

Tetra-arsenic tetra-sulfide induces cell cycle arrest and apoptosis in retinoic acid-resistant acute promyelocytic leukemia cells

YUAN WANG¹, PENG-CHENG HE¹, JUN QI², YAN-FENG LIU¹ and MEI ZHANG¹

¹Department of Hematology, The First Affiliated Hospital, Xi'an Jiaotong University Medical College;

²Institute of Xi'an Blood Bank, Shaanxi Blood Center, Xi'an, Shaanxi 710061, P.R. China

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Abstract. Previous studies have shown that the therapeutic action of tetra-arsenic tetra-sulfide (As_4S_4) is effective for acute promyelocytic leukemia. However, the molecular mechanism of the action of As_4S_4 in retinoic acid-resistant acute promyelocytic leukemia (APL) therapy remains unclear. In the present study, the signaling of the cytotoxic effects induced by As_4S_4 on retinoic acid-resistant APL NB4-R1 cells was investigated. A time-dependent increase in cell death and DNA cleavage was observed following As_4S_4 treatment. Changes in B-cell lymphoma 2 and Bax accompanied by the activation of caspase-3 and cleavage of poly ADP-ribose polymerase were observed as actions of As_4S_4 . As_4S_4 induced an accumulation of NB4-R1 cells in the S and G₂/M phases, as detected by flow cytometry. Therefore, the present results suggest that As_4S_4 -mediated apoptosis in NB4-R1 cells involves a mitochondria-dependent pathway.

Introduction

Acute promyelocytic leukemia (APL) is a form of acute myeloid leukemia, which has been identified as the M₃ subtype. A unique chromosome translocation t(15; 17)(q22; q21) found in the majority of APL patients leads to the formation of the promyelocytic leukemia retinoic acid receptor α (PML-RAR α) fusion gene (1). The fusion protein encoded by the PML-RAR α gene polymerizes and combines with retinoid-X receptor. The resultant protein complexes enhance histone deacetylase, thus repressing the transcription of the gene and disrupting the retinoic acid signal pathway under physiological concentrations of retinoic acid (1). This change results in the excessive growth

of malignant promyelocytes and an inhibition of granulocyte differentiation.

All-trans retinoic acid (ATRA) as a successful model of differentiation therapy has improved the curative effect and extended the survival time of patients with APL. Clinical data has shown that the application of ATRA combined with chemotherapy increases the clinical complete response rate to $\leq 95\%$ and the 2-year event-free survival rate was 86% (2). However, fatal retinoic acid syndrome and ATRA resistance in the majority of patients ultimately leads to treatment failure. Additionally, 31% of patients administered ATRA combined with chemotherapy relapse within 4-5 years after complete remission (3). Therefore, it is essential to identify new drugs for APL patients.

Arsenic trioxide (As_2O_3) and tetra-arsenic tetra-sulfide (As_4S_4), as traditional medicines, have been used widely for the treatment of newly diagnosed and relapse APL. The side effects, such as fluid retention, skin rashes, leukocytosis, gastrointestinal discomfort, pulmonary infiltrates, neuropathy, prolongation of the corrected QT interval, liver function abnormality and sudden death, make it difficult for APL patients to accept As_2O_3 as a single agent for long-term treatment (4). Therefore, As_2O_3 should be incorporated into combination medications with ATRA or used as a salvage therapy for relapse APL patients.

As_4S_4 , which exerts similar effectiveness and less toxicity, provides not only a better quality-of-life, but is also advantageous in cytogenetic remission and PML-RAR α reversion for newly diagnosed and hematological relapse patients. Following a single application of As_4S_4 , the leukemia-free survival rate (LFS) for 1 and 3 years reached 86.1 and 76.6%, respectively, among newly diagnosed APL patients, with a median follow-up time of 13.5 months. In addition, the LFS for 1 and 6 years was 96.7 and 87.4% for the hematological complete remission group, with a median follow-up of 23 months (5). A previous study suggested that the LFS of APL patients at 2 years treated with As_4S_4 is higher than for those treated with As_2O_3 (6). Therefore, oral As_4S_4 is not inferior to intravenous As_2O_3 as an effective treatment for APL and may be considered a routine treatment option for the appropriate patients. The exact molecular mechanism of the drug's action remains unclear and warrants further investigation. The aim of the present study was to characterize the toxicity and apoptosis induced by As_4S_4 in a specific human APL NB4-R1 cell line that exhibits resistance to ATRA.

Correspondence to: Professor Mei Zhang, Department of Hematology, The First Affiliated Hospital, Xi'an Jiaotong University Medical College, 277 Yanta West Road, Xi'an, Shaanxi 710061, P.R. China
E-mail: meizhang_med@163.com

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Materials and methods

Cell culture and reagents. The NB4-R1 APL-derived cells from an RA-resistant promyelocytic cell line were generously supplied by Shanghai Second Medical College (Shanghai, China). The NB4-R1 cells were cultured in RPMI-1640 (Gibco-BRL, Carlsbad, CA, USA) medium supplemented with 10% heat-inactivated (at 56°C for 30 min) fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin, and maintained in a 5% CO₂ humidified atmosphere at 37°C. The cells were grown at an optimal cell density between 5×10⁵ and 1×10⁶/ml. Cell viability was evaluated via trypan blue dye exclusion assays and the cell survival rate was >95%. As₄S₄ (Xi'an Traditional Chinese Drug Company, Xi'an, China) stock solution was obtained by dissolving in the 1.0 M NaOH assistant agent. According to the IC₅₀ of NB4-R1 cells in our previous study (7), the exponentially growing cells were treated with 25 µmol/l As₄S₄ for 0, 24 or 48 h and the cells were analyzed via flow cytometry, DNA ladder electrophoresis and western blot analysis.

Measurement of apoptosis

Annexin V-FLUOS/propidium iodide (PI) binding study using flow cytometry. Flow cytometric analysis using Annexin V-FLUOS and PI (Roche Custom Biotech, Indianapolis, IN, USA) was performed to differentiate between live, apoptotic and necrotic cells following treatment with As₄S₄. Subsequent to the treatment with 25 µmol/l As₄S₄ for 0, 24 or 48 h, 1×10⁶ cultured cells were harvested and washed twice with cold phosphate-buffered saline (PBS). The cells were centrifuged at 200 × g for 5 min at 4°C and re-suspended in 100 µl of Annexin V-FLUOS/PI labeling solution for 10–15 min in the dark at room temperature. The stained cell suspension was immediately analyzed using a flow cytometer (BD Biosciences FACSCalibur double laser flow cytometer; BD Biosciences, Franklin Lakes, NJ, USA). The data analysis was performed using the CellQuest software program (BD Biosciences).

DNA ladder agarose gel electrophoresis. DNA ladder fragmentation reflecting the endonuclease activity is a characteristic feature of apoptosis. After incubation for 0, 24 or 48 h with As₄S₄, the NB4-R1 cells were collected and washed twice with PBS. Subsequently, 1×10⁶ cells were solubilized and the chromosomal DNA was extracted and purified using an Apoptotic DNA Ladder kit (Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer's instructions. The DNA samples were electrophoresed on a 1.5% agarose gel containing 1 mg/ml ethidium bromide at 60 V for 2 h. The apoptotic DNA fragments were analyzed and photographed using a Quantity One gel image analysis system (ChemIDOC XRS; Bio-Rad, Richmond, CA, USA).

Cell cycle analysis. The cell cycle distribution was analyzed via flow cytometry (BD Biosciences FACSCalibur double laser flow cytometer). Following treatment with As₄S₄ for 0, 24 or 48 h, the NB4-R1 cells (1×10⁶) were harvested and washed twice with ice-cold PBS. The cells were suspended gently in 70% chilled ethanol at -20°C overnight. After washing with PBS, the cells were re-suspended in 500 µl

PBS containing PI (50 µg/ml) and RNase (50 µg/ml), and were incubated for 30 min at room temperature in the dark. The cell cycle phase distribution of each experiment was analyzed using 10,000 cells per sample. The proportion of cells in the G₀/G₁, S and G₂/M phases were represented as DNA histograms.

Western blot analysis. After treatment with 25 µmol/l As₄S₄ for 0, 24 or 48 h, the cultured cells were harvested and washed three times with cold PBS. Subsequently, the cells were solubilized in radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail (Sigma, St. Louis, MO, USA). After incubation on ice for 10 min, the cell suspension was centrifuged for protein at 15,500 × g for 15 min at 4°C. The protein (30 µg) was separated on 10% SDS-PAGE and transferred to a nitrocellulose membrane at 110 V for 2 h. The non-specific binding sites on the membranes were blocked with 5% (w/v) skimmed milk in Tris-buffered saline (TBS): [20 mmol/l Tris-HCl and 200 mmol/l NaCl (pH 7.6)] for 2 h under gentle agitation at room temperature. Subsequently, the membranes were incubated with the relevant primary antibodies [poly ADP-ribose polymerase (PARP) rabbit monoclonal, 1:10,000; Cell Signaling Technology, Inc., Danvers, MA, USA; B-cell lymphoma 2 (Bcl-2) mouse monoclonal, 1:1,000; Bax rabbit monoclonal, 1:1,000; caspase-3 rabbit monoclonal, 1:1,000; GAPDH mouse monoclonal, 1:10,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA] directed against the protein or enzyme of interest for 1 h at room temperature and subsequently at 4°C overnight. The membranes were washed extensively with TBS containing 0.05% Tween-20 (v/v) (TBST) and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Following washing with TBST, the membranes were incubated under chemiluminescence and wrapped in clear plastic wrap for film exposure. The bands on the immunoblots were quantified using Quantity One version 4.6.2 software (Bio-Rad). The protein expression of each sample was internally normalized to GAPDH and the quantity was compared with the expression of the control groups.

Statistical analysis. Experiments were performed in duplicates or triplicates of ≥3 independent experiments and the results are presented as the mean ± standard deviation. Statistical analysis between groups was carried out via a one-way analysis of variance using SPSS 19.0 software (IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

As₄S₄ induces NB4-R1 cell apoptosis in a time-dependent manner. Apoptotic characterization was performed in the As₄S₄-treated cells via Annexin V-FLUOS and PI double staining and the samples were analyzed via flow cytometry. The data revealed that the untreated cells showed normal cell viability. In contrast to the control cells, the percentage of early apoptotic cells treated with 25 µmol/l As₄S₄ for 24 or 48 h (Annexin V⁺/PI⁻, in the lower right quadrant) significantly increased from 0.00 to 24.49 and 47.41% (P<0.05),

Table I. Flow cytometric data showing the effect of As_4S_4 ($25 \mu\text{mol/l}$) on Annexin V/PI binding in the NB4-R1 cells.

Action time, h	Viable cells	Early apoptotic cells	Late apoptotic cells
0	99.53 \pm 0.39	0.00 \pm 0.00	0.08 \pm 0.13
24	60.27 \pm 5.12 ^a	24.49 \pm 4.05 ^a	14.72 \pm 1.82 ^a
48	31.60 \pm 1.48 ^a	47.41 \pm 4.78 ^a	20.70 \pm 3.89 ^a

^aP<0.05, significant difference compared to the control group. Data are mean % \pm SD; n=3. As_4S_4 , tetra-arsenic tetra-sulfide; PI, propidium iodide; SD, standard deviation.

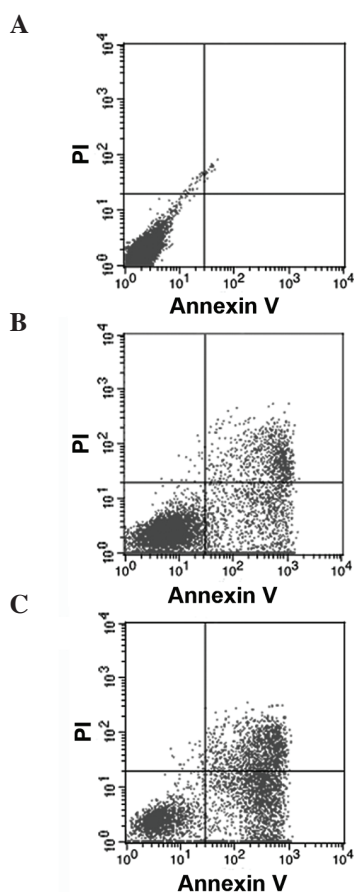


Figure 1. Apoptosis of the NB4-R1 cells treated with tetra-arsenic tetra-sulfide (As_4S_4) via flow cytometry. (A) Control; (B) As_4S_4 treatment for 24 h; and (C) As_4S_4 treatment for 48 h. PI, propidium iodide.

respectively. In addition, the percentage of late apoptotic cells (Annexin V⁺/PI⁺, shown in the upper right quadrant) significantly increased from 0.08 to 14.72 and 20.70% ($P<0.05$), respectively. As shown, a progressive increase in the number of apoptotic cells was observed, which suggests time-dependent cytotoxicity (Fig. 1, Table I).

DNA ladder agarose gel electrophoresis was used to distinguish apoptotic cells from necrosis. Apoptosis is characterized by internucleosomal DNA ladder fragmentation through agarose gel electrophoresis to show a 'ladder' pattern at 180-base pair (bp) intervals due to the activation

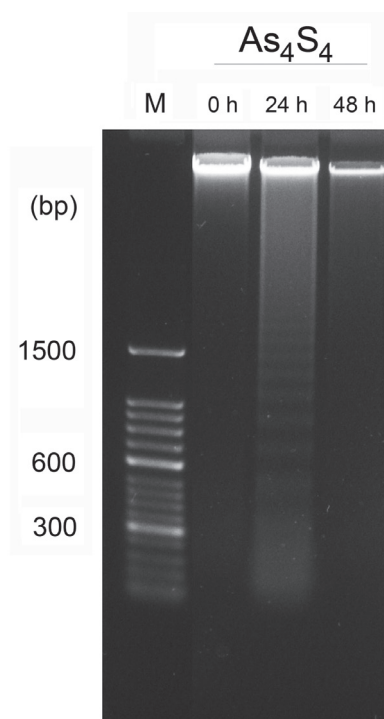


Figure 2. Apoptosis in the NB4-R1 cells treated with tetra-arsenic tetra-sulfide (As_4S_4) assessed by analyzing the DNA ladder fragmentation. The NB4-R1 cells were treated for 0, 24 or 48 h. M, DNA marker; bp, base pairs.

of endogenous endonucleases, whereas random DNA fragmentation is a typical manifestation of necrosis following electrophoretic separation. The untreated cells contained only high-molecular-weight genomic DNA. Compared with the control group, the NB4-R1 cells inoculated with As_4S_4 exhibited the characteristic pattern of nucleosomal laddering specific to apoptosis, which was visible as faint bands on the gel. As_4S_4 produced DNA fragments of low molecular weight consisting of multimers of 180-200 bp in the NB4-R1 cell line in the 24-h treatment groups. In the 48-h treatment groups, DNA degradation failed to form the typical bands but formed random DNA fragmentation, which indicates necrosis of cells at this time point (Fig. 2).

Effect of As_4S_4 on the proteins associated with NB4-R1 cell apoptosis. Based on the above result, the association between apoptosis factor expression and apoptosis induction was investigated in the NB4-R1 cells treated with As_4S_4 . The expression of several apoptosis-related factors was studied. Bax levels increased significantly over the control, whereas Bcl-2 showed a clear reduction. All the factors exhibited variations in a time-dependent manner.

As changes of Bax/Bcl-2 have been reported to play significant roles in the activation of caspase signaling, the activation of caspase-3 was detected in the following set of experiments. Incubation of the NB4-R1 cells with As_4S_4 for 24 h induced activation of caspase-3. Pro-caspase-3 was cleaved into small active fragments of 19 or 17 kDa under apoptotic stimulation. The 113 kDa PARP, as the specific substrate of caspase-3, was cleaved into 89 and 24 kDa fragments after treatment for 24 h. No cleavage of PARP in the control group was detected (Fig. 3).

Table II. Flow cytometric data showing the effect of As₄S₄ (25 μ mol/l) on the progression of the cell cycle in NB4-R1 cells.

Action time, h	G ₀ /G ₁	S	G ₂ /M
0	57.30 \pm 0.35	31.85 \pm 0.91	10.79 \pm 0.67
24	37.56 \pm 1.85 ^a	42.53 \pm 2.71 ^a	19.91 \pm 1.83 ^a
48	28.51 \pm 2.53 ^a	55.12 \pm 0.13 ^a	17.01 \pm 1.44 ^a

Data are mean % \pm SD. n=3. ^aP<0.05, significant difference compared to the control group. As₄S₄, tetra-arsenic tetra-sulfide; SD, standard deviation.

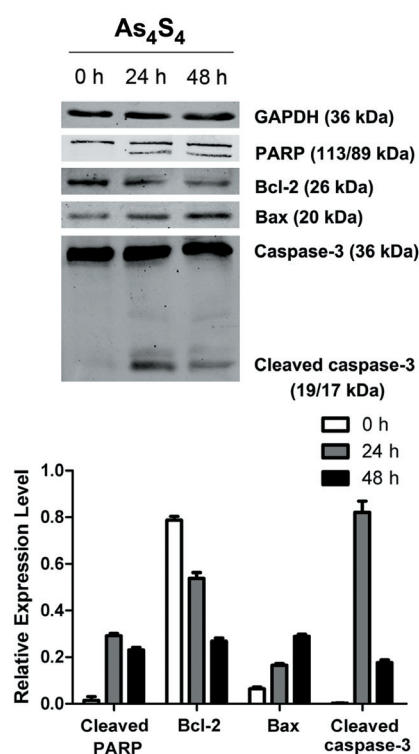


Figure 3. Expression of cleaved poly ADP-ribose polymerase (PARP), B-cell lymphoma 2 (Bcl-2), Bax and cleaved caspase-3 via western blot analysis. The NB4-R1 cells were treated with tetra-arsenic tetra-sulfide (As₄S₄) for 0, 24 or 48 h.

As₄S₄ induces cell cycle arrest in NB4-R1 cells. Flow cytometry assays were performed to analyze the effects of As₄S₄ on the cell cycle distribution. According to the DNA histogram results, the drug induced a significant increase in the cell population in the S phase. Cells in the S phase increased from 31.85% of untreated cells to 42.53 and 55.12% of cells in the experimental groups (P<0.05), where the action of As₄S₄ induced nearly a 2-fold increase. Compared to the control group, the distribution of NB4-R1 cells in the G₀/G₁ phases decreased from 57.29 to 37.57 and 28.51% (P<0.05), respectively. The percentage of G₂/M phase cells detected at different time points increased from 10.79 to 19.91 and 17.01%, representing a slight increase compared to the controls (P<0.05). These results show that the inhibitory effect on the growth of NB4-R1 cells induced by As₄S₄ was partially mediated by

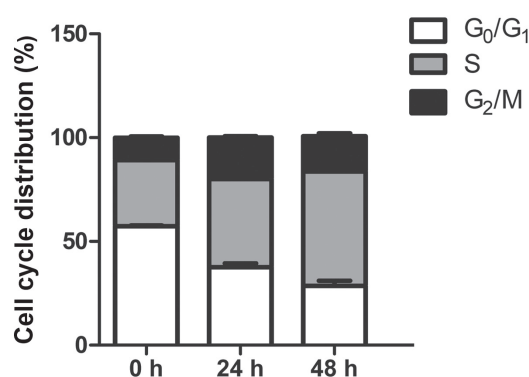


Figure 4. Influence of tetra-arsenic tetra-sulfide on the cell cycle of NB4-R1 cells.

reducing the number of cells in the G₀/G₁ phases and arresting the cell cycle in the S phase and G₂/M phases (Table II, Fig. 4).

Discussion

APL accounts for 10-15% of acute myeloid leukemia in adults (1). As₄S₄ has gained importance in the treatment of the APL. Previous studies have shown that the therapeutic action of As₄S₄ is also effective for other tumor therapies (8,9). However, the molecular mechanism of the action of As₄S₄ in RA-resistant APL therapy remains unknown. The present results reveal a time-dependent toxic action of As₄S₄ on RA-resistant NB4-R1 cells. Flow cytometric analyses and DNA ladder agarose gel electrophoresis confirmed that As₄S₄ inhibited tumor cell growth via inducing apoptosis. To probe the cell signaling pathways involved in this As₄S₄-induced apoptosis, the protein expression levels of Bcl-2, Bax, caspase-3 and PARP were detected via western blot analysis.

The Bcl-2 family of pro- and anti-apoptotic proteins plays an important role in apoptosis that is induced by a variety of stimuli. Bcl-2 proteins modulate the integrity of the mitochondrial and endoplasmic reticulum membranes, cytochrome *c* release, caspase activation and cell death (10). A reduction in Bcl-2 expression can lead to a loss of signals that are required for survival. Bax is a major pro-apoptotic member that is required for apoptotic cell death. Previous evidence has indicated that Bcl-2 can constitute homodimers and heterodimers with Bax, leading to an inhibition of the formation of Bax/Bax pro-apoptotic homodimers (11,12). The ratio between anti-apoptotic and proapoptotic members of the Bcl-2 family may determine the susceptibility of the cell to apoptosis. The present study reported a decrease in Bcl-2 and an increase in Bax following treatment of the NB4-R1 cells with As₄S₄. The decrease in the Bcl-2/Bax ratio leads to the translocation of Bax from the cytoplasm to mitochondria, promoting the release of cytochrome *c* and the activation of caspase. Variations in the levels of Bax and Bcl-2 can be deduced by apoptosis that is initiated via the intrinsic pathway.

Caspase-3, as the most important executor of apoptosis, participates in DNA degradation, nuclear condensation, plasma membrane blebbing and proteolysis of certain caspase substrates (13,14). Caspases are synthesized as relatively inactive precursors (zymogens) that require proteolytic processing for activation. As discovered in the NB4-R1

cells, As₄S₄ cleaves the 36-kDa pro-caspase-3 into small 17 or 19 kDa active fragments, leading to caspase-dependent apoptosis. Subsequently, the cleaved caspase-3 activates endonuclease caspase-activated DNase, leading to fragmentation of the chromosomal DNA at internucleosomal sites (15). The present results show that cleaved caspase-3 significantly increased after As₄S₄ incubation for 24 h while the DNA degradation revealed characteristic DNA 'ladder' bands. The activity of this endonuclease can be inhibited by PARP and the cleavage of PARP by activated caspase-3 reverses the activity of the endonuclease (16). In the present study, the 113-kDa PARP could be cleaved onto an 89-kDa C-terminal catalytic fragment and an N-terminal 24-kDa fragment after 24 h of As₄S₄ treatment, leading to a loss of DNA repair function.

Various chemotherapy drugs inhibit the growth of tumor cells by blocking the cell cycle. Numerous investigators have reported that As₄S₄ blocks tumor cells at different stages of the cell cycle (17,18). Variations in experimental results may be associated with drug concentration, action time and cell types. In the present study, the accumulation of cells in the S and G₂/M phases was observed for NB4-R1 cells, suggesting that As₄S₄ may exert its cytotoxic effects on NB4-R1 cells through cell cycle arrest and cell apoptosis.

In conclusion, the present study revealed that As₄S₄, a traditional medicine, inhibited the growth of NB4-R1 cells *in vitro*. As₄S₄ induced cell apoptosis through changes in Bcl-2 and Bax, activation of caspase-3 and cleavage of PARP. The results suggested the apoptosis of NB4-R1 cells via a mitochondria-dependent pathway. In addition, As₄S₄ may exert its cytotoxic effects on NB4-R1 cells through blocking the cell cycle in the S and G₂/M phases. Thus, As₄S₄ may be a potential anticancer drug candidate. The development of cell apoptosis is a multi-factor, multi-step and multi-gene interactive process. The signaling pathways and molecular mechanisms of As₄S₄ in apoptotic regulation require further investigations.

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