AIM: To investigate the effect of all-trans-retinoic acid (ATRA) on arsenic trioxide (As$_2$O$_3$)-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$_2$O$_3$ 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$_2$O$_3$ 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$_2$O$_3$ or As$_2$O$_3$+ATRA, while AGZY-83-a with higher GSH or GST activities was less sensitive to As$_2$O$_3$ or As$_2$O$_3$+ATRA. Treatment with 2 µmol/L As$_2$O$_3$ 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$_2$O$_3$ on intracellular GSH levels, but intracellular GST levels were not significantly affected by the combination of As$_2$O$_3$ and ATRA for 72 h as compared to As$_2$O$_3$ alone.

CONCLUSION: ATRA can strongly potentiate As$_2$O$_3$-induced growth-inhibition and apoptosis in each of the cell lines, and two drugs can produce a significant synergic effect. The sensitivity to As$_2$O$_3$ or As$_2$O$_3$+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$_2$O$_3$ 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

Lever, 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$_2$O$_3$ alone or the combination of all-trans-retinoic acid (ATRA) and As$_2$O$_3$ for patients with acute promyelocytic leukemia (APL), have achieved great success. 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.

Other studies have also shown that As$_2$O$_3$ or ATRA alone has antiproliferative and apoptotic activities in some solid tumors, including human hepatoma and breast cancer. Because of the drug resistance of solid tumors to As$_2$O$_3$, it has not been widely used in the treatment of solid tumors. Therefore, this study was to investigate the effect of ATRA on As$_2$O$_3$-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$_2$O$_3$ and ATRA for solid tumors.

The glutathione (GSH) redox system is known to modulate the growth-inhibitory and apoptotic effect of As$_2$O$_3$. 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$_2$O$_3$.$^{[9]}$ 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$_2$O$_3$, ATRA, or As$_2$O$_3$+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 mM L-glutamine, and 10% heat-inactivated fetal bovine serum in a humidified atmosphere of 5% CO$_2$ at 37°C. Cells in logarithmic growth were seeded at 1×10$^5$ cells/mL for studies performed in duplicate or repeated at least thrice. As$_2$O$_3$ 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 cell proliferation tests, cells were plated onto six-well plates (2×10$^3$ cells/well, 200 µL cell suspension per well) and cultured overnight to allow for cell attachment. They were then treated in the absence (control) or presence of As$_2$O$_3$ (2 µmol/L), ATRA (1 µmol/L), or As$_2$O$_3$+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$^6$ cells/dish) and grown overnight to allow for cell attachment. They were then treated in the absence (control) or presence of As$_2$O$_3$ (2 µmol/L), ATRA (1 µmol/L), or As$_2$O$_3$+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°C, 60 µL supernatant, 60 µL solution R1, 2 µL solution R2, and 1 µL 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 I-chloro-2,4-dinitrobenzene (CDNB) and GSH as substrates. Cells were plated onto six-well dishes (1×10$^6$ cells/dish) and grown overnight to allow for cell attachment. They were then treated in the absence (control) or presence of As$_2$O$_3$ (2 µmol/L), ATRA (1 µmol/L), or As$_2$O$_3$+ATRA for 72 h. After the indicated time, cells were harvested. The cell pellets were resuspended in 300 µL of 100 mM potassium phosphate buffer, pH 6.8, sonicated for 10 s at 4°C 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$_2$O$_3$ 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$_2$O$_3$ (0.5, 1, 1.5, and 2 µmol/L), ATRA (0.1, 1, and 10 µmol/L), or As$_2$O$_3$+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$_2$O$_3$ 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$_2$O$_3$ 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$_2$O$_3$ 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.

Interestingly, the combination of ATRA (0.1, 1, and 10 µmol/L) and As$_2$O$_3$ 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$_2$O$_3$ 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).

Figure 1 Effects of ATRA and As$_2$O$_3$ on growth inhibition in cancer cell lines HepG2 (A), Hep3B (B), MCF-7 (C), and AGZY-83-a (D).
As 2O3. Exposure of the cells to the combination of As2O3 (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 As2O3 alone (40.8±5.6%) or ATRA (45.6±3.0%) alone in the treatment of the three cell lines. Furthermore, treatment with 10 µmol/L As2O3 for 72 h significantly decreased the intracellular GSH and GST levels in each of the cell lines, and ATRA alone in the presence of As2O3 (2 µmol/L) was superior to either As2O3 or ATRA (10 µmol/L) for 72 h as compared to control cells.

Our in vitro studies showed that the combination of As2O3 and ATRA was statistically superior to either As2O3 or ATRA alone in the treatment of the three cell lines. Furthermore, ATRA (0.1, 1, and 10 µmol/L) could synergistically potentiate As2O3 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 As2O3 or the combination of 1 µmol/L As2O3 and 0.1 µmol/L ATRA for 72 h.

Apoptosis is important for the development and

### Table 1 Basal activities of GSH or GST and As2O3+ATRA or As2O3-induced apoptosis in different cell lines (mean±SD)

| Cell lines | GSH        | GST        | As2O3 | Apoptosis (%) |
|------------|------------|------------|-------|---------------|
|            | Control    | ATRA       | As2O3 | As2O3+ATRA     |
| HepG2      | 60.7±7.1   | 22.3±4.4   | 29.3±3.2 | 61.1±5.6   |
| Hep3B      | 62.4±5.4   | 21.4±2.8   | 27.3±6.5 | 57.1±4.9   |
| MCF-7      | 76.0±5.1   | 31.0±2.6   | 22.6±3.3 | 45.5±3.0   |
| AGZY-83-a  | 104.8±8.1  | 50.6±4.5   | 19.0±3.8 | 40.8±5.6   |

### Table 2 Effect of As2O3 on intracellular GSH levels (mean±SD)

| Cell lines | Control    | ATRA       | As2O3 | As2O3+ATRA     |
|------------|------------|------------|-------|---------------|
| 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±3.6   |
| AGZY-83-a  | 104.8±8.1  | 99.8±9.4   | 89.8±5.6 | 80.4±1.0   |

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

| Cell lines | Control    | ATRA       | As2O3 | As2O3+ATRA     |
|------------|------------|------------|-------|---------------|
| 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±1.6 | 10.3±3.4   |
| MCF-7      | 31.0±2.6   | 29.7±3.0   | 16.4±1.6 | 19.3±3.4   |
| AGZY-83-a  | 50.6±5.5   | 49.3±7.7   | 36.3±0.9 | 39.3±3.1   |

Changes of intracellular GSH and GST

HepG2 and Hep3B with low intracellular GSH or GST activities, were remarkably sensitive to 2 µmol/L As2O3 or As2O3 (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 As2O3 or As2O3+ATRA, the apoptotic rate was 19.0±3.8% and 40.8±5.6%, respectively (Table 1). The sensitivity to As2O3 or As2O3+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 As2O3 (2 µmol/L), or As2O3 (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 As2O3 (2 µmol/L), ATRA (1 µmol/L), or As2O3+ATRA for 72 h, with HepG2 being 48.4±4.1%, Hep3B being 49.3±4.2%, MCF-7 being 40.8±3.3%, AGZY-83-a being 32.9±3.0% respectively (Figure 2B). All these findings indicated that ATRA could synergistically potentiate As2O3 to induce apoptosis in each of the cell lines.
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$_2$O$_3$ 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$_2$O$_3$. 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$_2$O$_3$.[20] 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.[21] Our study showed that HepG2 and Hep3B with low intracellular GSH or GST activities were remarkably sensitive to As$_2$O$_3$ or As$_2$O$_3$+ATRA, while AGZY-83-a with higher GSH or GST activities was less sensitive to As$_2$O$_3$ or As$_2$O$_3$+ATRA. The sensitivity to As$_2$O$_3$ or As$_2$O$_3$+ATRA was inversely proportional to GSH or GST levels in each of the cell lines. Arsenic 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$_2$O$_3$ 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$_2$O$_3$ on intracellular GSH levels, but intracellular GST levels were not significantly affected by the combination of As$_2$O$_3$ (2 µmol/L) and ATRA (1 µmol/L) for 72 h as compared to As$_2$O$_3$ alone. These findings indicate that GSH redox system may be the plausible mechanism by which ATRA synergistically potentiates As$_2$O$_3$ to induce apoptosis or to exert a dose-dependent inhibition of growth. Either As$_2$O$_3$ alone or in combination with ATRA may become a useful adjuvant therapy for liver, breast, and lung cancers.

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