

## Arsenic Trioxide Has Additive Cytotoxic Effects on MCF-7 Breast Cancer Cell Line With Taxanes

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**Abstract:** Breast cancer is the most prevalent cancer in women and an important cause of both morbidity and mortality. Although long-term disease-free survival is offered to women with early stage disease by current treatment modality, some, 30-40% of patients will have metastatic disease. Treatment of patients with metastatic disease is a great challenge for oncologists. Therefore, new drugs and therapeutic options for women with metastatic breast cancer are urgently required.

Arsenical compounds have cytotoxic effects on several cancer cell lines including promyelocytic leukemia, esophageal carcinoma, megakaryocytic leukemia, and malignant lymphocytic cell lines. In addition, recent studies showed that arsenic trioxide ( $As_2O_3$ ) can induce a clinical remission in patients with acute promyelocytic leukemia.

In this study, we evaluated the cytotoxic effects of arsenic trioxide ( $As_2O_3$ ) alone and combined with Taxane compounds (paclitaxel and docetaxel) on human breast carcinoma cell line MCF-7. It is clearly demonstrated that  $As_2O_3$  has a significant cytotoxic effect on breast carcinoma cells.  $IC_{50}$  for  $As_2O_3$  was  $5 \times 10^{-6}$  M after 72 h. Apoptosis of carcinoma cells was responsible for the cytotoxicity of  $As_2O_3$ . The additive effects of taxane compounds with  $As_2O_3$  were shown. These in vitro results may suggest that  $As_2O_3$  is a potential new cytotoxic agent for the treatment of breast cancer.

**Key Words:** breast cancer, MCF-7, arsenic trioxide, docetaxel, paclitaxel

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### Introduction

Breast cancer accounts for 30% of all cancers in women and still remains the leading cause of cancer-related mortality in females (1). Metastatic breast cancer (MBC) develops in 30-40% of patients with breast carcinoma and is essentially incurable with standard therapy. Patients with MBC have a median survival of about 2 years (2,3) and, therefore, new anticancer agents are urgently required.

Arsenical compounds have been used as anticancer agents in traditional Chinese medicine. In the 1970s, the effective component in the remedy was identified as  $As_2O_3$ . Long-term clinical trials have indicated that  $As_2O_3$  is effective for the treatment of several types of leukemia, including APL (4,5). It has been shown that  $As_2O_3$  can induce clinical remission in patients with APL, including in those who have relapsed after retinoic acid treatment (6). However, the mechanism whereby  $As_2O_3$  targets tumor cells is not clearly understood. Several studies indicated that  $As_2O_3$  may be an oxidative agent that induces damage

to DNA and causes DNA mutations (7). Ex vivo studies on the APL cell line NB4 demonstrated that  $As_2O_3$  down-regulates Bcl-2 expression and induces apoptosis in the absence of apparent differentiation (8). Moreover, a recent study showed that  $As_2O_3$  has a specific effect on APL tumor cells by inducing the degradation of the nuclear receptor for retinoic acid fusion protein. Although  $As_2O_3$  may display a specific biological action on APL tumor cells, the apoptotic effect of  $As_2O_3$  in leukemic cells has led investigators to propose that  $As_2O_3$  may also induce apoptosis in other types of cancer cells.

Paclitaxel (PAC) and docetaxel (DOC) are clinically effective antineoplastic agents and excellent choices for the first- and second-line treatment of patients with MBC (9-11). Taxanes bind to tubulin at the same site, retard microtubule depolymerization, impair mitosis, and block progression through the cell cycle. In addition, the taxanes inactivate the Bcl-2 protein and induce apoptosis in breast cancer cells in vitro (12,13).

In patients with anthracycline-resistant MBC, PAC produced response rates (RR) of 6% to 48%. As front-line therapy in patients not previously exposed to chemotherapy, the RR were 32% to 62%. Several different doses and schedules of paclitaxel have been investigated and the regimen for optimal administration has yet to be determined (14,15,16).

DOC is a highly effective agent for MBC. In previously untreated patients, RR range from 40% - 68%, better than any other single-agent chemotherapy (17,18). DOC is particularly active in patients with anthracycline-resistant breast cancer (16).

Despite several attempts to improve further the efficacy of current therapeutic options for patients with MBC, no regimen until now has improved the chance of cure. For this reason, in this study, we evaluated the cytotoxic and apoptotic effects of a new candidate cytotoxic compound, As<sub>2</sub>O<sub>3</sub>, alone and in combination with taxanes on breast cancer cell line MCF-7 as a model system.

## Materials and Methods

### Tumor Cells

The human breast carcinoma cell line MCF-7 was kindly provided by Dr J.R. Bertino from the Memorial Sloan-Kettering Cancer Center. MCF-7 cell line is maintained in RPMI 1640 (Sigma Chemical Co., St. Louis, Missouri) plus 10% heat inactivated fetal calf serum (FCS) (Sigma Chemical Co., St. Louis, Missouri) added to 1% L-glutamine (Sigma Chemical Co., St. Louis, Missouri), 1% non-essential amino acids (Sigma Chemical Co., St. Louis, Missouri), 10,000 units/ml penicillin (Sigma Chemical Co., St. Louis, Missouri), and 10 mg/ml streptomycin (Sigma Chemical Co., St. Louis, Missouri) as adherent cells. The cell line was grown in a humidified atmosphere at 37 °C in 5% CO<sub>2</sub>. Tumor cells were used as target cells; for this purpose, adherent cells were treated with tyripsin-EDTA (Sigma Chemical Co., St. Louis, Missouri), washed, and resuspended in complete medium.

### Reagents

As<sub>2</sub>O<sub>3</sub> was kindly supplied by F. Lermioglou of Ege University, School of Pharmacy, Izmir, Turkey. In its lyophilized form, the 99.5% pure, inorganic compound corresponds to the reported extracted drug used in

Chinese herbal medicine. Paclitaxel, MTT, DMSO, and PBS were purchased from Sigma Chemical Co. St. Louis, Missouri. Docetaxel was kindly provided by W. Tong from Memorial Sloan-Kettering Cancer Center, New York, USA, at HPLC quality. Stock solutions of paclitaxel and docetaxel were prepared in DMSO (Sigma Chemical Co., St. Louis, Missouri) and the DMSO concentration in the assay did not exceed 0.1% and was not cytotoxic to MCF-7 cells in comparison to media control.

### The determination of doubling time of MCF-7 cells:

The trypan blue dye exclusion assay was used to determine the doubling time of MCF-7 cells as described previously (19). Each well of the six well plates included 5 x 10<sup>5</sup> cells in duplicate with control and total volume was made to 2 ml with medium. The plates were incubated in a humidified 5% CO<sub>2</sub> atmosphere. Cell viability was determined at 24, 48, 72, and 96 h.

### Cytotoxicity Assay

The trypan blue dye exclusion test was used to determine drug-mediated cytotoxicity as described previously. Briefly, 25 x 10<sup>4</sup> target tumor cells were resuspended in 1 ml. Two ml of cell suspension were distributed into each well of a 6-well plate, and then a 2 ml reagent solution or medium at the desired concentration was added into each well. Each plate was incubated for 72 h at 37 °C and 5% CO<sub>2</sub> atmosphere. Following the incubations, 100 µl of the trypan blue dye was added into 100 µl of cell suspension. After this process, viable and dead cells were counted. The percentage of cytotoxicity was calculated as follows:

(%) cytotoxicity = 100 - [100 x (total cell number in the experimental well / total cell number in the control well)]

Assays were performed at least three times.

### DNA fragment detection by ELISA

A cell death detection ELISA kit (Cell Death Detection ELISA<sup>plus</sup>; Boehringer Mannheim, Germany) was used according to the manufacturer's instructions for the DNA fragments detection (20). The principle of this test is based on the detection of mono- and oligo-nucleosomes in the cytoplasmic fractions of cell lysates by using biotinylated antihiston- and peroxidase-coupled anti-DNA antibodies. Enrichment factor is used as a parameter of apoptosis and is shown on the y-axis as mean ± standard

deviation (SD) of triplicates. Assays are performed at least three times, and data shown are representatives of those assays.

## Results

### Doubling time of MCF-7 cells

The doubling time of MCF 7 cells was 72 h.

The determination of cytotoxicity with  $As_2O_3$ , PAC and DOC on the MCF-7 breast cancer cell line:

#### 1. $IC_{50}$ concentration of $As_2O_3$

The stock solution of  $As_2O_3$  was prepared as  $1 \times 10^{-3}$  M and final concentrations were planned to be  $1 \times 10^{-6}$ ,  $2 \times 10^{-6}$ ,  $5 \times 10^{-6}$ ,  $8 \times 10^{-6}$ , and  $1 \times 10^{-5}$  M. For each concentration, cell viability was determined after 72 h incubation.  $IC_{50}$  value for  $As_2O_3$  was  $5 \times 10^{-6}$  M (Figure 1).

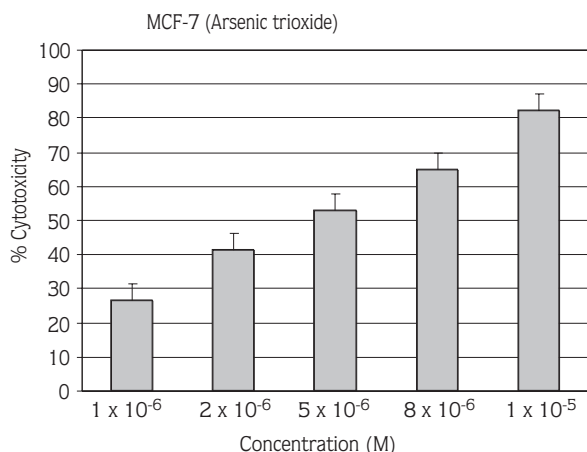


Figure 1. Cytotoxicity of tumor cells treated with different concentrations of  $As_2O_3$ . Cytotoxicity was assessed by the trypan blue dye exclusion test following 72 h culture. The data represent the mean of three different experiments with SD not exceeding 5%.

#### 2. The apoptotic effect of $As_2O_3$ on MCF-7 cells

To evaluate the induction of apoptosis of breast carcinoma cells by  $As_2O_3$ , the cell suspensions were treated by various concentrations of  $As_2O_3$ . Mono- and oligo- nucleosomes were measured. It was shown that the enrichment factor of mono- and oligo-nucleosomes increased in correlation with the increasing concentrations of  $As_2O_3$  (Figure 2).

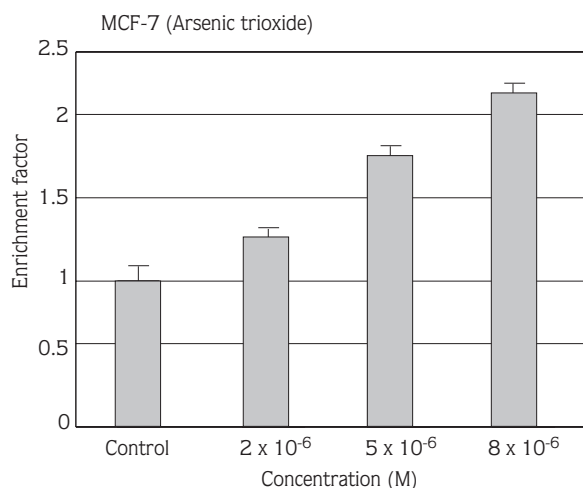


Figure 2.  $As_2O_3$ -induced apoptosis in human breast cancer cell line MCF-7. Cells were treated with different concentrations of  $As_2O_3$  for 24 h. Apoptosis was examined by determining nucleosomal DNA fragmentation using an apoptosis determination kit. Rate of apoptosis is reflected by enrichment of nucleosomes in the cytoplasm shown on the y-axis. Data presented show one representative of three independently performed experiments.

#### 3. $IC_{50}$ concentrations of PAC and DOC

PAC and DOC were prepared as 40 ng/ml stock solution and final concentrations were 2.5, 5, 10, 15, and 20 ng /ml. For each concentration, cell viability was determined after 72 h incubation.  $IC_{50}$  value for PAC was 10 ng/ml and for DOC was 5 ng/ml (Figures 3 and 4).

#### 4. The Cytotoxic Effect of $As_2O_3$ in Combination with Taxanes on MCF-7 Breast Carcinoma Cell Line

Figures 5 and 6 present data related to the combined effects of  $As_2O_3$  with PAC and DOC on MCF-7 cells. The graphs in Figures 5 and 6 clearly show that the effects of these combined drugs were additive and not synergistic. The data suggest that no dose lowering is possible with the combined  $As_2O_3$  regimens, but these agents can be used in combination for an augmented cytotoxic effect in the treatment of breast cancer.

## Discussion

Several in vitro and clinical investigations clearly showed that  $As_2O_3$  has a strong potential usage in different types of cancer treatment. Our only clinical experience was with patients with acute promyelocytic leukemia; there is strong investigational evidence that show its potent cytotoxic effects on different cancer cell

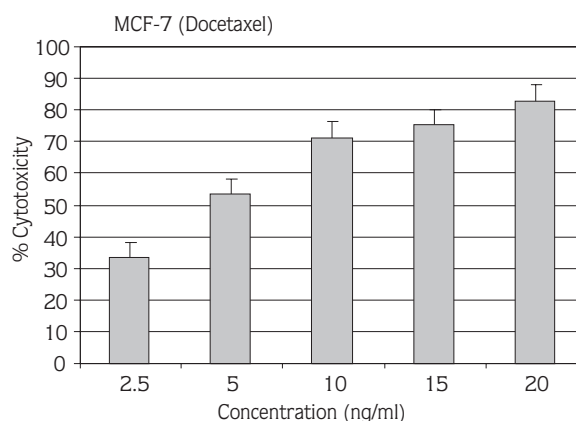
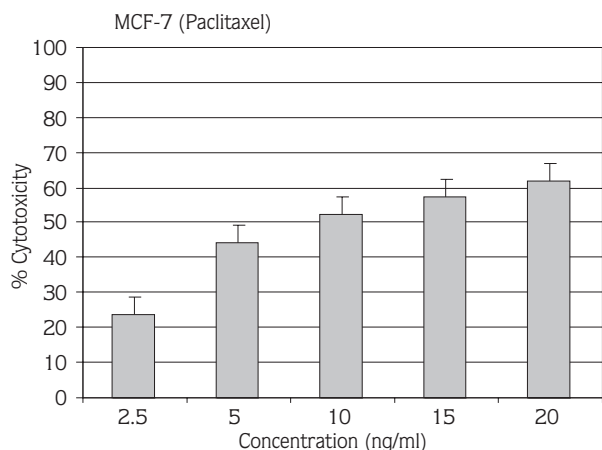


Figure 3 and 4. Cytotoxic effects of paclitaxel and docetaxel on MCF-7 cells. Cytotoxicity was assessed by the trypan blue dye exclusion test following 72 h culture. The data represent the mean of three different experiments with SD not exceeding 5%.

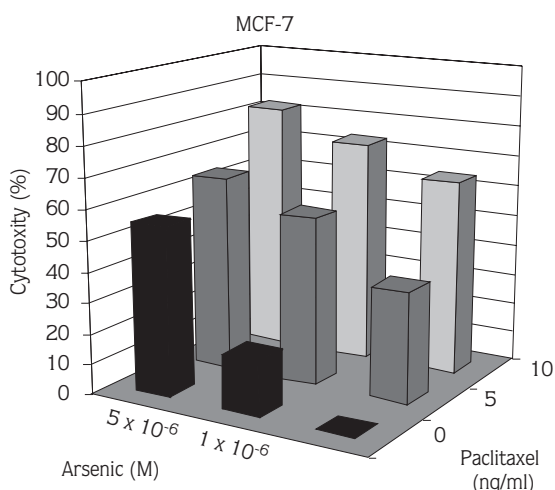


Figure 5. Cytotoxicity analysis of treatment with combination of As<sub>2</sub>O<sub>3</sub> and paclitaxel of MCF-7 cells. Cytotoxicity was determined by the trypan blue dye exclusion test in a 72 h culture. The results are expressed as the mean of three different experiments (SD < 5).

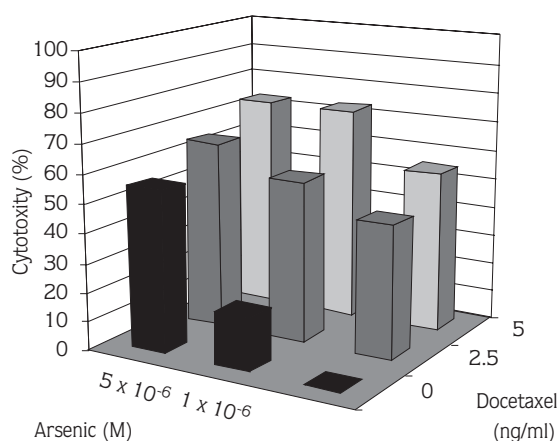


Figure 6. Cytotoxicity analysis of treatment with combination of As<sub>2</sub>O<sub>3</sub> and docetaxel of MCF-7 cells. Cytotoxicity was determined by the trypan blue dye exclusion test in a 72 h culture. The results are expressed as the mean of three different experiments (SD < 5).

lines in vitro. In this study, we have evaluated the cytotoxic effect of As<sub>2</sub>O<sub>3</sub> on the MCF-7 breast cancer cancer line. We have selected this cell line as a model system because of clinical requirements for the better treatment of metastatic breast carcinoma. As<sub>2</sub>O<sub>3</sub> exerts an important cytotoxicity on MCF-7 cells. The major underlying mechanism of its cytotoxicity seems to be the induction of cell apoptosis. The cytotoxic effects of As<sub>2</sub>O<sub>3</sub> in combination with two well-known active agents, PAC and DOC, were also investigated. Both PAC and DOC are

very active agents and have been widely used clinically. In combination with either of the taxanes additive effects were found. No synergism between As<sub>2</sub>O<sub>3</sub> and taxanes was investigated.

The effective cytotoxic dose of As<sub>2</sub>O<sub>3</sub> (IC<sub>50</sub> 5nM) on MCF-7 cells was found to be in the range of acceptable clinical dose levels. In patients with APL, plasma levels were about 10nM and this level was very well tolerated.

Although the precise mechanism of arsenic trioxide action is unknown, a variety of in vitro studies suggest

that several mechanisms may contribute to its effectiveness in vivo. These mechanisms include induction of apoptosis, partial cellular differentiation, degradation of specific APL fusion transcripts, antiproliferation, and inhibition of angiogenesis (4).

It has been shown that in certain leukemia cell lines, thiol diester bonds were lysed by  $As_2O_3$ , and that was the main mechanism underlying cytotoxicity (5).  $As_2O_3$  may also induce its apoptotic effects by means of its interaction with the cytoskeleton and tubuli system of the cell, which has been reported in leukemic cell lines (21). In APL, it has been shown that  $As_2O_3$  degrades abnormal PML-RARa fusion protein and induces Bcl-2 down-regulation (8).

$As_2O_3$  has mostly been used in hematological malignancies. Especially,  $As_2O_3$  is used in APL treatment refractory to other treatment modalities. There are limited data about the cytotoxicity of  $As_2O_3$  on solid

tumors, all of them include in vitro studies. Our group reported that arsenic trioxide has a cytotoxic effect on ovarian and prostate cancer cell lines (22).  $As_2O_3$  has also been reported to be effective on cervical cancer (23), bladder cancer (24), and esophageal cancer (25) cell lines.

In conclusion, the results presented in this report indicate that  $As_2O_3$  is cytotoxic on breast cancer cells. Paclitaxel and docetaxel showed promising effects in combination with  $As_2O_3$ , and clinical trials with these combinations are warranted.

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