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Comparison of As_2O_3 and As_4O_6 in the Detection of SiHa Cervical Cancer Cell Growth Inhibition Pathway

Yong Wook Kim, M.D.[1], **Su Mi Bae,** M.S.[2], **Keun Ho Lee,** M.D.[1], **Joon Mo Lee,** M.D.[1], **Sung Eun Namkoong,** M.D.[1], **Insu P. Lee,** M.D.[2], **Chong Kook Kim,** Ph.D.[3], **Jeong-Sun Seo,** M.D.[4], **Jeong-Im Sin,** Ph.D.[5], **Yong-Wan Kim,** Ph.D.[2] and **Woong Shick Ahn,** M.D.[1]

[1]Department of Obstetrics and Gynecology, [2]Catholic Research Institutes of Medical Science, College of Medicine, The Catholic University of Korea, Seoul, [3]College of Pharmacy, [4]Department of Biochemistry and Molecular Biology, Seoul National University, Seoul, [5]Department of Medicine, Catholic University of Daegu, Daegu, Korea

Purpose: **An arsenical compound, As_2O_3, has been reported to be effective for treating acute leukemia and inducing apoptosis in many different tumor cells. In this study, the ability of As_4O_6 to suppress cell growth and induce gene expression patterns was tested using a cDNA microarray in HPV16 immortalized cervical carcinoma cells, SiHa cells, along with As_2O_3.**

Materials and Methods: **A novel arsenical compound, As_4O_6, was designed and its ability to induce cell growth inhibition as well as gene expression profiles along with As_2O_3 in HPV16 infected SiHa cervical cancer cells was compared. Both As_2O_3 and As_4O_6 induced apoptosis in SiHa cells, as determined by DNA ladder formation. To further compare the gene expression profiles between these two drugs, a 384 cDNA microarray system was employed. Also, the gene expression profiles were classified into the Gene Ontology (GO) to investigate apoptosis-related cellular processes.**

Results: **As_4O_6 was more effective i suppressing the growth of SiHa cells in vitro compared to As_2O_3. In the case of treatment with As_2O_3, 41 genes were up- or down-regulated at least 2 fold compared to non-treatment. However, 65 genes were up- or down-regulated by As_4O_6 treatment. In particular, 27 genes were commonly regulated by both arsenic compounds. Also, the GO analysis indicated that down-regulation of cell-regulatory functions, such as cell cycle, protein kinase activity and DNA repair, induced anti-tumor effect.**

Conclusion: **These data support that As_4O_6 could be more effective than As_2O_3 in inhibiting the growth of HPV16 infected cervical cancer cells. This appears to be mediated through a unique, but overlapping regulatory mechanism(s), suggesting that the regulated genes and cellular processes could be further used as a new potential drug approach for treating cervical cancer in clinical settings.** ***(Cancer Research and Treatment 2004; 36:255-262)***

Key Words: **Cervical cancer, Arsenic compound, Apoptosis, cDNA microarray, Gene ontology**

INTRODUCTION

Arsenical compounds are distributed as natural toxicants, with no color, taste or smell. Arsenic trioxide, As_2O_3, has been reported to induce almost complete remission from acute promyelocytic leukemia (APL) (1). Cytopathological studies have also shown that As_2O_3 induces apoptosis in APL cells. Recent reports have shown that As_2O_3 down-regulates the bcl-2 gene expression and induces the expression of apoptosis related protein caspases, as well as degradation of PML and PML/PAR alpha proteins in APL cells. Similarly, arsenic trioxide suppresses the tumor cell growth by cell cycle arrest, cyclin-dependent kinase induction and apoptosis in MC/CAR myeloma cells (2). Arsenic trioxide-induced apoptosis is also enhanced in the presence of dithiothreitol in NB4 cells (3). Along with APL cells, arsenic trioxide can also induce apoptosis and cell growth inhibition in HPV16 infected cervical carcinoma cells (4). These collective studies suggest that arsenic trioxide has an anti-tumor activity in a variety of tumor cells. It has recently been reported that arsenic trioxide induces p53 accumulation via an ATM-dependent pathway in human fibroblast cells (5). Subsequently, p53 induces the expression of WAF1/p21 that has a growth-inhibitory ability, and elicits cell cycle arrests in the G1 phase, resulting in apoptosis (6). Furthermore, DNA breaks and DNA-protein cross-links are formed by arsenite treatments in a variety of cells (7). Although the exact mechanism(s) of arsenic trioxide to suppress cancer cell growth is still unclear, recent findings support the notion that it induces apoptosis of tumor cells, resulting in cancer cell

Correspondence: Woong Shick Ahn, Department of Obstetrics and Gynecology, College of Medicine, The Catholic University of Korea, 505 Banpo-dong, Seocho-gu, Seoul, Korea. (Tel) 82-2-590-2409, (Fax) 82-2-599-4120, (E-mail) ahnws@catholic.ac.kr

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growth inhibition. However, in one study, sodium arsenite (iAs^{3+}) was found to promote cell growth and the expression of certain genes in bladder epithelial cells via activation of AP-1 transactivation (8). Clinically, arsenite should be tumor-specific and have low toxicity (9), so it is likely to be beneficial to develop more effective and less toxic arsenites for the treatment of cervical carcinomas. However, no studies on As_4O_6 have been reported.

Cervical carcinomas are mostly caused by infection with a high-risk group of human papillomavirus (HPV) (10). After a high risk HPV infection, two viral oncogenic proteins, E6 and E7, play a critical role in inducing cervical cancers by interacting with p53 and pRB for the inactivation of these cellular regulatory proteins, respectively (11). Current surgical, radiation, immune and gene therapies approaches have met with limited success. Furthermore, the early detection of cervical cancer using the Pap smear has contributed to a reduction in the incidence of cervical cancers. However, relapsing cervical cancers have been problematic, adding importance to the development of anti-cervical cancer drugs.

The recent development of cDNA microarray technology has allowed to the simultaneous monitoring of the expression profiles of thousands of genes. Also, the annotation project directed by the Gene Ontology (GO) Consortium (http://www.geneontology.org) has been used to quantitatively understand multiple relationships between differentially regulated genes and pathogenesis. Gene expression profiles obtained from patients from cDNA microarray analyses could be useful for predicting the outcome of chemotherapy as well as the duration of patient survival. A generated fingerprint of gene expression profiles could be useful in elucidating cellular changes for any anti-cancer drug treatment. This is likely to shed light on the determining effects of chemotherapeutic regimens on tumor suppression.

Herein, a new arsenical compound, As_4O_6, has been designed and its ability to suppress cell growth and induce gene expression patterns tested using a cDNA microarray in HPV16 immortalized cervical carcinoma cells, SiHa cells, and compared to As_2O_3. It was observe that As_4O_6 was more effective in suppressing the SiHa cell growth at a lower dose compared to that of As_2O_3. Differential gene expression profiles of SiHa cells were also observed on treatments with As_2O_3 and As_4O_6, suggesting that further investigation of the identified genes is warranted. Thus, these data suggest that As_4O_6 could be a more potential drug candidate for the treatment of cervical cancers.

MATERIALS AND METHODS

1) Cell culture

Immortalized HPV16 human cervical carcinoma cell lines, SiHa cells containing wild type p53, 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.

2) Chemical reagents

The As_2O_3 was purchased from Sigma and the As_4O_6 was synthesized and provided by Chonjisan., Co., Seoul, Korea. These chemicals were diluted in H_2O to a final concentration of 10^{-3} M and kept at 4°C. MTT was purchased from Sigma and dissolved in PBS to a final concentration of 5 mg/ml.

3) DNA fragmentation assay

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

4) 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 a 96 well plate in 100 μl of cDMEM. After 24 hr incubation, the cells were treated with a different amounts of arsenic compounds. After 4 day incubation, 20 μl of MTT solution (Sigma, St. Louis, MO) was added to each well of the plates, which were then incubated for 4 hrs at 37°C. The cell media were replaced with 100 μl of DMSO (Sigma) per well and the plates shaken for 10 sec. The optical density (OD) was then measured at 570 nm using an ELISA-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×100.

5) RNA and probe DNA preparation

Total RNA was prepared using Trizol reagent (MRC, Cincinnati, Ohio), according the manufacturer's protocol. For the RT reaction, 50 μg of total RNA was mixed with 1 μl of control mRNA (lambda bacteriophage mRNA, 0.5 μg/ul) and 1.5 μg of oligo dT primer (1.5 μg), to a final volume of 20.5 μl. The reaction mixture was heated for 5 min at 70°C, and the denatured RNA reacted for 1 hr at 42°C in AMV buffer (low dT NTP, Cy3 or Cy5-dUTP, RNase inhibitor and AMV reverse transcriptase). Ten μl of 1 M NaOH was added to the reaction mixture for 10 min at 37°C, followed by the addition of 25 μl of 1 M Tris- HCl (pH 7.5). Probes were purified using Sephacryl S-100 columns And the ethanol-precipitated DNA probes solubilized in 15 μl of hybridization buffer (6×SSC, 0.2% SDS, 5×Denhardt solution and 0.1 μg of salmon sperm DNA).

6) Probe hybridization

The probes were denatured for 2 min at 95°C and then kept on ice. These were centrifuged for 10 min at 14,000 rpm, and the supernatant containing probes added drop wise to a 384 cDNA chip (Macrogen, Seoul, Korea). After sealing with a cover glass, the hybridization reaction was performed for 12 ~ 16 hrs at 62°C. After hybridization, the slides were washed twice for 30 min in 60°C washing buffer (2×SSC, 0.2% SDS) and then dried at the room temperature.

7) Northern blotting

Twenty micrograms of each RNA sample were electrophoresed through 1.1% formaldehyde-agarose gels and transferred to Hybond-N nylon membranes (Amersham Pharmacia Biotech). First strand cDNAs were incorporated with 32P-dATP during reverse transcription from total RNAs using oligo (dT) as primer. Hybridization was carried out overnight at 68°C in Rapid Hyb-buffer (Amersham Co.). After hybridization, the membrane was washed twice at room temperature in 2×SSC and 0.1% SDS, and a further twice at 65°C in 0.1×SSC and 0.1% SDS and then radiophotographed at -70°C for 24 ~ 48 hr.

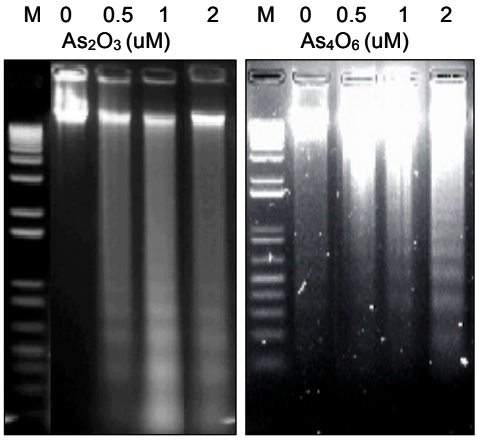

Fig. 1. Induction of DNA ladder by As_2O_3 and As_4O_6 in SiHa cells. Cells were treated with the indicated amounts (0, 0.5, 1 and 2 μM) of As_2O_3 and As_4O_6 for 48 hrs. The DNA was analyzed on a 2% agarose gel and photographed under UV light.

8) Scanning and data analyses

The DNA chip was scanned using a Generation III Array Scanner (Amersham Pharmacia Biotech.) and then analyzed via ImaGeneTM Version 3.0 (TaKaRa, Kyoto, Japan), according to the manufacturer's protocols. To classify the gene profiles into the gene ontology, cellular process analysis was carried out, as previously described (12). All files, including the results of the microarray experiment and the Gene Ontology analysis were downloaded from our anonymous FTP site: ftp://160.1.9.42/work/arsenic/

RESULTS

1) Both As_2O_3 and As_4O_6 induced apoptosis in SiHa cell lines

To investigate whether a new arsenical compound, As_4O_6, could induce apoptosis in SiHa cells, increasing amounts (0.5, 1 and 2 μM) of As_4O_6 were used to treat the cells for 48 hrs. As a positive control, cells were also incubated with As_2O_3. As shown in Fig. 1, both As_2O_3 and As_4O_6 induced DNA ladder formation at all concentrations tested (0.5, 1 and 2 μM), suggesting that As_4O_6 can also induce apoptosis in SiHa cells.

2) Comparison of effects of As_2O_3 and As_4O_6 on SiHa cell growth in vitro

To the compare anti-growth effects of As_2O_3 and As_4O_6 in SiHa cells, increasing amounts of arsenical compounds were added. As shown in Fig. 2, the growth of SiHa cells was decreased by the increasing amounts (0.5, 1, 2, 3, 4 and 5 μM) of both As_2O_3 and As_4O_6, as determined by the OD values. In the case of As_2O_3, the 1 μM treatment resulted in approximately 50% cell growth inhibition over the time periods, whereas the doses from 2 to 5 μM showed complete suppression of cell growth. In the case of As_4O_6, however, the 0.5

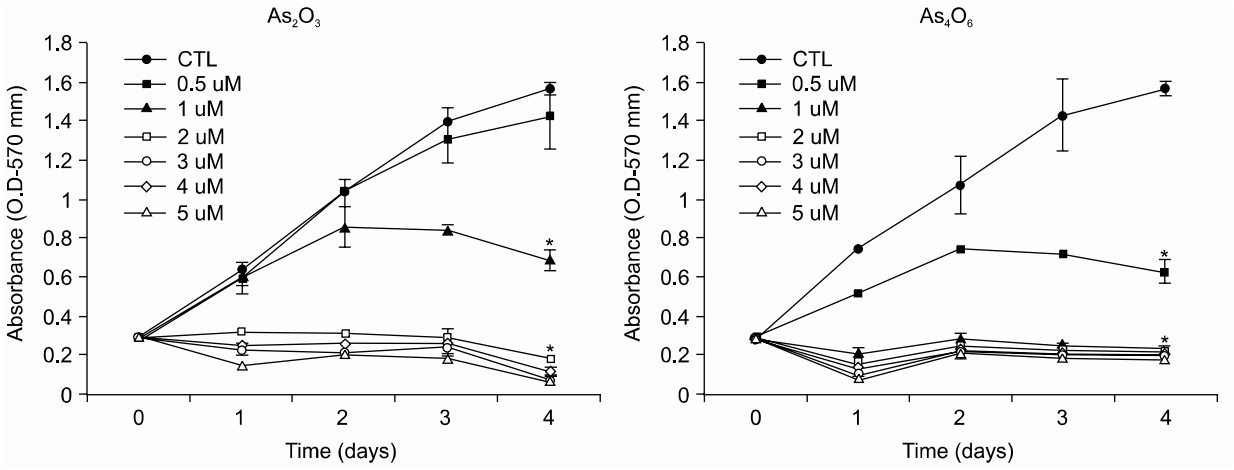

Fig. 2. Effects of As_2O_3 and As_4O_6 on the growth suppression of SiHA cells in vitro. Cells were treated with the indicated amount of the arsenic compounds, As_2O_3 and As_4O_6, and incubated for 4 days. Cell growth suppression was measured as described in 'Methods and Materials'. The OD was measured at 405 nm. The assay was performed in triplicate and average OD values and SD were recorded. This was repeated two more times with similar results. *Statistically significant at $p < 0.05$, using the paired Student's t test, compared to no drug treatment (control, CTL).

μM treatment resulted in approximately 50% cell growth inhibition over the time periods, whereas the doses ranging from 1 to 5 μM showed complete suppression of the SiHa cell growth. The cell growth inhibition rates (%) of As_2O_3 were calculated as 9.2, 56, 89, 93, 96 and 96% at concentrations of 0.5, 1, 2, 3, 4 and 5 μM, respectively. In contrast, the cell growth inhibition rates (%) of As_4O_6 were calculated as 54, 84, 84, 85, 85 and 87 at concentrations of 0.5, 1, 2, 3, 4 and 5 μM, respectively.

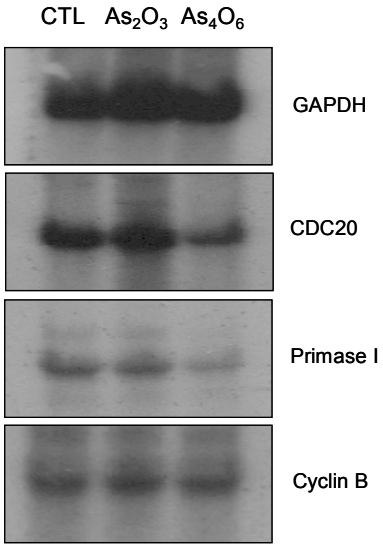

Fig. 3. Confirmations of cDNA chip experiment by Northern blot analysis. Total RNA obtained from As_2O_3 (lane 2) and As_4O_6 (lane 3) treated cells subjected to Northern analysis, as described in 'Materials and methods'.

3) Differentially regulated gene expression profiles of SiHa cells by As_2O_3 and As_4O_6

To determine the gene expression patterns of SiHa cells due to As_2O_3 and As_4O_6 treatment, the cells were incubated with 1 μM of As_2O_3 or As_4O_6, and their gene expression profiles analyzed using the 384 cDNA microarray system and compared to cells with no drug treatment. Northern blots were performed to confirm the gene expression patterns, and showed the consistency of the assays (Fig. 3). Table 1, 2 show the differentially expressed gene expression profiles due to the As_2O_3 and As_4O_6 treatments, respectively. More gene expressions were up- or down-regulated by As_4O_6 compared to As_2O_3. With the As_2O_3 treatment, 41 genes were more than 2 fold up- and down-regulated in their expressions. In the case of As_4O_6, 65 genes showed differential expressions greater than 2 fold. Among these genes, 5 were enhanced in their common expressions by both As_2O_3 and As_4O_6 treatments. These genes might possibly be associated with resistance to the arsenical drugs. These included UDP-glucose dehydrogenase, tumor differentially expressed 1, dual specificity phosphatase 1, cathepsin D and snuportin-1. In particular, 22 genes were commonly down-regulated by both As_2O_3 and As_4O_6. This suggests that these identified genes might be associated with drug sensitivity in SiHa cells. These included CDC20 (cell division cycle 20), cyclin B1, thymidylate synthetase and primase, as well as others (Table 3). In particular, the expressions of genes coding for thymidylate synthetase, primase, cyclin B1 and CDC20 were highly down-regulated by the As_4O_6 treatment.

Table 1. Summary of gene expression changes by As_2O_3 treatment

Unigene number	Gene symbol	Fold change	Unigene No.	Gene symbol	Fold change
Hs.387995	C1orf17	-7.4	Hs.57101	MCM2	-2.3
Hs.29475	TYMS	-5.6	Hs.406631	CDC27	-2.3
Hs.512102	GPS1	-5.2	Hs.446683	CGB	-2.3
Hs.23960	CCNB1	-5.0	Hs.17883	PPM1G	-2.3
Hs.409515	MCC	-3.6	Hs.347963	CSH1	-2.2
Hs.79241	BCL2	-3.2	Hs.105751	SLK	-2.2
Hs.82906	CDC20	-3.2	Hs.235557	ATP5J2	-2.1
Hs.240534	AGPAT1	-3.2	Hs.411098	NPM1	-2.1
Hs.78888	DBI	-2.9	Hs.225767	IDN3	-2.1
Hs.78996	PCNA	-2.9	Hs.74561	A2M	-2.0
Hs.73737	SFRS1	-2.9	Hs.343475	CTSD	2.1
Hs.511397	MCAM	-2.8	Hs.171695	DUSP1	2.3
Hs.82741	PRIM1	-2.8	Hs.77837	UGP2	2.4
Hs.232115	COL1A2	-2.8	Hs.408312	TP53	2.5
Hs.79197	CD83	-2.7	Hs.422215	MPP1	2.8
hs.177766	ADPRT	-2.6	Hs.111577	ITM2C	3.0
hs.440394	MSH2	-2.5	Hs.446352	ERBB2	3.0
Hs.95424	MAP4K1	-2.5	Hs.272168	TDE1	3.5
Hs.173724	CKB	-2.5	Hs.335293	DKFZP564C186	15.8
Hs.154672	MTHFD2	-2.3	Hs.21577	RNUT1	17.5
Hs.6880	GORASP2	-2.3			

Table 2. Summary of geneexpression changes by As_4O_6 treatment

Unigene number	Gene symbol	Fold change	Unigene No.	Gene symbol	Fold change
Hs.82906	CDC20	-31.1	Hs.105751	SLK	-2.3
Hs.23960	CCNB1	-12.0	Hs.75348	PSME1	-2.2
Hs.82741	PRIM1	-12.0	Hs.74561	A2M	-2.2
Hs.29475	TYMS	-11.5	Hs.169919	ETFA	-2.2
Hs.409515	MCC	-6.5	Hs.111779	SPARC	-2.2
Hs.177766	ADPRT	-5.5	Hs.132994	DKFZP434C171	-2.2
Hs.435974	MTHFD1	-4.7	Hs.73625	KIF20A	-2.2
Hs.95424	MAP4K1	-4.4	Hs.335918	FDPS	-2.1
Hs.78996	PCNA	-4.0	Hs.279784	PREB	-2.1
Hs.232115	COL1A2	-3.4	Hs.44	PTN	-2.0
Hs.263812	NUDC	-3.3	Hs.17428	BCAA	-2.0
Hs.57101	MCM2	-3.2	Hs.75890	MBTPS1	-2.0
Hs.511397	MCAM	-3.1	Hs.411098	NPM1	-2.0
Hs420563	NDUFS1	-3.1	Hs.77793	CSK	-2.0
Hs.24529	CHEK1	-3.0	Hs.435800	VIM	-2.0
Hs.235557	ATP5J2	-2.9	Hs.95577	CDK4	-2.0
Hs.79197	CD83	-2.9	Hs.211594	PSMC4	2.0
Hs.73737	SFRS1	-2.8	Hs.209061	RIOK3	2.3
Hs.440394	MSH2	-2.8	Hs.76253	SCA2	2.3
Hs.17883	PPM1G	-2.7	Hs.211612	SEC24A	2.4
Hs.273063	HSPC133	-2.6	Hs.116237	VAV1	2.5
Hs.169407	SACM2L	-2.6	Hs.250666	HES1	2.5
Hs.171734	PPP2R5C	-2.6	Hs.27299	HCNGP	2.6
Hs.225767	IDN3	-2.5	Hs.343475	CTSD	2.6
Hs.445240	FBLN1	-2.5	Hs.272168	TDE1	3.3
Hs.356427	PAI-RBP1	-2.5	Hs.443120	CD36	3.5
Hs.409209	ACAD9	-2.5	Hs.69855	D1S155E	3.8
Hs.173724	CKB	-2.4	Hs.77837	UGP2	3.8
Hs.112049	SBF1	-2.4	Hs.226372	DKFZP434J154	4.4
Hs.369579	CAPZA2	-2.3	Hs.171695	DUSP1	8.2
Hs.420269	COL6A2	-2.3	Hs.21577	RNUT1	12.0
Hs.446683	CGB	-2.3	Hs.439200	KIAA0090	12.7
Hs.414099	TNRC5	-2.3			

The Gene ontology classification of the differentially expressed genes revealed that most were functionally related to apoptotic regulation, as shown in Tables 3 and 4. The cellular processes identified as significant by the GO are listed in the Tables, including the expression patterns. There were several significant differences between the expression profiles of the functions in As_2O_3 and As_4O_6, including 3 significantly up-regulated genes coding for the proteins involved in membrane activity, 1 coding for oxidative stress response, 2 for proteolysis and peptidolysis, and other miscellaneous genes. In contrast, significantly down-regulated genes were found coding for DNA metabolism, humoral immune response, nucleus activity, cell cycle regulation and cell proliferation, and other miscellaneous genes. In particular, genes coding for the proteins involved in cell cycle and nucleic acid metabolism, such as CDC20 (cell division cycle 20) and cycline B1, had completely decreased expressions.

DISCUSSION

The anti-tumor functions of the arsenic compound, As_2O_3, have been reported in leukemia both in vivo and in vitro (1). It was also observed that As_2O_3 induced apoptosis and cell growth inhibition in SiHa cell lines. This supports the previous findings that arsenic trioxide induces anti-tumor effects through the induction of tumor cell apoptosis (2). In the case of promyeloleuckemic cells, As_2O_3 down-regulates the expressions of bcl-2 and PML/RAR α/PML protein, and are correlated with apoptosis (1). As_2O_3 also induces the production of p53 protein (5). The p53 protein subsequently induces cell cycle arrests and apoptosis through the induction of the WAF1/p21 expression (6). It was also reported that the glutathione redox system is associated with apoptosis due to As_2O_3 (13). It is likely that apoptosis is a mechanism by which arsenic trioxide suppresses tumor cell growth both *in vivo* and *in vitro*. This is also supported in that As_2O_3 was also found to inhibit cell growth

Table 3. Summarized cellularprocess ontology of down- and up-regulated transcripts by As_2O_3 treatment

Biological process Ontology	GO code	Down	Up
%Apoptosis	GO:0006915	2	1
%ATP binding	GO:0005524	3	1
%cell adhesion	GO:0007155	1	0
%cell cycle	GO:0007049	4	2
%cell growth and maintenance	GO:0008151	5	2
%cell proliferation	GO:0008283	2	1
%cell-cell signaling	GO:0007267	2	0
%CTD phophatase	GO:0008420	0	1
%cytokinesis	GO:0016288	2	0
%DNA binding	GO:0003677	4	1
%DNA repair	GO:0006281	3	1
%DNA replication	GO:0006260	3	0
%extracellular matrix	GO:0005578	2	0
%hormone activity	GO:0005179	2	0
%humoral immune response	GO:0006959	2	0
%hydrolase	GO:0016787	2	2
%integral membrane protein	GO:0005887	4	2
%kinase	GO:0016301	1	1
%membrane	GO:0016020	2	3
%mitosis	GO:0007067	3	0
%nucleus	GO:0005634	9	1
%oncogenesis	GO:0007048	2	2
%protein kinase	GO:0004612	2	1
%protein phophatase	GO:0004721	1	1
%proteolysis and peptidolysis	GO:0006508	1	1
%regulation of cell cycle	GO:0000074	4	0
%response to stress	GO:0006950	1	0
%signal transduction	GO:0007165	4	1
%skeletal development	GO:0001501	1	0
%transferase	GO:0016740	6	2
%tumor antigen	GO:0008222	1	0
%tumor suppressor	GO:0008181	1	1

Table 4. Summarized cellularprocess ontology of down- and up-regulated transcripts by As_4O_6 treatment

Biological process Ontology	GO code	Down	Up
%actin cytoskeleton activity	GO:0015629	1	0
%Apoptosis	GO:0006915	1	0
%ATP binding	GO:0005524	8	1
%calcium ion binding	GO:0005509	2	0
%cell adhesion	GO:0007155	2	1
%cell cycle	GO:0007049	5	1
%cell growth and maintenance	GO:0008151	5	1
%cell proliferation	GO:0008283	4	0
%cell-cell signaling	GO:0007267	2	0
%collagen activity	GO:0005202	2	0
%CTD phophatase	GO:0008420	1	1
%cytokinesis	GO:0016288	3	0
%defense response	GO:0006952	1	0
%DNA binding	GO:0003677	7	2
%DNA repair	GO:0006281	3	0
%DNA replication	GO:0006260	3	0
%extracellular matrix	GO:0005578	4	0
%Golgi apparatus	GO:0005794	2	0
%hormone activity	GO:0005179	1	0
%humoral immune response	GO:0006959	1	0
%hydrolase	GO:0016787	3	2
%immune response	GO:0006955	1	0
%integral membrane protein	GO:0005887	4	2
%intracellular protein transport	GO:0006886	3	1
%kinase	GO:0016301	1	1
%lipid metabolism	GO:0006629	1	1
%membrane	GO:0016020	1	1
%mitosis	GO:0007067	2	0
%mRNA processing	GO:0006397	1	0
%neurogenesis	GO:0007399	1	1
%nucleus	GO:0005634	13	3
%oncogenesis	GO:0007048	3	1
%oxidative stress response	GO:0006979	0	1
%oxidoreductase	GO:0016491	2	0
%protein kinase	GO:0004612	4	0
%protein phophatase	GO:0004721	4	1
%proteolysis and peptidolysis	GO:0006508	1	2
%regulation of cell cycle	GO:0000074	7	0
%response to stress	GO:0006950	1	0
%signal transduction	GO:0007165	1	0
%skeletal development	GO:0001501	3	0
%transferase	GO:0016740	10	1
%tumor antigen	GO:0008222	1	0
%tumor suppressor	GO:0008181	2	0
%vesicle targeting	GO:0005482	2	0

and induce apoptosis in different cancers, including leukemia, myelomas, lymphomas and solid tumors (14).

In contrast to As_2O_3, As_4O_6 was synthesized and evaluated for its anti-tumor effects in SiHa cells. As with As_2O_3, As_4O_6 also induced apoptosis and cell growth inhibition in vitro. In particular, As_4O_6 showed a significant anti-tumor activity (approximately 50% growth inhibition) at a dose of 0.5 μM, at which As_2O_3 displayed little anti-tumor growth effects. Instead, 1 μM of As_2O_3 showed approximately 50% growth inhibition, suggesting that As_4O_6 could be more potent in the control of cervical cancer cell growth. However, with doses ranging from 2 to 5 μM both As_2O_3 and As_4O_6 displayed a complete anti-growth effect. The dose effects of arsenic trioxide on growth inhibition are consistent with many previous reports (2,4). As_4O_6 was also injected into mice, along with As_2O_3, in order to compare their toxicities in animals. Mice injected with As_4O_6 survived longer than those injected with As_2O_3 (data not shown), suggesting that As_4O_6 is less toxic to animals than As_2O_3. These support the notion that As_4O_6 could be a less toxic, but more potent drug for controlling cervical cancers.

The cDNA microarray was further used to compare the gene expression profiles of these two drugs in SiHa cells. A new classification of cancer cell types has been proposed based on the altered gene expressions of cancer cells (15). It has also been reported that gene expression profiles might reflect drug resistance or sensitivity in tumor cells (16). Recently, cDNA

microarray analyses showed a dramatic difference in the gene expressions between normal liver tissues and liver tissues from arsenic-exposed patients (17). The differentially expressed genes included those involved in cell-cycle regulation, apoptosis, DNA damage response and intermediate filaments. A significant difference in the gene expression profiles was also observed between As_2O_3 and As_4O_6. For example, As_2O_3 influenced the expression of a total of 41 genes, whereas As_4O_6 influenced a total of 65 genes. This correlates well with the observed difference in the cell growth inhibitory abilities of these two drugs. These data also support the notion that As_4O_6 behaves differently from As_2O_3. Interestingly, the activity of response to oxidative stress (DUSP1) involved in the metabolism was completely up-regulated by As_4O_6. However, commonly regulated genes, such as up-regulated genes (UDP-glucose dehydrogenase, tumor differentially expressed 1, dual specificity phosphatase 1, cathepsin D and snuportin-1) and down-regulated genes (CDC20, cyclin B1, primase polypeptide 1, thymidylate synthetase, proliferating cell nuclear antigen) were also observed in the presence of both As_2O_3 and As_4O_6.

The functions of up-regulated genes are unclear, but the Gene Ontology analysis of the differentially regulated genes revealed that most of down-regulated genes play key functions in various cellular activities involved in cell death. Genes associated with DNA and cellular metabolisms are commonly down-regulated compared to their counterparts. The regulation of extracellular matrix expression is a key to the tissue remodeling in normal and pathologic processes that lead to tissue carcinogenesis (18). Interestingly, the genes in this cellular process were highly down-regulated by both arsenic compounds. Among the genes in this category were the collagens. The expression of collagens I that regulates its turnover has been demonstrated in tumorigenesis (19). Cytoskeleton integrity plays an important role in the cell cycle progression, cell death and cell differentiation. An abnormal cytoskeleton is often observed in cancer cells. In this study, the gene expression profiles involved with the cytoskeleton were completely down-regulated. Disruption of cell cycle progression pathways has been implicated in abnormal cell growth and carcinogenesis (20). In this study, it was observed that genes involved in the cell cycle, such as CCNB1 and MCM2, were highly down- regulated.

Cyclins, known as cell cycle regulators, bind to and activate the CDKs that are also regulators of the cell cycle in eukaryotic cells. The activation and subsequent inactivation of cyclins and CDKs are important in the control of the cell cycle (21). CDC20 is a regulator in mitotic (anaphase) checkpoints (22). Cyclin B1, a cell cycle checkpoint protein, regulates the transition from the G2 to M phase (23). Cyclin B1 protein has recently been reported to be a shared epithelial tumor associated antigen, showing a high error rate in the translation of cyclin B1 proteins in tumors. Similarly, primase has an important function in DNA replication, DNA repair and cell cycle checkpoints (24). Thymidylate synthetase catalyzes the conversion of dUMP to thymidylate, which is an essential component of DNA. Inhibition of this enzyme activity results in cell death. Proliferating cell nuclear antigen is also associated with DNA repair processes. In particular, the level of proliferating cell nuclear antigen correlates with DNA repair activity (25). In our cDNA microarray analyses, these cell regulatory genes (CDC20, cyclin B1, primase, thymidylate synthetase and proliferating cell nuclear antigen) were down-regulated by both As_2O_3 and As_4O_6. It is likely that these genes are associated with arsenite-mediated apoptosis and growth inhibition in SiHa cells. Down-regulation of the genes involved in the cell cycle, DNA repair and regulation was previously reported in arsenic-exposed patients. This further supports that these arsenical compounds might inhibit tumor cell growth at least through inhibition of expression of these regulatory genes. It also appears that As_2O_3 and As_4O_6 induce anti-tumor effects through a unique, but overlapping regulatory mechanism(s). This is based on our findings that two arsenical drug compounds displayed differential gene expression patterns with some commonly expressed genes.

CONCLUSIONS

The data presented herein suggest that As_4O_6 is more effective than As_2O_3 for inhibiting the growth of HPV16 infected SiHa cervical cancer cells. These two drugs appear to mediate the tumor cell growth inhibition at least through a unique, but overlapping regulatory mechanism(s), as determined by cDNA microarray and Gene Ontology analyses, reflecting evidence that indicates similarities and differences between the molecular environments of the cell growth suppression pathways. Thus, this study is likely to provide a new potential drug approach for treating cancers in clinical settings.

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