of leukaemia cell lines, suggesting that this signalling cascade negatively regulates the induction of antileukaemic responses by As₂O₃.¹⁷ Arsenic trioxide causes the dose-dependent activation of p38, JNK and caspase 3. Treatment with a p38 inhibitor and overexpression of dominant-negative JNK did not prevent As₂O₃-induced cell death.¹⁰ These diverse observations regarding the nature of the signalling pathways in As₂O₃-induced apoptosis represent the unusual characteristics of the mechanism of apoptosis induced by As₂O₃ in different cellular systems. In the present study, p38 was activated in As₂O₃-induced apoptosis, but the inhibition of p38 did not protect MCF-7 cells from As₂O₃-induced apoptosis. c-Jun N-terminal kinase 1/2 was not activated, even at a concentration of 10 µmol/L As₂O₃. These results indicate that p38 and JNK are not involved in As₂O₃-induced apoptosis in the MCF-7 cell line.

In mammalian cells, Raf/MEK/ERK was the first MAPK cascade to be identified and is the most extensively studied. Activation of Raf/MEK/ERK plays a pivotal role in the physiological regulation of many cellular processes, such as growth, proliferation, differentiation, survival, motility and angiogenesis.^{12,30,31} Although exceptions occur, the bulk of the evidence indicates that constitutive activation of the MEK/ERK signalling module increases the apoptotic threshold of cancer cells, consistent with its ability to regulate the expression and function of multiple anti-apoptotic players through transcriptional and non-transcriptional mechanisms.^{32–34} The MEK/ERK MAPK module has recently emerged as a potential target for anticancer therapies. Indeed, one of the most intriguing features of MEK inhibitors is their ability to lower the apoptotic threshold of leukaemic cells, thereby sensitizing them to the pro-apoptotic action of other biological agents, ionizing radiation and biological agents that modulate apoptosis.³⁵ Additive or synergistic antiproliferative and/or pro-apoptotic effects have also been reported recently in lymphoid and myeloid cells when inhibitors of the MEK/ERK MAPK module are combined with non-conventional cytostatics/cytotoxics, such as interferon-α.³⁶ In present study, treatment of MCF-7 cells with As₂O₃ resulted in the activation of ERK, so we questioned whether ERK activation by As₂O₃ may enhance cell proliferation and compromise the efficacy of this drug. To answer this question, a combination of As₂O₃ and the potent MEK1/2 inhibitors U0126 or PD98059 was used to treat the human breast carcinoma line. The results confirm that a combination of As₂O₃ with MEK inhibition enhances cell growth inhibition and apoptosis. The potential use of low-dose chemotherapy is important, because lower doses are more attainable during cancer therapy and are likely to cause less toxicity in patients. We performed a dose–response analysis to assess the minimal concentration of As₂O₃ that, when combined with U0126, causes enhanced cell death. Low doses of As₂O₃, starting in the 2 µmol/L range, combined with U0126 cause significantly enhanced cell growth inhibition of MCF-7 breast carcinoma cells. Annexin V–PI and Hoechst 33258 staining confirmed that the cell death observed above represented apoptosis. More recently, As₂O₃ has been reported to stimulate ERK activation and subsequent ERK-mediated BAD phosphorylation on Ser112, thereby inhibiting its pro-apoptotic function, in APL cells.²⁰ The inhibition of ERK1/2 and Bad phosphorylation by MEK1 inhibitors enhanced apoptosis

in As₂O₃-treated cells.²⁰ It remains to be clarified whether there was a similar mechanism operating in the present study.

Based on a molecular approach, the present study mainly documents that treatment with As₂O₃, combined with the inhibition of MEK1/2, led to enhanced apoptosis of a breast carcinoma cell line. The combination therapy of As₂O₃ and MEK inhibitor may allow the use of lower drug doses, likely leading to lower toxicity and enhanced breast tumour killing *in vivo*. There are broad implications for these findings in the potential clinical use of As₂O₃ and MEK inhibitors by improving the response rate and expanding the usefulness of As₂O₃ in the treatment of resistant tumours, which affect a large percentage of cancer patients.

ACKNOWLEDGEMENTS

This work was supported, in part, by grants from the National Natural Science Foundation of China (30471430), the Foundation of High-tech Key Project, Education Department, Jiangsu Province (JH01-049), the National Key Basic Research and Development Project⁹⁷³ (Grant No. 2002CB512900) and the Natural Science Research Project, Education Department, Jiangsu Province (02KJB330001).

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