Suppression of cell proliferation and regulation of estrogen receptor α signaling pathway by arsenic trioxide on human breast cancer MCF-7 cells

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Abstract

In recent years, breast cancers have aroused much concern. Together with a growing incidence all over the world, the development of drug resistance to tamoxifen, the most commonly prescribed chemotherapeutic drug for breast cancer patients, has highlighted the importance of developing a new chemotherapeutic drug in combating breast cancer. With the aim of treating breast cancers, the anti-tumor effects of arsenic trioxide in MCF-7 cells have been studied.

MCF-7 cells are estrogen responsive cells which mimic breast cancers at the early stage. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay and direct cell counting were used to measure cell proliferation. The mechanisms of action were elucidated through the measurement of estrogen receptor (ER) binding, mRNA and protein levels of ER and its activity.

We have demonstrated that arsenic trioxide was capable of reducing cell survival in MCF-7 cells via the suppression of the estrogen-induced growth stimulatory effects in MCF-7 cells. Arsenic trioxide was shown to suppress the action of estrogen through the regulation of the ERα signaling pathway. Arsenic trioxide could down-regulate ERα mRNA and protein levels without competing with estrogen for ERα binding. Arsenic trioxide also inhibited the transcription activity mediated by the ERα signaling pathway and ultimately it down-regulated c-myc protein expression and inhibited cell entry to S phase under estrogen’s stimulation.

In conclusion, arsenic trioxide could inhibit the growth of MCF-7 cells by reducing the growth stimulatory effect of estrogen. As estrogen is a primary risk factor in promoting the growth of breast tumor cells, the anti-estrogenicity exhibited by arsenic trioxide sheds light on the therapy of breast cancer.


Introduction

Arsenic trioxide (As2O3) is an arsenic compound existing as an odorless, tasteless, white crystal or powder. In the 1970s, the effects of As2O3 on a number of cancers were investigated and it was found to be most effective in killing acute promyelocytic leukemia (APL) cells. Later, another group from Shanghai found promising effects of As2O3 in clinical trials of APL patients. APL accounts for 10–15% of all acute myeloid leukemia in adults (Soignet et al. 1998). In earlier times, APL patients were treated with anthracyclines. This cytotoxic chemotherapy achieved 70–85% remission but also induced severe complications and drug resistance in APL patients (Warrell et al. 1993). It was not until the characterization of the molecular pathologies of APL that a group from Shanghai found that all-trans retinoic acid (ATRA) was effective in combating the disease. Clinical trials indicated a complete remission in 85–90% of cases (Huang et al. 1988). Although ATRA treatment showed improvement in coagulopathy and high complete remission in APL patients (Huang et al. 1988), adverse effects were also observed.

Hyperleukocytosis developed during the second and third weeks of treatment. It is attributed to the activation of leukocytes (Castaigne et al. 1990). Together with other syndromes such as respiratory distress, pleural effusions, weight gain, fever and occasionally renal failure, these adverse effects are collectively called 'retinoic acid syndrome'. Moreover, ATRA resistance was observed in patients following ATRA treatment. Since then, frequent clinical trials have been performed to assess the effectiveness of As2O3 in APL treatment. As2O3 achieved a complete remission rate of 57–98% in both de novo and relapsed APL patients. Moreover, patients receiving As2O3 treatment had improved disseminated intravascular coagulation, hypofibrinolysis and bleeding...
The present study mainly focused on the estrogen receptor-dependent signaling pathway. Studies were focused on using As$_2$O$_3$ in concentrations below 2 µM to elucidate the mechanism of action in mediating the anti-tumor effect on MCF-7 cells.

Materials and Methods

Cell culture

MCF-7 and MDA-MB-231 cell lines were purchased from the American Type Culture Collection (Baltimore, MD, USA). They were maintained in RPMI 1640 medium supplemented with 10% dextran-coated charcoal-stripped fetal bovine serum and 1% antibiotics (v/v) at 37 °C in a humidified atmosphere of 5% CO$_2$.

Preparation of As$_2$O$_3$

The stock solution of As$_2$O$_3$ was prepared by dissolving As$_2$O$_3$ powder in PBS at a concentration of 10 mM.

MTT assay

MCF-7 cells and MDA-MB-231 were seeded at 1 × 10$^4$ cells/well in 96-well plates. After treatment with the appropriate concentration of As$_2$O$_3$, suspension medium was removed and 30 µl MTT solution were added to each well and incubated at 37 °C for 2–4 h. After that, 100 µl DMSO were added to each well and incubated for a further 15 min. Optical density (O.D.) at 540 nm was measured. Percentage survival was defined as: % survival=100% × (O.D. of test sample/O.D. of control).

Direct cell counting by the trypan blue dye exclusion method

MCF-7 cells were seeded at 5 × 10$^4$ cells/well in 24-well plates. After treatment with the appropriate concentration of As$_2$O$_3$ and 17β-estradiol for 24, 48 or 72 h, the cell number in each well was counted by the trypan blue staining method using a hemocytometer.

Effect of As$_2$O$_3$ on estrogen binding to ERα by ERα competitive binding assay

The assay was carried out using an ERα competitive screening kit according to the manual provided by the manufacturer (Wako Chemical, Richmond, VA, USA; catalogue number 295-56301). In brief, human recombinant ERα was coated in microplates. When incubating samples with fluorescence-labeled estrogen, there is competition with estrogen for the binding sites on ERα. After removal of unbound substances or fluorescence-labeled estrogen, the retained estrogen was determined by
measuring the fluorescent intensity. A control of fluorescence-labeled estrogen alone was prepared to act as a positive control. Excitation of the sample mixture emitted high fluorescent intensity. The extent of binding depends on the capability of the substance in competing with estrogen and this is correlated to the affinity of that substance to ERα.

Assessment of the transcriptional activity of ERα

Estrogen response element (ERE) containing vector was constructed by insertion of a single stranded ERE oligonucleotide into pGL3 basic vector at NheI site upstream of the luciferase gene. The sequence of ERE oligonucleotide was constructed according to the previous report (Cullen et al. 2001).

The sequence is: 5'-CCAGGTCAGTGACCTGAGCTAAAATAACACATTCAG-3'. pGL3–control vector was used as a positive control which emitted strong luminescence upon activation. pGL3–basic vector was used as a negative control which is lacking in promoter and enhancer sequences. Renilla luciferase reporter plasmid was prepared to act as an internal control for the determination of transfection efficiency. The treatment scheme was as follows. Cells were seeded at 5 × 10⁴ cells/well in 24-well plates until confluence at 40–60%. Four wells were prepared for each treatment. Experimental medium without serum supplement (50 µl) was transferred to microtubes to which 300 ng ERE luciferase reporter plasmid, 100 ng Renilla luciferase reporter plasmids and 1 µg Fugene 6 transfection reagent were added. The mixture was incubated at room temperature for 30 min. After that, the mixture was discarded. The transfected cells were then treated with 10 nM 17β-estradiol, As₂O₃ at 1 µM, As₂O₃ at 2 µM or As₂O₃ plus 17β-estradiol. The cells were treated for either 48 or 72 h. Luciferase assay was carried out using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Procedures were followed according to the manual provided by the manufacturer. The luminescence of ERE luciferase measured is proportional to the luciferase activity of the ERα complex upon treatment. The luminescence of Renilla luciferase is proportional to the amount of transfected cells.

Normalized luciferase luminescence is calculated as follows: normalized luciferase luminescence = luminescence (Firefly luciferase)/luminescence (Renilla luciferase). Normalized luciferase activity (% control) = normalized luciferase luminescence (test sample)/normalized luciferase luminescence (untreated control)* 100%.

Normalized luciferase activity of untreated control was expressed as 100%.

Figure 1  Cell survival of MCF-7 cells following 17β-estradiol treatment for various time intervals. Cells were treated with different concentrations (0–1000 nM) of 17β-estradiol for 24, 48 and 72 h. The percentage cell survival was measured by MTT assay. Data are presented as means ± s.d. of 6 replicate measurements. The percentage survival is expressed relative to control which is defined as 100%. *P<0.05 (vs. control).
Detection of expression level of ERα by RT-PCR

Cells were seeded at 3 × 10^5 cells/well in 6-well plates. Cells were treated with 10 nM 17β-estradiol, 2 µM As₂O₃ or co-treated with both drugs for 24 and 48 h. Controls were prepared by incubating with experimental medium only. After treatment, RNA was isolated using the TRIzol reagent (Invitrogen) according to the procedures suggested by the manufacturer. cDNA was then synthesized using Superscript First-Strand Synthesis System (Invitrogen). In brief, RNA samples of 5 µg were mixed in 0.5 ml microtubes with 1 µl 10 mM dNTP mix, 1 µl Oligo(dT)₁₂–₁₈ (0.5 µg/µl) in diethy pyrocarbonate (DEPC) treated water to a final volume of 10 µl. The samples were incubated at 65 °C for 5 min and then placed on ice for at least 1 min. A reaction mixture composed of 1 µl RNase inhibitor, 2 µl 10 × RT buffer, 2 µl dithiothreitol (0.1 M), and 4 µl MgCl₂ (25 mM) was added to each sample and mixed gently. The samples were incubated at 42 °C for 2 min. One microliter Superscript reverse transcriptase was added and incubated for 50 min at 42 °C and then for 15 min at 70 °C. The mixture was then chilled on ice. Finally, 1 µl RNase H was added and further incubated for 20 min at 37 °C. The cDNA samples were stored at −20 °C until use. PCR was performed with primers flanking the ERα gene to produce a PCR product with a size of 490 base pairs. The sequences of the primers were as follows: forward 5′ CAG GGG TGA AGT GGG GTC TGC TC 3′; reverse 5′ ATG CGG AAC CGA GAT GAT GTA GC 3′.

As an internal control, PCR with primers specific for glyceraldehyde phosphate dehydrogenase (GADPH) was carried out. Samples (10 µl) were mixed with 2 µl 6× loading dye and electrophoresed in 1% agarose gel electrophoresis. The band intensities of PCR products were analyzed by ImageQuant program (Amersham Biosciences).

Detection of protein expression levels of ERα and c-myc by Western blot analysis

Cells were seeded at 1 × 10^6 cells/well in 100 mm culture plates and treated with different drugs for 48 h. Treatment groups in this assay included a control group with medium only, 2 µM As₂O₃ alone, 10 nM 17β-estradiol alone and 2 µM As₂O₃ together with 10 nM 17β-estradiol. After drug treatments, cells were collected and lysed.

Concentration of As₂O₃(µM)

Figure 2 Effect of As₂O₃ on cell survival of MCF-7 cells treated with 10 nM 17β-estradiol, measured by MTT assay. Cells were treated with 10 nM 17β-estradiol alone (E) and various concentrations (0.25–2 µM) of As₂O₃ simultaneously with 10 nM 17β estradiol (E) for 24, 48 and 72 h. Cells without any treatment were used as a negative control for comparison (CTL). The percentage cell survival was measured by MTT assay. Data are presented as means ± S.D. of 6 replicate measurements. The percentage survival was expressed relative to the untreated control which was defined as 100%. *P<0.05 compared with control; #P<0.05 compared with 17β-estradiol.
The protein content in each sample was determined by BCA assay (Sigma). Thirty micrograms protein of each sample were resolved by 10% SDS-PAGE. After electroblotting, the membrane was probed with anti-ERα antibody (Oncogene Science, Cambridge, MA, USA) or anti-c-myc antibody (BD Pharmingen, San Diego, CA, USA). Secondary antibody was conjugated with horse-radish peroxidase. Finally, the signal was detected by an enhanced chemiluminescence (ECL) kit (Amersham Biosciences).

*Effects of As$_2$O$_3$ on cell survival of MCF-7 cells treated with 10 nM 17β-estradiol, measured by direct cell counting. Cells were treated with 10 nM 17β-estradiol alone (E) and various concentrations (0.25–2 μM) of As$_2$O$_3$ simultaneously with 10 nM 17β-estradiol (E) for 24, 48 and 72 h. Cells without any treatment were used as negative control for comparison (CTL). The percentage cell survival was measured by trypan blue dye exclusion assay. Data are presented as means ± s.d. of 4 replicate measurements. *P<0.05 compared with control.*

The protein content in each sample was determined by BCA assay (Sigma). Thirty micrograms protein of each sample were resolved by 10% SDS-PAGE. After electroblotting, the membrane was probed with anti-ERα antibody (Oncogene Science, Cambridge, MA, USA) or anti-c-myc antibody (BD Pharmingen, San Diego, CA, USA). Secondary antibody was conjugated with horse-radish peroxidase. Finally, the signal was detected by an enhanced chemiluminescence (ECL) kit (Amersham Biosciences).

*Effects of As$_2$O$_3$ on cell cycle distribution of MCF-7 cells under estrogen stimulation*

Cells at $3 \times 10^5$ cells/well were seeded in 6-well plates and treated with different drugs for 48 or 72 h. Treatment groups in this assay included a control group with medium only, 2 μM As$_2$O$_3$ alone, 10 nM 17β-estradiol alone and 2 μM As$_2$O$_3$ together with 10 nM 17β-estradiol. After treatment, the cells were washed with PBS and fixed with 70% ethanol for at least 30 min. After fixation, cells were washed with PBS and stained with propidium iodide (PI) solution (2 mg/ml), RNase A (10 mg/ml) and 400 μl PBS for 30 min at 37°C under subdued light. Stained cells were analyzed using a FACSort flow cytometer (BD Biosciences, San Jose, CA, USA). With the CellQuest program, the cell population was targeted by forward light scatter (FSC) and side scatter (SSC). The fluorescence signal of PI was detected at channels of FL-2. The percentages of DNA content at different phases of the cell cycle were analyzed with Modfit software (Verity Software House, Topsham, ME, USA).

Statistical analyses

Data were expressed as means ± standard deviations (s.d.) for three replicate experiments. The Student’s t-test was used for statistical analyses.

Results

Effect of As$_2$O$_3$ and 17β-estradiol on cell viability of MCF-7 cells

The growth stimulatory effects of 17β-estradiol on MCF-7 cells in a range of concentrations were determined by MTT assay and direct cell counting. The percentage survival of MCF-7 cells increased upon incubation with 0·1 nM to 500 nM 17β-estradiol (Fig. 1) compared with the untreated control. Within 24 h incubation, no obvious survival stimulation was observed. It was not until 48 h
incubation that estrogen induced an increase in percentage survival from 100% in untreated controls to a maximum of 137% in 10 nM 17β-estradiol-treated cells. Increasing the incubation time to 72 h further increased the percentage survival. At this time period, 10 nM 17β-estradiol attained the maximum survival stimulation of 165% with respect to controls. On the other hand, 1000 nM 17β-estradiol reduced cell survival from 75% to 49% after 24 and 72 h treatment respectively. To assess the effect of As2O3 on 17β-estradiol-treated MCF-7 cells, MCF-7 cells were incubated with As2O3 and 17β-estradiol simultaneously. As seen in Fig. 2, when treated together with As2O3, the percentage survival of MCF-7 cells was reduced as compared with 17β-estradiol treatment alone. The reduction was dose- and time-dependent. For concentrations between 0·25 and 0·5 µM, As2O3 did not reduce the percentage survival. At a concentration of 2 µM As2O3, 25% reduction of survival was induced after 24 h treatment, and it was maximized after 72 h

treatment to 52% as compared with the untreated controls (Fig. 3).

Effect of As2O3 on cell survival of the hormone independent breast cancer cell line, MDA-MB-231

To compare the cell survival inhibiting effects of As2O3 on MCF-7 cells and MDA-MB-231 cells, IC50 after various time treatments was obtained and the results are shown in Table 1. Higher IC50 of As2O3 was obtained in MDA-MB-231 cells as compared with MCF-7 cells. In other words, As2O3 was more potent in combating cell survival in MCF-7 cells than in MDA-MB-231 cells.

Effect of As2O3 on estrogen binding to estrogen receptor α (ERα)

In assessing the competitive binding capacity of As2O3 on ERα, an ERα competitor screening kit was applied. The specific estradiol binding after incubation with various concentrations of 17β-estradiol, As2O3, tamoxifen and paclitaxel is shown in Fig. 4. 17β-Estradiol in concentrations of 0·1 nM to 200 nM reduced the specific estradiol binding dramatically in a concentration-dependent manner. Concentrations above 200 nM completely prevented the binding of fluorescent estradiol to ERα. The estradiol binding remained high in the presence of paclitaxel indicating that it did not compete with estradiol for ERα binding. The result was consistent with the fact that paclitaxel inhibited breast cancer survival through mechanisms other than ERα signaling. When incubated with tamoxifen in concentrations above 0·25 µM, the estradiol binding was reduced by more than 50%. Compared with As2O3, estradiol binding remained high in

Table 1 The inhibiting effects of As2O3 on cell survival of MCF-7 and MDA-MB-231 cells after different treatment times. The results are expressed as IC50 of As2O3 (µM)

<table>
<thead>
<tr>
<th>Treatment time (h)</th>
<th>IC50 MCF-7 (µM)</th>
<th>IC50 MDA-MB-231 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>8</td>
<td>17</td>
</tr>
<tr>
<td>48</td>
<td>1·8</td>
<td>7</td>
</tr>
<tr>
<td>72</td>
<td>1·2</td>
<td>4·8</td>
</tr>
<tr>
<td>96</td>
<td>0·8</td>
<td>3·4</td>
</tr>
<tr>
<td>120</td>
<td>0·5</td>
<td>2·1</td>
</tr>
</tbody>
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Figure 4 A graph showing the competitive binding ability of 17β-estradiol, As2O3, tamoxifen and paclitaxel to ERα. Data shown are representative of three separate experiments in each of which triplicate wells were assayed. Error bars are s.d. of triplicate wells. The wells incubated with solvent only are considered as control whose fluorescent intensity is expressed as 100%.

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concentrations below 2 µM; higher concentrations did not increase the ability of As₂O₃ to compete with estradiol for ER binding. This result suggested that As₂O₃ did not compete with 17β-estradiol for ER binding.

**Regulation of ERα mRNA levels following As₂O₃ treatment**

As ERα is the important mediator of the estrogen-stimulated signaling pathway, the alteration of ERα expression level in MCF-7 cells during As₂O₃ exposure may contribute to the interference exerted by As₂O₃ on the pathway. In this sense, the effect of As₂O₃ on ERα RNA levels was examined. Total RNA samples from the untreated control and after treatment with 2 µM As₂O₃ alone or simultaneously with 17β-estradiol were isolated and analyzed by RT-PCR. As seen in Fig. 5, in the first 24 h treatment, 10 nM 17β-estradiol down-regulated transcription levels by 52% relative to controls and further treatment to 48 h down-regulated the level by 72%. Twenty-four- and forty-eight-hour exposure to 2 µM As₂O₃ also induced a down-regulation of ERα expression but to a lesser extent. When MCF-7 cells were incubated with 2 µM As₂O₃ plus 17β-estradiol, a synergistic effect was observed at both time treatments (Fig. 5).

**Figure 5** Regulation of ERα mRNA level in MCF-7 cells following As₂O₃ treatment. MCF-7 cells were treated with 2 µM As₂O₃ alone and simultaneously with 10 nM 17β-estradiol for 24 and 48 h. After treatment, total RNA was isolated, transferred to cDNA and amplified with ERα primers. (A) Agarose gel of the RT-PCR amplified GADPH cDNA stained with ethidium bromide. GADPH with a size of 400 bp was used as internal control for normalization. (B) Agarose gel of the RT-PCR amplified ERα cDNA with a size of 490 bp. (C) Densitometric analysis of the amplified ERα PCR product after normalization with GADPH. Lanes 1–4, 24 h treatment: lane 1, untreated control; lane 2, As₂O₃ (2 µM); lane 3, 17β-estradiol (10 nM); lane 4, 17β-estradiol (10 nM) and As₂O₃ (2 µM); Lanes 5–8, 48 h treatment: lane 5, untreated control; lane 6, As₂O₃ (2 µM); lane 7, 17β-estradiol (10 nM); lane 8, 17β-estradiol (10 nM) and As₂O₃ (2 µM). Data were representative of three independent experiments.
Regulation of ERα protein levels following As$_2$O$_3$ treatment

In addition to the RNA levels, we have examined the ERα protein levels. Within 48 h of treatment, there were decreases in the ERα protein expression levels of 42% and 22% following treatment with 10 nM 17β-estradiol and 2 µM As$_2$O$_3$ when compared with controls without treatment. Co-treatment of both drugs resulted in a greater reduction in protein expression levels by 90%. This revealed that administration of 10 nM 17β-estradiol and 2 µM As$_2$O$_3$ together down-regulated ERα protein levels with greater effect than that induced by 17β-estradiol alone. Synergistic down-regulation was observed following both treatments together (Fig. 6).

Regulation of ERα transcriptional activity following As$_2$O$_3$ treatment

In this section, the regulation of the estrogen activated transcriptional activity by As$_2$O$_3$ was studied by examining the effect of As$_2$O$_3$ on ERα binding to ERE. In the study, a dual luciferase reporter system was adopted. Renilla luciferase control reporter vector and pGL3 reporter vector were co-transfected to MCF-7 cells. Results are expressed as percentage activation. The normalized luciferase activity of 10 nM 17β-estradiol, As$_2$O$_3$ at 1 µM and 2 µM concentrations, and As$_2$O$_3$ simultaneously added with 17β-estradiol are shown in Fig. 7. The luciferase activity was significantly enhanced 3.5-fold by 10 nM 17β-estradiol as compared with the controls. Upon treatment with both 1 µM and 2 µM As$_2$O$_3$, dose- and time-dependent reductions in percentage activity were observed. When exposed to both 17β-estradiol and 2 µM As$_2$O$_3$ over 72 h, the 3.5-fold induction by 10 nM 17β-estradiol was decreased to 28-77%. The results suggested that 2 µM As$_2$O$_3$ exerted an inhibitory effect on ERα-mediated luciferase activation over 48 and 72 h of treatment. In other words, As$_2$O$_3$ at 2 µM down-regulated the transcriptional activity of ERα. Most importantly, it also counteracted the transcriptional activity of ERα induced by 10 nM 17β-estradiol (Fig. 7).

Regulation of c-myc protein level by As$_2$O$_3$

By Western blot analysis, the protein expression of the c-myc gene was examined and the results are shown in Fig. 8. 17β-Estradiol up-regulated c-myc protein expression by 65% after 48 h treatment with respect to the untreated control. The up-regulation was partially suppressed by co-treatment with 2 µM As$_2$O$_3$ in MCF-7 cells over the same period of time, such that the protein level after co-treatment remained higher (131%) than that of controls (100%). After treatment with 2 µM As$_2$O$_3$ alone, the protein level of c-myc was down-regulated over 60% as compared with the controls without any treatment.

Effects of As$_2$O$_3$ on cell cycle distribution of MCF-7 cells under estrogen stimulation

The effect of As$_2$O$_3$ on cell cycle distribution of MCF-7 cells was analyzed after the cells were treated with 2 µM As$_2$O$_3$, 17β-estradiol, and 17β-estradiol simultaneously with 2 µM As$_2$O$_3$. Figure 9(A-D) shows the cell cycle distribution of MCF-7 cells after 48 h treatment. Compared with controls, 48 h treatment with 2 µM As$_2$O$_3$ markedly increased the proportion of cells in the G$_1$ phase from 46.7% to 67.4% while it reduced the proportion of cells in the S and G$_2$/M phases from 18.5% to 10.2% and from 34.6% to 22.4% respectively. Consistent results were obtained at 72 h treatment, with a greater percentage change in the phase distribution. Thus, As$_2$O$_3$, induced G$_1$ phase arrest in MCF-7 cells by inhibiting cell cycle progression to the S and G$_2$/M phases. Following incubation of MCF-7 cells with the same concentration of 17β-estradiol alone, a reduction of 35.5% of the cell population in the G$_1$ phase occurred while G$_2$/M phase and S phase cell populations were decreased by 9% and 79.8% respectively. Following incubation with 2 µM As$_2$O$_3$ together with 17β-estradiol, an increase in the cell population in the G$_1$ phase and a reduction in the cell population in the G$_2$/M phase and S phase of the same...
treatment time was also seen. This implied that As$_2$O$_3$ induced G$_1$ phase growth arrest of MCF-7 cells stimulated by 10 nM 17$\beta$-estradiol.

**Discussion**

It has been widely concluded that estrogen exposure is a prominent risk factor in breast cancer and its stimulatory effects can potentiate the growth of breast tumor cells (Henderson et al. 1988). In an attempt to treat breast cancer, estrogen withdrawal is one of the strategies used. To see whether As$_2$O$_3$ is able to block estrogen-stimulated cell growth in MCF-7 cells, the effect of As$_2$O$_3$ in estrogen withdrawal was assessed. Prior to the study, the growth stimulatory effect of estrogen was assessed in which 10 nM 17$\beta$-estradiol could induce a most dramatic cell growth stimulation. Different concentrations of 17$\beta$-estradiol have been used in studies of ER-regulated pathways or as a model for anti-estrogenicity. The concentrations range from 0·1 nM to 100 nM. Concentrations higher than this range induce toxic effects. Here, we observed that 1 nM 17$\beta$-estradiol could stimulate cell growth which was at its maximum at a concentration of 10 nM. So, 10 nM 17$\beta$-estradiol was used in the following experiments as a positive control (Fig. 1). With respect to the untreated controls, the percentage survival of MCF-7 cells was reduced from 160% with 10 nM 17$\beta$-estradiol treatment alone to 50% in co-treatment of 2 µM As$_2$O$_3$ together with 10 nM 17$\beta$-estradiol (Fig. 2). In addition, As$_2$O$_3$ was also found to suppress the stimulation of cell growth by estradiol in MCF-7 cells (Fig. 3).

The MDA-MB-231 cell line is an estrogen-independent cell line that does not depend on estrogen for growth and survival. As$_2$O$_3$ in concentrations below 2 µM was also shown to suppress the survival of MDA-MB-231 following 72 h exposure (Table 1). The lower sensitivity of MDA-MB-231 cells to As$_2$O$_3$ suggested an association of estrogen receptor status with growth inhibitory potency. Recent studies also revealed that tamoxifen induced apoptosis in both ER$^+$ and ER$^-$ cell lines via different mechanisms (Salami & Karami-Tehrani 2003). Only ER$^+$ cells could respond to low concentrations of tamoxifen. It might be concluded that there exists ER$^+$ and ER$^-$

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**Figure 7** Regulation of estrogen-activated ER$\alpha$ transcriptional activity following As$_2$O$_3$ treatment for 48 and 72 h. MCF-7 cells co-transfected with luciferase reporter plasmid were treated with various concentrations of As$_2$O$_3$ alone, 10 nM 17$\beta$-estradiol alone and 2 µM As$_2$O$_3$ simultaneously with 10 nM 17$\beta$-estradiol. Cells without any treatment (control) were prepared for comparison. Each value was normalized by Renilla luciferase control vector. The luciferase activity was expressed as the percentage of untreated control defined as 100%. The experiment was repeated three times and a single representative experiment is shown. Error bars represent the s.d. of four wells of each sample.
Effect of \( \text{As}_2\text{O}_3 \) on ER\( \alpha \) signaling

The role of ER\( \beta \) in breast cancer is not clearly known. Elucidating the role of ER\( \beta \) in the pathogenesis of breast cancer is important because many human breast tumors express both ER\( \alpha \) and ER\( \beta \). Recent studies showed that introducing ER\( \beta \) into MCF-7 cells caused an inhibition of proliferation in vitro and prevented tumor formation in response to estradiol in a mouse xenograft model (Paruthiyil et al. 2004). ER\( \beta \) inhibits the proliferation of MCF-7 cells by repressing c-myc, cyclin D1, and cyclin A gene transcription, and increases the expression of p21 and p27, which lead to a G2 cell cycle arrest (Paruthiyil et al. 2004). These results demonstrated that ER\( \alpha \) and ER\( \beta \) produced opposite effects on cell proliferation and tumor formation in MCF-7 cells. Here, we focused on ER\( \alpha \) in elucidating the mechanisms of how \( \text{As}_2\text{O}_3 \) inhibited breast cancer cell survival. The effects of \( \text{As}_2\text{O}_3 \) on ER\( \beta \) need further investigation.

Anti-estrogens, such as tamoxifen, elicited an estrogen withdrawal effect mainly by competitive binding to the hormone binding domain of ER\( \alpha \) and subsequent alteration of the conformation necessary for recruitment of transcription co-activators to transcription activation function 2 (AF2). Our study of the competitive binding ability of \( \text{As}_2\text{O}_3 \) on MCF-7 cells indicated that \( \text{As}_2\text{O}_3 \) did not compete with 17\( \beta \)-estradiol for ER binding (Fig. 4). The specific ER\( \alpha \) binding required hydrogen bond formation between an aromatic ring in the ligands and residues in the domain and a water molecule. The remaining residues in the binding cavity interacted with a variety of different hydrophobic groups of ligands (Brzozowski et al. 1997, Pike et al. 2001). The structure of \( \text{As}_2\text{O}_3 \) shows that the metal elements, arsenic and oxygen, are arranged in a polar ring structure. It was believed that the inability of \( \text{As}_2\text{O}_3 \) in binding to the ER\( \alpha \) ligand binding site could be attributed to its non-hydrophobic structure.

By RT-PCR and Western blot analysis, both ER\( \alpha \) gene transcription and protein expression levels were down-regulated following 10 nM 17\( \beta \)-estradiol treatment (Figs 5 and 6). Our results are consistent with other studies in which various concentrations of 17\( \beta \)-estradiol were used (Berthois et al. 1990, Alarid et al. 1999). Some reports have indicated an association of ER\( \alpha \) mRNA levels and protein levels after 17\( \beta \)-estradiol and other antiestrogen treatments (Saceda et al. 1988, Santagati et al. 1997). ER\( \alpha \) protein degradation was reported to be dependent on estrogen-induced proteasome-mediated proteolysis instead of DNA transcription (Alarid et al. 1999). Here, we demonstrated the down-regulation of ER\( \alpha \) mRNA levels followed by the down-regulation of ER\( \alpha \) protein levels following 48 h treatment with \( \text{As}_2\text{O}_3 \). The mechanism of action remains elusive but there is no doubt that following 2 \( \mu \text{M \text{As}}_2\text{O}_3 \) treatment together with 10 nM 17\( \beta \)-estradiol, both ER\( \alpha \) protein levels and mRNA levels were further down-regulated as compared with treatment with 17\( \beta \)-estradiol alone (Figs 5 and 6). The result might be responsible for the reduced estrogen stimulatory effect in MCF-7 cells by reducing the number of ER\( \alpha \) sites available for 17\( \beta \)-estradiol binding and subsequent activation of the ER\( \alpha \) signaling pathway.

After binding to ER\( \alpha \), the estrogen–ER\( \alpha \) complex will translocate to the target DNA binding site called estrogen responsive element (ERE) in the promoter region of the target gene for gene transcription activation (Klinge 2000).

Figure 8 Regulation of c-myc protein levels in MCF-7 cells following \( \text{As}_2\text{O}_3 \) treatment for 48 h. MCF-7 cells were treated with 2 \( \mu \text{M \text{As}}_2\text{O}_3 \) alone and in combination with 10 nM 17\( \beta \)-estradiol. (A) Western blot analysis of c-myc protein. (B) Relative protein levels of different samples after quantification. Lane 1, 10 nM 17\( \beta \)-estradiol; lane 2, untreated control; lane 3, 10 nM 17\( \beta \)-estradiol + 2 \( \mu \text{M \text{As}}_2\text{O}_3 \); lane 4, 2 \( \mu \text{M \text{As}}_2\text{O}_3 \).

Figure 9 Regulation of cell cycle distribution of MCF-7 cells following treatment with 2 \( \mu \text{M \text{As}}_2\text{O}_3 \) and 10 nM 17\( \beta \)-estradiol. MCF-7 cells were treated with various concentrations of \( \text{As}_2\text{O}_3 \) alone or together with 10 nM 17\( \beta \)-estradiol for 48 and 72 h. Cell cycle distribution of MCF-7 cells was studied by flow cytometry with propidium iodide (PI) staining. The Y-axis represents the number of events (total=10,000), whereas the X-axis represents the fluorescence intensity of PI (FL2-A). (A–D) Distribution after 48 h treatment; (E–H) distribution after 72 h treatment. (A) and (E) Untreated control; (B) and (F) 2 \( \mu \text{M \text{As}}_2\text{O}_3 \); (C) and (G) 10 nM 17\( \beta \)-estradiol; (D) and (H) 2 \( \mu \text{M \text{As}}_2\text{O}_3 \) and 10 nM 17\( \beta \)-estradiol. The data are from a representative experiment of three independent experiments.
Effect of As$_2$O$_3$ on ERα signaling

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% G1 : 46.69%
% G2 : 34.64%
% S phase : 18.48%
Sub-G1 : 1.91%

% G1 : 43.57%
% G2 : 36.11%
% S phase : 20.32%
Sub-G1 : 2.48%

% G1 : 67.43%
% G2 : 22.42%
% S phase : 10.15%
Sub-G1 : 6.04%

% G1 : 67.06%
% G2 : 24.04%
% S phase : 8.90%
Sub-G1 : 10.08%

% G1 : 35.95%
% G2 : 31.65%
% S phase : 32.39%
Sub-G1 : 1.18%

% G1 : 28.20%
% G2 : 35.36%
% S phase : 36.53%
Sub-G1 : 1.66%

% G1 : 57.03%
% G2 : 33.03%
% S phase : 9.94%
Sub-G1 : 5.3%

% G1 : 69.73%
% G2 : 25.41%
% S phase : 4.85%
Sub-G1 : 7.89%

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Here, we showed the suppressing effect of $\text{As}_2\text{O}_3$ on 10 nM 17$\beta$-estradiol-stimulated transcription activation by using a luciferase reporter system containing the ERE element. Following 10 nM 17$\beta$-estradiol treatment, the activation was dramatically enhanced indicating that the action of the 10 nM 17$\beta$-estradiol dose on MCF-7 cells was mediated by the estrogen–ER$\alpha$ complex binding to DNA and the stimulation of target gene transcription. When treated with 2 µM $\text{As}_2\text{O}_3$ simultaneously with 10 nM 17$\beta$-estradiol, transcription activation was not enhanced. Instead, it was suppressed twofold and fivefold over the 48 and 72 h treatment periods respectively, as compared with that of 10 nM 17$\beta$-estradiol treatment alone (Fig. 7). Thus, $\text{As}_2\text{O}_3$ at 2 µM suppressed 17$\beta$-estradiol-induced transcription of ERE bearing target genes.

It is of interest to examine the mechanism of how $\text{As}_2\text{O}_3$ suppressed 17$\beta$-estradiol-stimulated cell growth and even elicited growth inhibition in MCF-7 cells. In the present study, c-myc protein expression was studied. c-myc is an oncogene responsible for cell growth (Escot et al. 1986, Dang 1999). Previous studies have reported that c-myc expression was elevated in estrogen-treated cells (Dubik et al. 1987, Dubik & Shiu 1988). Moreover, down-regulation of c-myc expression was sufficient to block 17$\beta$-estradiol-stimulated cell growth. This result was also observed in our study as c-myc protein expression was enhanced by 10 nM 17$\beta$-estradiol after 48 h treatment. At this time, a dramatic increase in the percentage survival of MCF-7 cells following 10 nM 17$\beta$-estradiol treatment over 48 h was observed (Fig. 8). So, c-myc was responsible for the growth stimulation of MCF-7 cells as demonstrated in the previous study. Upon exposure to 2 µM $\text{As}_2\text{O}_3$ together with 10 nM 17$\beta$-estradiol, the expression level of c-myc was reduced as compared with that stimulated by 10 nM 17$\beta$-estradiol alone (Fig. 8). From our study, survival of MCF-7 cells under the same conditions was reduced to 70% of control. Following a longer incubation to 72 h, $\text{As}_2\text{O}_3$ suppressed cell survival to 48% of control (data not shown). Therefore, it was possible that the blocking of the 17$\beta$-estradiol-stimulated cell growth was associated with down-regulation of c-myc protein expression. As the promoter region upstream of c-myc gene included half the ERE, the down-regulation of c-myc protein expression might be attributed to the inhibition of ER$\alpha$ transcription activation.

The growth inhibition induced by $\text{As}_2\text{O}_3$ in MCF-7 cells was further explored by studying the effects of $\text{As}_2\text{O}_3$ on the estrogen-regulated cell cycle. 17$\beta$-Estradiol stimulated cell growth by accelerating G1→S phase progression and recruiting cells from the G0 phase to enter the cell cycle (Sutherland et al. 1983). Here, we showed that the cell population after 10 nM 17$\beta$-estradiol treatment showed an increase in numbers in the S phase and a decrease in numbers in the G1 phase, i.e. the stimulatory cell cycle progression in MCF-7 cells (Fig. 9). When co-treated with 2 µM $\text{As}_2\text{O}_3$ and 17$\beta$-estradiol, the cell population accumulated in the G1 phase, indicating that 2 µM $\text{As}_2\text{O}_3$ opposed 17$\beta$-estradiol-induced cell cycle progression to S phase in MCF-7 cells. The opposition may be due to the regulation of cell cycle proteins in the G1 and G1/S phases such as down-regulation of cyclin D1 mRNA and protein expression, as up-regulation of cyclin D1 levels were shown to be responsible for 17$\beta$-estradiol-stimulated cell cycle progression (Prall et al. 1997, Charpentier et al. 2000). By cDNA microarray analysis, cyclin D1 gene was found to be regulated by estrogen with the ERE sequence in the promoter regions (Gruvberger et al. 2001). So, $\text{As}_2\text{O}_3$ induced cell growth inhibition in MCF-7 cells by G1 phase arrest in MCF-7 cells; this might be related to cyclin D1 and awaits further studies.

As estrogen is a primary risk factor of promoting the growth of MCF-7 cells and estrogen was also found to promote resistance of chemotherapeutic drugs in MCF-7 cells (Teixeira et al. 1995), our finding that $\text{As}_2\text{O}_3$ could exhibit anti-estrogenicity on MCF-7 cells may shed light on the therapy of breast cancer in the initial stages of tumor development.

**Funding**

This study was supported by direct grants from Research Grants Council, Hong Kong and the Department of Biochemistry, The Chinese University of Hong Kong, Hong Kong. There is no conflict of interest that would prejudice its impartiality of this paper.

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Received 7 April 2004

Accepted 7 May 2004


Received 7 April 2004

Accepted 7 May 2004