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Synergistic effect of all-trans-retinoic acid and arsenic trioxide on growth inhibition and apoptosis in human hepatoma, breast cancer, and lung cancer cells *in vitro*

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AIM: To investigate the effect of all-trans-retinoic acid (ATRA) on arsenic trioxide (As_2O_3)-induced apoptosis of human hepatoma, breast cancer, and lung cancer cells in an attempt to find a better combination therapy for solid tumors.

METHODS: Human hepatoma cell lines HepG2, Hep3B, human breast cancer cell line MCF-7, and human lung adenocarcinoma cell line AGZY-83-a were treated with As_2O_3 together with ATRA. Cell survival fraction was determined by MTT assay, cell viability and apoptosis were measured by annexin V-fluorescein isothiocyanate (FITC) and PI staining, and intracellular glutathione (GSH) and glutathione-S-transferase (GST) activities were determined using commercial kits.

RESULTS: Cytotoxicity of ATRA was low. ATRA (0.1, 1, and 10 μmol/L) could synergistically potentiate As_2O_3 to exert a dose-dependent inhibition of growth and to induce apoptosis in each of the cell lines. HepG2 and Hep3B with low intracellular GSH or GST activities were remarkably sensitive to As_2O_3 or As_2O_3+ATRA, while AGZY-83-a with higher GSH or GST activities was less sensitive to As_2O_3 or As_2O_3+ATRA. Treatment with 2 μmol/L As_2O_3 for 72 h significantly decreased intracellular GSH and GST levels in each of the cell lines, and 1 μmol/L ATRA alone reduced minimal intracellular GSH and GST levels. ATRA potentiated the effect of As_2O_3 on intracellular GSH levels, but intracellular GST levels were not significantly affected by the combination of As_2O_3 and ATRA for 72 h as compared to As_2O_3 alone.

CONCLUSION: ATRA can strongly potentiate As_2O_3-induced growth-inhibition and apoptosis in each of the cell lines, and two drugs can produce a significant synergic effect. The sensitivity to As_2O_3 or As_2O_3+ATRA is inversely proportional to intracellular GSH or GST levels in each of the cell lines. The GSH redox system may be the possible mechanism by which ATRA synergistically potentiates As_2O_3 to exert a dose-dependent inhibition of growth and to induce apoptosis.

Key words: Arsenic trioxide; All-trans-retinoic acid; Hepatocellular carcinoma; Cancer of breast and lung; Glutathione; Glutathione-S-transferase

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Liver, breast, and lung cancers are the most common malignant diseases in the human race. Surgical resection or radiation therapy is potentially curative for localized diseases. Advanced liver, breast, and lung cancers are associated with a poor prognosis, and conventional chemotherapies and radiation therapy are still of limited effectiveness. Innovative approaches for advanced disease are necessary.

During the last decade, investigators using As_2O_3 alone or the combination of all-trans-retinoic acid (ATRA) and As_2O_3 for patients with acute promyelocytic leukemia (APL), have achieved great success[1-3]. Because of the many pathways involved in mediating the effects of arsenic, the potential exists for synergism with other agents to provide enhanced therapeutic benefits. Retinoic acid and arsenic synergize to eradicate leukemic cells in a mouse model of APL[4,5].

Other studies have also shown that As_2O_3 or ATRA alone has antiproliferative and apoptotic activities in some solid tumors, including human hepatoma and breast cancer[6-8]. Because of the drug resistance of solid tumors to As_2O_3, it has not been widely used in the treatment of solid tumors. Therefore, this study was to investigate the effect of ATRA on As_2O_3-induced cell apoptosis in human

hepatoma, breast cancer, and lung cancer in an attempt to find a better combination therapy for solid tumors. To our knowledge, this is the first report on the effects of the combination of As_2O_3 and ATRA for solid tumors.

The glutathione (GSH) redox system is known to modulate the growth-inhibitory and apoptotic effect of As_2O_3. Different kinds of malignant cells have different GSH levels. It was reported that the GSH redox system is relative to sensitivity of malignant cells to As_2O_3[9]. Elevated GSH levels are associated with the chemoresistance of malignant cells. Optimal therapies for chemoresistant malignant cells should overcome or bypass the increased intracellular GSH levels. Glutathione-S-transferase (GST), an enzyme involved in metabolic detoxification of a variety of xenobiotics, is increased in an arsenic-resistant CHO cell line[10,11]. Therefore, we also measured intracellular GSH and GST levels in malignant cells, when they were treated in the absence or presence of As_2O_3, ATRA, or As_2O_3+ATRA.

Materials

Human hepatoma cell lines HepG2, Hep3B, human breast cancer cell line MCF-7 (American Type Culture Collection, Rockville, MD, USA) and human lung adenocarcinoma cell line AGZY-83-a (Harbin Medical University) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mmol/L L-glutamine, and 10% heat-inactivated fetal bovine serum in a humidified atmosphere of 50 mL/L CO_2 at 37 ¡æ Cells in logarithmic growth were seeded at 1×10^5 cells/mL for studies performed in duplicate and repeated at least thrice. As_2O_3 solution (0.1%) was purchased from Harbin Medical University (Harbin, China). ATRA, MTT, and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell proliferation and cell survival rate tested with MTT

Cell proliferation was measured by a MTT assay. For proliferation assays, cells were plated onto 96-well plates (2×10^3 cells/well, 200 μL cell suspension per well) and cultured overnight to allow for cell attachment. Cells were then treated with drugs of different concentrations in the absence (control) or presence of As_2O_3 (0.5, 1, 1.5, and 2 μmol/L), ATRA (0.1, 1, and 10 μmol/L), or As_2O_3+ATRA. All groups had concentrations of four compound wells. After incubation for 72 h, 20 μL of 0.5% MTT was added to each well and incubated for another 4 h. The supernatant was discarded and 150 μL of DMSO was added. When the stain was dissolved, the optical density ABS (absorbance) value of each well was read on Minireader II at 490 nm. Cell survival rate was calculated with the following equation: average *A* value of experimental group/ average *A* value of control group×100%. Each experiment was repeated at least thrice.

Quantitation of cell viability and apoptotic cells

Cell viability and apoptosis were measured by annexin V-fluorescein isothiocyanate (FITC) (Becton Dickinson) and PI staining. Cells were plated onto six-well dishes (1×10^5 cells/dish) and grown overnight to allow for cell attachment. They were then treated in the absence (control) or presence of As_2O_3 (2 μmol/L), ATRA (1 and 10 μmol/L), or As_2O_3+ATRA (1 and 10 μmol/L) for 72 h. After the indicated time, cells were harvested, washed once in PBS and stained with annexin V-FITC (Biovision) and PI (2 mg/mL) according to manufacturer's instructions. Samples were acquired on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) and analyzed with CellQuest software (Becton Dickinson).

Intracellular glutathione measurements

Intracellular GSH levels were determined in all cell lines. Cells were plated onto six-well dishes (1×10^5 cells/dish) and grown overnight to allow for cell attachment. They were then treated in the absence (control) or presence of As_2O_3 (2 μmol/L), ATRA (1 μmol/L), or As_2O_3+ATRA for 72 h. After the indicated time, cells were harvested, and intracellular GSH was measured using the GSH assay kit (Calbiochem, San Diego, CA, USA). Briefly, cells were pelleted, resuspended in 500 μL ice-cold 5% metaphosphoric acid, and homogenized with a Teflon pestle and an overhead stirrer. After centrifugation at 3 000 r/min for 10 min at 4 ¡æ, 60 μL supernatant, 60 μL solution R1, 2 μL solution R2, and 1 mL solution R3 were combined according to the manufacturer's instructions. Samples were incubated at room temperature for 5 min in the dark, and the final absorbance was measured at 412 nm and compared to a GSH standard curve. To quantitate the total protein, the pellet from the above centrifugation was resuspended in 1 mol/L NaOH, and the protein concentration was measured using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA, USA). Intracellular GSH was normalized to total protein content.

Measurement of intracellular GST

GST activity was determined using commercial kits (Calbiochem, San Diego, CA, USA). GST activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) and GSH as substrates. Cells were plated onto six-well dishes (1×10^5 cells/dish) and grown overnight to allow for cell attachment. They were then treated in the absence (control) or presence of As_2O_3 (2 μmol/L), ATRA (1 μmol/L), or As_2O_3+ATRA for 72 h. After the indicated time, cells were harvested. The cell pellets were resuspended in 300 μL of 100 mmol/L potassium phosphate buffer, pH 6.8, sonicated for 10 s at 4 ¡æ and centrifuged at 14 000 *g* for 30 min. Supernatant was used for GST measurement according to the manufacturer's instructions. The absorbance at 412 nm was continuously recorded for 2 min. The pellet was dissolved in 1 mol/L NaOH and analyzed for protein by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). The GST content was expressed as nanomoles per milligram protein per minute.

Statistical analysis

Data were presented graphically as mean±SD. Treatment groups were compared by independent *t*-test or paired *t* test as appropriate, with *P* reported in each figure legend. Statistical analyses were performed using SPSS 10.1 software.

Effects of ATRA and As_2O_3 on growth inhibition in each cell line

To examine the possible relationship between serum concentration and growth-inhibitory effects of the two drugs, cells were treated with drugs of different concentrations in the absence (control) or presence of As_2O_3 (0.5, 1, 1.5, and 2 μmol/L), ATRA (0.1, 1, and 10 μmol/L), or As_2O_3+ATRA for 72 h, and then cell growth was measured by MTT assays.

The ATRA alone at 0.1, 1, and 10 μmol/L could only moderately inhibit cell growth in each of the cell lines. As_2O_3 exerted a dose-dependent growth inhibition of the HepG2, Hep3B, MCF-7, AGZY-83-a cells (Figures 1A-D).

Interestingly, the combination of ATRA (0.1, 1, and 10 μmol/L) and As_2O_3 had at least an additive effect on growth inhibition in each of the cell lines. ATRA (0.1, 1, and 10 μmol/L) could synergistically potentiate As_2O_3 to exert a dose-dependent growth inhibition in each of the cell lines. High concentration of ATRA (10 μmol/L) exerted greater synergistic effects on growth inhibition than low concentration (0.1 μmol/L, Figures 1A-D).

Effects of two drugs on apoptosis in each cell line

Cytotoxicity of ATRA was low. The apoptotic rate of 1 or 10 μmol/L of ATRA approached to that of control in each of the cell lines, and the difference was not significant

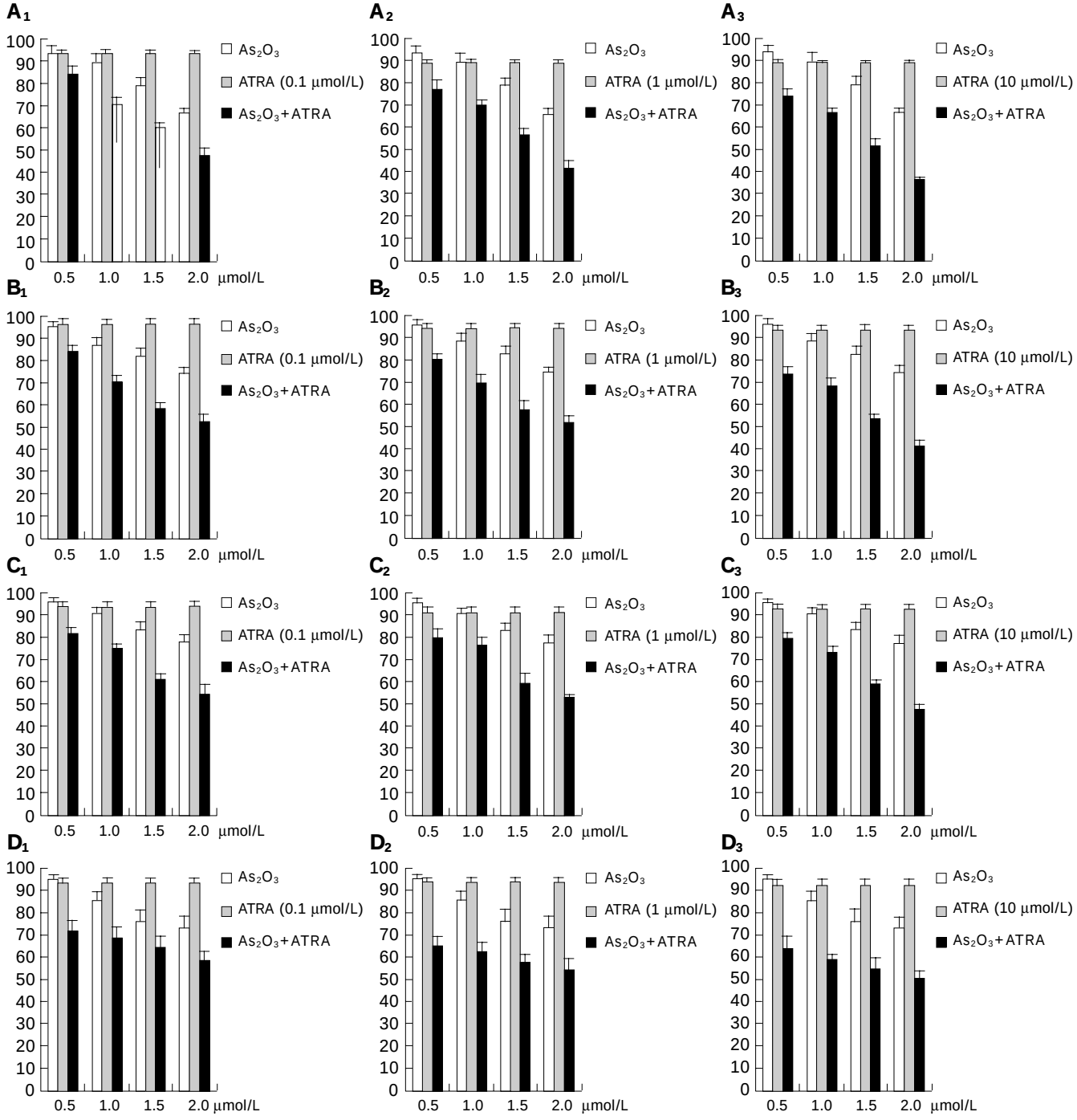

Figure 1 Effects of ATRA and As_2O_3 on growth inhibition in cancer cell lines HepG2 (A), Hep3B (B), MCF-7 (C), and AGZY-83-a (D).

Table 1 Basal activities of GSH or GST and As_2O_3+ ATRA or As_2O_3-induced apoptosis in different cell lines (mean±SD)

Cell lines	GSH	GST	As_2O_3	Apoptosis (%)	
				As_2O_3+ATRA (10 μmol/L)	As_2O_3+ ATRA (1 μmol/L)
HepG2	60.7±7.1	22.3±1.4	29.3±3.2	61.1±5.6	48.4±4. 1
Hep3B	62.4±5.4	21.4±2.8	27.3±6.5	57.1±4.9	49.3±4.2
MCF-7	76.0±5.1	31.0±2.6	22.6±3.3	45.5±3.0	40.2±3.3
AGZY-83-a	104.8±8.1	50.6±4.5	19.0±3.8	40.8±5.6	32.9±3.0

(P>0.05). ATRA greatly potentiated the apoptosis induced by As_2O_3. Exposure of the cells to the combination of As_2O_3 (2 μmol/L) and ATRA (10 μmol/L) for 72 h synergistically induced apoptosis in HepG2 (61.1±5.6%), Hep3B (57.1±4.9%), MCF-7 (45.5±3.0%), AGZY-83-a (40.8±5.6%) respectively, as measured by annexin V-FITC and PI staining as compared to exposure of the cells to either agent alone (Figure 2A). A similar, dramatic effect was observed, when they were treated in the absence (control) or presence of As_2O_3 (2 μmol/L), ATRA (1 μmol/L), or As_2O_3+ATRA for 72 h, with HepG2 being 48.4±4.1%, Hep3B being 49.3±4.2%, MCF-7 being 40.2±3.3%, AGZY-83-a being 32.9±3.0% respectively (Figure 2B). All these findings indicated that ATRA could synergistically potentiate As_2O_3 to induce apoptosis in each of the cell lines.

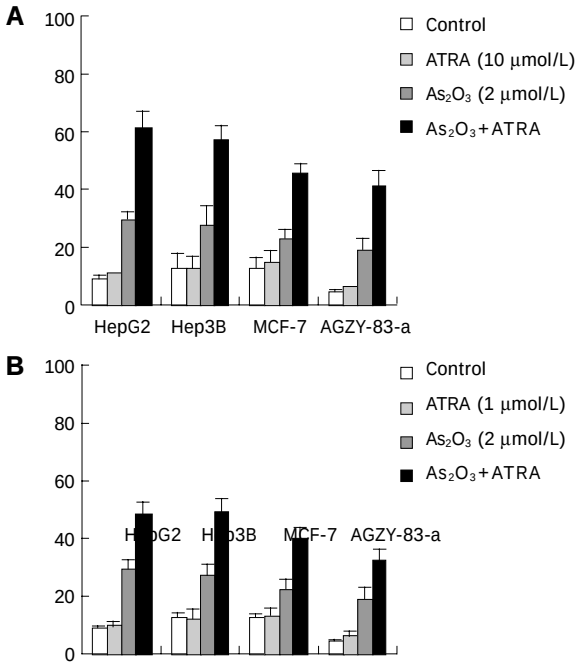

Figure 2 Effects of combined ATRA (10 mol/L: A, 1 mol/L: B) and As_2O_3, and either agent alone on apoptosis in cancer cell lines.

Changes of intracellular GSH and GST

HepG2 and Hep3B with low intracellular GSH or GST activities, were remarkably sensitive to 2 μmol/L As_2O_3 or As_2O_3 (2 μmol/L)+ATRA (10 μmol/L), the apoptotic rate was 29.3±3.2% and 61.1±5.6%, respectively. AGZY-83-a with higher GSH or GST activities was less sensitive to As_2O_3 or As_2O_3+ATRA, the apoptotic rate was 19.0±3.8% and 40.8±5.6%, respectively (Table 1). The sensitivity to As_2O_3 or As_2O_3+ATRA was inversely proportional to intracellular GSH or GST levels in each of the cell lines. A similar, dramatic effect was observed when they were treated in the presence of As_2O_3 (2 μmol/L), or As_2O_3 (2 μmol/L)+ATRA (1 μmol/L) for 72 h (Table 1).

Dramatic changes of intracellular GSH or GST activities were observed when the cell lines were treated in the absence (control) or presence of As_2O_3 (2 μmol/L), ATRA (1 μmol/L), or As_2O_3+ATRA for 72 h. Treatment with 2 μmol/L As_2O_3 for 72 h significantly decreased the intracelluar GSH and GST levels in each of the cell lines, and 1 μmol/L ATRA alone reduced minimal intracellular GSH and GST levels. ATRA potentiated the effect of As_2O_3 on intracellular GSH levels, but intracellular GST levels were not significantly affected by the combination of As_2O_3 (2 μmol/L) and ATRA (1 μmol/L) for 72 h as compared to As_2O_3 alone (Tables 2 and 3).

Table 2 Effect of As_2O_3 on intracellular GSH levels (mean±SD)

Cell lines	Control	ATRA[a]	As_2O_3[c]	As_2O_3+ATRA[e]
HepG2	60.7±7.1	54.4±4.9	46.7±9.0	26.8±4.4
Hep3B	62.4±5.4	55.6±0.5	35.3±3.0	25.9±3.6
MCF-7	76.0±5.1	69.9±1.7	52.5±2.4	44.1±3.8
AGZY-83-a	104.8±8.1	99.8±9.4	89.8±5.6	80.4±1.0

[a]P>0.05, [c]P<0.05 *vs* the control cells, [e]P<0.05 *vs* As_2O_3-treated cells.

Table 3 Changes in intracellular GST activities (mean±SD)

Cell lines	Control	ATRA[a]	As_2O_3[c]	As_2O_3+ATRA[e]
HepG2	22.3±1.4	21.2±1.7	5.5±1.8	8.6±2.3
Hep3B	21.4±2.8	20.4±4.4	8.6±1.8	10.3±3.4
MCF-7	31.0±2.6	29.7±3.0	16.4±1.6	19.3±1.4
AGZY-83-a	50.6±4.5	49.3±4.7	36.3±0.9	39.3±3.1

[a]P>0.05, [c]P<0.05 *vs* the control cells, [e]P>0.05 *vs* As_2O_3-treated cells.

Our *in vitro* studies showed that the combination of As_2O_3 and ATRA was statistically superior to either As_2O_3 or ATRA alone in the treatment of the three cell lines. Furthermore, ATRA (0.1, 1, and 10 μmol/L) could synergistically potentiate As_2O_3 to exert a dose-dependent growth inhibition in each of the cell lines. Cell survival rate could be reduced from 89.5±3.9% to 70.5±3.3% in HepG2 cells exposed to 1 μmol/L As_2O_3 or the combination of 1 μmol/L As_2O_3 and 0.1 μmol/L ATRA for 72 h.

Apoptosis is important for the development and

homeostasis of multicellular organisms. Specific therapies have been designed to enhance the susceptibility of human cancers to apoptosis. We showed that the combination of As_2O_3 and ATRA dramatically and significantly increased the number of apoptotic cells in each of the cell lines, especially the human hepatoma cell lines HepG2 and Hep3B.

Drug resistance of cancer cells and toxicity of cell apoptotic agents are the major factors contributing to the failure of chemotherapy. At least four distinct mechanisms contribute to the chemoresistance. Cellular response to steroids typically depends on the expression of the glucocorticoid receptor, and resistance to steroid therapies is classically associated with downregulation or loss of glucocorticoid receptor expression in some malignant cells[12,13]. The overexpression of drug efflux pumps, such as *mdr* gene product P-glycoprotein (PgP), is also a common trait of chemoresistant malignant cells[14,15]. In addition, it was reported that the expression of the antiapoptotic protein Bcl-xL is higher in some chemoresistant malignant cells[16]. Furthermore, increased expression or activity of GSH and GSH-related enzymes confers resistance to antineoplastic agents[17,18]. GSH is the major auto-oxidant of the cells, and functions to scavenge free radicals and to detoxify toxins and chemotherapeutic agents. GSH can bind to arsenic by the formation of a transient $As(GS)_3$ complex[19].

The GSH redox system is known to modulate the growth-inhibitory and apoptotic effect of As_2O_3. Different kinds of malignant cells have different GSH levels. It was reported that the GSH redox system is relative to sensitivity of malignant cells to As_2O_3[9]. Elevated GSH levels are associated with the chemoresistance of malignant cells. Optimal therapies for chemoresistant malignant cells should overcome or bypass the increased intracellular GSH levels. It is known that GST, an enzyme involved in metabolic detoxification of a variety of xenobiotics, is increased in an arsenic-resistant CHO cell line[10]. Our study showed that HepG2 and Hep3B with low intracellular GSH or GST activities were remarkably sensitive to As_2O_3 or As_2O_3+ATRA, while AGZY-83-a with higher GSH or GST activities was less sensitive to As_2O_3 or As_2O_3+ATRA. The sensitivity to As_2O_3 or As_2O_3+ATRA was inversely proportional to GSH or GST levels in each of the cell lines.

Arsenite can decrease GSH levels which can result in DNA damage as a result of increased intracellular reactive oxygen molecules. Our intracellular GSH and GST assays showed that treatment with 2 μmol/L As_2O_3 for 72 h significantly decreased intracellular GSH and GST levels in each of the cell lines, and 1 μmol/L ATRA alone reduced minimal intracellular GSH and GST levels (Tables 2 and 3). ATRA potentiated the effect of As_2O_3 on intracellular GSH levels, but intracellular GST levels were not significantly affected by the combination of As_2O_3 (2 μmol/L) and ATRA (1 μmol/L) for 72 h as compared to As_2O_3 alone. These findings indicate that GSH redox system may be the possible mechanism by which ATRA synergistically potentiates As_2O_3 to induce apoptosis or to exert a dose-dependent inhibition of growth. Either As_2O_3 alone or in combination with ATRA may become a useful adjuvant therapy for liver, breast, and lung cancers.

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