Arsenic trioxide re-sensitizes ERα-negative breast cancer cells to endocrine therapy by restoring ERα expression *in vitro* and *in vivo*

WEIJIE ZHANG[1*], LIUXING WANG[1*], QINGXIA FAN[1], XINAI WU[1], FENG WANG[1],
RUI WANG[1], ZHIJUN MA[1], JIANHUA YANG[1] and SHIH HSIN LU[1,2]

[1]Department of Oncology, the First Affiliated Hospital of Zhengzhou University, ErQi District, Zhengzhou 450052;
[2]Cancer Institute and Hospital, Chinese Academy of Medical Sciences, Chaoyang District, Beijing 100021, P.R. China

Abstract. Approximately one-third of breast cancers lack estrogen receptor α (ERα) because of the hypermethylation of the CpG island in the receptor's promoter. These tumors are associated with poorer histological differentiation, a higher growth fraction, are rarely responsive to endocrine therapy and have a worse clinical outcome. Thus, re-expression of ERα in ERα-negative breast cancers may restore the sensitivity of antiestrogen therapy. The ERα-negative breast cancer cell line MDA-MB-435s was treated with different concentrations of arsenic trioxide (As_2O_3). MS-PCR was used to detect the change in the methylation status of ERα. RT-PCR, immuno-histochemistry and Western blot analyses were used to detect changes in the mRNA and protein expression of DNA methyltransferase-1 (DNMT1) and ERα. Cell proliferation was examined using the MTT assay. A xenograft model in nude mice was used to further examine the results we observed *in vitro*. The ERα gene was demethylated after As_2O_3 treatment of MDA-MB-435s cells. RT-PCR, immunohistochemistry and Western blot analyses revealed that DNMT1 expression was inhibited and ERα was re-expressed in a concentration-dependent manner after As_2O_3 treatment. The MTT assay showed that cell proliferation was significantly suppressed after exposure to different concentrations of As_2O_3. Addition of tamoxifen (TAM) further suppressed levels of cell proliferation. *In vivo*, the xenograft tumor volumes of As_2O_3-treated mice were smaller than those observed in untreated and TAM-treated mice. Treatment with a combination of As_2O_3 + TAM resulted in further suppression. As_2O_3 can act as a demethylation agent to restore ERα expression in ERα-negative breast cancer cells and re-sensitize these cells to endocrine therapy *in vitro* and *in vivo*.

Correspondence to: Dr Shih Hsin Lu, Cancer Institute and Hospital, Chinese Academy of Medical Sciences, 17 South Panjiayuan, Chaoyang District, Beijing 100021, P.R. China
E-mail: shlu1212@gmail.com

*Contributed equally

Introduction

Among women in the developed world, breast cancer is the leading cause of cancer and the second leading cause of cancer-related deaths. The American Cancer Society estimates that in 2010, 207,090 new breast cancer cases (accounting for 28% of all new cancer cases) and 39,840 deaths (accounting for 15% of all cancer-related deaths) in women will be reported in the USA alone (1). Experimental, clinical and epidemiologic data suggest that estrogens bind to the estrogen receptor (ERα or ERβ) and stimulate the transcription of target genes involved in cell proliferation, which contribute to the development of breast cancer (2). ERα has a greater affinity for estrogen and is associated with comparatively higher levels of transcriptional activity (3). Clinically, the presence or absence of ERα in breast tumors is an important prognostic indicator of the disease. The presence of ERα correlates with increased disease-free survival and an overall better prognosis (4). However, one-third of breast cancers lack ERα. These tumors rarely respond to endocrine therapy and are associated with poorer histological differentiation, a higher growth fraction and worse clinical outcome (5). Thus, re-expression of the ERα receptor in breast tumors that do not express ERα could allow their antiestrogen sensitivity to be restored.

Although several sequence mutations for the ERα gene have been identified and shown to be related to decreased ERα expression and estradiol binding, they are uncommon and do not explain the loss of ERα expression in a significant fraction of human breast cancers (6). This finding suggested that mechanisms other than genetic changes might also contribute to the loss of ERα expression. In addition, the absence of ERα protein expression has been frequently associated with loss of ERα transcription (7). The pattern of altered gene expression or epigenetic change is important in common malignancies (8). DNA is methylated by DNA methyltransferases (DNMTs), which transfer the methyl group from S-adenosylmethionine (SAM) to generate patterns of genomic methylation that silence

gene expression (9-11). To date, the following DNMTs have been identified: DNMT1, DNMT2, DNMT3a, DNMT3b and DNMT3L (12). DNMT1 is mainly responsible for the maintenance of DNA methylation. DNMT1 levels and activity are increased 2- to 10-fold in ERα-negative breast cancer cell lines, consistent with the levels of methylation observed (13).

These findings raise the possibility that the absence of ERα expression is associated with ERα gene hypermethylation (14,15). Furthermore, inhibition of DNA methylation might be a therapeutic strategy in those cancers with ERα-negative phenotypes (16). Two well-characterized and clinically relevant DNMTs inhibitors, 5-aza-2'-cytidine (5-aza-dC) and 5-aza-2'-deoxycytidine (5-aza-CdR), have some toxicity and instability due to their incorporation into either DNA or both DNA and RNA *in vitro* and *in vivo*. They are also unstable in neutral solutions (17-19). Hence, a nontoxic, highly stable, effective DNMTs inhibitor would be an ideal epigenetic therapeutic agent (20).

Arsenic has been used as a medicinal agent for more than 2,400 years. The National Cancer Institute (NCI) is working cooperatively with research centers across the USA to evaluate the clinical activity of arsenic/arsenic trioxide (As_2O_3) in hematologic malignancies, such as APL, AML, CML, NHL, HL, CLL, myelodysplastic syndrome and multiple myeloma. The NCI is also supporting research in solid tumors, such as advanced hormone-refractory prostate cancer, renal cell cancer, cervical cancer and refractory transitional cell carcinoma of the bladder (21-23). The results of these ongoing studies should provide important insights into the clinical utility of As_2O_3 in these diseases. The emerging consensus is that induction of differentiation and apoptosis are the principal modalities involved in the antitumor effect of As_2O_3 (24,25), as well as reactivation of silenced tumor suppressor genes through DNA demethylation (26,27).

Previously, we have confirmed that As_2O_3 could restore ERα re-expression *in vitro* (28). In this study, we found that As_2O_3 treatment inhibited DNMT1 resulting in decreased methylation of the ERα gene and re-expression of ERα in the ERα-negative human breast cancer cell line MDA-MB-435s. We also observed increased treatment efficacy of combined tamoxifen (TAM) and As_2O_3 therapy *in vitro* and *in vivo*.

Materials and methods

Cell culture and treatment protocols. The human ERα-negative MDA-MB-435s and ERα-positive MCF-7 breast cancer cell lines (obtained from ATCC) were routinely cultured at 37˚C in an atmosphere consisting of 5% CO_2 in RPMI-1640 medium (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, USA). MDA-MB-435s cells were treated with 0, 0.5, 1, 2 and 4 μmol/l As_2O_3 (Harbin, Iraq) and 10 μmol/l TAM (Sigma, USA) for 24, 48 and 72 h. MCF-7 cells were used as the positive control.

In vivo studies. Six-week-old female BALB/c nude mice (Slaccas, Shanghai, China) were housed in laminar-flow cabinets under specific pathogen-free (SPF) conditions. The study protocol was approved by the ZhengZhou University Medical Experimental Animal Care Committee. Each nude mouse was injected into the left flank with $2x10^6$ untreated MDA-MB-435s human breast cancer cells in 0.2 ml cell culture medium. Tumors were allowed to grow to approximately 0.1 cm in diameter before treatment. Thirty-six nude mice were randomized into the following six groups: A, negative control group; B, 5 mg/kg/day TAM group; C, 2 mg/kg/day As_2O_3 group; D, 2 mg/kg/day As_2O_3 combined with 5 mg/kg/day TAM group; E, 4 mg/kg/day As_2O_3 group; and F, 4 mg/kg/day As_2O_3 combined with 5 mg/kg/day TAM group. TAM (1 ml) was administered orally and As_2O_3 (0.2 ml) was administered by intraperitoneal injection, continuously for 28 days. Animals were sacrificed and autopsied at 6 weeks post-inoculation. Once the tumors became palpable, their diameters were measured with a caliper each week after subcutaneous implantation. Tumor volume was calculated according to the equation: Volume (cm^3) = $LxS^2x0.5$. Tumor volumes and weights are presented as mean ± SE.

MTT assay. MCF-7 and MDA-MB-435s cells were plated in minimal medium at 1,000 cells/well into 96-well plates (Costar, USA). After treatment with the appropriate concentration of As_2O_3 (0, 0.5, 1, 2 and 4 μmol/l) for 24 and 48 h, TAM (10 μmol/l) was added for a further 24 h. Cell viability was measured using the MTT assay. Briefly, the medium was removed and 10 μl MTT solutions was added to each well and incubated at 37˚C for 4 h. Then, 100 μl DMSO was added to each well and incubated for a further 15 min. The optical density (OD) was measured at 595 nm. The percentage survival was defined as: % survival = 100% x (OD of test sample/OD of control). At least three independent experiments were performed for each study.

Methylation-specific PCR (MS-PCR). Genomic DNA samples from both MDA-MB-435s and MCF-7 cells that were either untreated or treated with different concentrations of As_2O_3 were isolated using a DNA extraction kit (Axygen, China). Genomic DNA (1 μg) was treated with sodium bisulfite using the CpGenome™ DNA modification kit (Epigentek, USA). Methylation-specific PCR was performed in the thermal cycler (GeneAmp PCR System 9700, ABI, USA). ERα unmethylated DNA was amplified by using 5'-GGG GTT GGA TGT AGT GGT TTA T-3' as the forward primer and 5'-TAA AAC TAC AAA TAC CCA CCA-3' as the reverse primer. ERα methylated DNA was amplified by using 5'-GGG TTG GAC GTA GTG GTT TAC-3' as the forward primer and 5'-AAA ACT ACA AAT ACC CGC CG-3' as the reverse primer. Both the primers amplified a 170-base pair (bp) fragment. PCR amplification of the ERα gene was carried out as follows: 94˚C for 5 min, followed by denaturation at 94˚C for 30 sec, annealing at 58˚C for 45 sec, and extension at 72˚C for 90 sec, followed by a final 5 min extension at 94˚C for 35 cycles. The reaction products were loaded onto 2% agarose gels containing ethidium bromide and visualized under the Biospectrum 600 imaging system (UVP, USA). The band intensities of the PCR products were analyzed by the UVP VisionWorks LS 6.6a (UVP), and are expressed as the means ± SD.

RNA isolation and semi-quantitative RT-PCR analysis. Total-RNA was extracted from both MDA-MB-435s and MCF-7 cells that were either untreated or treated with different concentrations of As_2O_3 using TRIzol reagent (Gibco-BRL,

Gaithersburg, MD, USA). RNA purity and concentrations were determined by measuring the A260/A280 absorption. cDNA was synthesized from 1 μg of RNA using a Thermoscript reverse transcriptase (RT-PCR) System (Fermentas, Canada). ERα mRNA was amplified by using 5'-TGA TGA AAG GTG GGA TAC GAA A-3' as the forward and 5'-GGC TGT TCT TCT TAG AGC GTT TG-3' as the reverse primer. DNMT1 mRNA was amplified by using 5'-CTA CCA GGG AGA AGG ACA GG-3' as the forward and 5'-CTC ACA GAC GCC ACA TCG-3' as the reverse primer. β-actin mRNA was amplified by using 5'-AGG CAT TGT GAT GGA CTC CG-3' as the forward and 5'-AGT GAT GAC CTG GCC GTC AG-3' as the reverse primer. β-actin was used as an internal control. The primer pair amplified a 168-base pair (bp) fragment as ERα, a 152-bp fragment as DNMT1, and a 301-bp fragment as β-actin. PCR was performed in the thermal cycler (GeneAmp PCR System 9700, ABI) for 35 cycles consisting of denaturation at 94°C for 30 sec, annealing at 65°C (β-actin) for 45 sec or 54°C (ERα, DNMT1) for 30 sec, and extension at 72°C for 90 sec, followed by a final 5-min extension at 94°C. The reaction products were loaded onto 1.5% agarose gels containing ethidium bromide and visualized under the Biospectrum 600 imaging system. The band intensities of the PCR products were analyzed using the UVP VisionWorks LS 6.6a, and are expressed as the means ± SD.

Immunohistochemistry. MDA-MB-435s and MCF-7 cells, untreated or treated with different concentrations of As$_2$O$_3$, were cultured in 24-well chamber slides (Corning, USA) for 48 h, then fixed in 2% paraformaldehyde for 30 min at room temperature. The cells were permeabilized with PBS containing 0.2% Triton X-100 for 10 min at room temperature. Endogenous peroxidase activity was blocked by incubation with 0.03% hydrogen peroxide in methanol for 5 min. Sections were incubated with a monoclonal mouse anti-ERα antibody (Abcam, UK) for 30 min at room temperature. Sections were gently rinsed three times with washing buffer, then incubated with the peroxidase-labeled polymer conjugated to goat anti-mouse IgG for 30 min followed by staining with 3,3'-diaminobenzidine tetrahydrochloride (DAB) for 5 min. Samples were counterstained with hematoxylin, dehydrated and mounted in Diatex. A known ERα-positive cell line MCF-7 was used as a positive control while the same concentration of non-immune mouse IgG was applied as the negative control. All controls gave satisfactory results. Paraffin sections (4 μm) of the transplanted tumor tissues were dewaxed and stained as described above. ERα-positive staining was seen as light-yellow to brown granules, and mainly located in the cytoplasm and nuclei.

Western blot analysis. Equal amounts of protein sample (20 μg) were separated on 4% SDS acrylamide gel (Bio-Rad, USA) for 0.5 h at 60 V and 10% SDS acrylamide gel for 1.5 h at 150 V. The samples were then transferred onto a nitrocellulose membrane for 1.5 h at 60 V (Whatman, UK). After blocking in 5% fat-free milk, the membrane was incubated with the primary antibody (anti-ERα mouse monoclonal antibody, Abcam, 1:500; anti-DNMT1 mouse monoclonal antibody, Abcam, 1:500) overnight at 4°C, followed by incubation with the secondary IgG-horseradish peroxidase (HRP)-conjugated antibody for 1 h at room temperature (1:5,000). The antibody
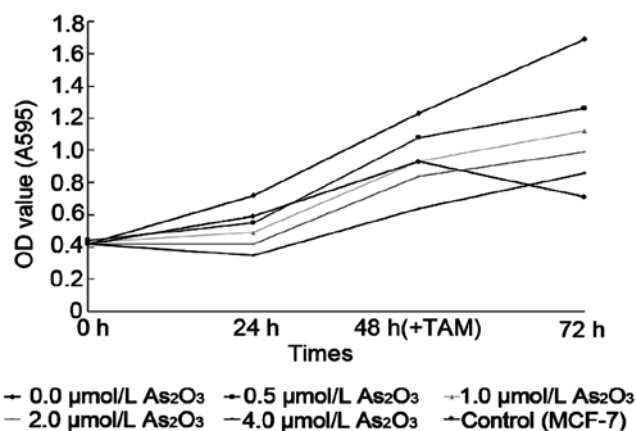

Figure 1. Treatment with As$_2$O$_3$ significantly suppresses cell proliferation in ERα-negative MDA-MB-435s cells. The MTT assay was used to measure the cell viability of MDA-MB-435s cells after treatment with As$_2$O$_3$ followed by tamoxifen (TAM). MCF-7 cells, which express ERα, served as the control.

was diluted in PBS containing 5% Blotto (Santa Cruz Biotechnology, USA) and 0.1% Tween-20. The stained membranes were visualized by enhanced chemiluminescence reaction using the ECL Plus kit (GE Healthcare, USA). The mouse monoclonal β-actin antibody (Abcam) was used as the loading control for Western blot analysis at a dilution of 1:1000. The band intensities of the PCR products were analyzed using the UVP VisionWorks LS 6.6a (UVP), and are expressed as the means ± SD.

Statistical analysis. For all experiments, data are presented as the mean ± SD. Significance between groups was determined by one-way analysis of variance followed by the Bonferroni multiple-comparisons test. In all comparisons, differences were considered to be significant at P<0.05 and all P-values were two-sided.

Results

Treatment with As$_2$O$_3$ and TAM significantly suppress cell proliferation in ERα-negative MDA-MB-435s cells. The MTT assay showed that the level of proliferation in the ERα-negative MDA-MB-435s cell line was significantly suppressed after treatment with different concentrations of As$_2$O$_3$ for 24 and 48 h (P<0.05) (Fig. 1). Addition of TAM for an additional 24 h led to further suppression of MDA-MB-435s proliferation (Fig. 1). The effects of TAM on MDA-MB-435s cells treated with As$_2$O$_3$ were not greater than the effects observed in the control ERα-positive MCF-7 cell line, suggesting that ERα was re-expressed in MDA-MB-435s cells but at lower levels to MCF-7 cells.

As$_2$O$_3$ treatment decreases DNMT1-dependent methylation of the ERα gene and restores mRNA expression. Assessment of the methylation status of the ERα CpG islands in MDA-MB-435s and MCF-7 cells revealed that they were hypermethylated and unmethylated , respectively, with a corresponding absence and presence of ERα mRNA expression (Figs. 2A and 3B). After treatment of MDA-MB-435s cells with different concentrations of As$_2$O$_3$ (1.0, 2.0 and 4.0 μmol/l) for 48 h, we observed

Figure 2. As$_2$O$_3$ treatment decreases methylation of the ERα gene *in vitro* and *in vivo*. (A) The methylation status of the ERα gene was examined in MDA-MB-435s and MCF-7 cells by MSP analysis. The lanes are as follows: 1, water control for contamination in the PCR reaction; 2-6, MDA-MB-435s cells treated with 0 (lane 2), 0.5 (lane 3), 1.0 (lane 4), 2.0 (lane 5) and 4.0 μmol/l As$_2$O$_3$ (lane 6); 7, MCF-7 cells (control). M, methylated band (170 bp); U, unmethylated band (170 bp). (B) MSP analysis of ERα in transplanted tumor and ERα-positive breast cancer tissue. The lanes are as follows: 1, water control for contamination in the PCR reaction; 2, treatment with 2 mg/kg/day As$_2$O$_3$; 3, treatment with 2 mg/kg/day As$_2$O$_3$ + tamoxifen (TAM); 4, treatment with 4 mg/kg/day As$_2$O$_3$; 5, treatment with 4 mg/kg/day As$_2$O$_3$ + TAM; 6, treatment with TAM alone; 7, NS, normal saline; 8, positive control.
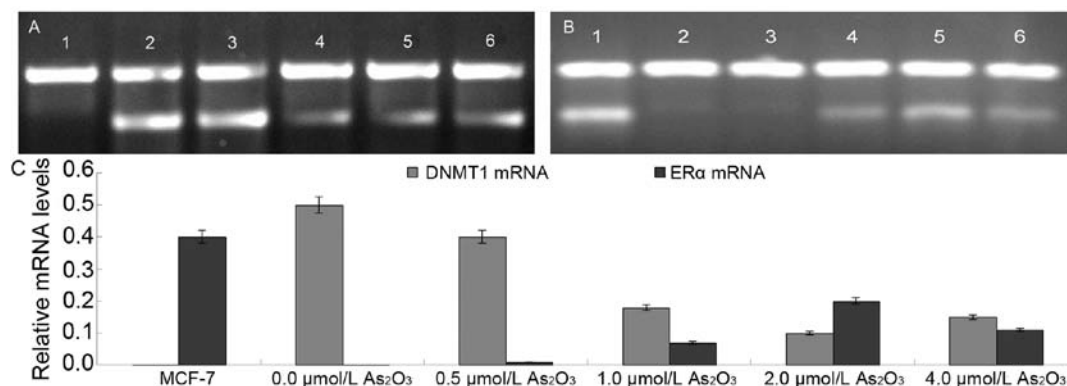

Figure 3. Effect of As$_2$O$_3$ treatment of MDA-MB-435s cells on ERα and DNMT1 mRNA expression levels. mRNA expression of (A) DNMT1 (152 bp) and (B) ERα (168 bp). The lanes are as follows: 1, MCF-7 cells; 2-6, MDA-MB-435s cells treated with 0 (lane 2), 0.5 (lane 3), 1.0 (lane 4), 2.0 (lane 5) and 4.0 μmol/l As$_2$O$_3$ (lane 6). (C) The relative mRNA expression levels of DNMT1 and ERα. Semiquantitative values of three independently repeated RT-PCR experiments were statistically analyzed by densitometry using VisionWorks LS 6.6a, and are expressed as means ± SD. P<0.05 compared to the untreated group.

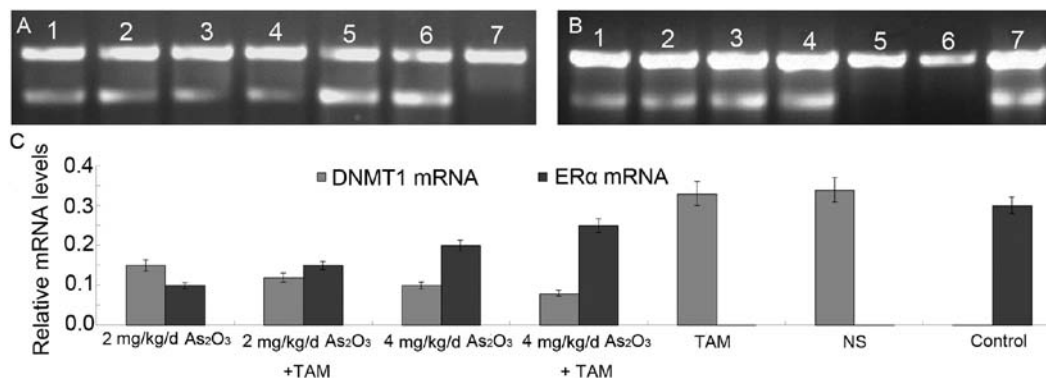Figure 4. Effect of As$_2$O$_3$ and tamoxifen (TAM) treatment of xenografted tumors on ERα and DNMT1 mRNA expression levels. (A and B) mRNA expression of (A) DNMT1 (152 bp) and (B) ERα (168 bp). The lanes are as follows: 1, 2 mg/kg/day As$_2$O$_3$; 2, 2 mg/kg/day As$_2$O$_3$ + TAM; 3, 4 mg/kg/day As$_2$O$_3$; 4, 4 mg/kg/day As$_2$O$_3$ + TAM; 5, TAM alone; 6, NS; 7, positive control. (C) The relative mRNA levels of DNMT1 and ERα. Semiquantitive values of three independently repeated RT-PCR experiments were statistically analyzed by densitometry using VisionWorks LS 6.6a, and are expressed as means ± SD. P<0.05 compared to the untreated group.

a decrease in the ERα methylation-specific bands. In contrast, the unmethylation-specific bands were enhanced. In addition, we found that the hypermethylation status of MDA-MB-435s cells treated with 0 or 0.5 μmol/l As$_2$O$_3$ cells remained unchanged (Fig. 2A). Furthermore, the re-expression of ERα mRNA was increased in a concentration-dependent manner (Fig. 3B and C). We also found that the marked increase in ERα mRNA levels was accompanied by a significant reduction in DNMT1 mRNA levels compared with the untreated control group (P<0.05) (Fig. 3A and C). DNMT1 mRNA was not expressed in MCF-7 cells (Fig. 3A and C). ERα mRNA was not expressed in MDA-MB-435s cells treated with 0 and

0.5 μmol/l As$_2$O$_3$ (Fig. 3B and C) Similar findings were observed in the transplanted tumor tissues treated with or without As$_2$O$_3$ (P<0.05) (Figs. 2B, 4A and B). Surprisingly, ERα mRNA expression was lower in cells treated with 4 than with 2 μmol/l As$_2$O$_3$ *in vitro* (Fig. 3B and C).

As$_2$O$_3$ treatment significantly enhances ERα expression in MDA-MB-435s cells. We examined the effects of As$_2$O$_3$ on ERα gene expression by immunohistochemistry *in vitro* (Fig. 5) and *in vivo* (Fig. 6). Loss of ERα expression was observed in untreated MDA-MB-435s cells. In contrast, a significant increase in nuclear immunostaining of ERα was induced in
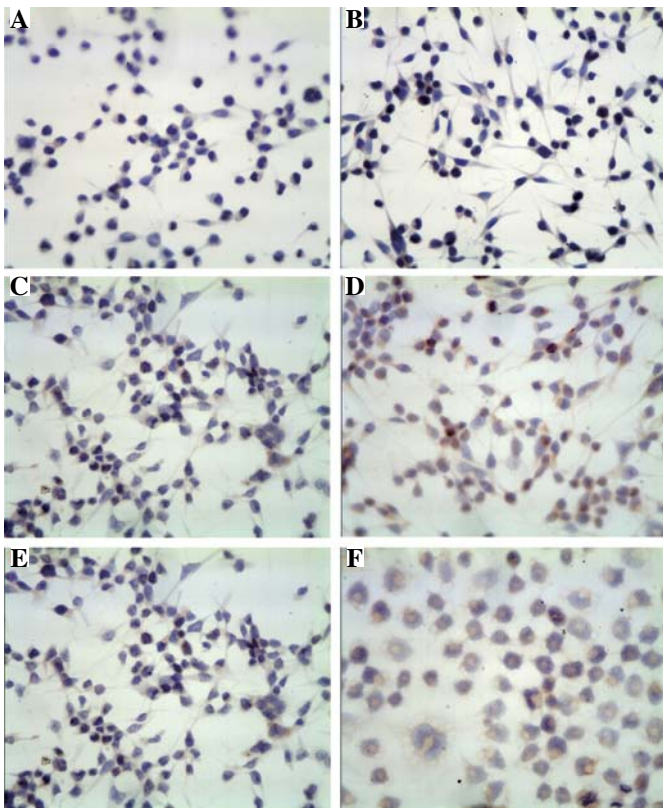
Figure 5. Immunohistochemical analysis of the re-expression of ERα protein in MDA-MB-435S cells after treatment with As$_2$O$_3$. (A-E) MDA-MB-435S cells were treated with (A) 0, (B) 0.5, (C) 1.0, (D) 2.0 and (E) 4.0 μmol/l As$_2$O$_3$; (F) MCF-7 cells. (x400).

Figure 7. Western blot analysis of DNMT1 (183 kDa) and ERα (67 kDa) protein levels in MDA-MB-435s cells after treatment with different concentrations of As$_2$O$_3$. (A) Lanes: 1-5, MDA-MB-435s cells treated with 0 (lane 1), 0.5 (lane 2), 1.0 (lane 3), 2.0 (lane 4) and 4.0 As$_2$O$_3$ (lane 5); 6, MCF-7 cells. (B) Semiquantitive values of three independently repeated Western blot analyses were statistically analyzed by densitometry using VisionWorks LS 6.6a, and are expressed as the means ± SD. P<0.05 compared to the untreated group. Levels of β-actin (43 kDa) are shown for equal loading conditions.
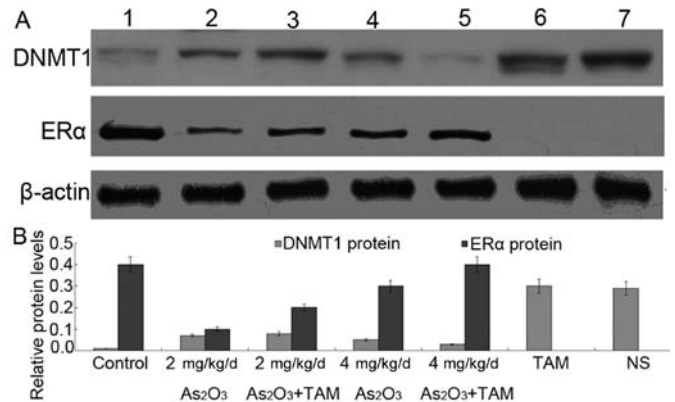
Figure 8. Western blot analysis of DNMT1 (183 kDa) and ERα (67 kDa) protein levels in xenografted tumors after treatment with As$_2$O$_3$ and tamoxifen (TAM). (A) Lanes: 1, positive control; 2, 2 mg/kg/day As$_2$O$_3$; 3, 2 mg/kg/day As$_2$O$_3$ + TAM; 4, 4 mg/kg/day As$_2$O$_3$; 5, 4 mg/kg/day As$_2$O$_3$ + TAM; 6, TAM alone; 7, NS. (B) Semiquantitive values of three independently repeated Western blot analyses were statistically analyzed by densitometry using VisionWorks LS 6.6a, and are expressed as the means ± SD. P<0.05 compared to the untreated group. Levels of β-actin (43 kDa) are shown for equal loading conditions.
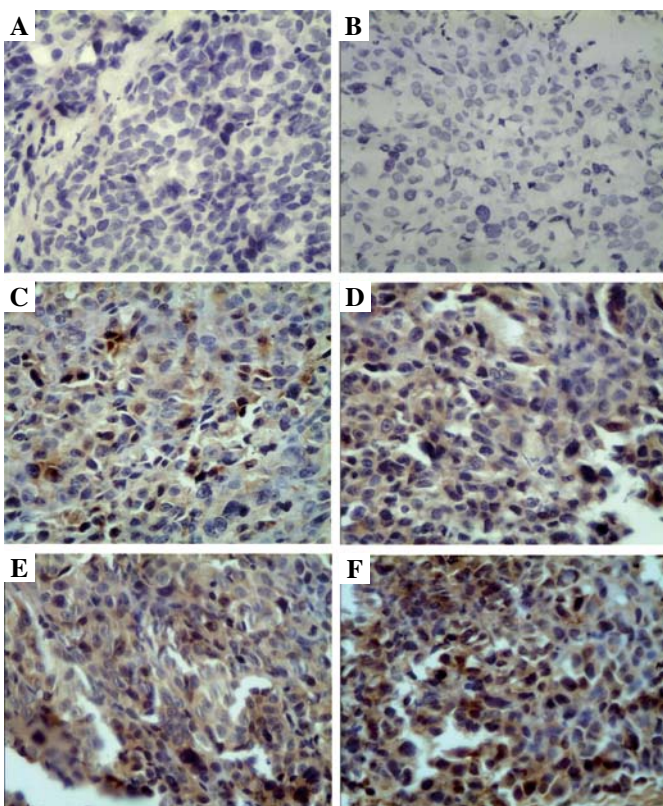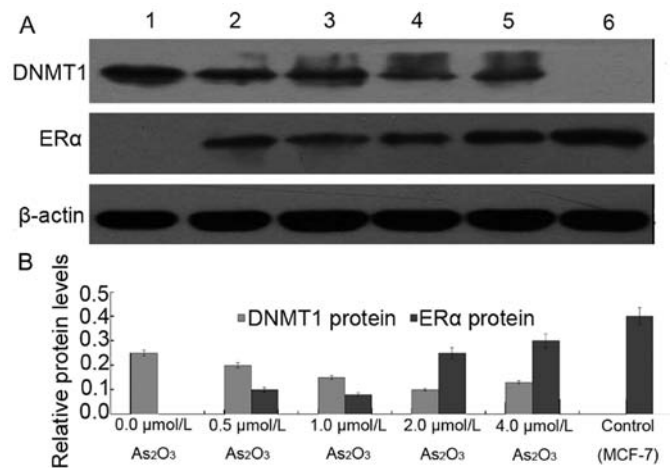

Figure 6. Immunohistochemical analysis of the re-expression of ERα protein in xenografted tumors after treatment with As$_2$O$_3$ and tamoxifen (TAM). (A) NS; (B) TAM alone; (C) 2 mg/kg/day As$_2$O$_3$; (D) 2 mg/kg/day As$_2$O$_3$ + TAM; (E) 4 mg/kg/day As$_2$O$_3$; (F) 4 mg/kg/day As$_2$O$_3$ + TAM.

MDA-MB-435s cells treated with different concentrations of As$_2$O$_3$ (0.5, 1, 2 and 4 μmol/l). MCF-7 cells served as the positive control (Fig. 5). *In vivo*, the immunostaining of ERα was also dramatically enhanced in all As$_2$O$_3$ treatment groups and stronger staining was observed after combined treatment with As$_2$O$_3$ and TAM. No ERα staining was observed in the negative control and TAM-treatment alone groups (Fig. 6).

As$_2$O$_3$ treatment of MDA-MB-435s cells increases ERα and decreases DNMT1 protein expression levels. Western blot analysis revealed that the re-expression of ERα protein was markedly increased after treatment with different concentrations
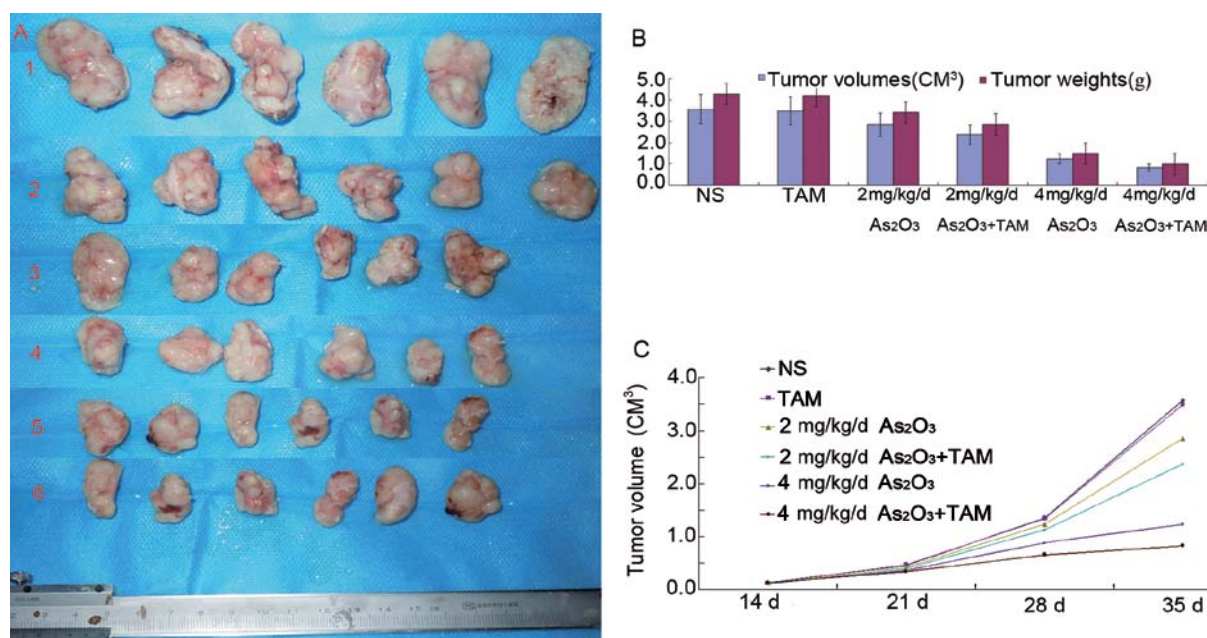
Figure 9. Effect of As$_2$O$_3$ treatment alone or in combination with tamoxifen (TAM) on tumor growth in nude mice. (A) Micrograph of the tumors at 35 days. 1, NS; 2, TAM alone; 3, 2 mg/kg/day As$_2$O$_3$; 4, 2 mg/kg/day As$_2$O$_3$ + TAM; 5, 4 mg/kg/day As$_2$O$_3$; 6, 4 mg/kg/day As$_2$O$_3$ + TAM. (B) The tumor volumes and weights of all groups are presented as the mean ± SD (P<0.05). (C) The growth curves of the tumors of all groups of nude mice.

of As$_2$O$_3$ (0.5, 1, 2 and 4 μmol/l) in a concentration-dependent manner (Fig. 7). This was accompanied by a significant reduction in DNMT1 protein expression compared with the untreated control (Fig. 7) (P<0.05). DNMT1 protein expression was not observed in MCF-7 cells. This phenomenon was also observed *in vivo*: ERα protein was re-expressed in the As$_2$O$_3$-treated mice (Fig. 8). Significantly increased re-expression of ERα protein was also observed in mice treated with a combination of As$_2$O$_3$ and TAM (P<0.05). ERα re-expression was not observed in untreated mice and in mice treated with TAM alone (Fig. 8). Surprisingly, ERα protein levels were lower in the 4 μmol/l group than in the 2 μmol/l group *in vitro*, but higher after combined treatment with TAM *in vivo* (P<0.05).

Combined treatment with As$_2$O$_3$ and TAM inhibits tumor growth in human breast cancer xenografts in nude mice. An obvious decrease in the volumes and weights of tumors in the As$_2$O$_3$-treated group compared with the untreated group was observed in human breast cancer xenografts in nude mice. A further reduction in tumor size was observed after combined treatment of As$_2$O$_3$ with TAM compared to the untreated and TAM-treatment alone groups (P<0.05) (Fig. 9).

Discussion

In industrialized countries, breast cancer is the most common type of cancer among women and the leading cause of death among women aged 40 to 55 years. Considering the current estimates of the incidence of breast cancer, a woman living in the USA today has approximately a one in eight chance of developing breast cancer during her lifetime (1,29).

Decades of research have led to considerable understanding of the factors involved in the development of breast cancer, However, it has become clear in recent years that epigenetic regulation is another mechanism (rather than loss of hetero-

zygosity or homozygous deletion) that leads to transcriptional silencing. Epigenetic changes differ from genetic changes as they are not based upon a change in primary DNA sequence. They arise at a higher frequency, are reversible upon treatment with pharmacological agents and occur at defined regions within a gene (30).

The human ERα cDNA and gene were cloned in 1986. This 140 kb ERα gene has eight exons and is located on chromosome 6q25.1 (31). Numerous experimental and clinical studies have established that estrogen plays a major role in the initiation and progression of breast cancer (32). Approximately one third of breast cancers lack detectable ERα protein and are rarely responsive to hormonal treatment (33). Thus, many studies have focused on elucidating the possible molecular genetic mechanisms that mediate the loss of ERα expression in breast cancer.

Interestingly, the ERα gene has a CpG island in its promoter and first exon regions, which are marked by a clustering of sites responsive to methylation-sensitive restriction endo-nucleases (34). This mechanism has been most extensively studied in breast cancer cell lines and tissues to explain the loss of ERα expression. Studies have consistently demonstrated that, whereas normal breast tissues and ERα-positive breast cancer cell lines lack methylation of the ER gene, ER-negative breast cancer cell lines and tumors display extensive methylation (5,13). These new findings suggest that epigenetic changes might play a crucial role in ER gene inactivation.

Arsenic compounds have been used as medicinal agents for many centuries. The detailed mechanisms of As$_2$O$_3$-induced cytotoxicity include cellular differentiation, induction of apoptosis, degradation of specific APL transcripts, inhibition of proliferation and inhibition of angiogenesis (35). Inorganic arsenic is known to be enzymatically metabolized to monomethylated and dimethylated arsenics in mammals (27). Thus, cells continuously exposed to arsenic would produce

greater quantities of S-adenosylhomocysteine (SAH). SAH is an effective competitive inhibitor of DNMTs (36). In fact, a low concentration of As_2O_3 significantly suppressed the activity of DNMTs.

In our study, we observed that after treatment of MDA-MB-435s cells with different concentrations of As_2O_3 (1.0, 2.0 and 4.0 μmol/l) for 48 h, the methylation-specific bands of the ERα gene were decreased. In contrast, the unmethylation-specific bands were enhanced. This phenomenon was also observed in the transplanted tumor tissues. We also found that the marked increase in ERα mRNA levels was accompanied by a significant reduction in DNMT1 mRNA levels. Similar results were obtained for ERα and DNMT1 protein levels using immunohistochemical and Western blot analyses. In addition, the re-expression of ERα mRNA and protein was increased in a concentration-dependent manner. Surprisingly, we found that the expression of ERα in the 4 μmol/l As_2O_3-treated group was lower than in the 2 μmol/l As_2O_3-treated group in vitro but higher after combined treatment with TAM in vivo ($P<0.05$). We hypothesize that As_2O_3 has an effect on gene restoration at relatively low concentrations (2 μmol/l), but at higher concentrations As_2O_3 inhibits ERα gene expression with severe cytotoxicity. Although the possible mechanism of in vitro toxicity with the higher concentration of As_2O_3 is unclear, it is clear that the cells are unable to efficiently metabolize arsenic. These data indicate that high concentrations of As_2O_3 have the ability to cause significant cytotoxicity, and thus, can only be used at a relatively low concentration to reactivate silenced tumor suppressor genes (28).

We observed that the TAM-unresponsive MDA-MB-435 cells restored their response to TAM after the re-expression of functional ERα following As_2O_3 treatment. Furthermore, we found that the extent of growth suppression was parallel to the ERα re-expression level. These findings indicated that the re-expression of ERα might at least partially mediate the inhibitory effects of TAM on tumor growth. However, there may be other mechanisms involved in this pathway. We also hypothesize that TAM has synergism with As_2O_3, since TAM enhanced the inhibition of tumor growth when used in combination with As_2O_3. Differences in the effects of different As_2O_3 concnetrations in vivo and in vitro are probably due to the complexities associated with in vivo studies. Surprisingly, partial hypermethylation of the ERα CpG island was maintained even at high concentrations of As_2O_3 and when combined with TAM. Even so, the partial demethylation of the ERα gene coincided with decreased DNMT1 expression, suggesting a causal link.

In summary, we demonstrated that functional ERα could be re-expressed in ERα-negative breast cancer cells following treatment with As_2O_3. In addition, treatment with As_2O_3 restored sensitivity to TAM treatment both in vitro and in vivo, and may therefore provide a novel therapeutic approach for ERα-negative breast cancer patients in clinical practice.

Acknowledgements

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