Arсенный триоксид-медиаторная цитотоксичность и апоптоз в клетках предстательной и яичниковокарциномы

Ручан Услу, Улус Али Санли, Цанфеза Сезгин, Бурлет Карабулат, Эндер Терзиоглу, Сердар Бедий Омай, и Эрдем Гокер

Департаменты онкологии и гематологии [Р. У., У. А. С., С. С., Б. К., С. Б. О., Е. Г.], и Департамент иммунологии [Е. Т.], Египетский университет школа медицины, 35100, Извим, Турция

Абстракт

Мы изучили эффект арсената триоксида (As₂O₃) на клетки предстательной и яичниковокарциномы. As₂O₃ был показан как эффективный в лекарственной, и ампутация промиелобластной лейкемии, особенно в in vitro и in vivo. Как модельных клеток, мы использовали DU145 и PC-3 для предстательной и яичниковокарциномы. Новые модальности лечения являются основным в этих видах рака, которые приводят к высокой смертности. Метаболический подход, используемый бутоксиданисолом показал, что цитотоксическое действие As₂O₃ не происходило через супероксидный путь. Исследования метаболического подхода показали, что цитотоксическое действие As₂O₃ может быть апоптозом. Было доказано, что As₂O₃ может иметь значительное цитотоксическое действие на клетки предстательной и яичниковокарциномы. 

Введение

Овариальный и предстательный рак являются наиболее часто диагностируемыми опухолями в человеческом организме, с высокой смертностью и плохим прогнозом, особенно в стойкой форме (1, 2). Тumor cells very often develop resistance to chemotherapy (3). Whereas increased dosages of chemotherapeutic agents or high-dose ablative regimens are frequently more effective at tumor cell cytodestruction, progressive dose escalation of these agents is ultimately limited by their nonspecific end-organ toxicity.

Овариальный рак - это модельный рак для изучения химиотерапевтической рефрактерности из-за своей природы и приобретенной рефрактерности к химиотерапии. Приблизительно 75% пациентов имеют хирургически incurable disease at the time of diagnosis. Aggressive treatment of these patients with platinum-based combination chemotherapy usually results in severe adverse effects, and it is rarely curative (4). Therefore, new effective and subtoxic therapeutic modalities are needed.

Предстательный рак является наиболее часто диагностируемой опухолью в мире, с приблизительно 41,000 американцев умирают ежегодно от этого заболевания (2). Хормональная аблация является основной терапией для метастатического, хормон-независимого рака. Дальнейшее, андроген-независимые опухоли также становятся резистентными к широкому спектру цитотоксических препаратов. В настоящее время нет терапии, которая позволила улучшить выживаемость этих пациентов (6). Поэтому, новые терапевтические подходы должны быть разработаны для метастатического, хормон-независимого предстательного рака.

Арсенные препараты были использованы как антитumorальные агенты в традиционной китайской медицине. В 1970-х годах, эффективным компонентом в ряду был As₂O₃. Long-term clinical trials have indicated that As₂O₃ is very effective in the treatment of several types of leukemia, including APL (7). It has been shown that As₂O₃ can induce clinical remission in patients with APL, including in those who have relapsed after retinoic acid treatment (8). However, the mechanism whereby As₂O₃ targets the tumor cells is not clearly understood. Several studies indicated that As₂O₃ may be an oxidative agent that induces damage to DNA and causes DNA mutations (9). Ex vivo studies on the APL cell line NB4 demonstrated that As₂O₃ down-regulates bcl-2 expression and induces apoptosis in the absence of apparent differentiation (10). Moreover, a recent study showed that As₂O₃ has a specific effect on APL tumor cells by inducing the degradation of the nuclear receptor for retinoic acid fusion protein (10). Although As₂O₃ may display a specific biological action on APL tumor cells, the apoptotic effect of As₂O₃ in leukemic cells has led investigators to propose that As₂O₃ may also induce apoptosis in other types of cancer cells.

The apoptotic effect of As₂O₃ on other myeloid leukemic cell lines (11), megakaryositisic leukemic cell lines (12), and esophageal carcinoma cell lines (13) have been reported. In this study, we examined the cytotoxic and apoptotic effects of As₂O₃ on prostate and ovarian carcinoma cell lines. For this purpose,
we used the ovarian carcinoma cell line MDAH 2774 and hormone-independent prostate carcinoma cell lines PC-3 and DU145. The rationale underlying the selection of ovarian and prostate carcinoma cell lines as model systems are the characteristic patterns of recurrences of these types of carcinomas. Ovarian cancer very often causes disseminated peritoneal metastasis, and prostate cancer recurs as an advanced local disease. Studying As$_2$O$_3$ cytotoxicity on these cell lines would allow the clinical investigators to try locoregional use of As$_2$O$_3$ in these tumors, which may cause less systemic toxicity. As$_2$O$_3$ is also foreseen as an optional treatment with these special indications, e.g., i.p. treatment. As$_2$O$_3$ is tolerated as a less systemically toxic drug by leukemia patients; hence, it may be an easy method of chemoprotection as well. With these factors in mind, we performed experiments using As$_2$O$_3$ in combination with other chemotherapeutic agents and alone. We also tried to bring to light the role of superoxide radical generation in As$_2$O$_3$-induced cytotoxicity by using BHA, an antioxidant, in these cancer cells.

MATERIALS AND METHODS
Tumor Cells
The human hormone-independent and drug-resistant prostatic carcinoma cell lines DU145 and PC-3 were purchased from American Type Culture Collection (Manassas, VA). The human ovarian carcinoma cell line MDAH 2774 was generously supplied by Dr. E. Aktar, Istanbul University, Istanbul, Turkey. These tumor cell lines were maintained in culture as adherent cells and cultured in RPMI 1640 (Sigma Chemical Co., St. Louis, MO) plus 10% heat inactivated FCS (Sigma Chemical Co.) added to 1% L-glutamine (Sigma Chemical Co.), 1% nonessential amino acids (Sigma Chemical Co.), 10,000 units/ml penicillin (Sigma Chemical Co.), and 10 mg/ml streptomycin (Sigma Chemical Co.). All lines were grown in a humidified atmosphere at 37°C in 5% CO$_2$. When the tumor cell lines were used as target cells, they were treated with trypsin-EDTA (Sigma Chemical Co.), washed, and resuspended in complete medium.

Reagents
Cisplatin, Adriamycin, etoposide, BHA, MTT, DMSO, and PBS were purchased from Sigma Chemical Co. As$_2$O$_3$ was kindly supplied by F. Lermoglu, Ege University, Izmir, Turkey. It is a lyophilized, 99.5% pure, inorganic compound corresponding to the reported extracted drug used in Chinese herbal medicine. CycleTest-Plus DNA reagent KIT was purchased from Becton Dickinson (Mountain View, CA). Cell Death Detection ELISA$^{\text{plus}}$ KIT was purchased from Boehringer Mannheim (Mannheim, Germany). Stock solution of As$_2$O$_3$, was prepared in distilled water. Stock solutions of cisplatin, adriamycin, and etoposide were prepared in DMSO (Sigma Chemical Co.); the DMSO concentration in the assay did not exceed 0.1% and was not cytotoxic to the tumor cells.

Cytotoxicity Assay
The MTT assay was used to determine drug-mediated cytotoxicity, as described previously (14, 15). For MTT assay, briefly, target tumor cells were resuspended in medium at $1 \times 10^5$ cells/ml after verifying cell viability by trypsin blue dye (Sigma Chemical Co.) exclusion assay. One hundred µl of cell suspension were distributed into each well of 96-well flat-bottomed microtiter plate (Greiner Labortechnik, Frickenhausen, Germany), and each plate was incubated for 24 h at 37°C and 5% CO$_2$ atmosphere. After the incubations, 100-µl reagent solutions or media at the desired concentrations were distributed into each well. The well containing
only media served as a positive control. Treatments were performed in triplicate. Two hundred μl of the medium alone without cells and reagent were used as a negative control. The microtiter plate was incubated for the desired period of time. Thereafter, 20 μl of the MTT dye (5 mg/ml) were added into each well. The unreactive supernatants in the well were carefully aspirated and replaced with 100 μl of isopropanol (Sigma Chemical Co.) supplemented with 0.05 N HCl to solubilize the reactive dye. The absorbance (A) values of each well at 540 nm were read using an automatic multiwell spectrophotometer (Bio-Rad-Coda, Richmond, CA). The negative control well was used for zeroing absorbance. The percentage of cytotoxicity was calculated using the background-corrected absorbance as follows:

\[
\% \text{ cytotoxicity} = \left(\frac{1 - A \text{ of experimental well}}{A \text{ of positive control well}}\right) \times 100
\]

Experiments were performed at least three times with representative data presented.

*Fig. 2* As₂O₃ induces apoptosis of human ovarian carcinoma cell line MDAH 2774. Cells were treated with \(5 \times 10^{-6}\) M As₂O₃ for 72 h, and As₂O₃-mediated apoptosis was determined by flow cytometric analysis. a, control; b, As₂O₃, \(5 \times 10^{-6}\) M).
Analysis of DNA Fragmentation

Flow Cytometric Analysis. Commercially available CycleTest-Plus DNA reagent KIT (Becton Dickinson, Mount View, CA) was used to stain the cells by propidium iodide. Fluorescence data related to the DNA content of the cells in different cell cycles was collected with the Becton Dickinson FACSCalibur flow cytometer. Apoptotic DNA was gated and evaluated in relation to the cytotoxicity assay. Assays were performed at least three times, and data shown are representative of those assays.

DNA Fragment Detection by ELISA. A cell death detection ELISA Kit (Cell Death Detection ELISA<sup>Plus</sup>; Boehringer Mannheim) was used according to the manufacturer’s instructions for DNA fragment detection (16). The DNA fragments are discrete multiples of a 180-bp subunit, which is detected as a DNA ladder on agarose gel. The enrichment of mono- and oligonucleosomes in the cytoplasm of the apoptotic cells is attributable to the fact that DNA degradation occurs several hours before plasma membrane breakdown. The principle on which this test is based is the detection of mono- and oligonucleosomes in the cytoplasmic fractions of cell lysates by using biotinylated antihistone- and peroxidase-coupled anti-DNA antibodies. The enrichment of mono- and oligonucleosomes released into the cytoplasm is calculated as absorbance of sample cells/absorbance of control cells. The enrichment factor was used as a parameter of apoptosis and is shown on the Y axis as mean ± SD of triplicates. Assays were performed at least three times, and data shown are representative of those assays.

Statistical Analysis

All assays were set up in triplicate, and the results were expressed as the mean ± SD. Statistical analysis was determined by Student’s t test. For synergy, isobolograph analysis was performed according to Berenbaum (17). The isobole has been used to evaluate the presence of synergism or antagonism in many fields. It requires experimental data for agents used alone and in different dose combinations at equieffective levels. These data are plotted on isoeffective graphs with the axis representing the doses of each agent. If two agents do not interact, the line forming the point corresponding to the combination of those on the axis representing doses isoeffective with the combination will be a straight line. When agents in combination are more effective than what might be expected from their dose-response curves (synergy), smaller amounts will be needed to produce the effect under consideration, and a concave-up isobole results. On the other hand, when agents in combination are less effective than expected (antagonism), greater doses than expected will be needed to produce the same effect, and a concave-down isobole is generated. The fraction of inhibitory concentrations was calculated as the percentage of each treatment to effect a fixed level of cytotoxicity.

RESULTS

Sensitivity of Human Prostate Carcinoma Cell Lines and Human Ovarian Carcinoma Cell Line to As<sub>2</sub>O<sub>3</sub>. Two hormone- and drug-resistant human prostate carcinoma cell lines, DU145 and PC-3, and an ovarian carcinoma cell line, MDAH 2774, were selected for the study. They were treated with different concentrations of As<sub>2</sub>O<sub>3</sub> and cytotoxicity was determined by the MTT assay. Cytotoxicity was determined every 24 h for three days. All cell lines were sensitive to As<sub>2</sub>O<sub>3</sub>-mediated cytotoxicity, and maximum cytotoxicity was achieved at 72 h. With the increasing concentrations of As<sub>2</sub>O<sub>3</sub>, cell death increased. At 5 \times 10^{-6} M concentrations of As<sub>2</sub>O<sub>3</sub>, more than 50% of the cells were dead in all cell lines at 72 h, as shown in Fig. 1. The experiments clearly showed that As<sub>2</sub>O<sub>3</sub> induces cytotoxicity in DU145 and PC-3 prostatic carcinoma cell lines and in the MDAH 2774 ovarian carcinoma cell line in direct proportion to the time and concentration.

As<sub>2</sub>O<sub>3</sub> Induces Apoptosis in Human Ovarian and Prostate Carcinoma Cell Lines.

We further investigated whether the cytotoxic effect was by apoptosis. For this purpose, cell suspensions with deferring
concentrations of As$_2$O$_3$ were stained with propidium iodide at 72 h and evaluated by flow cytometry. As seen in the flow cytometric photographs in Fig. 2, representative of the three cell lines, in contrast to the control, there was a clear shift in the degraded DNA with a nonspecific cell cycle phase pattern. Also, the mono- and oligonucleosomes were evaluated at 24 h by ELISA. The reason for the different time points between flow cytometry and ELISA was the emergence of nucleosomes approximately 24 h before the complete DNA fragmentation, which can be detected by flow cytometry. With ELISA, the increasing concentrations of As$_2$O$_3$ demonstrated an increased enrichment factor, indicating the increased mono- and oligonucleosomes with the incubation time. Fig. 3 presents the data related to MDAH 2774 and is representative of the apoptosis also occurring in prostatic cancer cell lines DU145 and PC-3.

Effect of BHA on Cytotoxicity Mediated by As$_2$O$_3$ on Human Prostate and Ovarian Carcinoma Cell Lines. The phenolic compound 2,6-di-tert-butyl-4-methoxy-phenol (BHA) is a potent antioxidant that is widely used to prevent foods from oxidative deterioration and rancidity (18). BHA is also commonly used as an antioxidant in tissue culture experiments. In addition to its preventive effect from peroxidation of lipids and proteins, BHA has been shown to inhibit the mitochondrial electron transport system, especially at the complex I and complex II sites (19, 20). BHA has also been shown to increase glutathione and glutathione-S-transferases levels and to increase $\gamma$-glutamylcysteine synthetase activity (21, 22). These studies suggest that BHA plays an important role in both inhibition of the production of free radicals and in the detoxification of free radicals. Fig. 4 presents the data related to DU145 representative of the effect of BHA on As$_2$O$_3$-mediated cytotoxicity as well as that occurring in PC-3 and MDAH 2774 cancer cell lines. As seen in Fig. 4, nontoxic concentrations of BHA in the cell cultures exerted no effect on the As$_2$O$_3$-mediated cytotoxicity. It suggests that As$_2$O$_3$-mediated cytotoxicity on DU145, PC3, and MDAH 2774 is not related to As$_2$O$_3$-mediated superoxide radical generation.

The Cytotoxic Effect of As$_2$O$_3$ in Combination with Various Chemotherapeutic Drugs against DU145 and PC-3 Prostate Carcinoma Cell Lines and MDAH 2774 Ovarian Carcinoma Cell Line. Adriamycin, etoposide, and cisplatin are the most commonly used chemotherapeutic agents in the treatment of metastatic hormone refractory prostate cancer and ovarian cancer. However, the overall response rates induced by these drugs are very poor, and the duration of response is very short with limited impact on survival. Therefore, we evaluated the combined effect of As$_2$O$_3$ with adriamycin, etoposide, and cisplatin. Figs. 5, 6, and 7 present the data related to the combined effect of As$_2$O$_3$ with adriamycin, etoposide, and cisplatin on PC-3 cells as well as on DU145 and MDAH 2774 cells. The graphs and isobologram analysis in Figs. 5, 6, and 7 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$_2$O$_3$ regimens, but these agents can be used in combination for an augmented cytotoxic effect in the treatment of prostate and ovarian cancer.

**Effect of Sequential Treatment of DU145, PC-3, and MDAH 2774 Cells with Either As$_2$O$_3$ or Various Chemotherapeutic Drugs and Subsequent Treatment with the Second Agents.** We also evaluated whether sequential treatment of prostate cancer cells can result in augmented cytotoxicity. For this purpose we treated DU145, PC3, and MDAH 2774 cells with the deferring concentrations of As$_2$O$_3$ and drug combinations by washing the first drug out of the cells and incubating with the second agent at time intervals of 24–48 h. Figs. 8, 9, and 10 present the effect of sequential treatment with either As$_2$O$_3$ or adriamycin, etoposide, and cisplatin on DU145 cells as well as in PC-3 and MDAH 2774 cells. As can be seen in Figs. 8, 9, and 10, there was no difference on total cytotoxicity compared with the concomitant effects of the combined drugs.

**DISCUSSION**

In this study, we have first shown that As$_2$O$_3$ exerts a significant degree of cytotoxicity on prostate and ovarian carcinoma cell lines. These cell lines are very good models in which to study the effects and mechanisms of various drugs because they represent the highly aggressive nature of metastatic human prostate and ovarian cancers. For prostate cancers we used DU145 and PC-3, which are human hormone-independent and drug-resistant prostatic carcinoma cell lines. For ovarian cancer we used MDAH 2774, which is also derived from an aggressive ovarian carcinoma. As$_2$O$_3$ was cytotoxic in all three cell lines in a very near concentration and time range. This concentration was in the $10^{-6}$ m region, which is a very acceptable dose for the *in vivo* administration as a parenteral drug (8).

The mechanisms underlying cytotoxicity and differentiation seem to be completely different from each other. At higher concentrations with a considerable cytotoxicity, apoptosis appeared to be the main phenomenon resulting in significant cell

---

**Fig. 7** Cytotoxicity and isobolograph analysis of treatment of PC-3 cells with combination of As$_2$O$_3$ and etoposide. Cytotoxicity was determined by MTT assay in a 72-h culture. The results are expressed as the mean of three different experiments (SD <5).

**Fig. 8** Effect on cytotoxicity of sequential treatment with either As$_2$O$_3$ or cisplatin. DU145 tumor cells were pretreated with medium or with the desired concentrations of As$_2$O$_3$ for 24 h and thereafter, the medium was aspirated, and the tumor cells were washed twice with RPMI 1640 and subsequently incubated with medium or with the desired concentrations of cisplatin for 48 h; cytotoxicity was assessed by MTT assay (a). Then, DU145 cells were pretreated with medium or with the desired concentrations of cisplatin for 24 h and thereafter, the medium was aspirated, and the tumor cells were washed twice with RPMI 1640 and subsequently incubated with medium or the desired concentrations As$_2$O$_3$ for 48 h; cytotoxicity was assessed by MTT assay (b). The data represent the mean of three different experiments with a SD not exceeding 5%.
In our experiments, we have clearly demonstrated that apoptosis was induced both in prostate and ovarian carcinoma cell lines with significant cytotoxicity. It is not known how As$_2$O$_3$ induces apoptosis in these cell lines. In previous reports, it was shown that superoxide generation was the main mechanism of apoptosis by As$_2$O$_3$ (9, 23, 24). However, our experiments using BHA, which is a strong antioxidant agent (18), demonstrated that BHA could not prevent cytotoxicity by As$_2$O$_3$, indicating that in prostate and ovarian carcinoma cell lines, apoptosis is induced by a different mechanism, rather than by superoxide generation. For example, it has been shown that in certain leukemia cell lines, thiol diester bonds were lysed by As$_2$O$_3$, and that was the main mechanism underlying cytotoxicity (7). As$_2$O$_3$, 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 (25).

In APL, it has been shown that As$_2$O$_3$ degrades abnormal PML-RAR$\alpha$ fusion protein and induces bcl-2 down-regulation (10). There is the possibility that in prostate and ovarian car-
cinoma cell lines, bcl-2 down-regulation can be induced by As$_2$O$_3$.

At the beginning of our study, we thought that As$_2$O$_3$ would interact with conventional chemotherapeutic agents synergistically, but we could not demonstrate any synergism with the cisplatin, adriamycin, and etoposide. However, the additive effect demonstrated with the isobologram analysis opens a window for new combined modality approaches to achieve augmented cytotoxicity in treating prostate and ovarian carcinomas. But these combinations may result in greater toxicity, and thus, before strong recommendation or use with patients, they should be tried in animal models. Also, we showed that treatment of cancer cells with As$_2$O$_3$ and the other chemotherapeutic agents, whether sequential or nonsequential, did not cause a significant difference in the total cytotoxicity. This suggests ease of use of different chemotherapeutic agents with As$_2$O$_3$ resulting in the desired total net effect but with fewer adverse effects.

Ovarian and prostate cancers are very common and account for a considerable death toll. Although life expectancy has increased thanks to conventional chemotherapeutic drugs, the number of patients unresponsive to such treatments is also increasing. New modalities of treatment are essential for these types of patients. As$_2$O$_3$ is a new option for these types of cancers, as has been shown in our study. A second use for As$_2$O$_3$ may be the i.p. local administration in these desperate cases, as has been shown in our study. A second use for As$_2$O$_3$ i.p. may lessen systemic toxicity while increasing cytotoxicity against ovarian carcinoma. The high molecular weight of As$_2$O$_3$ provides the opportunity for this type of administration. Additional pharmacokinetic studies are warranted.

In conclusion, As$_2$O$_3$ is a very promising new drug for use in prostate and ovarian cancers.

ACKNOWLEDGMENTS

We thank Drs. F. Buyukkececi, G. Saydam, and C. Cal for their generous support of our study.

REFERENCES

Arsenic Trioxide-mediated Cytotoxicity and Apoptosis in Prostate and Ovarian Carcinoma Cell Lines
