

Tetraarsenic Oxide-mediated Apoptosis in a Cervical Cancer Cell Line, SiHa

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Purpose: Diarsenic oxide, As₂O₃, has been reported to be effective in treating acute leukemia, and induce apoptosis in many tumor cells. In this study, the ability of a novel arsenical compound, As₄O₆ (tetraarsenic oxide), along with As₂O₃, for its ability to induce cell growth inhibition, as well as apoptosis, in human cervical cancer cells, SiHa cells, were evaluated *in vitro*.

Materials and Methods: To examine the levels of apoptosis, SiHa cells were given two sensitive doses, 0.5 and 1 μ M, of arsenical compounds, and a DNA fragmentation assay and FACS analysis were then conducted. In addition, a Western blotting assay was performed to identify target molecules for apoptosis.

Results: Both As₂O₃ and As₄O₆ induced dose-dependent inhibition of SiHa cell proliferation. In particular, As₄O₆ was more effective at suppressing SiHa cell growth than As₂O₃. In parallel with the inhibition of cell proliferation, As₄O₆ caused a signif-

icantly greater increase in the sub-G₁ cell population than As₂O₃, as determined by propidium iodide DNA staining. This was confirmed by a DNA fragmentation assay and annexin V staining. The Western blotting analysis also showed that the expression of proliferating cell nuclear antigen (PCNA) was suppressed to a significantly greater extent by As₄O₆ than As₂O₃ at a dose of 0.5 μ M. However, the apoptosis-related protein, Bax, was expressed to a significantly greater extent due to As₄O₆ than As₂O₃.

Conclusion: Taken together, these findings suggest that a novel arsenic compound, As₄O₆, possesses more potent anti-proliferative effects on human cervical cancer cells, with the induction of apoptosis also, at least via the activation of Bax protein *in vitro*. (Cancer Res Treat. 2005;37:307-312)

Key Words: Tetraarsenic oxide, Cervix neoplasms, Apoptosis

INTRODUCTION

Arsenical compounds are distributed as a natural toxicant, with no color, taste or smell. Diarsenic trioxide, As₂O₃, has been reported to induce almost complete remission of acute promyelocytic leukemia (APL) (1~3). Cytopathological studies have also shown that As₂O₃ induces apoptosis in APL cells.

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Received August 18, 2005, Accepted September 21, 2005

This work was supported by a Korea Research Foundation Grant (KRF-2000-015-FP0047).

Recent reports have shown that As₂O₃ down-regulates the expression of the bcl-2 gene and induces the expression of the apoptosis-related proteins, caspases, as well as the degradation of promyelocytic leukemia (PML) and promyelocytic leukemia gene/retinoic acid receptor (PML/RAR) α proteins in APL cells (2,4). Similarly, arsenic trioxide has been shown to suppress the growth of tumor cells by cell cycle arrest, induction of cyclin-dependent kinase (CDK) inhibitors and apoptosis in a myeloma cell line, MC/CAR (5). Arsenic trioxide also causes cell death through apoptosis in a human leukemia cell line, NB4 (6), human papillomavirus (HPV) 16 infected cervical carcinoma cells (7) and human pancreatic cancer cells (8,9), which collectively suggest that arsenic trioxide has anti-tumor activity in a variety of tumor cells.

Cervical cancer is mostly caused by infection within the high risk HPV group (10~12). After a high risk HPV infection, two viral oncogenic proteins, E6 and E7, play a critical role in the induction of cervical cancers by their interaction with p53 and

retinoblastoma protein (pRb), respectively, for the inactivation of these cellular regulatory proteins (13~15). Presently, surgical, radiation and chemo- therapies have been used as approaches, but with limited success. However, the early detection of cervical cancer using the Pap smear has contributed to reducing the incidence of cervical cancers. However, relapsing cervical cancers have been problematic, adding importance to the development of anti-cervical cancer drugs.

Here, the ability of a new arsenical compound, As_4O_6 (tetra-arsenic oxide), along with As_2O_3 , were evaluated for their ability to suppress cell growth in human cervical carcinoma cells, SiHa cells. As_4O_6 was observed to be more effective at inhibiting SiHa cell growth than As_2O_3 . Furthermore, As_4O_6 caused significantly greater increases in the sub-G1 cell population, DNA ladder formation and annexin V staining than As_2O_3 . Furthermore, Western blotting analysis showed the expression of a cell proliferation marker was more significantly suppressed by As_4O_6 than As_2O_3 . However, the expression of apoptosis-related protein was significantly increased by As_4O_6 than As_2O_3 . Thus, these data suggest that a novel arsenic compound, As_4O_6 , possesses more potent anti-proliferative effects on human cervical cancer cells, also with the induction of apoptosis, *in vitro*, compared to As_2O_3 .

MATERIALS AND METHODS

1) Cell culture

HPV 16, immortalized a human cervical carcinoma cell lines, SiHa cells, containing wild type p53, were incubated at 37°C in DMEM supplemented with 5% fetal bovine serum, 0.37% sodium bicarbonate, 30μM HEPES and 100μg/ml streptomycin/penicillin (cDMEM), in a CO₂ incubator.

2) Chemical reagents

The As_2O_3 was purchased from Sigma and the As_4O_6 (16~19) was provided from Chonjisan, Co., Seoul, Korea. The chemical structures of both As_2O_3 and As_4O_6 are shown in Fig. 1. These chemicals were diluted in phosphate-buffered saline (PBS), to a final concentration of 10⁻³ M, and kept at 4°C. The

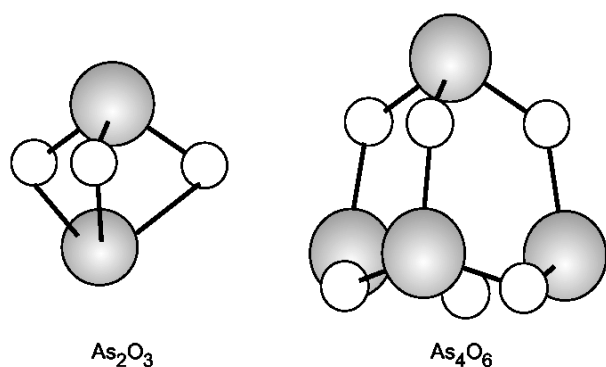


Fig. 1. The chemical structures of As_2O_3 and As_4O_6 . The shaded bigger circles and empty smaller circles represent As and O, respectively.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma, which was dissolved in PBS to a final concentration of 5 mg/ml.

3) Cell growth inhibition assay

As a cell growth inhibition assay, the MTT assay was performed. First, SiHa cells (1×10^3 cells/well) were divided into 96 well plates in 100μl of cDMEM. After 24 h incubation, the cells were treated with different amounts of the two arsenic compounds. After 4 days incubation, 20μl of MTT solution was added to each well of the plates, which were further incubated for 4 h at 37°C. The cell media were replaced with 100μl of dimethyl sulfoxide (Sigma) per well. The plate was shaken for 10 sec and the optical density (OD) then measured at 570 nm, using an enzyme-linked immunosorbent assay-Reader (Spectromax 250, Molecular Devices). The growth inhibition rate (%) was calculated as follows: OD of non-treatment-OD of drug treatment/OD of non-treatment $\times 100$.

4) DNA fragmentation assay

SiHa cells were divided into 5×10^5 cells per 100 mm dish plate. After 24 h incubation, different amounts of arsenic compounds were added to the cells, which were further incubated for 48 h at 37°C. The cells were then centrifuged, with lysis buffer [0.8% SDS, 0.1 M NaCl, 0.1 M EDTA, 50μM Tris-HCl (pH8.0)] added to the subsequent cell pellets, followed by the addition of 20μg/ml proteinase K (Sigma) and further incubated for 4 h at 56°C. DNA was extracted by phenol/chloroform treatment. Five mg of extracted DNA was analyzed on a 2% agarose gel containing ethidium bromide (0.1μg/ml). The DNA ladder formation was visualized under UV light.

5) FACS analysis

Cells were washed twice with PBS and then resuspended in 1 \times binding buffer (10μM HEPES/NaOH, pH 7.4, 140μM NaCl, 2.5μM CaCl₂). 5μl of Annexin V-FITC and 10 ml of propidium iodide (BD, San Jose, CA) were added to 1×10^5 cells per tube, followed by incubation at 22°C for 15 min. 100 μl of 1 \times binding buffer was added to each tube, and the cells then analyzed by flow cytometry (BD). To analyze for the DNA contents, ethanol-fixed cells were incubated with RNase A (10 mg/ml) and propidium iodide (400μg/ml), and then shaken for 1 h at 37°C in the dark. The samples were read using flow cytometry (BD). The cell debris and fixation artifacts were gated out, and the G0/G1, S and G2/M populations were quantified using the CellQuest program.

6) Western blotting analysis

SiHa Cells were treated with 0.5 and 1μM of As_2O_3 and As_4O_6 for 48 h. The cell lysates (approximately 30μg of protein) was separated on 12% polyacrylamide SDS-gels and transferred to the nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membrane was then immersed in blocking buffer (5% skimmed milk and 0.1% Tween 20 in PBS, pH 7.4) for 1 h, at room temperature, and then incubated overnight with the primary antibodies (SantaCruz Biotechnology, Inc., Santa Cruz, CA), PCNA (1 : 200), CDK4 (1 : 200), p21 (1 : 200), Bax (1 : 200), Bcl-XL/Bcl-XS (1 : 500) and actin (1 : 5000), prepared in blocking buffer at 4°C. After

incubation, the membrane was probed with the horseradish peroxidase-labeled anti-mouse IgG antibody (1 : 5000), prepared in PBS (containing of 0.05% Tween 20 and 5% skim milk powder), for 30 min at room temperature. The proteins bands in the membrane were detected using an enhanced chemiluminescence detection system (Amersham, Buckinghamshire, UK), and visualized using autoradiography with an X-ray film (Amersham).

RESULTS

1) As_4O_6 inhibited SiHa cell growth to a more significant level than As_2O_3

To investigate whether a new arsenical compound, As_4O_6 , could suppress the growth of human cervical cancer cells, SiHa cells were treated with increasing amounts (0.5~5 μM) of As_4O_6 for 48 h. As a positive control, cells were also treated

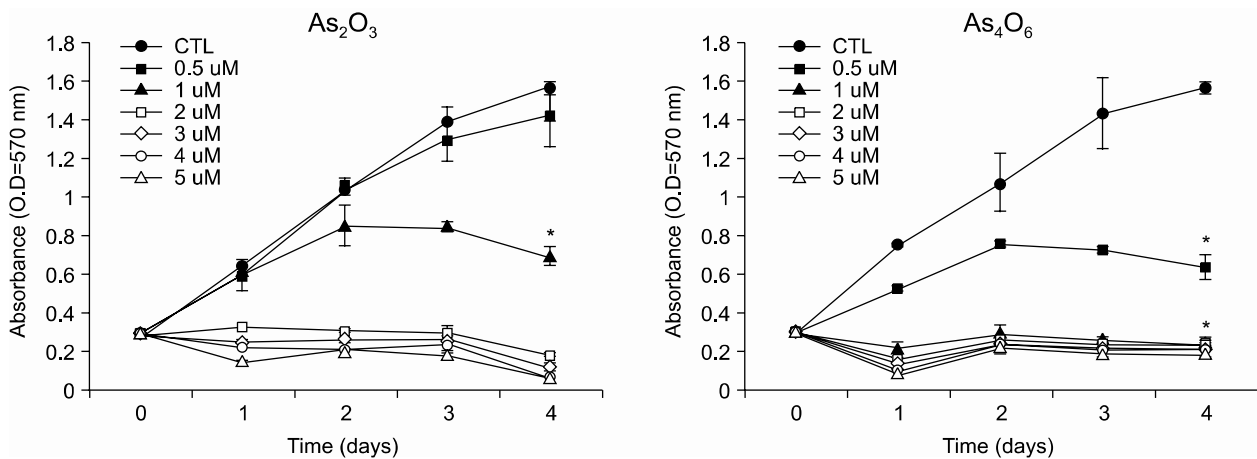


Fig. 2. Growth inhibition patterns of As_2O_3 and As_4O_6 in SiHa cells *in vitro*. Cells were treated with the indicated amount of the two arsenic compounds, As_2O_3 and As_4O_6 , over 4 day incubation periods. Cell growth suppression was measured as described in "Methods and Materials." OD was measured at 570 nm. The assay was performed in triplicate, with the average OD values and SD recorded, which was repeated twice more, with similar results. *Statistically significant at $p < 0.05$, using the paired Student's *t*-test, compared to no drug treatment (control, CTL).

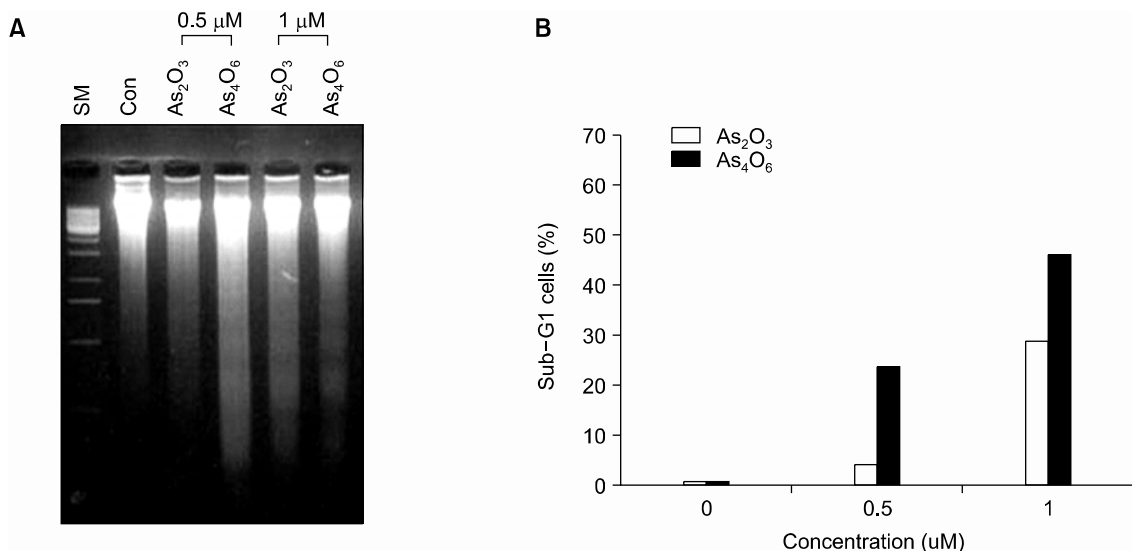


Fig. 3. Induction of DNA ladder formation (A) and sub-G1 cell population (B) in SiHa cells due to treatment with As_2O_3 and As_4O_6 . Cells were treated with 0.5 and 1 μM of As_2O_3 or As_4O_6 for 48 h. (A) DNA was analyzed on a 2% agarose gel and photographed under UV light (Con: Control, SM: Size marker). (B) Cells were stained with propidium iodide, and analyzed using flow cytometer, for the detection of the sub-G1 population.

with As_2O_3 . As shown in Fig. 2, the growth of SiHa cells was unaffected by their treatment with $0.5\mu\text{M}$ As_2O_3 . However, $1\mu\text{M}$ As_2O_3 showed approximately 50% cell growth inhibition, while As_2O_3 , ranging from 2 to $5\mu\text{M}$, displayed complete growth inhibition of the cancer cells. With As_4O_6 , $0.5\mu\text{M}$ displayed approximately 50% growth inhibition of the cancer cells, but within the range 1 to $5\mu\text{M}$ showed complete growth inhibition of the cancer cells. This illustrates that As_4O_6 is more effective than As_2O_3 at inhibiting the growth of human cervical cancer cells *in vitro*.

2) As_4O_6 induced apoptosis more significant than As_2O_3 in SiHa cells

Next, the levels of apoptosis due to the addition of the two most sensitive doses, 0.5 and $1\mu\text{M}$, of the arsenical compounds (As_2O_3 and As_4O_6) were examined. As shown in Fig. 3A, As_4O_6 induced DNA ladder formation at $0.5\mu\text{M}$, indicative of apoptosis. However, few DNA ladders were formed on the addition of $0.5\mu\text{M}$ As_2O_3 . This suggests that As_4O_6 is more effective for the induction of apoptosis compared to As_2O_3 . This pattern of apoptosis was confirmed by flow cytometry (Fig. 3B). In particular, As_4O_6 displayed 25% sub-G1 cell

populations at $0.5\mu\text{M}$. However, few sub-G1 cell populations were observed on the addition of $0.5\mu\text{M}$ As_2O_3 . Similarly, $1\mu\text{M}$ of each As_2O_3 and As_4O_6 showed 30 and 50% sub-G1 cell populations, respectively. Therefore, this data confirms that As_4O_6 is more potent at inducing apoptosis in SiHa cells.

3) As_4O_6 induced more early and late apoptotic cell populations in SiHa cells, as compared to As_2O_3

Next, the different apoptotic cell populations induced by these two drugs were counted. As shown in Fig. 4, the propidium iodide (+)/Annexin V (+) double positive cell populations (late apoptotic group) were 3.7, 3.7 and 4.8% at 0.0, 0.5 and $1\mu\text{M}$ As_2O_3 , respectively. The propidium iodide (-)/Annexin V (+) cell populations (early apoptotic group) were 1.4, 1.9 and 4.4% at 0.0, 0.5 and $1\mu\text{M}$ As_2O_3 , respectively. However, the propidium iodide (+)/Annexin V (+) double positive cell populations were 3.7, 8.0 and 11.5% at 0.0, 0.5 and $1\mu\text{M}$ As_4O_6 , respectively. Similarly, the propidium iodide (-)/Annexin V (+) cell populations were 1.4, 2.5 and 8.1% at 0.0, 0.5 and $1\mu\text{M}$ As_4O_6 , respectively. This shows that As_4O_6 induces greater increases in early and late apoptotic cells compared to As_2O_3 .

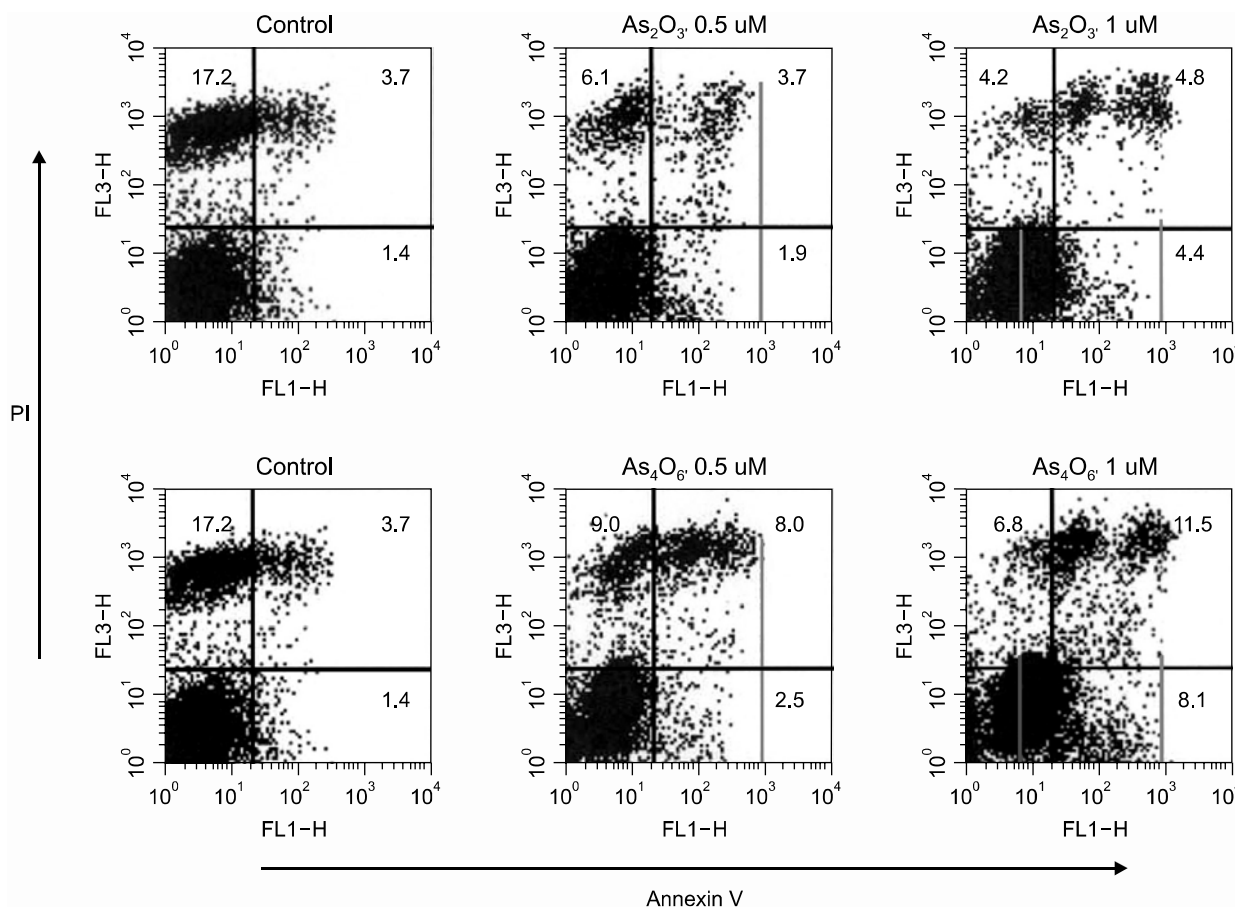


Fig. 4. The induction of early and late apoptotic cells in SiHa cells due to treatment with As_2O_3 and As_4O_6 . Cells were treated with 0.5 and $1\mu\text{M}$ of As_2O_3 or As_4O_6 for 48 h. Cells were stained with both annexin V-FITC and propidium iodide, and then analyzed for different apoptotic cell populations using flow cytometry.

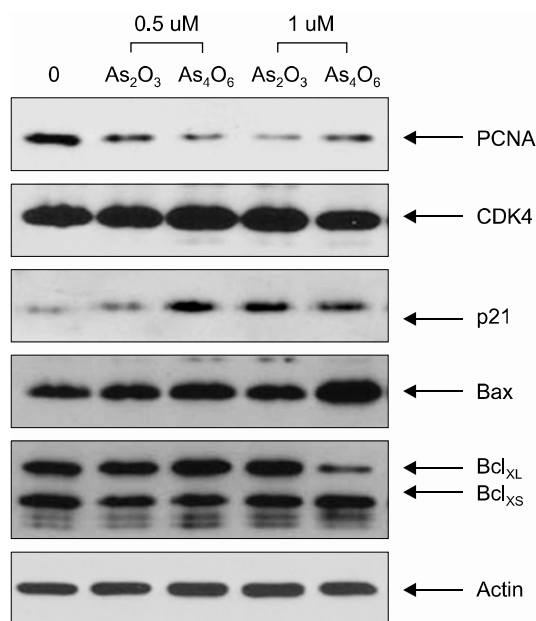


Fig. 5. Western blots of the cell proliferation marker and apoptosis-related proteins in SiHa cells due to treatment with As_2O_3 and As_4O_6 . Cells were treated with 0.5 and $1\mu\text{M}$ of As_2O_3 or As_4O_6 for 48 h. The cells were harvested and the cell lysates run on 12% SDS-PAGE. Subsequent protein bands were transblotted onto a nitrocellulose membrane for an immunoblot assay.

4) Comparison of expression of cell growth- and apoptosis-related proteins by As_2O_3 and As_4O_6 in SiHa cells

To compare the anti-growth effects induced by As_2O_3 and As_4O_6 at the cell proliferation- and apoptosis-related protein levels, the cells were treated with the two arsenical compounds at 0.5 and $1\mu\text{M}$. As shown in Fig. 5, the expression of the cell proliferation marker, PCNA, was decreased by both arsenic compounds, whereas those of the apoptosis-related proteins, Bax and p21, were conversely increased. In particular, As_4O_6 was found to significantly inhibit the expressions of PCNA and Bcl_{XL} compared to As_2O_3 at $0.5\mu\text{M}$ and $1\mu\text{M}$, respectively. Similarly, the expressions of Bax and p21 were significantly increased by As_4O_6 compared to As_2O_3 at $1\mu\text{M}$ and $0.5\mu\text{M}$, respectively. However, the expressions of CDK4, Bcl_{XS} and actin were unaffected by the two arsenic compounds. This illustrates that As_4O_6 can induce apoptosis, at least via the activation of the apoptosis-related protein, Bax, to a significantly greater level compared to As_2O_3 .

DISCUSSION

The anti-tumor functions of the arsenic compound, As_2O_3 , have been reported in leukemia both *in vivo* and *in vitro* (1~4). Here, As_2O_3 was also observed to induce apoptosis and cell growth inhibition in SiHa cell lines. This supports the previous findings where arsenic trioxide was found to induces anti-tumor

effects via the induction of tumor cell apoptosis (1~5, 8, 9). In the case of promyeloleukemic cells, As_2O_3 has been found to down-regulate the expressions of bcl-2 and PML/RAR α /PML proteins, which correlated with apoptosis (2,4). As_2O_3 also induces apoptosis in human pancreatic cancer cells, via changes in the cell cycle and caspase activation (8,9). Dai et al. also reported that the glutathione redox system was associated with apoptosis due to As_2O_3 (20). In one article, As_2O_3 was reported to inhibit cell growth and induce apoptosis in different cancers, including leukemia, myeloma, lymphoma and other solid tumors (21), suggesting apoptosis is a likely mechanism of arsenic trioxide in the suppression of tumor cell growth both *in vivo* and *in vitro*.

Herein, As_4O_6 was observed to be more effective at suppressing SiHa cells proliferation *in vitro* compared to As_2O_3 . In particular, As_4O_6 showed significant anti-growth activity (approximately 50% cell growth inhibition) at a dose of $0.5\mu\text{M}$, but As_2O_3 , at this concentration displayed few anti-growth effects. However, $1\mu\text{M}$ As_2O_3 showed approximately 50% growth inhibition, which was consistent with our observation, where at a dose of $0.5\mu\text{M}$ both DNA fragments and a sub-G1 cell population were significantly generated by As_4O_6 compared to As_2O_3 . This is in line with the greater induction of early and late apoptotic cells observed in this study due to As_4O_6 . These data support the notion that As_4O_6 could be a more potent drug against cervical cancer cell growth via apoptosis. The dose effects of arsenic trioxide on growth inhibition were consistent with many previous reports (5,7). The expression of the cell proliferation marker, PCNA (22), was also observed to be decreased by the two arsenic compounds. In particular, As_4O_6 significantly inhibited the expression of PCNA expression compared to As_2O_3 . This is in line with our observation where As_4O_6 was shown to significantly inhibit the growth of cancer cells compared to As_2O_3 . Furthermore, the expressions of the apoptosis-related proteins, Bax (23) and p21 (24), were increased by both arsenic compounds. In particular, the expression of Bax was significantly increased by As_4O_6 compared to As_2O_3 . This correlates well with our observation that As_4O_6 significantly induced apoptosis in SiHa cells compared to As_2O_3 , which is also compatible with the previous finding where As_2O_3 increased the expressions of the p21 and Bax proteins in human pancreatic cancer cells (9). However, the expressions of CDK4, Bcl_{XS} and actin were unaffected by the two arsenic compounds. Thus, these data suggest that As_4O_6 mediates apoptosis, at least via the activation of the Bax pathway, in human cervical cancer cells, SiHa cells. Conversely, our thinking was that the Bcl_{XL} (25) and p21 proteins were more sensitive to changes in the concentrations of arsenic compounds.

CONCLUSIONS

The data presented in this study suggest that tetraarsenic oxide, As_4O_6 , is more effective at suppressing the growth of SiHa cell growth compared to As_2O_3 . In parallel with the inhibition of cell proliferation, As_4O_6 caused significant increases in the sub-G1 cell population, DNA ladder formation and annexin V staining compared to As_2O_3 . Furthermore, the Western blotting analysis showed the expression of the apop-

tosis-related protein, Bax, was significantly increased by As_4O_6 compared to As_2O_3 . Taken together, these findings suggest that a novel arsenic compound, As_4O_6 , possesses more potent anti-growth effects toward human cervical cancer cells, with the induction of apoptosis *in vitro*, which might provide a new drug choice in the treatment of HPV-associated cervical cancer.

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