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## Report

# Arsenic trioxide as a novel anticancer agent against human transitional carcinoma—characterizing its apoptotic pathway

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**Arsenic trioxide ($As_2O_3$) has been shown to be an active agent against acute promyelocytic leukemia. Little is known about its therapeutic efficacy in human transitional carcinomas. In this study, the arsenic-mediated apoptotic pathway in transitional carcinoma cells was investigated. Three bladder transitional carcinoma cell lines were used, including a parental sensitive line and two resistant daughter lines (cisplatin and $As_2O_3$ resistant). The $As_2O_3$-mediated cytotoxicity to the three cell lines was studied *in vitro* in the presence or absence of buthionine sulfoximine (BSO), a chemotherapy modulator. In results, although a lesser extent of apoptosis was seen in cells treated with $As_2O_3$ alone, more significant apoptotic events were observed in the combined treatment of $As_2O_3$ and non-toxic concentrations of BSO (up to 10 $\mu$M). These included the accumulation of sub-$G_1$ fractions and internucleosomal DNA breakdown, which were preceded by production of reactive oxygen species, loss of mitochondrial membrane potential and activation of caspase-3. In conclusion, $As_2O_3$ in the presence of BSO may be an active agent against both chemonaive and cisplatin-resistant transitional carcinomas. The $As_2O_3$-mediated cytotoxicity appeared to go through the conventional apoptotic pathway. Our results have clinical implications and warrant further investigation. [© 2002 Lippincott Williams & Wilkins.]**

*Key words:* Bladder neoplasms, buthionine sulfoximine, caspases, glutathione, NTUB1 cells, reactive oxygen species.

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## Introduction

Although arsenic compounds are known as poisons, they have been used in traditional Chinese medicine for centuries. Interestingly, arsenic compounds, such as arsenic trioxide ($As_2O_3$) and arsenic disulfide, were recently shown to be effective in the treatment of acute promyelocytic leukemia (APL).[1] The mechanisms of action were shown to be associated with the induction of apoptosis and differentiation.[2] Moreover, *in vitro* studies revealed that clinically achievable concentrations of $As_2O_3$ could trigger apoptosis of leukemia[3] and lymphoma[4] cells as well as some solid tumor cells, including esophageal cancer,[5] neuroblastoma,[6] prostate cancer,[7] ovarian cancer,[7] etc. This suggests that $As_2O_3$-induced apoptosis may also be seen in a variety of tumor models. Although $As_2O_3$-mediated apoptosis has been explored in many tissue systems, little is known about the cytotoxic effects of $As_2O_3$ on human transitional carcinoma cells.

About 30–50% of advanced transitional cell carcinomas do not respond to cisplatin-based chemotherapy. Treatment failure is not uncommon and an effective salvage therapy for patients who failed cisplatin-based regimens is urgently needed. If $As_2O_3$ is to be used as a second-line agent against transitional carcinoma, apoptosis should be seen in arsenic-treated cisplatin-resistant cells. Data of this kind are also lacking.

We have previously shown that intracellular glutathione (GSH) content has a decisive effect on $As_2O_3$-induced apoptosis.[8] Cells that have a low GSH

content are highly sensitive to $As_2O_3$ and experimental up-modulation of GSH content may decrease the sensitivity to $As_2O_3$.[4] It was also demonstrated that ascorbic acid or buthionine sulfoximine (BSO) may sensitize cells to $As_2O_3$ by depleting the GSH content.[4] It will be of clinical significance if modulators like BSO are proved capable of potentiating $As_2O_3$ cytotoxicity in human transitional carcinomas.

This study was therefore designed to explore cytotoxic effects, typically those in the apoptotic pathway, of $As_2O_3$ on transitional carcinoma cells *in vitro*. By doing this, we hope this study can be of help in substantiating the clinical use of arsenic compounds in the treatment of human transitional carcinoma.

## Materials and methods

### Cell lines and chemicals

Three bladder transitional carcinoma cell lines were used: NTUB1, NTUB1/P (cisplatin-resistant subline)[9] and NTUB1/As ($As_2O_3$-resistant subline). NTUB1/P and NTUB1/As were generated by culturing NTUB1 in progressively increasing concentrations of each drug, and could thrive at 14 $\mu$M cisplatin and 0.4 $\mu$M $As_2O_3$, respectively. The cisplatin $IC_{50}$ of NTUB1/P and $As_2O_3$ $IC_{50}$ of NTUB1/As were 36.6 and 5.0 $\mu$M, respectively, being 15.3- and 4.2-fold higher than those of NTUB1. All cells were maintained in an RPMI 1640 medium (Gibco/BRL, Gaithersburg, MD) containing 10% heat-inactivated fetal calf serum (Gibco/BRL) at 37°C in humidified air with 5% $CO_2$. $As_2O_3$ was purchased from Sigma (St Louis, MO), and was initially dissolved in hot distilled water as a high-concentration stock and then in culture medium immediately before use.

### Morphological study and sub-$G_1$ fraction analysis by DNA flow cytometry

The sub-$G_1$ fraction represents the proportion of a cell population that is undergoing apoptosis. NTUB1 ($8 \times 10^4$ cells), NTUB1/P ($1.4 \times 10^5$ cells) and NTUB1/As ($1 \times 10^5$ cells) were treated with $As_2O_3$ at concentrations comparable to their respective $IC_{20}$s (drug concentrations inhibiting 20% of cell growth) with or without BSO at concentrations of 3 (NTUB1) and 10 (NTUB1/P and NTUB1/As) $\mu$M for up to 72 h in six-well plates. The $As_2O_3$ $IC_{20}$s for NTUB1, NTUB1/P and NTUB1/As were 0.6, 1 and 3 $\mu$M, respectively. Morphological changes were recorded every 12 h for up to 72 h. Cells were then harvested by trypsinization, washed with 1 × PBS, resuspended in 200 $\mu$l PBS and fixed in 800 $\mu$l of ice-cold 100% ethanol at −20°C. After overnight incubation, the cell pellets were collected by centrifugation, resuspended in 1 ml of the hypotonic buffer (0.1% Triton X-100 and 50 $\mu$g/ml RNase A) and incubated at 37°C for 30 min. Then, 1 ml of propidium iodide solution (50 $\mu$g/ml) was added and the mixture was allowed to stand on ice for 30 min. The DNA contents of the nuclei were analyzed with a flow cytometer (FACScan; Becton Dickinson, San Jose, CA).

### Induction of internucleosomal DNA fragmentation

The three cell lines were cultivated in 10-cm Petri dishes with the same treatment conditions as described in the sub-$G_1$ experiments, harvested after varied culture intervals (24, 48 and 72 h), resuspended in 100 $\mu$l of the lysis buffer (10 mM EDTA, 50 mM Tris–HCl, pH 8.0, 0.5% sarcosyl and 0.5 $\mu$g/ml proteinase K) and incubated at 50°C for 3 h. The mixture was then incubated with RNase A (500 $\mu$g/ml) at 50°C for 1 h. DNA fragments were extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and the supernatant was collected by centrifugation for 30 min at 14 000 *g*. DNA fragments were separated by electrophoresis in 1.8% agarose gels and stained with ethidium bromide.

### Determination of reactive oxygen species (ROS)

Production of ROS has been shown to be a critical step in apoptotic cell death. ROS production was studied by flow cytometric analysis using a non-polar dye, dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR) that readily diffuses into cells and is hydrolyzed by intracellular esterase to yield DCFH that is then trapped in the cells. Hydrogen peroxide or low-molecular-weight peroxides present in the cells oxidize DCFH to the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF).[10] The fluorescence intensity is proportional to the amount of peroxide present in the cells. By quantifying the intracellular capability to oxidize DCFH and release the fluorescent compound by flow cytometry, we determined the amount of ROS production in $As_2O_3$-treated cells. The three cell lines were treated with $As_2O_3$ with or without BSO at concentrations identical to those in the sub-$G_1$ experiments for different time intervals and incubated with 100 $\mu$M DCFH-DA for 1 h. The green fluorescence of intracellular DCF was detected by a

flow cytometer (FACScan) with a 525-nm band pass filter. Fold induction of ROS was determined by the ratio of mean fluorescence intensity of treated cells over that of control cells.

### Mitochondrial membrane potential by flow cytometry

Several studies have shown that cellular apoptosis is accompanied by loss of mitochondrial membrane potential following production of ROS.[6] Relative mitochondrial membrane potential was determined by flow cytometric analysis. Briefly, the three cell lines were treated with $As_2O_3$ with or without BSO at concentrations identical to those in the sub-$G_1$ experiments for different time intervals and then 40 nM 3,3′-dihexyloxacarbocyanine ($DiOC_6(3)$; Molecular Probes) was added to stain the cells for 15 min at 37°C. $DiOC_6(3)$, a lipophilic cationic fluorescent dye, is known to be able to anchor on the inner surface of the mitochondrial membrane and the amount of dye anchorage is positively proportional to the membrane potential. Loss of the mitochondrial membrane potential is associated with the reduction of dye anchorage and hence the reduction of green fluorescence that can be detected by flow cytometry (FACScan) with a 525-nm band pass filter. The magnitude of reduction of mitochondrial membrane potential was calculated by [1−(mean fluorescence intensity of treated cells/mean fluorescence intensity of control cells)] × 100%.

### Caspase-3 activity assay

Apoptotic pathways are drug and cell type-specific and are associated with the activation of specific caspases that lead to cell death. In this study, we examined the role of the caspase-3 activation pathway in $As_2O_3$-induced apoptosis in the three transitional carcinoma cells. After concurrent treatment with $As_2O_3$ and BSO at concentrations identical to those in the sub-$G_1$ experiments for different time intervals, cells were collected, washed with 1 × PBS and resuspended in 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothreitol, 2 mM phenylmethlysulfonyl fluoride, 10 μg/ml pepstatin A and 10 μg/ml leupeptin. Concentrations used for each cell were titrated to induce 50% cell death in the chemosensitivity assay. The caspase-3 activity assay was done using the CaspACE Assay System kit (Promega, Madison, WI). Cell lysates were centrifuged at 12 000 r.p.m. for 5 min and aliquots of clear lysate containing 50 μg of protein were incubated with 50 μM acetyl-Asp-Glu-Val-Asp-7-amino-4 methyl coumarin (Ac-DEVD-AMC) as the substrate at 30°C for 1 h. Upon cleavage by activated caspase-3, the substrate releases a yellow-green fluorescent compound, 7-amino-4 methyl coumarin (AMC) which can be detected by a spectrofluorometer (F-4500; Hitachi, Hitachinaka-Shi, Japan) with excitation and emission at 360 and 460 nm, respectively. The amount of fluorescence produced is proportional to the amount of caspase-3 activity present in the sample.[11]

### Statistical methods

All symmetrical numeric data were presented as mean ± SEM and compared with the Student's *t*-test.

## Results

### Morphological study

Cellular death with formation of apoptotic bodies and micronuclei was clearly seen in cells treated with both $As_2O_3$ and BSO (Figure 1). Those treated with

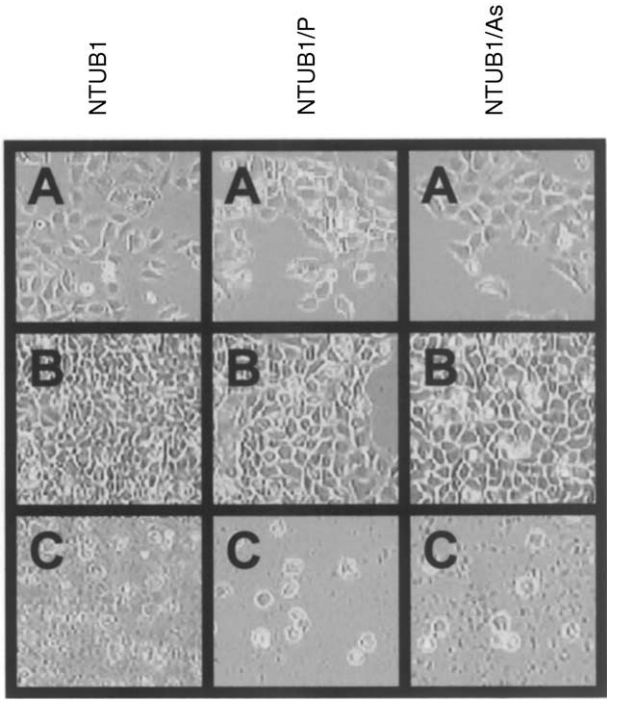


**Figure 1.** Morphological evaluation of transitional carcinoma cells treated with $As_2O_3$ and BSO ( × 380). (A) Control cells at time 0. (B) Control cells at 72 h without treatment. (C) Treatment with $As_2O_3$ and BSO for 72 h. Apoptotic bodies and micronuclei can be seen in the combined treatments for all three cell lines.

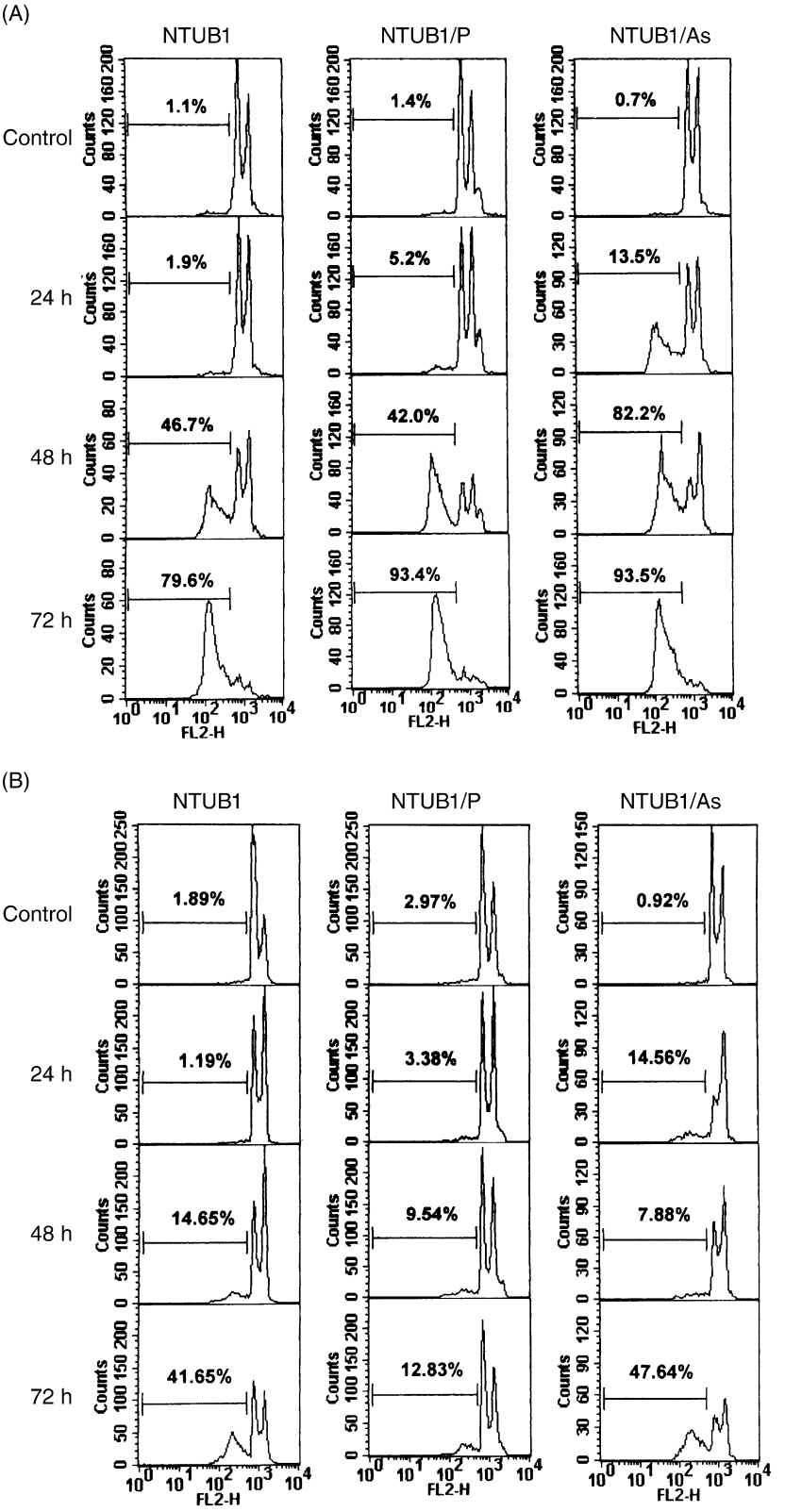


**Figure 2.** Sub-$G_1$ fraction analyses of NTUB1, NTUB1/P and NTUB1/As treated with $As_2O_3$ with (A) or without (B) BSO by using DNA flow cytometry. The sub-$G_1$ fractions that represent the apoptotic populations (given in percentages) increased dramatically over time in all three cell lines, which indicates that $As_2O_3$ exerted its toxic effect through the apoptotic pathway. Moreover, treatments with BSO induced more significant apoptosis than those without.

$As_2O_3$ alone at the respective $IC_{20}$s showed much less cytotoxicity than the combined treatment. BSO was non-toxic to the three cell lines at concentrations up to 100 $\mu$M (data not shown).

### Sub-$G_1$ fraction analysis

With the combination of BSO and $As_2O_3$, the sub-$G_1$ fractions in flow cytometric analyses increased dramatically in all three cell lines, which indicates that combined treatments induced significant apoptosis in both the sensitive and resistant cells (Figure 2A). The apoptotic fractions increased over time (from 24 to 72 h) after $As_2O_3$ exposure. Among the three cell lines, NTUB1/As appeared to be more sensitive to the combined treatments than the others as shown by higher sub-$G_1$ fractions. In contrast, although evident sub-$G_1$ fractions were seen in all three cell lines treated with $As_2O_3$ alone at 72 h, these fractions remained smaller at 48 h of exposure to $As_2O_3$ alone than the combined treatment (Figure 2B).

### Induction of the internucleosomal DNA fragmentation

Combined treatments with $As_2O_3$ and BSO induced evident apoptosis in the three cell lines as shown by the internucleosomal DNA fragmentation or DNA laddering (Figure 3). Combined treatments (Figure 3A) induced significant DNA fragmentation in NTUB1/As at as early as 24 h and in the other two cells at 48 h. The intensity of the DNA laddering appeared to be much weaker with $As_2O_3$ alone (Figure 3B) than that with combined treatments at 48 h. At 72 h, significant DNA laddering can also be seen in the three cell lines treated with $As_2O_3$ alone (data not shown).

### Production of ROS and loss of mitochondrial membrane potential

The relative amount of ROS and mitochondrial membrane potential was determined by flow cytometric analysis. While $As_2O_3$ alone generated only minimal ROS in the three cell lines, the combination of $As_2O_3$ and BSO induced a significant amount of ROS at as early as 18 h (Figure 4). The ROS inductions by the combined treatments were 3.9-, 17.9- and 2.8-fold higher in NTUB1, NTUB1/P and NTUB1/As, respectively, as compared to the controls.

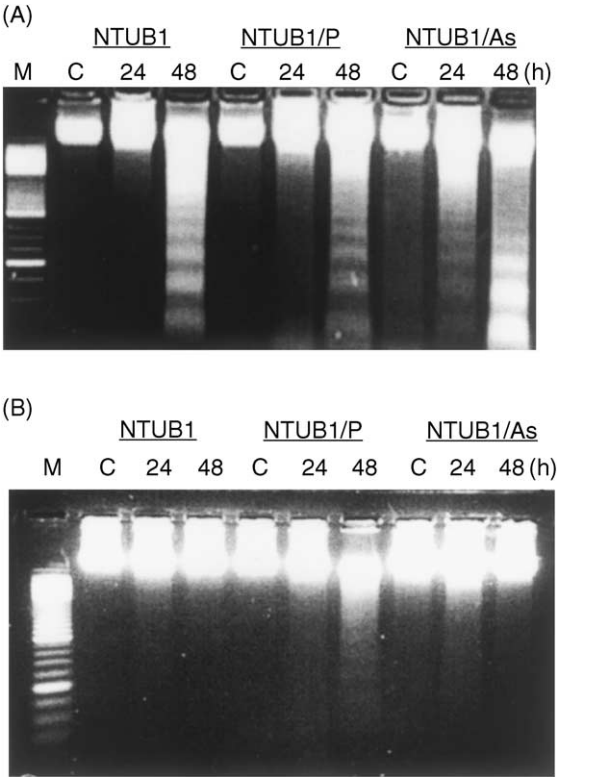


**Figure 3.** Induction of the internucleosomal DNA fragmentation in transitional carcinoma cells by $As_2O_3$ with (A) or without (B) BSO. Concurrent treatment of $As_2O_3$ and BSO for 48 h induced evident apoptotic DNA fragmentation in NTUB1/As at 24 h and in all three cell lines at 48 h, while treatment with $As_2O_3$ alone brought on only minimal DNA fragmentation. M: marker (at 100 bp increments). C: control.

$As_2O_3$ alone was able to reduce the mitochondrial membrane potential at 48 h or more of treatment in the two resistant cells. The reduction of the membrane potential was greater with the combination of $As_2O_3$ and BSO than with $As_2O_3$ alone. The magnitudes of reduction with the combined treatments were 53, 45 and 49% in NTUB1, NTUB1/P and NTUB1/As, respectively, as compared to 1, 22 and 38%, respectively, with $As_2O_3$ alone at 48 h (Figure 5).

### Cellular caspase-3 activation

In the presence of BSO, $As_2O_3$ induced significant caspase-3 activation in all three cell lines in a time-dependent manner (Figure 6). At 48 h, the caspase-3 activity was increased 9.7-, 4.4- and 11.8-fold for NTUB1, NTUB1/P and NTUB1/As, respectively, as compared to the controls (all $p < 0.001$). Among the three cell lines, caspase-3 levels were significantly higher in NTUB1/As than in the other two cells after

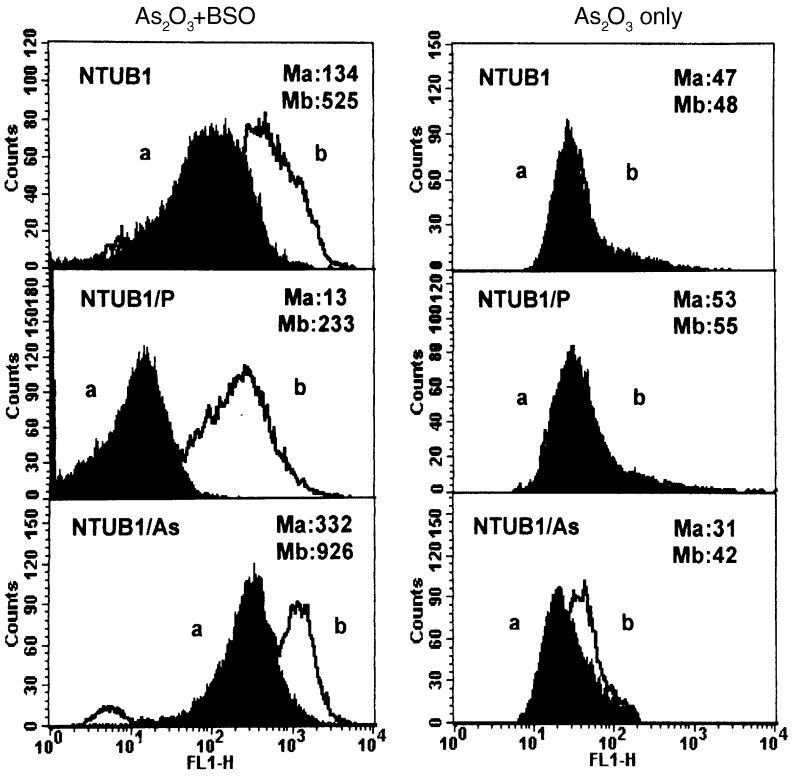


**Figure 4.** Flow cytometric analyses of relative amounts of ROS induced in three transitional carcinoma cell lines by $As_2O_3$ with or without BSO. Solid histograms (a) indicate the controls (treatment with drug-free medium for 18 h) and open histograms indicate treatment with $As_2O_3$ for 18 h. While $As_2O_3$ alone brought on little ROS induction, combined treatments with $As_2O_3$ and BSO induced greater amounts of ROS in all three cell lines. Ma and Mb: the mean fluorescence intensity of histograms a and b, respectively.

activation at 24 and 48 h. These data indicate that $As_2O_3$-mediated apoptosis in the presence of BSO was associated with the activation of the caspase-3 cascade in transitional carcinoma cells.

## Discussion

Although arsenic compounds have been applied clinically in the treatment of APL, little is known about its potential use in transitional carcinomas. Cells exposed to arsenic compounds *in vitro*, typically $As_2O_3$, go through conventional apoptotic pathways that can be found in many tissue systems. Our data showed that $As_2O_3$ also induced apoptosis in transitional carcinoma cells.

Since most regimens of first-line systemic chemotherapy for advanced or metastatic transitional tumors are cisplatin-based, the possible cross-resistance between cisplatin and any second-line chemotherapeutic agents should be taken into consideration before the initiation of salvage treatment. Our data indicated that $As_2O_3$, especially in the presence of BSO, showed significant activity against not only sensitive transitional carcinoma cells but also those resistant to cisplatin. Evident apoptotic events can be readily induced in these resistant cells by the combined treatment of $As_2O_3$ plus BSO. This may warrant further investigations on its role in the salvage therapy for cisplatin-refractory transitional carcinomas.

Accumulating evidence showed that $As_2O_3$-induced apoptosis involves classical pathways that are associated with ROS inductive signals.[12] In brief, $As_2O_3$ elicits ROS production, rapid collapse in mitochondrial membrane potential, release of cytochrome *c*, caspase-3 activation, DNA fragmentation and, finally, morphologic evidence of apoptosis. However, in prostate and ovarian cancer cell models, it was shown that $As_2O_3$-mediated cytotoxicity did not involve superoxide generation.[7] $As_2O_3$-mediated apoptotic pathways have never been explored in transitional carcinoma before. We have shown that the $As_2O_3$-induced apoptosis in transitional carcinoma cells also went through the classical pathway as shown by the appearance of the sub-$G_1$ fraction and

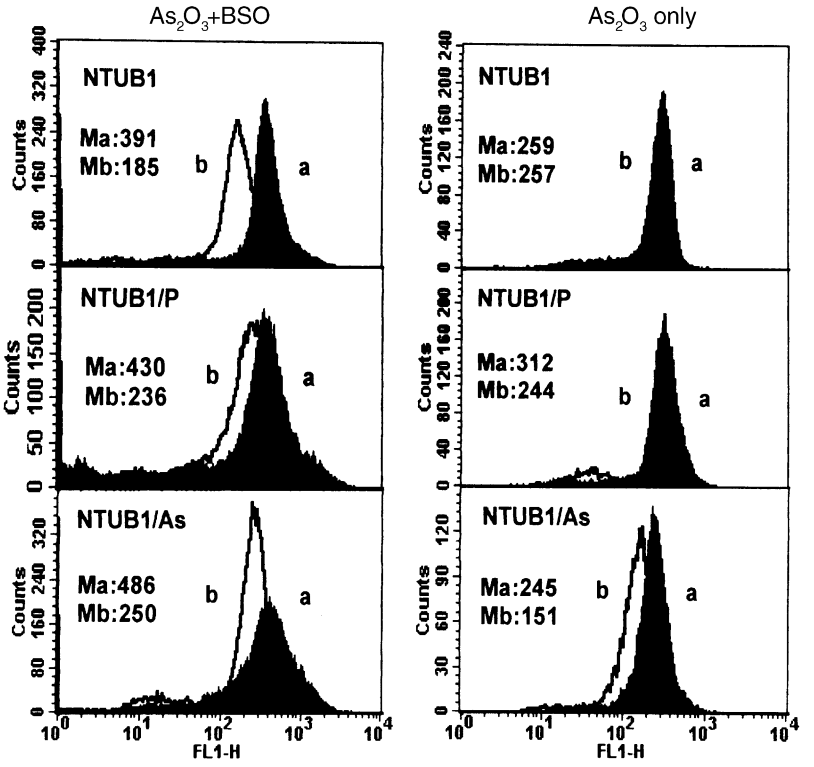


**Figure 5.** Flow cytometric analyses of relative levels of the mitochondrial membrane potential in three transitional carcinoma cells treated with $As_2O_3$ with or without BSO. Solid histograms (a) indicate the controls (treatment with drug-free medium for 48 h) while open histograms indicate treatment with $As_2O_3$ for 48 h. Reduction of the membrane potential was greater with the combined treatments than with $As_2O_3$ alone. Ma and Mb: the mean fluorescence intensity of the histograms a and b, respectively.

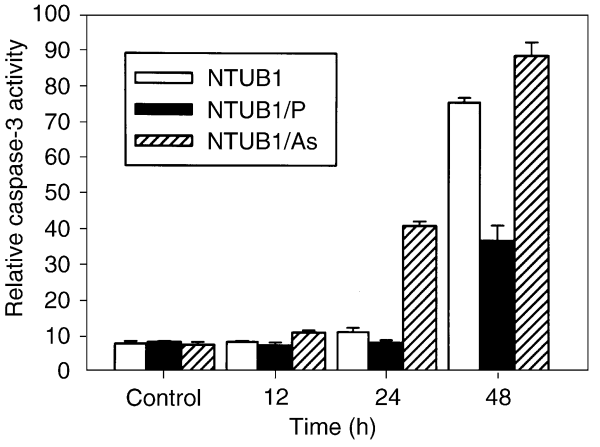


**Figure 6.** Relative caspase-3 activity induced by $As_2O_3$ in the presence of BSO. At 48 h, the caspase-3 activity was increased 9.7-, 4.4- and 11.8-fold for NTUB1, NTUB1/P and NTUB1/As, respectively, as compared to the controls (all $p < 0.001$). Data are presented as mean $\pm$ SEM of three separate experiments.

internucleosomal DNA fragmentation upon exposure to $As_2O_3$. The upstream events included the production of ROS, loss of mitochondrial membrane potential and activation of caspase-3. These events may take place in a sequence as shown here since arsenic-mediated ROS production occurred at as early as 18–24 h, yet the caspase-3 activity was not seen in NTUB1 and NTUB1/P until 48 h, and in NTUB1/As until 24 h after $As_2O_3$ exposure. The apoptotic events can be demonstrated not only in parental cells, but also in cells that are resistant to cisplatin or arsenic. Of note, cells treated with $As_2O_3$ and BSO showed a significantly greater extent of apoptosis than those with $As_2O_3$ alone within the same treatment duration. Since $As_2O_3$-mediated apoptosis is time dependent, all three cell lines would show evident apoptosis with longer exposure to $As_2O_3$ alone. We only used one parental cell line in the present study. Additional studies using more transitional carcinoma cell lines are needed to generalize the notion that transitional carcinoma is sensitive to $As_2O_3$.

In conclusion, $As_2O_3$ may serve as an active agent against human transitional carcinoma. $As_2O_3$ exerts its cytotoxic effect via the conventional apoptotic pathway that involves ROS production, loss of mitochondrial membrane potential, activation of

caspase-3 and internucleosomal DNA breakdown. Our results have clinical implications and represent one of the few efforts to substantiate the clinical use of arsenic compounds in the treatment of human transitional carcinomas.

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