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Anti-Proliferation of MDA-MB-231 Human Breast Tumour Cells by Arsenic Trioxide via Induction of Apoptosis

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Abstract: Arsenic Trioxide (As_2O_3) has been explored for its use as medicine in both Western and Chinese societies. In 1990s, As_2O_3 was reported to treat patients with acute promyelocytic leukemia (APL). Until recently, research on other solid tumour cells was emerged. In the present study, the mechanism of As_2O_3 treatment of human breast tumour MDA-MB-231 cells was investigated. It was found that As_2O_3 inhibited the cell proliferation of MDA-MB-231 cells in a time- and dose-dependent manner. Mechanistic study indicated that the inhibition was induced via cell cycle arrest and apoptosis. As_2O_3 induced apoptosis via both extrinsic and intrinsic apoptotic pathways by regulating the pro- or anti-apoptotic molecules. Moreover, As_2O_3-induced cell cycle arrested at G_2 phase in MDA-MB-231 cells. The study revealed that As_2O_3 was a potent candidate for further investigation for combating against human breast tumour including the late stage breast tumour.

Key words: Arsenic trioxide, breast tumour, MDA-MB-231, apoptosis

INTRODUCTION

Breast tumour is the fifth most common tumours in the world. Estrogens have been found to be the primary stimulant of the breast tumour. Mechanistic study demonstrated that estrogen promoted cell proliferation and prevented apoptosis of breast tumour cells (Lippman *et al*., 1976; Umans *et al*., 1984; Hajek *et al*., 1997). The anti-estrogen drug for breast tumour treatment that has been used most often is tamoxifen. Tamoxifen is also used to treat metastatic breast tumour and to prevent the development of breast tumour in women at high risk. Tamoxifen works as an anti-estrogen to treat human breast tumour and prevents the induction and growth of estrogen-receptor (ER) positive carcinogen-induced rat mammary carcinomas (Jordan, 1976). Tamoxifen is effective in pre-menopausal women but is ineffective in ER negative patients (i.e., advanced breast tumour patients). However, tamoxifen may cause some severe side effects, including blood clots (thrombosis), endometrial tumour (tumour of the uterine lining), abnormal growth of uterine tissue (endomertriosis), stroke and fertility issues (Osborne, 1998; Clemons *et al*., 2002). Alternative treatment for breast tumour is urgently needed.

In early 1970s, a group of researchers from Harbin Medical University in Northeast China found that intravenous administration of arsenic trioxide (As_2O_3) with relatively small doses (10 mg day^{-1}) was effective in treating patients with acute promyelocytic leukemia (APL) (Zhang *et al*., 1998; Li *et al*., 1988; Sun *et al*., 1992). Then, Sun *et al*. (1992) reported promising results of 32 cases of APL treated with As_2O_3. Later, a trial performed at the Shanghai Second Medical University indicated that complete remission could be achieved in 14 of 15 investigated patients that had relapsed after prior treatment with all-trans retinoic acid (ATRA) or conventional chemotherapy (Shen *et al*., 1997). In Western countries, the population clinical efficacy of As_2O_3 was proven by studies performed by Soignet *et al*. (1998, 2001). Recently, the drug Trisenox™ was formally approved by the State Drug Administration in China (1999) and the Food and Drug Administration of the United States (2000). Apart from APL, As_2O_3 were reported to inhibit growth and promote apoptosis in other hematological tumour cell lines (Konig *et al*., 1997). As_2O_3 also exhibits anti-proliferative effect and induces apoptosis on solid tumour cells such as prostate tumour, renal tumour, cervical tumour and hepatoma (Ling *et al*., 2002; Li *et al*., 2004). We recently found that As_2O_3 is

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effective in suppressing growth of ERα positive human breast tumour cell line MCF-7 (Chow *et al.*, 2004a, b), which mimic the early stage of breast tumour.

In the study, we further investigate the effects of As_2O_3 on another human breast tumour cell line, MDA-MB-231, which is ERα negative, mimicking the late stage of human breast tumour. The aim of this study is to explore the potential anti-tumour effect of As_2O_3 in MDA-MB-231 cells and to study the underlying mechanisms.

MATERIALS AND METHODS

Cell culture and chemicals: MDA-MB-231 cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were maintained in RPMI 1640 medium, which was supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. All of these reagents were purchase from Invitrogen, USA. The cells were maintained at 37°C in a humidified atmosphere of 5% carbon dioxide (CO_2).

Arsenic Trioxide (As_2O_3) was purchased from Sigma Chemical Company. Stock As_2O_3 solution was prepared by dissolving powder As_2O_3 in boiled phosphate buffered saline (PBS) at 10 mM. Boiling was continued until As_2O_3 powder was completely dissolved. The stock solution was sterilized using 0.22 μM syringe filter (Millipore) and stored at -20°C.

Cell viability assay: Total 1×10^4 cells/well were seeded onto a 96-well plate in RMPI 1640 medium. After 24 h incubation at 37°C, the cells were treated with various concentrations of As_2O_3 in a fresh medium. After 24, 48 or 72 h, the medium in each well was removed and washed with PBS. Negative control was prepared by treating cells with me dium alone. Then, 30 μL of MTT solution (5 mg mL^{-1}) was added to each well and the plate was incubated at 37°C for 3 h. After incubation, MTT solution was discarded and 100 μL DMSO was added to dissolve the crystals in the cells. Then, absorbance at 540 nm was measured using an ELISA plate reader (BIO-RAD). Percentage cell survival of each treatment was calculated as:

$$\text{Percent cell survival} = (\text{O.D. treatment/ O.D. negative control}) \times 100\%$$

Detection of DNA fragmentation: Total 5×10^5 cells/well were seeded onto 60 mm culture dish and incubated at 37°C, 5% CO_2. After appropriate treatment with As_2O_3, cells were lysed with 400 μL of DNA lysis buffer and vortexed until no cell debris was left. Twenty microliter of 10 mg mL^{-1} proteinase K was added and incubated at 37°C for 2 h. Then, 150 μL of saturated NaCl was added and the sample was shaken vigorously. The mixture was centrifuged at 6500× g for 15 min. The supernatant was collected; 1 mL of cold ethanol was added and centrifuged again at 15000×g for 20 min. After rinsing with cold 75% ethanol, the pellet was dried at room temperature. Finally, 20 μL of RNase A solution in Tris-EDTA buffer (0.2 mg mL^{-1}) was added to each sample and further incubated at 37°C for 90 min. Equal amount of sample was electrophoresed in 1.5% agarose gel and visualized by ethidium bromide staining.

Cell cycle analysis: Total 3×10^5 cells/well were seeded onto 6-well culture plate and incubated at 37°C, 5% CO_2. Twenty-four h later, the medium was discarded and appropriate concentrations of As_2O_3 were added. After incubation for 72 h, the cells were harvested and washed with PBS. The cells were then fixed with 1 mL of 70% ethanol at 4°C overnight. After fixation, the cells were centrifuged at 3000 × g for 5 min. to remove the ethanol. Cells were then resuspended in 0.46 mL freshly prepared propidium iodide solution containing propidium iodide (43 μg mL^{-1}) and RNase A (1 mg mL^{-1}) and incubated in dark at 37°C for 30 min. After incubation, the cells were analyzed by FACSort flow cytometer (Becton Dickinson) (Chow *et al.*, 2004a).

Detection of apoptotic cells by Annexin V-PI staining: The detection of apoptosis was performed by using BD Pharmingen™ Annexin V-FITC kit (Bio-Gene Technology Ltd.). Total 5×10^5 cells were seeded in a 60 mm culture dish. Twenty four hour later, medium was discarded and As_2O_3 was added with fresh medium. Then after 72 h, cells were harvested and washed with PBS. And 1×10^5 cells were used for the assay. Ten micro liters of 10×binding buffer, 10 μL of PI, 3 μL of Annexin V-FITC conjugate and 77 μL of dH_2O were added to a sample and incubated in the dark at room temperature for 15 min. After incubation, 400 μL of 1 × binding buffer was added to the sample and the sample was analyzed by FACSort flow cytometer (Becton Dickinson) as soon as possible. A computer program, WinMDI, was used for data analysis (Chow *et al.*, 2004a).

Western blot analysis: Total 1×10^6 cells were seeded on 100 mm culture dish with 10 mL RPMI 1640 medium. Twenty-four hour later, appropriate concentrations of As_2O_3 were added. After 72 h, cells were collected and washed twice with PBS. Then, 100 μL of lysis buffer was added and allowed to stand on ice for 2 h. After that, samples were boiled for 10 min and centrifuged at 13600 × g for 10 min at 4°C. Finally, the supernatant was collected and protein concentration of each sample was determined by BCA assay. Equal amount (50 μg) of each protein sample was dissolved in 12.5% SDS-PAGE. The protein was then transferred to PVDF membrane by an electro-blotter (Bio-Rad). The membrane was blocked with 10% non-fat milk for 2 h. Then, appropriate amount of each of primary antibody was added and incubated at 4°C overnight. After that, the membrane was washed with TBS-T for 3 times and probed with secondary antibody (conjugated with horseradish peroxidase) at room temperature for 1 h. The membrane was washed again with TBS-T for 3 times and finally the signal was detected using Enhanced Chemiluminescence (ECL) detection kit and developed on X-ray film (Chow *et al*., 2004a).

RESULTS DISCUSSION

Anti-proliferative effects of As_2O_3 on MDA-MB-231 cells: MDA-MB-231 cells were treated with various concentrations of As_2O_3 for 24, 48 and 72 h, respectively. After treatment, MTT assay was performed. The results were shown in Fig. 1. IC_{50} values when MDA-MB-231 cells were treated with As_2O_3 for 24, 48 and 72 h were found to be 20, 18.4 and 12.1 μM, respectively.

DNA fragmentation induced by As_2O_3 treatment: MDA-MB-231 cells were incubated with various concentrations of As_2O_3 for 72 h. After treatment, the DNA contents were extracted and separated on 1.5% agarose gel. Laddering pattern was observed when the MDA-MB-231 cells were treated with 6.25, 12.5 and 25 μM As_2O_3, respectively, for 72 h (Fig. 2).

Increase in sub-G_1 phase by As_2O_3 treatment: Cell cycle analysis was performed after MDA-MB-231 cells were treated with different concentrations of As_2O_3 for 72 h. The results showed an increase in sub-G_1 phase upon As_2O_3 treatment indicating that As_2O_3 might inhibit the growth of MDA-MB-231 cells by inducing apoptosis. Increase in S and G_2/M phase observed from the results indicated that cell cycle arrest occurred in As_2O_3-treated MDA-MB-231 cells (Fig. 3).

Increase in percentage of apoptotic cells by As_2O_3 treatment: From Fig. 4, the percentage of apoptotic cells as shown in the lower right quadrant of the dot plot increased from 6.5-12.5% after treatment with 25 μM of As_2O_3 for 72 h.

Regulation of apoptosis-related proteins by As_2O_3 treatment: The MDA-MB-231 cells were treated with various concentrations of As_2O_3 for 72 h. Then, Western bolt analysis of apoptosis-related proteins was performed. There is an obvious decrease in expression level of bcl-2 while there is no significant change in expression level of bax protein (Fig. 5a and b). Results of Fig. 5 also showed an increase of cytochrome c released to cytosol while the expression level of pro-caspase 3, pro-caspase 8 and pro-caspase 9 decreased after treatment with As_2O_3 (Fig. 5c-f). Expression level of FasL was up-regulated while that of p53 was down-regulated in the MDA-MB-

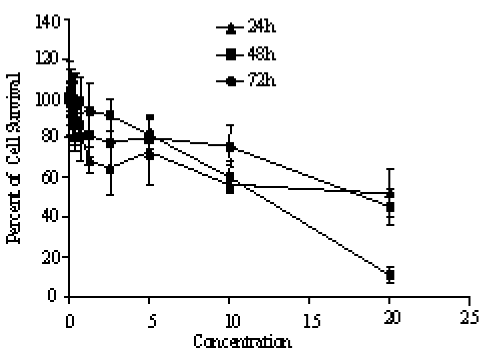


Fig. 1: The anti-proliferative effect of As_2O_3 on MDA-MB-231 cells. MDA-MB-231 cells were treated with various concentrations of As_2O_3 for 24, 48 or 72 h. Data was expressed in mean±standard deviation of four replicates

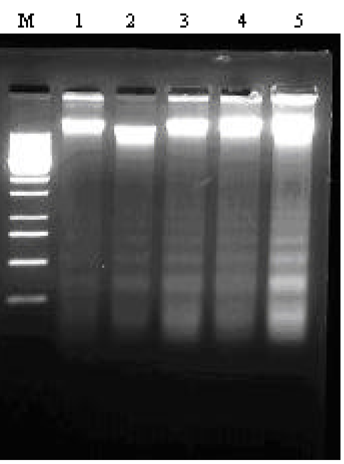
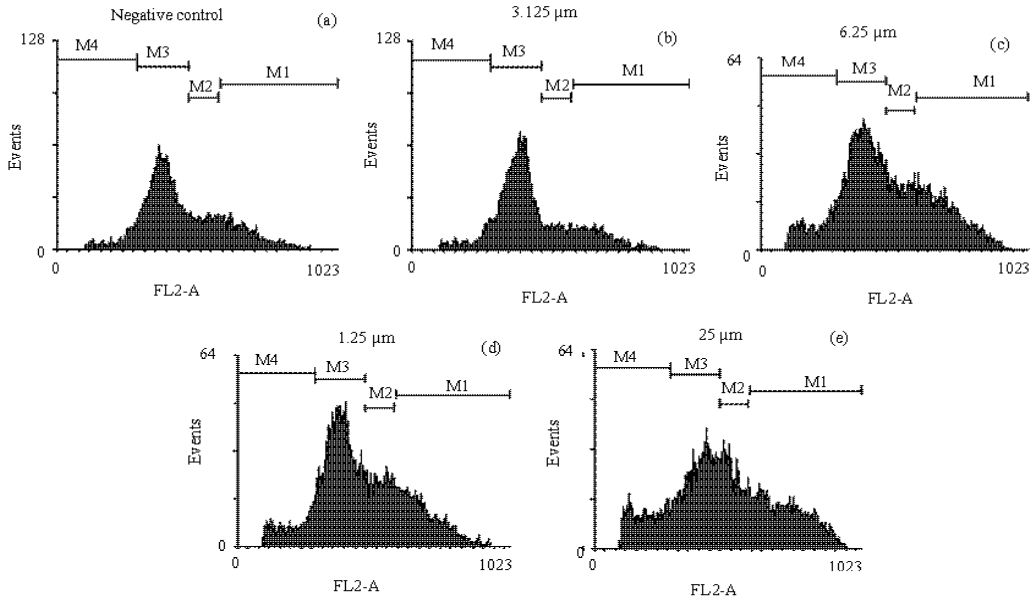


Fig. 2: DNA fragmentation induced by As_2O_3 on MDA-MB-231 cells. DNA prepared from MDA-MB-231 cells after treated with As_2O_3 for 72 h. Lane 1 is negative control without As_2O_3 treatment. Lanes 2, 3, 4 and 5 were treatment with 3.125 μM, 6.25 μM 12.5 μM and 25 μM of As_2O_3, respectively. M is 100 basepair marker. Laddering patterns were observed at concentrations 6.25 μM, 12.5 μM and 25 μM. This figure showed a representative of three independent trials



As_2O_3 (μM)	Percentage of cell population (%)			
	Sub-G_1	G_0/G_1	S	G_2/M
0	7.94	63.65	11.77	17.25
3.125	8.03	56.34	17.03	19.26
6.25	9.33	49.03	19.97	22.57
125	9.83	47.57	18.56	25.16
25	14.59	36.04	21.80	28.79

Fig. 3: Cell cycle analysis of MDA-MB-231 cells treated with As_2O_3 for 72 h. (Upper panel) 3×10^5 cells were seeded in each well of 6-well plates. After treated with various concentrations of As_2O_3 for 72 h, the cells were stained with PI. The populations of cells in sub-G_1, G_0/G_1, S and G_2/M phases were determined by FACSort flow cytometer and analysed by WinMIDI (Lower panel). The percentage of MDA-MB-231 cells distributions in different cell cycle were summarized in the table. This figure showed a representative of three independent trials

231 cells treated with As_2O_3 when compared with the untreated cells (Fig. 5g and h).

As shown by the cell cycle analysis by PI staining (Fig. 3), there was an increase in cell population in G_2/M phase after MDA-MB-231 cells were treated with different concentration of As_2O_3, indicating that As_2O_3 induced a cell cycle arrest at G_2/M phase. The expression level of cyclin B was examined by Western boltting. The result showed that there was a marked increase in expression level of cyclin B with increasing concentrations of As_2O_3. For cyclin E, there is no significant change in the protein expression level (Fig. 5i and j).

In addition to the significant anti-tumour effect on acute promyelocytic leukemia (APL), As_2O_3 has been studied on its potential use in other types of leukemia as well as solid tumours. In the previous study of our group, As_2O_3 was found to be able to inhibit the proliferation of ERα positive MCF-7 cells *in vitro* (Chow *et al.*, 2004a). Further mechanistic study indicated that As_2O_3 induced apoptosis and cell cycle arrest on MCF-7 cells and these effects were found to be related to the regulation of ERα signaling pathway (Chow *et al.*, 2004a). Patients of breast tumour at late stage always have their breast tissues contain ERα negative cells. To study the anti-tumour effect of As_2O_3 on late stage breast tumour, MDA-MB-231 cell line, an ERα negative breast tumour cell line, was used to mimic the late stage of breast tumour in this study. Our results indicated that the As_2O_3 could inhibit MDA-MB-231 cell growth in a dose and time dependent manner (Fig. 1).

Induction of apoptosis and cycle arrest are the most common mechanisms studied on the chemotherapeutic drugs in recent years. As_2O_3 was reported to induce apoptosis in ATRA-resistant APL cells (Shao *et al.*, 1998; Kinjo *et al.*, 2000). Apart from apoptosis, regulation of cell cycle is another important mechanism involved in the anti-tumour effect of As_2O_3 in solid tumour cells (Park *et al.*, 2003; Wu *et al.*, 2004). In our study, As_2O_3 at

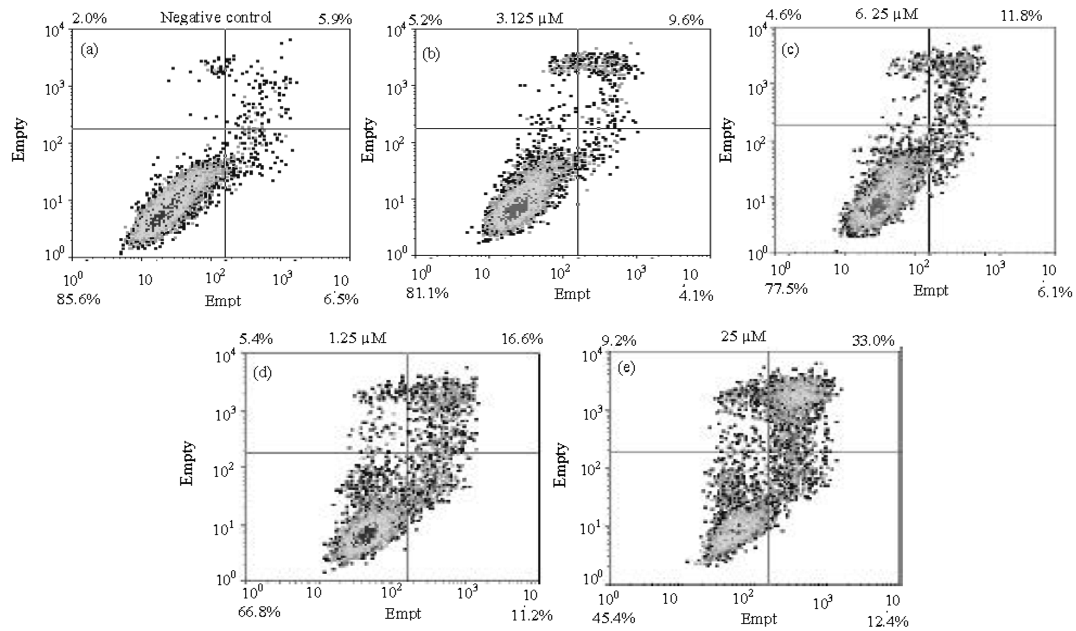


Fig. 4: Percentage of apoptotic cells detected by PI and Annexin V-FITC staining. Cells (3×10^5) were seeded in each well of 6-well plates. After treated with As_2O_3 for 72 h, the cells were stained with PI and Annexin V-FITC conjugate. A shift of cell population to the right bottom quadrant was observed after As_2O_3 treatment. This figure showed a representative of three independent trials

concentration more than 3.125 μM induced apoptosis in MDA-MB-231 cells. The apoptosis was preliminarily proven by the typical DNA ladder (Fig. 2). To further confirm the result, we examined the externalization of PS by Annexin-V and PI staining. An increase in apoptotic cell population was observed after As_2O_3 treatment (Fig. 4). Together with the detection of increase in sub-G_1 phase during cell cycle analysis (Fig. 3), it was suggested that As_2O_3 could inhibit the proliferation of MDA-MB-231 cells via induction of apoptosis. Meanwhile, the increase of cell population in G_2/M phase indicated that As_2O_3 induced G_2/M cell cycle arrest in MDA-MB-231 cells (Fig. 3).

In previous study, As_2O_3 has been found to induce apoptosis in different human tumour cell lines by regulating the expression level of apoptosis related proteins. However, detailed mechanisms involved were quite different. Chow *et al.* (2004a) reported that As_2O_3-induced apoptosis in ERα positive breast tumour cell line MCF-7 was due to the collapse of mitochondrial membrane potential, up-regulation of tumour suppressor gene p53 and regulation of ERα signaling pathway. As the MDA-MB-231 cells are known to be ERα negative, the effect of As_2O_3 in these cells does not involve ERα. Also, the features of MCF-7 cells and MDA-MB-231 cells are quite different. Caspase 3, a key factor for initiation of DNA fragmentation was not expressed in MCF-7 cells but expressed in MDA-MB-231 cells. In our study, the involvement of extrinsic apoptotic pathway in the apoptosis in MDA-MB-231 cells treated by As_2O_3 was confirmed by the findings that the expression level of Fas receptor Ligand (FasL) was increased, caspase 8 was activated and the ratio of bax/bcl-2 was increased. The increased level of cytochrome c released to cytosol and activation of caspase 9 suggested the involvement of intrinsic apoptotic pathway. p53 was involved in both cell cycle arrest and apoptosis. p53 was found to be up-regulated and induced apoptosis through regulating bax protein (Miyashita and Reed, 1995). From our study, the expression level of p53 was decreased which indicated that p53 did not directly trigger apoptosis in MDA-MB-231 cells. Since the function of p53 is cellular repair from DNA damage, lacking of p53 may cause prolonged DNA damage in the MDA-MB-231 cells. DNA damage may then activate the intrinsic apoptotic pathway by down-regulating the expression level of bcl-2 (Fig. 5).

The induction of cell cycle arrest on various tumour cell lines by As_2O_3 is mostly related to the arrest in G_0/G_1 and G_2/M phase. Cyclin B1 associates with cdk1 and remains inactive in cytosol until it is activated by

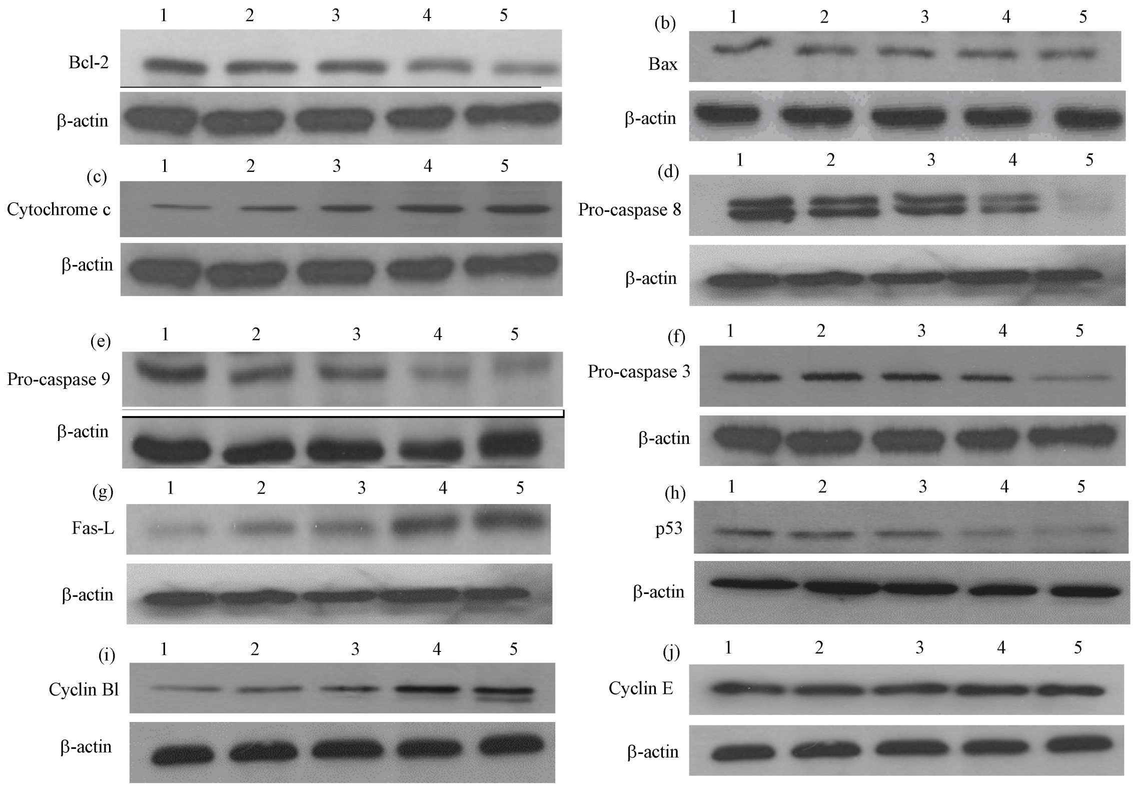


Fig. 5: Expression level of apoptosis and cell cycle arrest related proteins in MDA-MB-231 cells were examined by Western blot analysis. Antibodies probing various proteins were used (A-J) and β-actin was used to normalize the protein contents in each sample. Lane 1 is negative control without As_2O_3 treatment. Lanes 2, 3, 4 and 5 were treatment with 3.125, 6.25, 12.5 and 25 µM of As_2O_3, respectively. This figure showed a representative of three independent trials

phosophorylation and cyclinB-cdk1 induced G_2/M phase arrest by prevention of the dephosphorylation of cyclinB-cdk1 complex (Halloran and Fenton, 1998). In our study, the changes in expression levels of cyclin-B1 and p53 indicated that As_2O_3 induced G_2/M phase cell cycle arrest via prevention of dephosphorylation of cyclinB-cdk1 complex. Cyclin E-cdk2 regulates the transition from G_1 to S phase and p53 tumour suppressor gene inhibits the cyclin E-cdk2 activity after DNA damage (Di Leonardo *et al*., 1994). Our results showed that there was an significant decrease in percentage of MDA-MB-231 cells in G_1 phase, together with the decrease of p53 expression and no significant change of cyclin E expression in As_2O_3 treated MDA-MB-231 cells (Fig. 5), indicating that the p53 may not be involved in As_2O_3-induced cell cycle arrest.

For thousands of year, As_2O_3 was considered as a toxic agent in both Chinese and Western society. Pharmacokinetic study performed by Shen *et al*. (1997) during the treatment of APL suggested that it is safe to inject As_2O_3 with a dose of 10 mg day^{-1} because arsenic was rapidly eliminated in the plasma and continuous administration of As_2O_3 did not result in the accumulation of arsenic in plasma. The growth inhibition of As_2O_3 on normal human 184B5 breast cells was studied to investigate the toxic effect of As_2O_3 on normal human breast tissue. When incubation with As_2O_3 at IC_{50} concentration on MDA-MB-231 cells at 48 and 72 h were applied, the percentages of survival of 184B5 cells were over 70% for both 48h and 72h As_2O_3 treatment (data not shown). Our data on nude mice also showed that As_2O_3 at dosage 0.06 mg/kg/day to 0.12 mg/kg/day administered on every other days for 14 days has no apparent toxicity to the animal (data not shown).

CONCULSION

In conclusion, As_2O_3 was found to be effective in treating breast tumour cells including late stage breast tumour cells which are Erα negative and administration of As_2O_3 was found to be safe to normal breast cells and on nude mice. Our results indicated that As_2O_3 is a potential candidate to be further investigated as an anti-tumour drug for human breast tumours.

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