

Arsenic trioxide induces the apoptosis of human breast cancer MCF-7 cells through activation of caspase-3 and inhibition of HERG channels

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Abstract. Arsenic trioxide (As₂O₃) has been widely used to treat patients with acute promyelocytic leukemia and has also been shown to exhibit therapeutic effects on various types of solid tumors, including gastric cancer and lung carcinoma. Breast cancer is a type of solid tumor whose incidence has been increasing for many years. The present study was designed to investigate the effects of As₂O₃ on the human breast cancer cell line MCF-7, and to explore its potential mechanisms. The MTT assay demonstrated that As₂O₃ decreased the cellular viability of MCF-7 cells in a concentration-dependent manner. Morphological observation, the TUNEL assay and flow cytometric analysis revealed that apoptosis was involved in the process. An assay for caspase-3 activity suggested that the apoptosis was mediated through caspase-3 activation. Further investigation indicated that protein levels of the human *ether-a-go-go*-related gene (HERG) were markedly downregulated by As₂O₃. Taken together, the results indicate that arsenic trioxide induces the apoptosis of human breast cancer MCF-7 cells at least in part through the activation of caspase-3 and the decrease in HERG expression.

Introduction

Breast cancer is the most common solid tumor found in women, and is the main cause of mortality due to cancer (1). According to the American Cancer Society's estimation, over 40,000 patients in the US diagnosed with breast cancer succumbed to the disease in 2005. Presently, the treatment of breast cancer includes surgery and radiation, sometimes supported by adjuvant chemotherapy or hormone therapy (2).

Although major advances have been made in understanding the pathogenesis of this disease, therapeutic problems such as the unselective sacrificing of normal vs. tumor cells persists.

Arsenic trioxide (As₂O₃) is an arsenic compound found in nature and has been used as a medicinal agent for more than 2,400 years for conditions ranging from infectious diseases to cancer (3). In the 1970s, researchers at Harbin Medical University discovered its ability to cure acute promyelocytic leukemia (APL) (4,5). Since then, our studies and those of other research groups have demonstrated that As₂O₃ also inhibits many solid tumors, including gastric carcinoma and lung cancer. For example, As₂O₃ was found to induce apoptosis through a reactive oxygen species-dependent pathway and the loss of mitochondrial membrane potential in HeLa cells (6). In human gastric cancer MGC-803 cells, As₂O₃ was also found to inhibit cell growth and to induce cell apoptosis (7). Similar findings were observed in esophageal carcinoma (8), neuroblastoma (9), prostate and ovarian carcinoma (10), and breast cancer (11,12) cells. However, the molecular mechanisms underlying the As₂O₃-promoted apoptosis of solid tumor cells remain unknown.

HERG belongs to the family of voltage-gated potassium channels *ether-a-go-go* (EAG), and mutations in this gene can cause long Q-T syndrome-2 (LQT2) in humans (13). We previously found that As₂O₃ prolonged the QT interval and regulated several ion channels in the guinea pig heart (14). In particular, As₂O₃ was reported to downregulate the protein expression of cardiac potassium channel HERG and to decrease IKr in guinea pig ventricular myocytes (15). The reduced trafficking of HERG channels to the cell surface in patients treated with As₂O₃ contributed to the induction of QT prolongation and torsade de pointes (16). Notably, HERG expression was noted in a variety of tumor cell lines of varied histogenesis, but was absent from the healthy cells from which the respective tumor cells were derived (17-19). In our previous study, we showed that HERG expression facilitates tumor cell proliferation caused by tumor necrosis factor (TNF) ligand (TNF-α). Cisapride, a specific blocker for the human HERG, channel was shown to exhibit therapeutic effects on gastric cancer by inhibiting the growth of gastric cancer cells through the regulation of the cell cycle and the induction of apoptosis. Similarly, silencing of HERG protein expression

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by siRNA technology was found to decrease HERG currents and to inhibit proliferation, invasion and tumorigenicity, and to induce the apoptosis of gastric cancer cells by inhibiting their entry into the S phase from the G₁ phase (20). These findings prompted us to hypothesize that the HERG channel may be involved in the regulation of cell death by As₂O₃ in breast cancer. The present study was designed to investigate the anti-cancer effect of As₂O₃ on human breast cancer MCF-7 cells, and to examine the role of the HERG channel in this process.

Materials and methods

Materials. Dulbecco's minimal essential medium (DMEM), foetal bovine serum (FBS), penicillin, streptomycin and other cell culture reagents were obtained from Gibco (Grand Island, NY, USA). The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) detection kit was purchased from Roche (Penzberg, Germany). Apoptosis-FITC was from Bao Sai Company (Beijing, China). Anti-HERG and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Santa Cruz Biotechnology (USA). The CaspACE™ assay system, a fluorometric detection kit, was obtained from Promega (USA). As₂O₃ was from Yida (Harbin, China).

Cell line and cell culture. The MCF-7 cell line was provided by Dr Wang Zhiguo of the Montreal Heart Institute, Canada. The cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin in a humidified atmosphere with 5% CO₂ at 37°C. Cells were passaged regularly and subcultured to ~80% confluence before conducting the experimental procedures.

Cell proliferation assay. Cell proliferation was assessed using the MTT assay. Briefly, cells were treated with various concentrations of As₂O₃ for 24 h. Then, 15 µl of MTT reagent was added to each well. After 4 h of incubation at 37°C, the supernatants were discarded and the crystals were dissolved in dimethyl sulfoxide (DMSO). The absorbance was measured at 490 nm.

Fluorescence microscopy measurements. For the detection of apoptosis, cells were stained with acridine orange/ethidium bromide (AO/EB). The fluorescent dye AO readily enters either intact cells or cells with damaged membranes and stains them green. EB, which is impermeable to cells with preserved membranes, stains cells red. These dyes were used to detect apoptotic and necrotic cells. For the AO/EB procedure, cells were harvested with 10 µl of prepared AO/EB working solution (100 µg/ml AO and 100 µg/ml of EB) in phosphate-buffered saline (PBS) for 5 min and examined under a fluorescence microscope (Eclipse TE300, Nikon, Japan).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. DNA fragmentation of individual cells was detected *in situ* by TUNEL with the In Situ Cell Death Detection kit, Fluorescein. Cells grown on coverslips were washed with PBS containing (in mM) NaCl 137.0, KCl 2.7, Na₂HPO₄ 4.3, KH₂PO₄ 1.4 (pH 7.4), and were fixed in 4% paraformaldehyde solution for 1 h at 4°C. The cells were permeabilized in solution containing 0.1% Triton X-100

for 2 min on ice, followed by incubation in freshly prepared TUNEL reaction mixture for 1 h at 37°C in the dark. The coverslips were then washed with PBS and mounted on slides with anti-fading solution. TUNEL staining was analyzed using fluorescence microscopy (Olympus, Tokyo, Japan).

Flow cytometric analysis of apoptotic progression. Quantitative assessment of apoptosis was conducted using an Annexin V assay kit. MCF-7 cells were centrifuged at 1000 x g for 10 min at 4°C after trypsinization, then washed with ice-cold PBS twice. The pellet was resuspended in ice-cold binding buffer provided in the kit. Subsequently, 10 µl Annexin V-FITC and 5 µl propidium iodide (PI) were added to the cell suspension, which was maintained on ice in the dark for 15 min. The samples were then assessed for viable (Annexin V/PI⁻), early apoptotic (Annexin V⁺/PI⁻), late apoptotic (Annexin V⁺/PI⁺) and necrotic (Annexin V/PI⁺) cells using a flow cytometer (Fc500MDL; Beckman Coulter, USA).

Caspase-3 activity assay. Caspase-3 activity was measured using the CaspACE™ assay system, fluorometric kit, according to the manufacturer's instructions. Briefly, the cells were lysed and the supernatant was used for the assay. The fluorogenic substrates for caspase-3 were labeled with fluorochrome 7-amino-methylcoumarin (AMC). AMC was released from these substrates upon cleavage by caspase-3. Enzyme activity was determined by monitoring the fluorescence produced by free AMC using the GloMax™ 20/20ⁿ luminometer (Promega) at 360/460 nm.

Western blot analysis. The immunoblotting procedures were as follows: cells were incubated at 37°C in DMEM in the presence or absence of As₂O₃. After treatment for 24 h, the adherent cells were scraped off from the culture flask in ice-cold PBS and centrifuged at 2,500 x g for 10 min. RIPA buffer (Beyotime, Shanghai) containing (in mM) Tris 50.0 (pH 7.4), NaCl 150.0, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, sodium orthovanadate, sodium fluoride, EDTA and leupeptin was added to the pellets, and the cells were homogenized on ice for 45 min. The cells were then centrifuged at 13,500 x g for 30 min, and the cleared lysates were used for immunoblotting. Protein concentration was determined according to the Bradford method (Sigma) using BSA as a standard. Denatured protein was separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a PVDF membrane (Stratagene) and blocked in 5% nonfat milk overnight. The next day, the membrane was incubated with primary antibodies against GAPDH (1:1000 dilution) and the specific polyclonal rabbit anti-HERG antibody (dilution 1:200). Goat anti-rabbit Alexa Fluor 700 (1:4000 dilution, Molecular Probes) was used as a secondary antibody. The Odyssey infrared fluorescent scanning system (LICOR) was used to detect the protein bands. The intensity of the band was determined by densitometry using Odyssey v1.2 software.

Statistical analysis. Data are expressed as the mean ± standard error of the mean (SEM), with the exception of the 50% inhibitory concentration (IC₅₀). Statistical differences were analyzed by the Student's t-test. A two-tailed p<0.05 was considered statistically significant.

Results

As₂O₃ reduced the cell viability of MCF-7 cells. To detect cell viability, the mitochondrial-dependent reduction of MTT to formazan was measured. The cells were incubated with 2, 4, 8 and 10 μ M As₂O₃ for 24 h. As shown in Fig. 1, cell viability was markedly inhibited with increased As₂O₃ concentrations in a dose-dependent manner. As₂O₃ (2 and 4 μ M) treatment of MCF-7 cells resulted in 87.63 ± 4.90 and $75.67 \pm 4.46\%$ reduced cell viability, respectively, whereas 8 and 10 μ M As₂O₃ resulted in only 50.70 ± 2.84 and $42.41 \pm 2.66\%$ reduced cell viability, respectively, after a 24-h treatment compared to the controls. The IC₅₀ value of the inhibition of MCF-7 cells by As₂O₃ was 8.2 μ M upon treatment for 24 h.

As₂O₃ induced the apoptosis of MCF-7 cells. Since cell viability was decreased by As₂O₃, we hypothesized that apoptosis might be involved in this process. Several assays were used to determine whether As₂O₃ induced cellular apoptosis in the MCF-7 cells. Apoptotic morphological changes in the nuclear chromatin of cells were detected by AO/EB staining. The cells treated with As₂O₃ showed typical apoptotic morphology, which included condensed nuclei, membrane blebbing and the formation of apoptotic bodies (Fig. 2B). By contrast, control cells showed intact nuclear architecture (Fig. 2A). The number of apoptotic cells was increased by $33.47 \pm 2.30\%$ in the As₂O₃-treated group compared to the control group (Fig. 2C).

For the quantification of As₂O₃-induced apoptotic death, the number of apoptotic and necrotic cells was measured by flow cytometry with the Annexin V/PI assay. Apoptotic cells were detected by Annexin V binding to phospholipid phos-

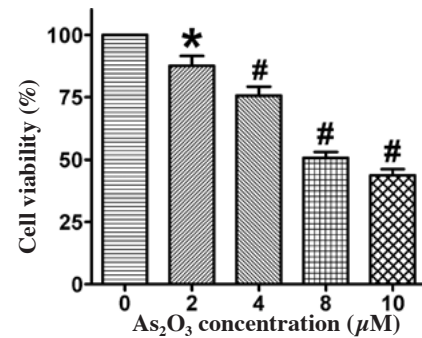


Figure 1. As₂O₃ inhibited the cell viability of MCF-7 cells. Adherent cells plated in 96-well plates (1×10^4 cells/well) were treated with various concentrations (2-10 μ M) of As₂O₃ for 24 h. Cell proliferation was determined by the MTT assay. Data are expressed as the percentage of cell proliferation relative to the proliferation of the control. Results are representative of three independent experiments. *P<0.05 vs. control, #P<0.01 vs. control.

phatidylserine (PS), which was translocated from the inner to the outer leaflet of the plasma membrane of the apoptotic cells. The cells were treated with 8 or 16 μ M As₂O₃ for 24 h. As shown in Fig. 3, treatment with all concentrations of As₂O₃ resulted in a statistically significant increase in the number of early apoptotic cells. At 8 μ M As₂O₃, the median values for Annexin V/PI⁻ (normal) the Annexin V/PI⁺ (early apoptotic) cells were 52.13 ± 6.14 and $45.10 \pm 5.19\%$, respectively. With increasing concentration, the number of normal cells decreased while the number of early apoptotic cells increased. When the cells were treated with 16 μ M As₂O₃ for 24 h, the median values of normal and early apoptotic cells were 39.60 ± 5.28 and $57.43 \pm 5.13\%$, respectively.

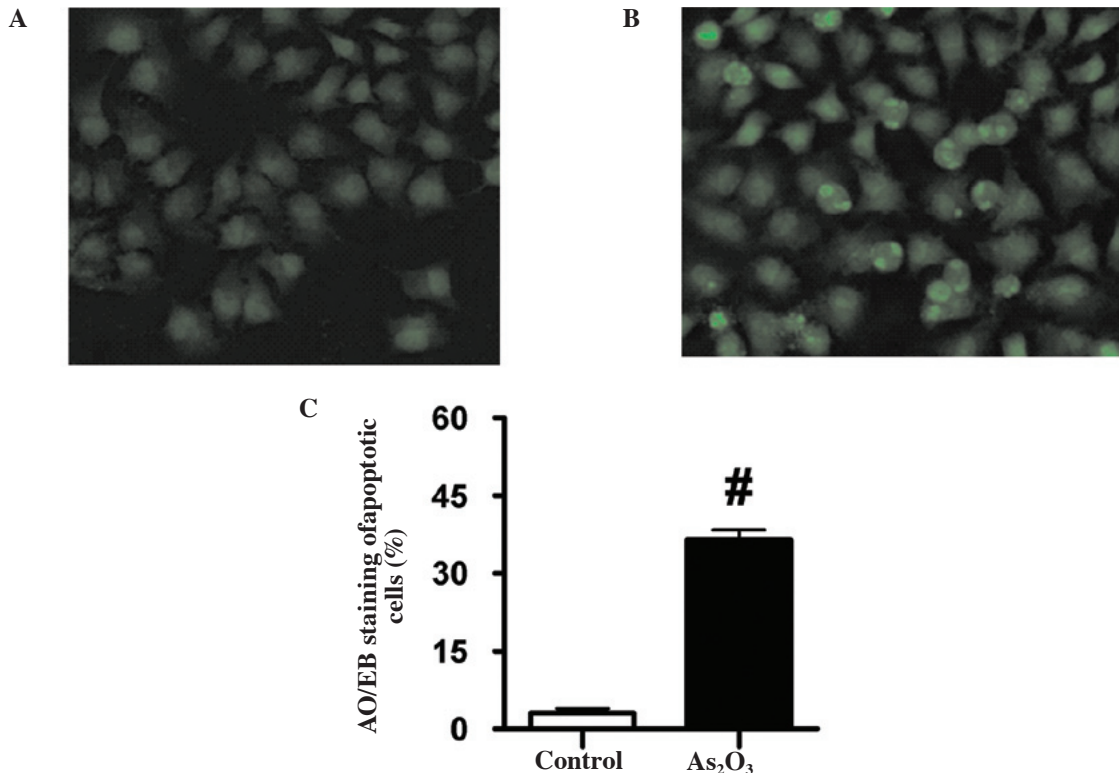


Figure 2. Apoptosis was determined by acridine orange/ethidium bromide (AO/EB) staining. AO/EB staining was performed 24 h after exposure of MCF-7 cells to 8 μ M As₂O₃. Morphological changes in the (A) control and (B) As₂O₃-treated MCF-7 cells. (C) Quantitative analysis of apoptotic cell nuclei. Results are representative of three independent experiments. #P<0.01 vs. control.

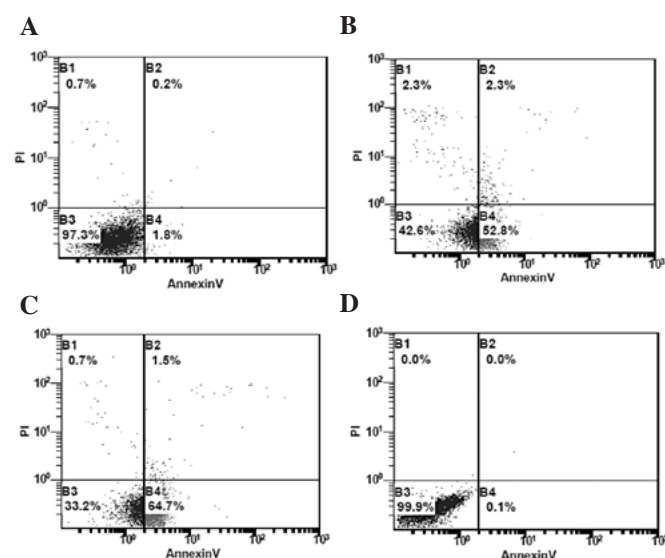


Figure 3. Apoptosis was determined by flow cytometry in Annexin V/PI-stained MCF-7 cells. Quantitative analysis of flow cytometry of Annexin V/PI-stained MCF-7 cells after treatment with 8 and 16 μ M of As₂O₃ for 24 h. Results are representative of three separate experiments. (A) control (B) As₂O₃ 8 μ M, (C) As₂O₃ 16 μ M, (D) negative control.

Typical apoptotic nuclear condensation is regarded as the morphological marker of apoptosis. When DNA strands are cleaved or nicked by nucleases, a large number of 3'-hydroxyl ends are exposed. The TUNEL assay was performed to detect cells containing massive DNA fragmentation, a hallmark of late apoptosis. As shown in Fig. 4, TUNEL-positive cells were seldom observed in the control MCF-7 cells, whereas in the As₂O₃-treated MCF-7 cells, the number of TUNEL-positive cells significantly increased by $29.52 \pm 2.83\%$. These obvious morphologic changes of apoptosis were markedly induced by treatment with 8 μ M As₂O₃.

As₂O₃ induced apoptosis through activation of caspase-3. Activation of caspase-3 is important in the initiation of apoptosis in diverse biological processes. In order to investigate apoptotic signaling, the activities of caspase-3 were examined. As shown in Fig. 5, when the cells cultured to ~80% confluence were exposed to 8 μ M As₂O₃ for 6 h, caspase-3 activity increased markedly. The activity of caspase-3 increased $55.67 \pm 0.12\%$ as compared to the control group.

Expression of HERG in MCF-7 cells treated with As₂O₃. HERG channel was reported to have oncogenic properties. The distribution is restricted in normal tissue and becomes ubiquitous in tumor cells. To examine whether HERG is involved in the cell growth and cell death induced by As₂O₃ in the MCF-7 cell line, we used Western blot analysis to detect the expression of HERG protein. The cells seeded in culture medium to 80% confluence were treated with As₂O₃ at 0 and 8 μ M, respectively. As shown in Fig. 6, the expression of HERG decreased by $41 \pm 0.11\%$ according to the level of non-treated cells.

Discussion

In the heart, HERG encodes the rapid delayed rectifier K⁺ current and undergoes marked developmental changes, predominating

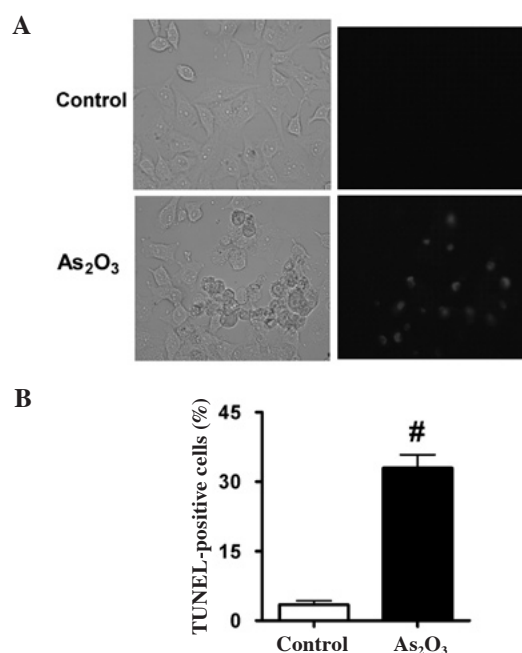


Figure 4. Apoptosis was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL). (A) MCF-7 cells were cultured with 8 μ M As₂O₃ or without (Control) for 24 h and (B) quantitative analysis of the apoptotic cell nuclei was carried out. Results are representative of three separate experiments. #P<0.01 compared to the control.

in the fetal heart and dissipating in the adult (21,22). Most strikingly, HERG is abundantly expressed in a variety of tumor cells but is not present in the corresponding healthy cells implicating HERG in the regulation of tumor cell proliferation. The present study was carried out to investigate whether As₂O₃ may induce an anticancer effect in MCF-7 cells and whether the HERG channel protein is involved in this process. Our results revealed that i) As₂O₃ induced apoptosis in MCF-7 cells and ii) expression of HERG decreased in the apoptotic process.

Recent studies have shown that As₂O₃ is effective in the inhibition of solid tumors and showed efficacy in a pilot treatment of APL patients (23). In our study, the cytotoxicity of As₂O₃ against human breast cancer MCF-7 cells was assessed using several parameters. A series of concentrations of As₂O₃ ranging from 1 to 100 μ M was tested in preliminary experiments. MTT assay showed that the cell viability was significantly inhibited in a dose-dependent manner by As₂O₃. The IC₅₀ was 8.2 μ M at 24 h. The concentration of 8 μ M As₂O₃ was chosen for subsequent experiments on the induction of apoptosis in MCF-7 cells.

Apoptosis, defined as programmed cell death or cell suicide, is believed to be an important mechanism and a target for treating APL cell lines and other solid human tumors (24). In our study, apoptosis occurred in MCF-7 cells upon treatment for over 24 h at a concentration of 8 μ M As₂O₃. Morphological changes are important features of cells undergoing apoptosis and are readily observed by microscopy. The images of AO/EB staining showed specific apoptotic morphological changes after treatment with As₂O₃ for 24 h.

To further confirm the induction of apoptosis by As₂O₃, flow cytometric analysis of Annexin V/PI-stained cells was carried out. Apoptotic cells were detected by Annexin V binding to

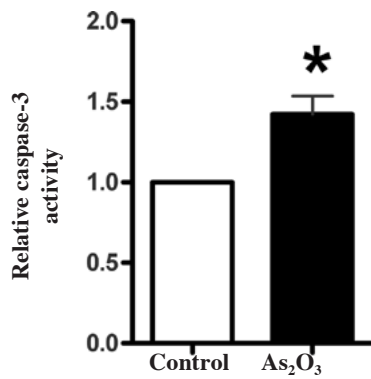


Figure 5. As₂O₃ induces apoptosis through caspase-3 activation. MCF-7 cells were exposed to As₂O₃ at a concentration of 8 μ M for 6 h. The cell protein was then extracted and the activity of caspase-3 was detected. *P<0.01 compared to the control, n=3.

phospholipid phosphatidylserine (PS), whose externalization was observed in the majority of treated cells. Meanwhile, TUNEL assay identified internucleosomal DNA fragmentation in apoptotic cells by attachment of a fluorescent indicator to the ends of fragmented DNA suggesting that decreased cell viability was due to As₂O₃-induced cell apoptosis.

Several action mechanisms involved in As₂O₃-induced apoptosis of MCF-7 cells have been identified, including signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK), p53, Bcl-2 (11,12). Here, we investigated caspase-3 and the expression of HERG in As₂O₃-induced apoptosis of MCF-7 cells.

Caspase-3 is a major executioner protease, responsible for initiating the apoptotic process (25). The expression of caspase-3 and poly ADP-ribose polymerase (PARP) which is the specific cleavage of its downstream substrates was observed in the breast carcinoma cell line MCF-7 by As₂O₃ treatment (26). Thus, caspase-3 present in the MCF-7 cells along with this enzyme contributed to the apoptotic signaling process. Our data demonstrated that As₂O₃ exposure significantly increased the level of caspase-3 and it was caspase-3 that mediated this apoptosis.

HERG belongs to an evolutionarily conserved multigenic family of voltage-gated K⁺ channels, the *eag* (*ether a-go-go*) family. It is expressed in many tumor cell lines of different histogenesis but is not present in the corresponding normal cells, which has highlighted the tight association between HERG and cancer (27-29). It is reported that the *HERG* gene and HERG protein are expressed with high frequency in primary human endometrial cancers, as compared to its absence in normal and hyperplastic endometrium (18). A similar expression pattern was observed in leukemia where almost all of the primary leukemia cells and K562 leukemia cell lines expressed HERG mRNA, while no expression was detected in normal bone marrow cells. Moreover, inhibition of the HERG channel can reduce leukemia cell proliferation by affecting the G1/S transition phase of the cell cycle, while not affecting the growth of cells which do not express HERG channels (30). HERG was also expressed at the protein and mRNA levels in MDA-MB-435S melanoma cells. Blockade of HERG channels and downregulation of HERG by siRNA can both induce an antiproliferative effect on melanoma cell lines

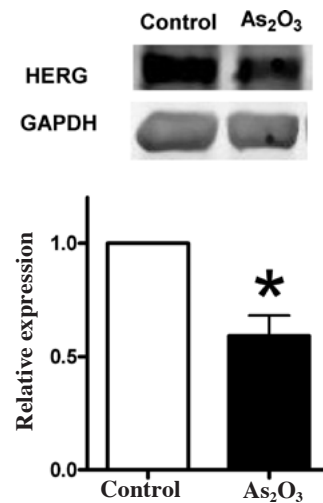


Figure 6. Western blotting of HERG proteins in MCF-7 cells treated with 8 μ M As₂O₃ for 24 h. Whole cellular lysates were electrophoresed on SDS-PAGE gels and proteins were transferred to PVDF membranes and probed with specific antibodies. Lane 1, control; lane 2, As₂O₃. The results are representative of three separate experiments.

(31). The same association was observed in human neuroblastoma SH-SY5Y cell lines. It was found that silencing of the *HERG* gene by shRNA suppressed the cellular growth rate, inhibited cell viability and reduced colony formation (32). Accumulating evidence indicates that the HERG channel promotes tumor cell proliferation. Therefore, inhibition of HERG channel functions or downregulation of HERG channel expression should inhibit tumorigenesis. The results of the present study demonstrated that the expression of HERG decreased as a result of the As₂O₃-induced antiproliferative effect. We speculate that inhibition of HERG contributed to the anticancer effect of As₂O₃ in MCF-7 cells. A recent study demonstrated that HERG is physically linked to β 1 integrins and thereby modulates adhesion-dependent signaling (33). Integrins are known to mediate numerous signaling pathways that are involved in cell proliferation, migration, differentiation and anti-apoptotic functions. The finding of the association between HERG protein and integrins may provide new clues for further study. The HERG channel has also been related to tumor cell invasion and neoangiogenesis apart from its activity in cell proliferation (34-36). Shao *et al* found that inhibition of HERG protein expression reduced the invasiveness of gastric cancer cells (37). A similar finding was also reported in colon cancer, but the mechanism was not clear (38). Moreover, blocking of the HERG channel significantly impaired VEGF secretion in HERG-expressing glioblastoma cells (39).

In conclusion, As₂O₃ induced apoptosis in MCF-7 cells through the activation of caspase-3 and downregulation of HERG protein. Because of the specific expression variation of HERG protein in tumor cells, the HERG channel is endowed with therapeutic potential.

Acknowledgements

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