Abstract. As$_2$O$_3$ has been reported to induce apoptosis and inhibit the proliferation of various human cancer cells. We evaluated the ability of a novel arsenic compound, As$_4$O$_6$, along with As$_2$O$_3$ in vitro and in vivo. To examine the levels of apoptosis of HPV 16-positive SiHa cervical cancer cell, flow cytometry and Western blotting were employed at various time intervals after two arsenic compound treatments. Ingenuity Pathway Analysis (IPA) was applied to investigate the differential cell death pathway of As$_4$O$_6$ and As$_2$O$_3$. The results showed that As$_4$O$_6$ was more effective in suppressing SiHa cell growth in vitro and in vivo compared to As$_2$O$_3$. In addition, the cell cycle was arrested at the sub-G$_1$ phase by As$_4$O$_6$. Western blot analysis showed that the proliferating cell nuclear antigen (PCNA) and Bcl-X$_L$ with sequence homology to Bcl-2 were significantly suppressed by As$_4$O$_6$. However, the apoptosis-related proteins such as p21 and Bax were overexpressed by As$_4$O$_6$. IPA suggested that there is a significant difference between As$_2$O$_3$- and As$_4$O$_6$-induced cell death pathways. Taken together, As$_4$O$_6$ has a specific cell death pathway and possesses more potent anti-tumor effects on human cervical cancer cells in vitro and in vivo.

Introduction

Arsenical compounds As$_2$O$_3$ and As$_4$O$_6$ have been demonstrated to possess life-preserving qualities in cancer treatment. Promising results with patients were reported showing that diarsenic oxide (As$_2$O$_3$) treatment could offer an alternative to chemotherapy for acute promyelocytic leukemia (APL) (1-3). Cytopathological studies showed induction of apoptosis in APL cells. Recent reports suggested that arsenical compounds inhibit the proliferation of human umbilical vein endothelial cells (HUVEC) via G$_1$ and G$_2$/M phase arrest of the cell cycle. In addition, these inhibitory effects on bFGF- or VEGF-stimulated cell proliferation suggest antiangiogenic potential of these arsenical compounds (4). It has been reported that diarsenic oxide suppresses 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). Diarsenic oxide also causes cell death through apoptosis in a human leukemia cell line, NB4 (6), a human papillomavirus (HPV) 16 infected cervical carcinoma cells (7), and a human pancreatic cancer cells (8). On the other hand, tetra-arsenic oxide (As$_4$O$_6$) was reported to have antiangiogenic effects on the new vessels induced by NGF in the rat cornea compared to control group and As$_2$O$_3$ group (9). It has been suggested that As$_4$O$_6$ might be a new arsenic compound as it induced apoptosis in U937 leukemic cells at much lower concentration than As$_2$O$_3$ (10). However, attempts to establish the efficacy of its anticancer activity in vitro and in vivo are technically challenging.

Human papillomaviruses (HPV) have been consistently implicated in causing cervical cancer. Especially high-risk types (HPV 16, 18, 31, 45) have been strongly associated with cervical cancer (11,12). Surgical, radiation, chemotherapies have had only limited success. Also, relapsing cervical cancers are problematic, adding importance to developing anti-cervical cancer drugs.

Here we evaluated the ability of As$_4$O$_6$ along with As$_2$O$_3$ to suppress cell growth in HPV 16-positive SiHa human cervical cancer cells. We observe that As$_4$O$_6$ is more effective in inhibiting the SiHa cell growth in vitro and in vivo compared to As$_2$O$_3$. In addition, there is a significant difference in
functional profiles between As$_2$O$_3$ and As$_4$O$_6$-induced cell cycle and cell death pathways. Thus, these data suggest that a novel arsenic compound, As$_4$O$_6$, possesses more potent anti-tumor effects on human cervical cancer cells in vitro and in vivo compared to As$_2$O$_3$.

Materials and methods

Cell culture. SiHa HPV 16-immortalized human cervical carcinoma cells were incubated in DMEM supplemented with 5% fetal bovine serum, 0.37% sodium bicarbonate, 30 mM HEPES, and 100 μg/ml streptomycin/penicillin (cDMEM) at 37°C in a CO$_2$ incubator.

Chemical reagents. As$_2$O$_3$ was purchased from Sigma (St. Louis, MO). As$_4$O$_6$ was provided from Chonjisan Co. (Seoul, Korea). These chemicals were diluted in phosphate-buffered saline (PBS) to a final concentration of 10$^{-3}$ M and kept at 4°C. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma and dissolved in PBS at a final concentration of 5 mg/ml.

FACS analysis. Cells were washed twice with PBS and then resuspended in 1X binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl$_2$). Per tube 1x10$^5$ cells were added with 5 μl of Annexin V-FITC and 10 μl of propidium iodide (PI). As shown in Fig. 1, the cell death significantly increased after arsenic induced by these two compounds by double staining the SiHa cells with annexin V and propidium iodide (PI). As reported previously in cDNA microarray analyses (13), the 108 genes that consistently displayed altered expression patterns in both arsenic compounds were newly analyzed using Ingenuity Pathway Analysis to identify how the transcripts identified by the gene expression signature are related to the cell death signaling pathways. The functional analysis was carried out as follows. Each gene was annotated by integrating the information on the Gene Ontology website (http://GenMAPP.org). First, each gene was associated with its corresponding current curated gene entry in UniGene (http://www.ncbi.nlm.nih.gov). Next, the Ingenuity Pathway Analysis software (IPA, Ingenuity Systems, Mountain View, CA) was utilized to identify networks of interacting genes and other functional groups. Semantically consistent pathway relationships are modeled based on a continual, formal extraction from the public domain literature and cover more than 10,300 human genes (www.ingenuity.com/products/pathways_knowledge.html). These genes were then used as a starting point for generating biologic networks. The resulting networks were represented in graphic format. The files, including results of the Ingenuity are available from our anonymous FTP site: ftp://160.1.9.42/work/arsenicIPA/

Statistical analysis. Statistical analysis was done using the paired Student’s t-test and ANOVA. Values between different groups were compared. A P<0.05 was considered significant.

Results

As$_4$O$_6$ induced more early and late apoptotic cell populations in SiHa cells. We counted different apoptotic cell populations induced by these two compounds by double staining the SiHa cells with annexin V and propidium iodide (PI). As shown in Fig. 1, the cell death significantly increased after arsenic compound treatment in the SiHa cells. Double positive cell populations (late apoptotic group) were 3.7, 3.7 and 4.8% at 0, 0.5 and 1 μM of As$_2$O$_3$, respectively. However, double positive cell populations were 1.4, 1.9 and 4.4% at 0, 0.5 and 1 μM of As$_4$O$_6$, respectively. Similarly, early apoptotic cell populations were 3.7, 3.7 and 4.8% at 0, 0.5 and 1 μM of As$_2$O$_3$, respectively. However, double positive cell populations were 1.4, 1.9 and 4.4% at 0, 0.5 and 1 μM of As$_4$O$_6$, respectively. On the other hand, lower sensitivity to As$_2$O$_3$ was shown in the SiHa cells compared to As$_4$O$_6$. This shows that As$_4$O$_6$ induced more early and late apoptotic cell populations compared to As$_2$O$_3$.

As$_4$O$_6$ induced apoptosis more significantly than As$_2$O$_3$. We were next interested in examining the levels of apoptosis achieved by addition of two most sensitive doses, 0.5 and...
1 μM of arsenical compounds (As₂O₃ and As₄O₆). As shown in Fig. 2, the apoptosis pattern was confirmed by flow cytometry. In particular, As₄O₆ displayed 25% sub-G₁ cell populations at 0.5 μM. However, little sub-G₁ cell populations were observed by 0.5 μM of As₂O₃. Similarly, 1 μM of As₂O₃ and As₄O₆ showed 30 and 50% sub-G₁ cell populations, respectively. Therefore, the data confirm that As₄O₆ significantly induced the levels of apoptosis.

Anti-tumor effects of As₄O₆ in mice. To investigate the anti-tumor effect of arsenic compounds in vivo, we treated As₄O₆ with the SiHa cell-xenografted nude mice and then measured each tumor for one month. As shown in Fig. 3, the tumor size increased almost linearly with time in the control group. On the other hand, in the case of As₂O₃ treatment, the tumor size was decreased significantly compared to As₂O₆ and control. It is, however, notable that the levels of tumor growth inhibition of As₂O₃ were not similar with the case of As₄O₆. No cytotoxicity was observed (after 20 days post treatment) with As₂O₃ treatment, followed by a rapid increase until the end of the observation period.

Comparison of expression of apoptosis-related proteins by As₂O₃ and As₄O₆. To compare anti-growth effects induced by As₂O₃ and As₄O₆ at the levels of cell proliferation- and apoptosis-related proteins, Western blot analysis was performed after treatment with arsenic compounds at 0.5 and 1 μM. As shown in Fig. 4, the expression of the cell
Figure 5. Network mapping of genes with >2-fold expression change using Ingenuity Pathway Analysis (IPA). At least 8 genes in the network are involved in the cell death pathway, including PCNA, CCNB1, PARP1, MCM2, CDK4, TP53, ERIIB2 and BCL2. Each was differentially regulated by As$_4$O$_6$ and As$_2$O$_3$, respectively. Nodes represent genes, with their shapes representing the functional classes of the gene products, and edges indicate the biologic relationships between the nodes, which include physical and functional interactions. Nodes are color-coded according to their expression levels (red, under-expression; green, over-expression). The fold change of the regulated gene is displayed under the corresponding node. (A) Cell death pathway by As$_2$O$_3$ treatment and (B) by As$_4$O$_6$ treatment.
proliferation marker (PCNA) was down-regulated by these compounds. And the anti-apoptotic protein (Bcl-XL) was only down-regulated at 1 μM of As4O6 compared to control. In contrast, the expression of apoptosis-related proteins (Bax and p21) was up-regulated compared to the control. Note that As4O6 inhibited PCNA and Bcl-XL expression significantly compared to As2O3 at 0.5 and 1 μM, respectively. Similarly, Bax and p21 expression was significantly increased by As4O6. However, expression of CDK4 and Bcl-XL was continually expressed by these two arsenic compounds. Taken together, As4O6 can induce apoptosis through activation of Bax and p21 to a more significant level than As2O3.

**Pathway identification.** We first placed the transcripts in the context of the present interactome knowledge using Ingenuity Pathways Analysis tools. Most of these genes were classified into multiple categories and pathways by the software and the Gene Ontology (P for all <0.01). In the case of As4O6 treatment, the main functional networks containing the most statistically robust candidates included the cell death of lymphoblastoid cells (P=0.000013), accumulation of cervical cancer cells (P=0.000104), G1 phase of tumor cells (P=0.00035), survival of tumor cells (P=0.000504), and transformation of fibroblasts (P=0.000557). The genes (13 of 19) encoding for apoptosis function were down-regulated (P=0.00043; CHEK1, CSK, MAP4K1, MCM2, MPL, MSH2, NPM1, PARP1, PCNA, PTN, SLK, SPARC, TYMS/ATXN2, CD36, DUSP1, HES1, SERINC3, VAV1). Also, the genes (11 of 13) encoding for cell cycle progression were down-regulated (P=0.000069; down-regulation of CDK4, CHEK1, CSK, MCM2, MPL, MSH2, NPM1, PCNA, PPM1G, PPP2R5C, SPARC/up-regulation of DUSP1, VAV1). In contrast, in the case of As2O3 treatment, the functional networks included arrest in G2 phase of fibroblasts (P=0.000004), ploidy of tumor cells (P=0.0031), and cell cycle progression (P=0.000041). The genes (9 of 12) encoding for apoptosis function were down-regulated (P=0.00167; MAP4K1, MCM2, MPL, MSH2, NPM1, PARP1, PCNA, SLK, TYMS/TP53, DUSP1, ERBB2). Also, the genes (7 of 10) encoding for cell cycle progression were down-regulated (P=0.000041; GPS1, MCM2, MPL, MSH2, NPM1, PCNA, PPM1G/TP53, DUSP1, ERBB2). The results suggested differential expression patterns of these gene products for the cell death pathway.

We searched the apoptosis-related transcript expression patterns for interaction of additional members of these functional networks. The results of the main network reconstruction by the highest Ingenuity score are shown in Fig. 5. In the case of As4O6 treatment (Fig. 5A), PCNA, PARP1, BCL2 and CCNB1 central nodes were among the genes constituting the cell death network and were significantly down-regulated at the level of transcription compared to significant up-regulation of P53 and ERBB2. In the case of As2O3 treatment (Fig. 5B), PCNA, PARP1, CCNB1, MCM2 and CDK4 were significantly down-regulated in the cell death network. In contrast, there is no comparable expression of p53 and ERBB2. The results showed that there is a significant difference in functional networks between As4O6- and As2O3-associated cell cycle and death pathways.

**Discussion.**

As4O6 has been reported to induce apoptosis and inhibit the proliferation of various human cancer cells derived from solid tumors as well as hematopoietic malignancies (8,14). This supports previous findings that diarsenic oxide induces anti-tumor effects through induction of tumor cell apoptosis (1-3). On the other hand, As4O6 has been reported as a novel antiangiogenesis and antimetastasis chemical agent (5). In this study, the results showed that As4O6 was more effective for suppressing the SiHa cell proliferation in vitro and in vivo compared to As2O3. We also injected As4O6 into mice along with As2O3 to compare their antitumor effects in mice. As there is no good animal model for inorganic arsenic-related human cancer, we used 10 μg/gBW arsenic doses, higher than the physiological criteria in dietary intakes of inorganic arsenic compounds (120 μg/day females and 214 μg/day males) (15). The dose effects of diarsenic oxide on tumor growth inhibition are consistent with many previous reports (16,17). Also, with these doses, it has been reported that no acute toxicity or effect on the body or organ weight of the mice was observed (16).

Anti-proliferative effect of As4O6 on tumor cells was accomplished by inhibition of cell cycle progression at the G1 phase, G2/M phase or both (14,18,19). In the case of As4O6, it has been reported that induction of cell cycle arrest at G1/M phase and inhibition of MMP-2 secretion were possible antiangiogenic mechanisms. This difference might be because of the differential role of the cell-specific apoptotic cell death. We reported the effect of cell-specific p53 adenosinergic vector on cervical cancer cells in vitro and in vivo, suggesting that for successful medical treatment of cervical cancer, understanding of the molecular-level of cell-specific growth suppression effects is required (20). In this study, however, the main concern with the study design is that only a single cell line was deployed both in the in vitro and in the animal model. First, in order to validate our experimental approach, these findings should be duplicated in other cervical cancer cell lines and compared with previously reported results. Also, an improved strategy for anti-cancer effect of As4O6 depending on the cancer cell-dependent pathway should be studied.

In the case of promyeloleukemic cells, As4O6 down-regulates the expression of bcl-2 and PML/RARα/PML proteins which are correlated with apoptosis (2). Also, As4O6 induces apoptosis in human pancreatic cancer cells through changes in cell cycle, caspase activation and glutathione redox system (8,21,22). In the case of cervical cancer cells, however, it has been reported that expression of human bcl-2 protein expression does not occur in the SiHa cells and induction (or repression) of the bcl-2 protein causes no change in the survival of HeLaS3 cells (23,24). Though, As4O6 is a potent antitumor agent, its precise mechanisms remain unclear. Recently, it has been reported that As4O6 inhibited the proliferation of human umbilical vein endothelial cells (HUVEC) by degradation of cyclin A, CDC2, and CDC25C. It could also inactivate CDK2 and CDK1 activities, suggesting that apoptosis is a likely mechanism of tetraarsenic oxide suppression of tumor cell growth in vitro and in vivo.
In this study, we observed that the expression of cell proliferation marker, PCNA, and the anti-apoptotic protein, Bcl-X<sub>1</sub>, was decreased by As,<sub>4</sub>O<sub>6</sub>. This is consistent with previously reported results (25,26). PCNA is known to associate with DNA repair processes. Thus, the level of PCNA is known to correlate with DNA repair activity (27). In particular, As,<sub>4</sub>O<sub>6</sub> inhibited PCNA and Bcl-X<sub>1</sub> expression. Also, the expression of apoptosis-related proteins, Bax and p21 (25,28), was significantly increased by As,<sub>4</sub>O<sub>6</sub> compared to As,<sub>2</sub>O<sub>3</sub>. This correlates well with our observation that As,<sub>4</sub>O<sub>6</sub> significantly induced apoptosis in the SiHa cells, in vivo and in vitro.

With the cDNA microarray expression with Ingenuity Pathway Analysis, the results showed that there is a significant difference in functional networks between As,<sub>2</sub>O<sub>3</sub>- and As,<sub>4</sub>O<sub>6</sub>-induced cell death pathways (P<0.01). Especially, as central nodes, proliferation markers MCM2 and CDK4 (cyclin-dependent kinase 4), which play an important role in onset of DNA replication and cell division were differentially down-regulated in the case of As,<sub>4</sub>O<sub>6</sub> treatment compared to As,<sub>2</sub>O<sub>3</sub> treatment. It has been reported that MCM2 expression was present only in normal and some reactive tissues, such as the uterine cervix (29). Also MCM2 became a candidate for an oncogene affected by chromosomal breaks in acute myeloid leukemia (AML) (30). It has been reported that the expression of CDK4 is essential for Ras-induced cancer development, regardless of p53 status or the presence of another frequently mutated tumor suppressor gene, Ink4a/Arf (31). CDK4 suppression has been suggested as a potential therapeutic tool to combat the ~30% of human tumors in which the Ras oncogene is activated. It has been accepted that genes are mutated in a large number of human cancers. For example, the Ras oncogene is activated in roughly one third of all human tumors, while the p53 tumor suppressor gene is inactivated in half. In light of the frequency with which the Ras oncogene is expressed and the p53 tumour suppressor pathway is disabled in human tumors, the suppression of CDK4 activity was suggested as an alternate point of entry to regulate the cell growth cycle and halt tumorigenesis (32).

In the case of As,<sub>4</sub>O<sub>6</sub> treatment, as a central node, the proliferation marker ERBB2 was differentially up-regulated. The ERBB2 protein is a member of a very important group of proteins called receptor tyrosine kinases. During the past decade the role of the ERBB2 oncogene as an important predictor of patient outcome and response to various therapies in breast cancer has been clearly established (33,34). Thus, As,<sub>4</sub>O<sub>6</sub> possesses more potent anti-tumor effects on human cervical cancer cells compared to As,<sub>2</sub>O<sub>3</sub>.

In order to understand the relationship of these specific genes to target cancer, it is important to understand the fundamental mechanisms underlying signal transduction by protein-protein interactions. It is proposed that proteins that directly interact with targets will function in important signaling mechanisms. Using Ingenuity Pathway Analysis, several molecules were identified that interact with differentially expressed genes. Understanding the different role of cell death networks is important in designing how therapeutic interventions can be used as novel anti-cancer therapies. The newly identified molecules will be tested to see if parts of the molecule actually has anti-tumor effects.

In conclusion, As,<sub>4</sub>O<sub>6</sub> is more effective for suppressing the SiHa cell growth in vitro and in vivo compared to As,<sub>2</sub>O<sub>3</sub>. In parallel with inhibition of cell proliferation, there is a significant difference in functional networks between As,<sub>2</sub>O<sub>3</sub>-and As,<sub>4</sub>O<sub>6</sub>-induced cell death pathways. These experiments provided important new information regarding the role of molecular network in mediating apoptosis, possibly through two different pathways. Thus, these findings suggest that As,<sub>4</sub>O<sub>6</sub> possesses more potent anti-tumor effects on human cervical cancer with induction of apoptosis, which might provide a new drug choice for treating HPV-associated cervical cancer.

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References


