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Humic acid enhances the cytotoxic effects of arsenic trioxide on human cervical cancer cells

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ABSTRACT

Cervical cancer is the second leading cancer affecting women, and recent studies have demonstrated arsenic trioxide (As_2O_3) has therapeutic effects on cervical cancer by promoting apoptosis and inhibiting metastasis *in vitro* and *in vivo*. Humic acid (HA) possesses various pharmacologic properties, including anti-inflammatory, anti-neoplastic, and anti-proliferative effects by inducing apoptosis. We examined the growth inhibition properties and the combined effects of HA and As_2O_3 in human cervical adenocarcinoma cell lines. Our results shown both As_2O_3 and HA-induced inhibition of cell growth, most likely by ROS-mediated cell damage and activation of the apoptosis pathway, and HA enhanced the anti-proliferative action of As_2O_3 in HeLa and SiHa cells, which reduced the LC_{50} about 57.62 or 73.52% (300 μg HA/mL) to 83.67 or 79.03% (500 μg HA/mL), respectively. This study is relevant to the development of chemotherapeutic approaches using As_2O_3 in treating human cervical cancer.

1. Introduction

Cervical cancer was the second leading cancer death cause among 20–39 years old women in 2006, with about 11,270 new cases and more than 4070 deaths estimated in the United States in 2009 (Jemal et al., 2009). In addition, cervical cancer was the 6th leading cause of deaths in females in Taiwan in 2006 (Department of Health, Executive Yuan, R.O.C.). Treatments for cervical cancer include radiation therapy, chemotherapy, and surgery, but new, more effective treatment methods are needed (Roomi et al., 2006).

In traditional Chinese medicine, arsenous acid or arsenic trioxide (As_2O_3) has been used as a devitalizing agent prior to teeth fillings, and until the 1940s in the treatment of other diseases and medical conditions, such as psoriasis, syphilis, hemorrhoids, and rheumatism (Shen et al., 1997). Arsenic was the mainstay for treating leukemia from the 1700s through the 1900s (Gallagher, 1998). With the development of modern chemotherapy and concerns about its toxicity, the use of arsenic in Western medicine diminished through the 20th century (Dilda and Hogg, 2007). Based on the studies of Chinese clinicians in 1971 (Shen et al., 1997; Chen et al., 1997; Dilda and Hogg, 2007), As_2O_3 was recognized as an ideal anticancer drug for treating acute promyelocytic leukemia (APL), and these results renewed interest in this metalloid for treating various forms of cancers. Recent studies have demonstrated As_2O_3 has therapeutic effects on cervical cancer by promoting apoptosis and inhibiting metastasis *in vitro* and *in vivo* (Woo et al., 2002; Yu et al., 2007a,b). Arsenic compounds represent apoptosis-inducing agents through reactive oxygen species (ROS) production, such as H_2O_2 (Woo et al., 2002), and induce disturbances of the assembly of spindle microtubules, resulting in mitotic arrest in HeLa cells (Huang et al., 2000). On the other hand, preliminary data from phase II clinical trials in patients with metastatic renal cell carcinoma, metastatic melanoma, advanced primary liver cancer, and

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stage IVB or recurrent cervical cancer have been completed, but the results are not yet available (Dilda and Hogg, 2007). Nevertheless, these results suggest the single agent As_2O_3 has limited effects on solid tumor growth, and As_2O_3 should be evaluated in combination with other therapeutic modalities in order to try to enhance its clinical activity against solid tumors (Ning and Knox, 2006).

Humic acid (HA) is a group of high-molecular-weight polymers originating from decomposition of organic matter, in particular, dead plants. HA exists abundantly in peat, soil, well water, and other sources (Hartenstein, 1981). A hypothetical structure for HA has been proposed as follows: a polymer composed of aromatic rings of the di- and tri-hydroxybenzene type bridged by ether, methylene, amine, imine, and other linkages (Cheng et al., 2003). Because of its ubiquitous presence in soil and groundwater, and its characteristic ability to dissolve or to suspend metals (Schulze et al., 1994), HA could be used to help solubilize metals for environmental toxicology studies (Tully et al., 2000). It has been shown that HA-metal complexes are more potent than metal ions alone in their effects on hepatic enzymes (Lu et al., 1988; Lu and Lee, 1992). HA has been shown to generate ROS, such as superoxide anion (Cheng et al., 2003), and deplete glutathione and several antioxidant enzymes (Cheng et al., 1999). The presence of free radicals in HA has been revealed using ESR spectroscopy (Lu et al., 1988). In addition, HA has been utilized in traditional Chinese medicine and possesses various pharmacologic properties, including anti-inflammatory, anti-hypertensive, anti-neoplastic, and hemostatic activities (Guan and Yang, 1999), and HA displays anti-proliferative effects by inducing apoptosis (Yang et al., 2004). In our previous studies, HA and As_2O_3 damaged pancreatic islet cells through increased oxidative stress in vitro and in vivo (Yen et al., 2007). In several cell types, changes in cellular redox potential due to enhanced generation of ROS, decreased detoxification, or depleted or reduced glutathione are sufficient to induce opening of the mitochondrial permeability transition pores and subsequent apoptosis (Dai et al., 1999).

In the present study, we examined the growth inhibition properties and the effects of combining HA with As_2O_3 in human cervical cancer cell lines in vitro by measuring cell proliferation, programmed cell death, intracellular ROS, and GSH.

2. Materials and methods

2.1. Chemicals

Fetal bovine serum, PBS (phosphate buffered saline), RPMI-1640 medium, penicillin-streptomycin (PS), and glutamine were obtained from Hyclone Co. (Logan VT, USA). Humic acid, agarose, phenol/chloroform/isoamyl alcohol, proteinase K, RNase A, Tris–HCl, ethidium bromide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), QuantiPro™ BCA kit, Annexin V-Cy3™ apoptosis detection kit (APO-AC), 2′-7′-dichlorofluorescein diacetate (DCFDA), and N-acetyl-cysteine (NAC) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Monochlorobimane (mBCL) was obtained from Molecular Probes Inc. (Fingene, OR, USA). CaspACE™ Assay System was purchased from Promega Co. (Madison, WI, USA).

2.2. Purification of HA

Chromatographic and fluorescence data indicate Sigma-Aldrich HA is similar to HA obtained from coral skeletons, sea water, river water, soil, and plant leaves (Susic and Boto, 1989). Therefore, all experiments were performed with the same batch of HA, which was obtained from Sigma–Aldrich Co.

HA was first dissolved in 1N NaOH solution (pH > 10), and any undissolved material was removed by filtration. The solution was then acidified with 1N HCl (pro-analysis grade, Merck, Darmstadt, Germany) to a pH < 2.0 to precipitate the HA, and dissolved metals which bind with HA. Any precipitate formed was collected by centrifugation at 3000 × *g* for 30 min, and redissolved in 1N NaOH. The alkaline-acid treatment was repeated three times to obtain the purest HA, as described by Schnitzers (1982). After the third round of acid precipitation, the precipitate was dissolved in 0.1N NaOH, and the pH of the resulting solution was adjusted to 7.2–7.4. The purified HA was then freeze-dried to a powder form. The HA was stored as a dried powder, and dissolved in PBS (pH 7.4) or double deionized water before the experiments.

2.3. Cell culture

Two different types of human cervical cancer cells, HeLa (CCL-2, human cervical epithelioid cell carcinoma) and SiHa cells (HTB-35, human cervical squamous cell carcinoma), were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in a DMEM medium containing 10% FBS, 100 unit/mL of penicillin, and 100 unit/mL of streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. The medium was exchanged every 2–3 days.

2.4. Cell viability

Cells (2×10^5 mL^{-1}) were seeded onto 24-well culture plates, and then cells were treated for 48 h with various concentrations of HA, As_2O_3, or both. The cells were washed two times with PBS, and then a new medium containing MTT (2 mg/mL) was added and incubated for another 4 h at 37 °C. Cell viability was determined using the MTT assay (Denozot and Lang, 1986). The values of viability are expressed in relation to the absorption of untreated control cells which were considered to be 100%.

2.5. Analysis of DNA fragmentation

SiHa cells (5×10^6 cells) were exposed to various concentrations of HA, As_2O_3, or As_2O_3 combined with 500 μg/mL HA in DMEM with 10% FBS for 48 h. After incubation, both floating and attached cells were harvested, washed with PBS, and dissolved in 100–200 μL of lysis buffer (10 mM EDTA, 0.1% SDS, and 0.5 mg/mL proteinase K in 50 mM Tris–HCl buffer, pH 8.0) at 50 °C for overnight. 10 μL of 2 mg/mL RNase A was added and incubated for an extra 3 h. The DNA was extracted using 200 μL of phenol/chloroform/isoamyl alcohol (25:24:1), and DNA fragments were separated by electrophoresis through the 1.8% agarose gel. The gel was visualized using stained with ethidium bromide (0.5 μg/mL) and detected using Alphalmager 3300 (Alpha Innotech Corp., San Leandro, CA).

2.6. Cell cycle

The cell cycle stages in the drug-treated groups were measured by flow cytometric analysis. After 24 h drug treatment, adherent and floating cells were pooled, washed with ice-cold PBS, fixed in PBS-methanol (1:2, volume/volume) solution, and finally maintained at 4 °C for at least 18 h. Following two more washes with PBS, the cell pellet was stained with a fluorescent probe solution containing PBS, 40 μg/mL of PI, and 40 μg/mL of DNase-free RNaseA for 30 min at room temperature in the dark. DNA fluorescence of the PI-stained cells was evaluated by excitation at 488 nm and monitoring through a 630/22-nm band pass filter using a Becton-Dickinson FACS-Calibur flow cytometer. A minimum of 10,000 cells were analyzed per sample, and the DNA histograms were gated and analyzed further using Modfit software on a Mac workstation to estimate the percentage of cells in various phases of the cell cycle.

2.7. Outer membrane phosphatidylserine labeling

In living cells, phosphatidylserine (PS) is transported to the inner plasma membrane (Kuypers et al., 1996), and during the onset of apoptosis or necrosis, the PS is transported to the external leaflet of the plasma membrane. Apoptotic cells were distinguished from necrotic cells by an apoptosis detection kit (Annexin V-Cy3 [Apo-AC]; Sigma–Aldrich). HeLa cells (5×10^6 cells) were exposed to various concentrations of As_2O_3 with or without HA in DMEM with 10% FBS for 16 h. After incubation, the cells were incubated with Annexin V-Cy3 (Ann Cy3) and 6-carboxy fluorescein diacetate (6-CFDA) simultaneously. After labeling at room temperature, the cells were immediately observed by fluorescence microscopy (Axiovert 200, Carl Zeiss Inc., Germany). The PS was available for binding to Ann Cy3, which was observed as red fluorescence. In addition, cell viability could be measured by 6-CFDA, hydrolyzed to 6-CF and appearing as green fluorescence. Live cells were labeled only with 6-CF (green), while necrotic cells were labeled with Ann Cy3 (red). Cells in the early stage of apoptosis were labeled with both Ann Cy3 (red) and 6-CF (green).

2.8. Caspase 3 activity assay

Caspase 3 activity in the cell lysate was determined using Caspase 3 assay kit (CaspACE™ Assay System Fluorometric, Promega). Briefly, following pretreatment with HA, As_2O_3 or both of them for 24 h, cells were washed with cold PBS. Then cells were lysed with lysis buffer on ice for 15 min. After centrifugation the lysed cells at 16,000 × *g* at 4 °C for 15 min, supernatants were transferred to 96 well plate and added with caspase 3 substrate AC-DEVD-AMC (7-amino-4-methyl coumarin) and assay buffer. The plate was incubated at 37 °C for 60–90 min and measured for fluorescence by a fluorescence microplate reader (FLx800, Bio-Tek Inc., Winooski, VT, USA), with a filter set of *Ex*/*Em* = 360 ± 40 nm/460 ± 40 nm.

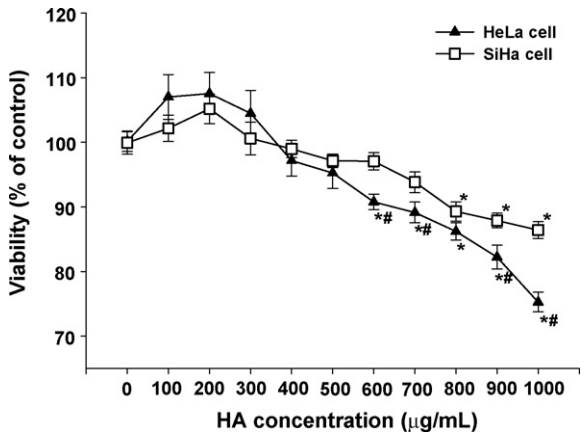

Fig. 1. Effects of humic acid (HA) on the viability of cervical cancer cells. The cells were exposed to different concentrations of HA for 48 h. Cells viability was determined by the MTT assay, as described in Section 2. The values (%) are expressed in relation to untreated control cells. Data are presented as the mean ± SEM (n = 8–15). $^{*}p < 0.05$ as compared with control cells; $^{\#}p < 0.05$ as compared with both cells.

2.9. Intracellular ROS

The amount of intracellular ROS, especially H_2O_2 produced by drug treatment, was determined using DCFDA (Gou et al., 1998). HeLa cells were treated for 1 or 2 h with various concentrations of HA and As_2O_3 or both of them. After removing the supernatant, cells were washed twice with PBS, and then a new medium containing 10 μM DCFDA was added and incubated for a further 20 min. Cells were lysed in 110 mM Tris and 0.25 M sucrose in 0.05% Triton X-100 solution (pH 7.5). The cytoplasmic fluorescence intensity was measured by a fluorescence spectrometer (PerkinElmer LS-30, PerkinElmer Inc., USA), with excitation and emission wavelengths of 405 nm and 520 nm, respectively.

2.10. Intracellular GSH levels

After treatment for 8 h, HeLa cells were washed with PBS, and then a new medium containing 60 μM mBCL, specified to GSH, was added and incubated for a further 30 min. Cells were lysed in lysing buffer (110 mM Tris and 0.25 M sucrose in 0.05% Triton X-100, pH 7.5). The mBCL-GSH related fluorescence in the cells was monitored by a fluorescence spectrometer (PerkinElmer LS-30) with excitation and emission wavelengths of 385 nm and 485 nm, respectively (Chatterzee et al., 1999).

2.11. The role of NAC (GSH donor) in the cell viability of HeLa cells

GSH is one of the most abundant cellular antioxidants, and it plays an important role in protection against ROS and toxic xenobiotics (Chatterzee et al., 1999). NAC, a precursor of GSH, has the same benefits as GSH, both through detoxifying and antioxidant functions (Belletti et al., 2002). In this study, both cancer cells were preincubated for 30 min in a medium with 5 mM NAC, and the cell viability of cells was assayed after incubation for 24 h with HA and As_2O_3 or both of them.

2.12. Statistical analysis

Student's t-test was used to determine the statistical significance between treatment groups. Differences were considered statistically significant at a value of $p < 0.05$.

3. Results

3.1. The cytotoxicities of HA and As_2O_3 on cancer cells

Cell viability was determined by the MTT assay as described in Section 2. HA in concentrations ranging from 100 to 1000 μg/mL with stepped increases was used to treat HeLa cells for 48 h. Fig. 1 shows that treatment with lower concentrations (≦500 μg/mL) of HA alone for 48 h did not result in a decrease in HeLa or SiHa cells viability.

Fig. 2A and B represent the cytotoxic effects of HA and As_2O_3 in HeLa and SiHa cells for 48 h, respectively. Treatments at lower concentrations of As_2O_3 alone (≦1–2 μM) slightly increased cell viability. In contrast, higher concentrations of As_2O_3 or co-exposure

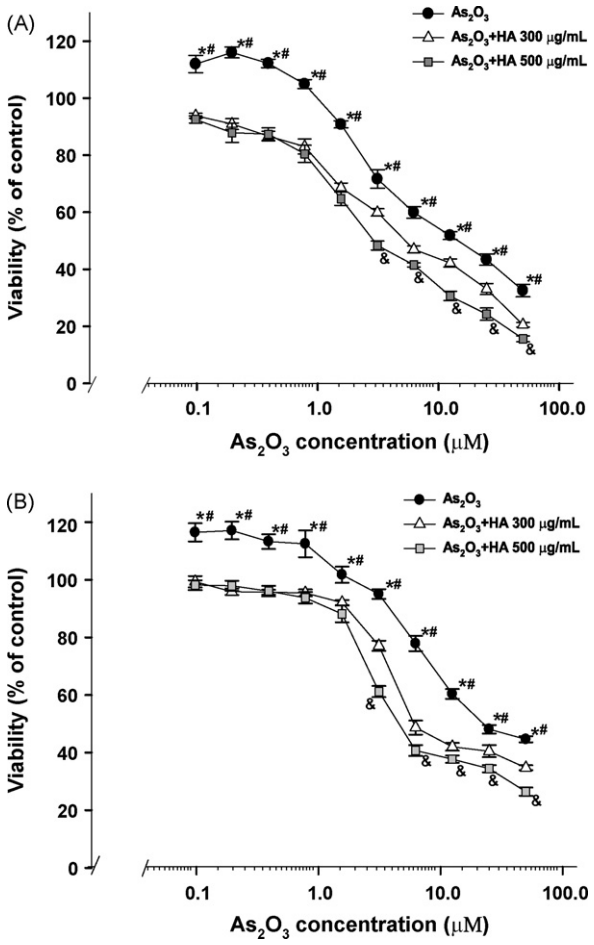

Fig. 2. Effects of As_2O_3 or As_2O_3 combined with HA on the viability of cervical cancer cells. (A) HeLa cells were exposed to As_2O_3 (-●-) or a combination of 300 μg/mL HA and As_2O_3 (-△-) or 500 μg/mL HA and As_2O_3 (-■-) for 48 h. (B) SiHa cells were exposed to As_2O_3 (-●-) or a combination of 300 μg/mL HA and As_2O_3 (-△-) or 500 μg/mL HA and As_2O_3 (-■-) for 48 h. Cells viability was determined by the MTT assays, as described in Section 2. The values (%) are expressed in relation to untreated control cells. Data are presented as the mean ± SEM (n = 6–10). $^{*,\#}p < 0.05$ as compared with As_2O_3 combined with 300 μg/mL or 500 μg/mL HA group, respectively. $^{\&}p < 0.05$ as compared within both As_2O_3 combined with HA group.

to As_2O_3 and HA inhibited cells growth of cervical cancer cells ($p < 0.05$). Interesting results were obtained in the HeLa cells treated with a combination of different concentrations of As_2O_3 and HA (the LC_{50} of As_2O_3 vs. As_2O_3 combined with 300 or 500 μg/mL of HA was about 13.04 μM vs. 5.53 or 2.13 μM for 48 h, respectively). Further, similar results were also obtained in SiHa cells after 48 h exposure, and the LC_{50} of As_2O_3 vs. As_2O_3 combined with 300 or 500 μg/mL of HA was about 23.07 μM vs. 6.11 or 4.84 μM for 48 h, respectively. Such co-exposed treatment successfully eliminated the growth-stimulating effects of As_2O_3 at lower concentrations, as well as enhanced the growth-inhibiting effects at higher concentrations.

3.2. Induction of programmed cell death by HA and As_2O_3

Fig. 3A and B shows DNA extracted from the HA or As_2O_3-treated SiHa cells showed faint fragmentation. In contrast, a significant amount of DNA fragmentation was observed in the cells co-treated with As_2O_3 and HA. The results indicated combining As_2O_3 with HA successfully triggered apoptosis, even at the non-responsive concentration of As_2O_3.

Flowcytometric analysis for the DNA content or cell cycle progression was performed to clarify the effects of HA on HeLa cells.

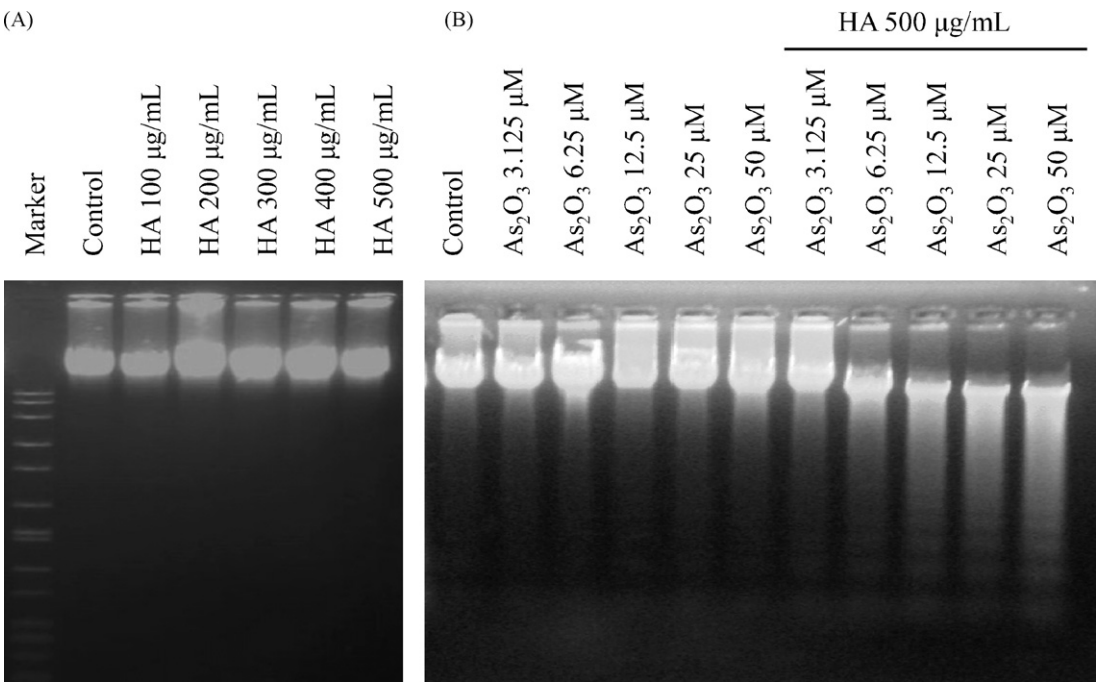

Fig. 3. Effect of HA and As_2O_3 on the integrity of DNA of SiHa cells. The cells were exposed to different concentrations of (A) HA, (B) As_2O_3 or As_2O_3 combined with 500 μg/mL HA for 48 h. DNA laddering, a typical characteristic of apoptosis was analyzed by agarose gel electrophoresis and DNA was visualized by ethidium bromide (0.5 μg/mL) and detection as described in Section 2.

Fig. 4 shows no clear alterations between the control and HA-treated groups. These results indicated HA had little influence on the growth in HeLa cells. On the other hand, As_2O_3 treatment alone resulted in an accumulation of G2 populations. Combined treatment of As_2O_3 and 500 μg/mL HA enhanced the degree of cell cycle retardation. In addition, a slight increase in sub-G1 population was also observed in combined As_2O_3 and HA treatment.

Fig. 5 shows the photographs of a characteristic population of HA- or As_2O_3-treated HeLa cells stained with 6-CFDA and AnnCy3 with an apoptosis detection kit. Microscopic examination showed after 16 h exposure showed an increased number of HeLa cells treated with HA and As_2O_3 were labeled with both green (6-CF) and red (AnnCy3) fluorescence, indicating the cells were in the early stage of apoptosis.

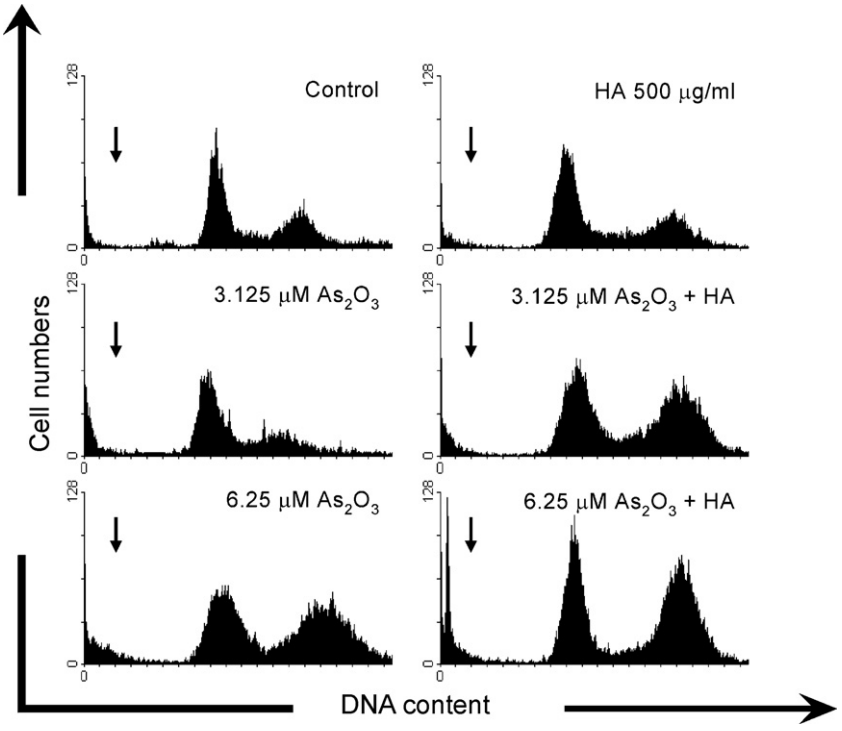

Fig. 4. Effects of HA, As_2O_3, or As_2O_3 combined with 500 μg/mL HA on the cell cycle progression of HeLa cells after 24 h exposure. Cellular DNA content was determined by flow cytometric analysis of propidium iodide-labeled cells, as described in Section 2. Sub-G1 stage was denoted by black arrow.

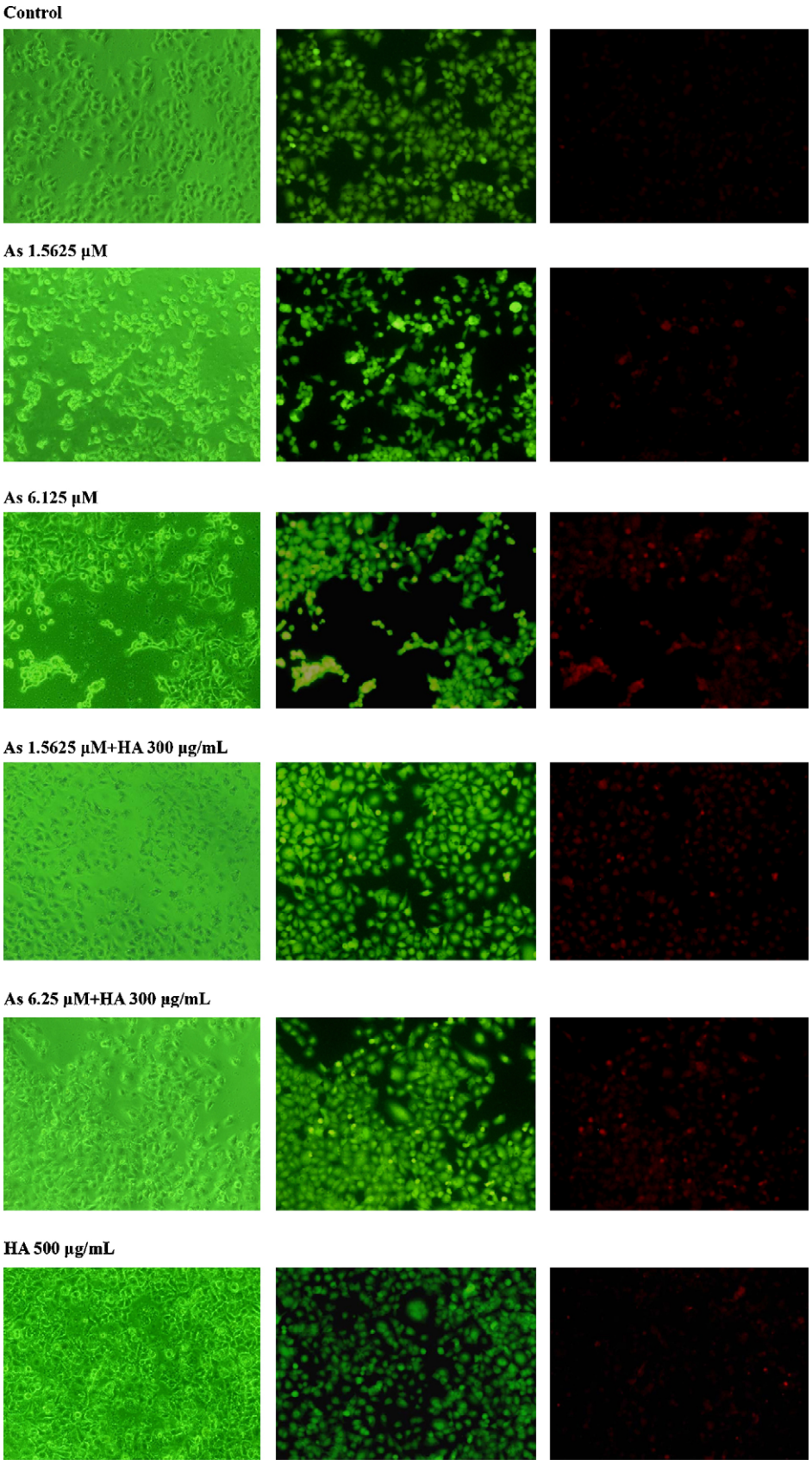

Fig. 5. Effect of HA and As_2O_3 on the morphology of HeLa cells. The cells were exposed to different concentrations of HA or As_2O_3 for 16 h. Apoptosis cells could be differentiated from necrotic cells by incubation with Annexin V-Cy3 (Ann Cy3) and 6-carboxy fluorescein diacetate (6-CFDA) simultaneously. After labeling at room temperature, the cells were immediately observed by fluorescence microscopy (100×). Live cells were labeled only with 6-CF (green fluorescence), while necrotic cells were labeled only with Ann Cy3 (red fluorescence). Cells in the early stage of apoptosis were labeled with both Ann Cy3 (red fluorescence) and 6-CF (green fluorescence). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

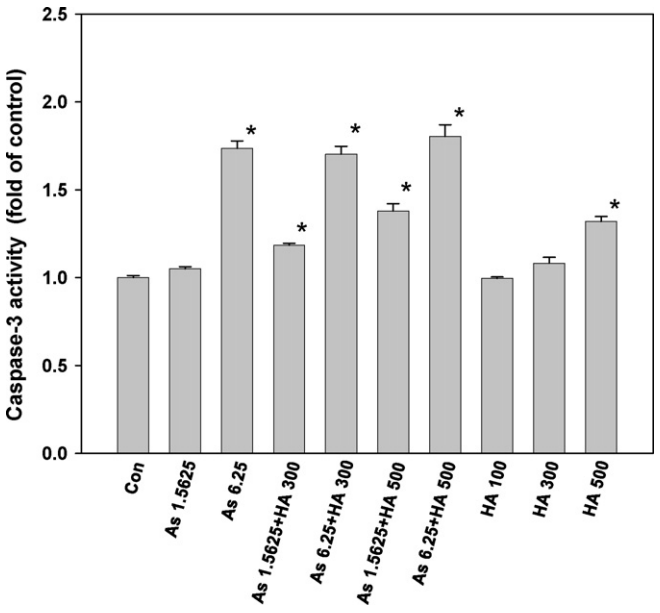

Fig. 6. Effect of HA and As_2O_3 on the caspase 3 activity of HeLa cells. Caspase 3 activity in the cell lysate was determined using Caspase 3 assay kit. Cells were lysed with lysis buffer on ice, and centrifuge at 16,000 × g at 4 °C for 15 min. Supernatants were transferred to 96 well plates and added with caspase 3 substrate AC-DEVD-AMC and assay buffer. The plate was incubated at 37 °C for 60–90 min and measured the fluorescence by a fluorescence microplate reader (FLx800, Bio-Tek Inc., Winooski, VT, USA), with a filter set of *Ex*/*Em* = 360 ± 40 nm/460 ± 40 nm. The average ratios of caspase 3 activity were compared with untreated control cells. Data are presented as mean ± SEM (n = 4–6). *$p < 0.05$ as compared with control.

As shown in Fig. 6, higher concentrations of HA or As_2O_3 significantly induce caspase 3 activity after 24 h exposure ($p < 0.05$). Based on these observations, the apoptosis pathway could be triggered in cervical cancer cells after exposure to HA, As_2O_3 or both of them.

3.3. Intracellular ROS

We used DCFDA as a free radical probe to study the intracellular oxidation of HeLa cells treated for 1 or 2 h with HA or As_2O_3. Exposures to HA, As_2O_3, or both of them significantly induced oxidative stress in the cells ($p < 0.05$, Fig. 7A and B). In addition, the oxidative properties of HeLa cells exposed to HA or As_2O_3 were concentration-dependent. Further, HA enhanced the oxidative stress induced by As_2O_3 or HA along at an early stage (1 h, Fig. 7A), and then decreased (2 h, Fig. 7B).

3.4. Intracellular GSH levels

GSH concentrations were significantly increased in HeLa cells after exposure to As_2O_3 or HA for 8 h, especially in lower concentrations of As_2O_3 combined with HA, but the effects of intracellular GSH accumulation were decreased at higher concentrations of As_2O_3 or a combination of As_2O_3 and HA ($p < 0.05$, Fig. 8).

3.5. The preventive effects of NAC (GSH donor) on the cytotoxicities of HA and As_2O_3 on HeLa cells

Fig. 9 shows when HeLa or SiHa cells were preincubated with NAC, a precursor of GSH, for 30 min, and then exposed to HA and As_2O_3 for an extra 24 h, it could restore the cell viability ranging from 5.63% to 54.47% (HeLa cells) or 10.61% to 56.16% (SiHa cells) compared to the cells not pretreated with NAC.

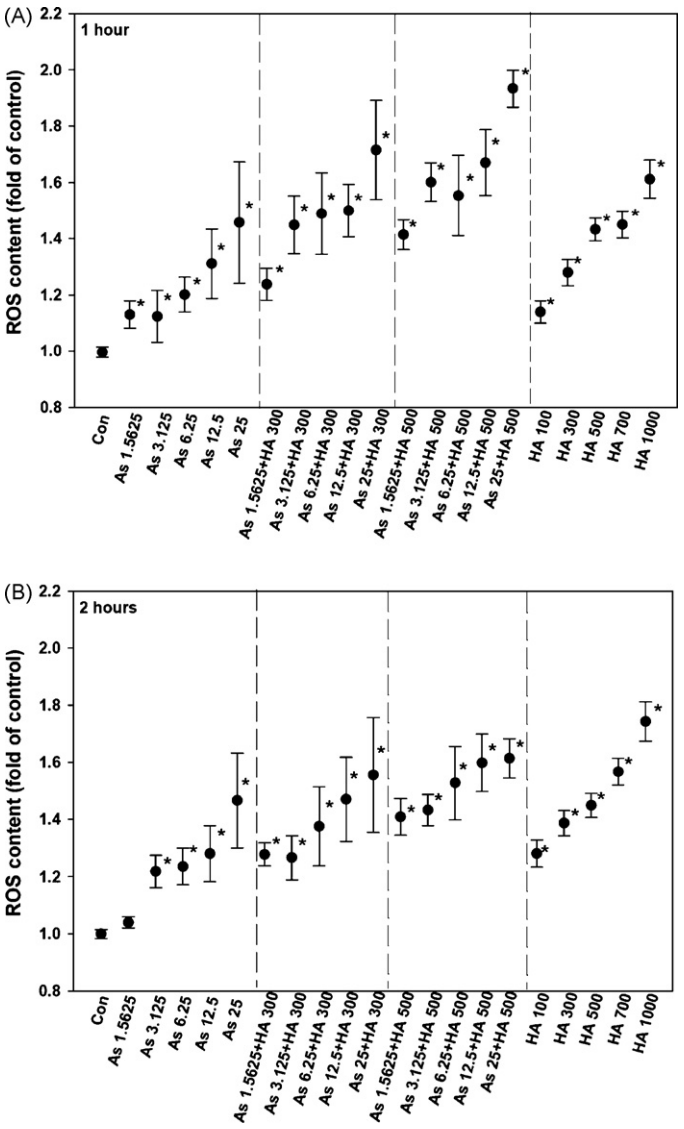

Fig. 7. Effect of HA and As_2O_3 on the intracellular oxidative property of HeLa cells. The cells were exposed to different concentrations of HA (100–1000 μg/mL), As_2O_3 (1.5625–25 μM), or both for (A) 1 or (B) 2 h. After removing the supernatant, the cells were washed two times with PBS, and the new medium which contained 10 μM DCFDA was added and incubated for a further 20 min. The cytoplasmic fluorescence intensity was measured by a fluorescence spectrometer (PerkinElmer LS-30) with excitation and emission wavelengths of 405 and 520 nm, respectively. The values are expressed in relation to untreated control cells. Data are presented as the mean ± SEM (n = 4–7). *$p < 0.05$ as compared with control.

4. Discussion

Apoptosis gives some clues to effective cancer therapy, with many chemotherapeutic agents reportedly exerting their antitumor effects by inducing apoptosis in cancer cells (Kamesaki, 1998). Recent studies have highlighted the relationship between apoptosis and cancer, with increasing evidence suggesting the processes of neoplastic transformation, progression and metastasis involve alteration of the normal apoptotic pathway (Bold et al., 1997; Yang et al., 2004).

With respect to its vascular effects, As_2O_3 has been shown to be anti-angiogenic at higher doses, such as those used in treating cancer, whereas it has also been shown lower, environmentally relevant doses of As_2O_3 are in fact pro-angiogenic and tumorigenic (Bishop and Kipling, 1978). Indeed, As_2O_3 has proven to be a promising anticancer agent for leukemia and some solid tumors

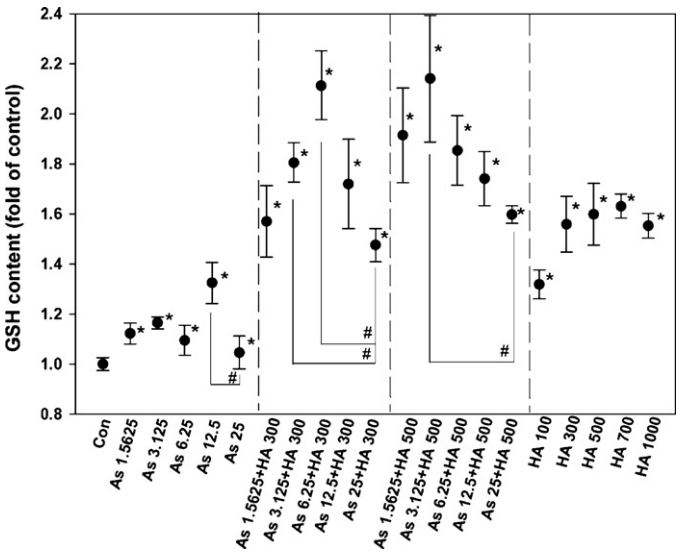

Fig. 8. Effect of HA and As_2O_3 on the intracellular GSH content of HeLa cells. The cells were exposed to different concentrations of HA (100–1000 μg/mL), As_2O_3 (1.5625–25 μM), or both for 8 h. Cells were washed with PBS after treatment, and then a new medium which contained 60 μM of mBCL, specific for GSH, was added and incubated for a further 30 min. Cells were lysed in lysing buffer, and the mBCL-GSH related fluorescence in the cells was monitored by a fluorescence spectrometer (PerkinElmer LS-30) with excitation and emission wavelengths of 385 and 485 nm, respectively. The values are expressed in relation to untreated control cells. Data are presented as the mean ± SEM (n = 4–6). $^*p < 0.05$ as compared with the control. $^\#p < 0.05$ as compared with two groups.

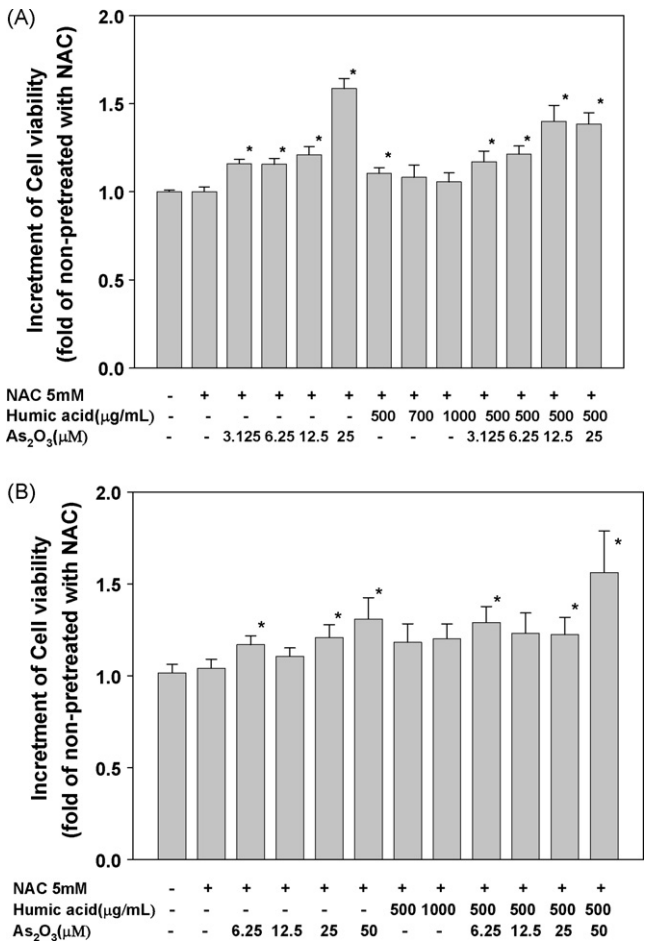

Fig. 9. The role of NAC (GSH donor) on the cell viability of HA, As_2O_3 or As_2O_3 combined with HA (500 μg/mL). HeLa cells (A) or SiHa cells (B) were preincubated for 30 min in a medium with 5 mM NAC, and then exposed to HA, As_2O_3 or a combination of HA and As_2O_3 for an extra 24 h. Cells viability was determined by MTT assays, as described in Section 2. The values are expressed in relation to the same exposure cells which were non-pretreated with NAC [(cell viability of pretreated with NAC)/(cell viability of non-pretreated with NAC)]. Data are presented as the mean ± SEM (n = 4–8). $^*p < 0.05$ as compared with non-pretreated NAC cells.

(Yu et al., 2007a,b; Wei et al., 2005; Chun et al., 2002; Murgo, 2001) Recent studies have shown As_2O_3 has apoptotic, anti-proliferative, antiviral, and antimetastatic effects (Woo et al., 2002; Dilda and Hogg, 2007).

On the other hand, As_2O_3, when used as a single anticancer agent, is able to inhibit the initial tumor growth in vivo, but after administration of As_2O_3 is stopped, no significant differences occur in the tumor growth delay time between the As_2O_3-treated and -untreated group (Ning and Knox, 2006). In addition, preliminary data from phase II clinical trials in patients with metastatic renal cell carcinoma, metastatic melanoma, advanced primary liver cancer, and stage IVB or recurrent cervical cancer has been completed, but the results are not yet available (Dilda and Hogg, 2007). These results suggest that the single agent As_2O_3 has limited effects on solid tumor growth, and As_2O_3 should be evaluated in combination with other therapeutic modalities to in order to try to enhance its efficiency in the clinical use against solid tumors (Ning and Knox, 2006).

Recently, it has been reported HA can induce apoptosis in HL-60 (Yang et al., 2004) and HIT-T15 cells (Yen et al., 2007), but we did not observe significant cytotoxicity of HA in HeLa and SiHa cells after 48 h of exposure (Fig. 1). Apoptosis is a highly regulated process involving activation of a cascade of molecular events leading to cell death. Further, dying HeLa and SiHa cells, following exposure to As_2O_3 or combined As_2O_3 with HA, display ultrastructural and biochemical features characteristic of apoptosis, as shown by the following: loss of cell viability (Fig. 2), increased the degree of DNA fragmentation as shown by the increase in DNA laddering as well as sub-G1 population (Figs. 3 and 4), 6-CFDA- and AnnCy3-positive staining (Fig. 5), and the activation of caspase 3 (Fig. 6). Our results show As_2O_3-induced inhibition of cell growth in cervical cancer cells, which was a similar finding to previous studies (Decaudin et al., 1998; Dai et al., 1999; Huang et al., 2000; Woo et al., 2002; Dilda and Hogg, 2007). In addition, HA enhanced the anti-proliferative action and growth inhibition of As_2O_3 in 2 different types of human cervical cancer cells, which down-regulated the LC_{50} about 57.62 or 73.52% (300 μg HA/mL) to 83.67 or 79.03% (500 μg HA/mL), respectively.

Recent reports suggest the generation of ROS is one of the major mediators of apoptosis of tumor cells by As_2O_3 (Woo et al., 2002; Kang et al., 2004). Due to the chemical properties of HA, it strongly interacts with both inorganic and organic agents, and thus might participate in redox regulation. As_2O_3 is well-known for its ability to induce the production of superoxide and H_2O_2 (Barchowsky et al., 1999; Woo et al., 2002). HA has been shown to generate ROS, such as superoxide anion (Cheng et al., 2003), and causes a depletion of glutathione and several antioxidant enzymes (Cheng et al., 1999). Latch and McNeill (2006) noted irradiated HA solutions can produce singlet oxygen in aquatic systems which can react with proteins, DNA, and other biomolecular, which have also been reported in our previous studies (Lu et al., 1988; Cheng et al., 1999, 2003; Yen et al., 2007). We used DCFDA as a free radical probe to study intracellular oxidation in HeLa cells treated for 1 and 2 h with HA or As_2O_3. Fig. 7 shows ROS (as H_2O_2) was significantly increased in HeLa cells exposed to HA and As_2O_3, and HA enhanced the oxidative stress induced by As_2O_3 at an early stage (1 h, Fig. 7A), and then decreased (2 h, Fig. 7B). On the other hand, ROS was constantly increased in HeLa cells to HA. It seems HA could be a more stable free radical donor compared with As_2O_3 in this study.

On the other hand, after exposure to different concentrations of HA and As_2O_3 in the medium for 8 h, the cellular GSH content rose to 1.05–2.14-fold of control levels (Fig. 8), especially when lower concentrations of As_2O_3 were combined with HA, which may be caused by up-regulation of GSH synthesis in the cells (Li and Chou, 1992). Intracellular GSH was significantly decreased after exposure to higher concentrations of As_2O_3 or As_2O_3 combined with HA for 8 h compared with exposure to lower concentrations of the drugs (Fig. 8). Based on our data, the increased oxidative stress induced by HA and As_2O_3 consumed the cellular GSH pool and damaged the HeLa cells, and combining HA and As_2O_3 intensified this effect, which agreed with previous studies (Cheng et al., 1999, 2003; Yen et al., 2007). Further, the anti-proliferative effects of HA and As_2O_3 in HeLa or SiHa cells could be restored by pretreatment with NAC, a precursor of GSH and an antioxidant (Fig. 9). Elevation of GSH in response to HA and As_2O_3 exposure may reflect a self-protective mechanism against cellular injury (Li and Chou, 1992; Yen et al., 2007).

The aim of this study was to examine the growth inhibitory properties and the effects of the combined use of HA and As_2O_3 in 2 different types of human cervical cancer cells in vitro. The results reported here show upon co-treatment of human cervical cancer HeLa or SiHa cells with As_2O_3 and HA, the anti-proliferative action and growth inhibition of cancer cells are significantly enhanced, most likely by ROS-mediated cell damage and activation of the apoptosis pathway. This study is of interest with respect to the development of chemotherapeutic approaches using As_2O_3 in treating human cervical cancer. In the future, we would like to clarify whether As_2O_3 or As_2O_3 combined with HA are able to inhibit invasion, and to describe the mechanisms by which HA enhances the sensitivity of human cervical cancer cells to As_2O_3-induced apoptosis.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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