Arsenic disulfide induces apoptosis of human diffuse large B cell lymphoma cells involving Bax cleavage

LING WANG1,2, XINYU LIU1, XIANGLU LI1, XIAO LV1, KANG LU1, NA CHEN1, PEIPEI LI1 and XIN WANG1,3

1Department of Hematology, Provincial Hospital Affiliated to Shandong University, Jinan, Shandong 250021; 2Department of Hematology, Taian City Central Hospital, Taian, Shandong 271000; 3Institute of Diagnostics, Shandong University, Jinan, Shandong 250012, P.R. China

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Abstract. The aim of the present study was to investigate the effect of arsenic disulfide (As2S2) on the proliferation and apoptosis of LY1 and LY8 human diffuse large B cell lymphoma (DLBCL) cells in an attempt to discover a more effective alternative therapy scheme. Human DLBCL cells LY1 and LY8 were treated with various concentrations of As2S2 for different time periods. Cell viability was detected by the CCK-8 assay; cell apoptosis was evaluated by flow cytometric analysis. The expression levels of Bax, Bcl-2 and caspase-3 were examined by quantitative PCR and western blotting. We found that the DLBCL cell viability was significantly decreased following treatment with As2S2 for 24, 48 and 72 h. Along with increasing As2S2 concentrations, the DLBCL cell viability was notably reduced when compared with the control group, and the results were statistically significant. Meanwhile, the apoptotic rates of DLBCL cells were significantly enhanced at 24, 48 and 72 h following treatment with increasing As2S2 concentration, and the results were also statistically significant. The quantitative PCR results showed that at the mRNA level, the Bax/Bcl-2 expression ratio was increased and caspase-3 mRNA expression was upregulated in As2S2-treated DLBCL cells. Western blot analysis revealed that at the protein level, As2S2 increased the Bax/Bcl-2 protein ratio in contrast to decreased pro-caspase-3 expression in the DLBCL cells. Our findings also demonstrated that 21-kDa Bax was proteolytically cleaved into the 18-kDa Bax in the DLBCL cells exposed to As2S2 at a concentration of 10 µM. As2S2 inhibited proliferation and induced apoptosis of LY1 and LY8 cells in a concentration- and time-dependent manner. The effect was partly due to the induction of mitochondrial-dependent apoptosis involving Bax cleavage.

Introduction

Non-Hodgkin lymphoma (NHL) is the most common hematological neoplasm in the United States, and accounts for 4% of all malignant tumors diagnosed each year (1). Diffuse large B cell lymphoma (DLBCL) is the most prevalent subtype of NHL worldwide and represents up to 40% of all NHL cases among adults in the Western world (2), and frequent in developing countries (3,4). DLBCL is a heterogeneous disease with variable cytogenetics and immunophenotypes, as well as clinical features (5). Although the initial standard therapy with rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone (R-CHOP) chemotherapy has improved outcomes for patients with DLBCL (6,7), ~40% of patients are either refractory to initial treatment or relapse after standard therapy (8). For refractory and relapsed DLBCL patients who do not respond to high-dose therapy (HDT) and autologous stem cell transplantation (ASCT), the outcome is poor. Therefore, the discovery of novel and alternative therapeutic approaches is urgent.

Since arsenic trioxide (As2O3) induces apoptosis in acute promyelocytic leukemia (APL) at a high concentration and mediates partial differentiation at a low concentration (9), arsenic drugs have become a ‘hot topic’ and have attracted increased attention in regards to malignant hematological neoplasms and other solid tumors (10-13). Arsenic drugs, including arsenic disulfide (As2S2), tetra-arsenic tetra-sulfide...
(As₂S₃) and arsenic trioxide (As₂O₃), have similar antitumor effects. As₂S₃, one of the main ingredients of realgar, has been chosen as an anticancer candidate due to its superior effectiveness, advantage of oral administration and low toxicity in Chinese traditional medicine.

Realgar has been found to induce both apoptosis and differentiation simultaneously in all-trans-retinoic acid (ATRA)-sensitive NB4 and ATRA-resistant MK2 FPLM-RARα APL cell lines (14). Previous studies have demonstrated that realgar induced apoptosis of HL-60, NB4 and K562 cell lines, which was associated with CD95/CD95L and MAPK pathway, inhibition of telomerase activity and decreased expression of BCL-2 and PNAS-2 (15-18). In addition, realgar induced the differentiation of the HL-60 cell line via not only the enhancement of the activity of serine/threonine protein phosphatase type 1 (PP1) and type 2A (PP2A) but also oxidative stress and stress-related mitochondrial transmembrane potential (MTP) (16,19).

In addition, previous studies have demonstrated that arsenic induced apoptosis in NHL cells by means of different mechanisms. They revealed that As₂O₃ inhibited proliferation and induced apoptosis in the human Burkitt lymphoma cell line Raji and in the human T lymphoma cell line Jurkat through cell cycle arrest, decrease in respiratory function and MTP, downregulating the expression of MCL-1 and subsequently activating caspase-3 (20-23). However Jurkat cells were less sensitive to As₂O₃-induced apoptosis than Raji cells, as Jurkat cells express high levels of glutathione S-transferase type 1 (GSTP1) (21,23). At the same time, realgar induced apoptosis of the human T lymphocyte leukemia cell line CEM through cell cycle arrest in the G2/M phase, a decrease in the expression of Bcl-2 and an increase in Apo2.7 protein expression (24). A recent study demonstrated that As₂O₃ inhibited the growth of mantle cell lymphoma (MCL) and induced apoptosis through a decrease in cyclin D1 expression (25).

However, whether As₂S₃ induces the apoptosis of DLBCL cells remains unknown. Since induction of apoptosis has been used as an important target with which to evaluate new antitumor drugs, it is necessary to study the antitumor mechanisms of As₂S₃. For the first time, our present study focused on the effects of As₂S₃ on the proliferation and apoptosis of DLBCL cell lines and the related mechanisms. We demonstrated that As₂S₃ inhibited the proliferation and induced the apoptosis of DLBCL cells in a concentration- and time-dependent manner. We also demonstrated that the effect was partly due to the induction of mitochondrial-dependent apoptosis involving Bax cleavage.

Materials and methods

Cell lines and cell culture. The human DLBCL cell lines LY1 and LY8 were maintained as suspension cells at 37°C in 5% carbon dioxide in a humidified atmosphere. LY1 and LY8 cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS) (both from HyClone, Logan, UT, USA). LY1 and LY8 cells in the exponential growth phase were seeded into 96-well plates or culture flasks.

Antibodies and reagents. As₂S₃ (99.53% in purity) was purchased from Alfa Aesar Company (Shanghai, China). For studies, As₂S₃ was dissolved in 0.1 M sodium hydroxide (NaOH), and hydrochloric acid (HCl) was used to adjust the pH to 7.35-7.45. The stock solution (1 mM As₂S₃) was then passed through a 0.22-µm filter. To prepare working solutions, aliquots were further diluted in IMDM supplemented with 10% FBS immediately before each experiment. Rabbit anti-Bcl-2, -Bax and -capsase-3 monoclonal antibodies were purchased from Cell Signaling Technology, Inc. (Boston, MA, USA). Mouse anti-β-actin polyclonal antibody was purchased from Zhongshan Golden Bridge (Beijing, China).

Assessment of the cytotoxic effect of As₂S₃ by Cell Counting Kit-8 (CCK-8). Cell proliferation was measured by the CCK-8 assay (Beyotime, China). LY1 and LY8 cell lines (1x10⁴ cells/100 µl/well, respectively) were seeded into 96-well plates, treated with various concentrations of As₂S₃ and cultured for 24, 48 and 72 h in a humidified atmosphere in a 5% carbon dioxide incubator. All experiments were performed in triplicate and repeated 3 times. Cells were incubated with 10 µl of CCK-8 at 37°C for 4 h. Then the optical density (OD) for each well was measured at 450 nm using an ELISA reader. The inhibitory concentration of 50% of cells (IC₅₀) was obtained using probit regression analysis method. The cell viability rate was calculated according to the following equation: Cell viability rate = (OD experiment - OD blank)/(OD control - OD blank) x 100%.

Assessment of apoptosis by Annexin V and propidium iodide. Induction of apoptosis was assessed using the Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (KeyGen Biotech, China). LY1 and LY8 cells were treated with As₂S₃ at 2, 4, 6, 8 and 10 µM. Cell culture medium without As₂S₃ was added to the untreated control. Dual staining with Annexin V-FITC and propidium iodide (PI) was carried out according to the manufacturer's instructions. The cells (5-10x10⁴) were analyzed by flow cytometry (Becton-Dickinson, USA). The acquired data were processed with FlowJo7.6 software. Annexin V-FITC and PI-negative cells were identified as viable cells. Cells exhibiting Annexin V-FITC-positive and PI-negative staining were considered to be early apoptotic cells while those with both Annexin V-FITC and PI-positive staining were considered late apoptotic cells. The sum of early and late apoptotic cells constituted the apoptotic cell population.

Gene expression study by quantitative real-time PCR. Total RNA was extracted from the As₂S₃-treated and untreated DLBCL cell lines using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA). Then reverse transcription reaction was conducted by means of Takara reverse transcription reagents (Takara, Dalian, China). Amplification reactions were performed using SYBR Premix Ex Taq (Takara) using the Roche LightCycler 480 Real-Time quantitative PCR system. Specific primers for RT-PCR were obtained from Sangon Biotech (Shanghai, China), and the primer sequences are listed in Table 1. Expression data were normalized to the geometric mean of housekeeping gene β-actin to control the variability in expression levels. For data analysis, the 2-ΔΔCt method was
used. Real-time PCR for each gene of each cDNA sample was assayed in triplicate.

$$\Delta Ct = Ct \text{ (target gene)} - Ct \text{ (}\beta\text{-actin gene)}$$

$$\Delta\Delta Ct = \Delta Ct \text{ (As}_2\text{S}_2\text{-treated cells)} - \Delta Ct \text{ (untreated control)}$$

**Protein expression study by western blot analysis.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were performed to evaluate the protein levels of Bax, Bcl-2 and caspase-3. Total protein was extracted from As$_2$S$_2$-treated and untreated DLBCL cells using RIPA and 1% PMSF (Shenergy Biocolor, Shanghai, China). The protein concentration of the samples was determined by the BCA assay (Shenergy Biocolor). Proteins were detected using the chemiluminescence detection kit (Millipore, Billerica, MA, USA). Western blotting results were analyzed using Las-4000 Image software and Multi Gauge Ver. 3.0 software (Fujifilm Life Science, Japan).

**Statistical analysis.** Statistical analysis was performed using SPSS 17.0. The data are reported as means ± standard deviation (SD). ANOVA was used to evaluate data from the cell viability assays and for the cell apoptotic rates. Other statistical analyses of data were performed using the Student's t-test. Statistical significance was defined as P<0.05.

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**Table I. Primers used for the quantitative real-time PCR.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Product (bp)</th>
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| β-actin    | Forward: 5’-TGACGTGGACATCCGCAAAG-3'  
Reverse: 5’-CTGGAAGGTGGACAGCGAGG-3' | 205          |
| Bax        | Forward: 5’-CCCGAGAGGTCTTTTTTCCAG-3'  
Reverse: 5’-CCAGCCCATGATGGTTCTGAT-3' | 155          |
| Bcl-2      | Forward: 5’-ATGTGTGTGGAGAGCGTCAA-3'  
Reverse: 5’-ACAGTTCCACAAAGGCAATCC-3' | 136          |
| Caspase-3  | Forward: 5’-GACTCTGGAAATATCCGCTGGACAAACA-3'  
Reverse: 5’-AGGTTTTCATCGACATCTG-3' | 140          |

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**Results**

**As$_2$S$_2$ inhibits the proliferation of DLBCL cells.** The effect of As$_2$S$_2$ on the cell viability of DLBCL cells was evaluated by CCK-8 assay. A significant decrease in cell viability was observed after the LY1 and LY8 cells were incubated with different doses of As$_2$S$_2$ (2, 4, 6, 8 and 10 µM) for 24, 48 and 72 h. CCK-8 assay was used to evaluate the cell viability. The data are presented as the percentages of the corresponding untreated control. The data are from untreated cells (control) and from As$_2$S$_2$-treated cells at different concentrations (2-10 µM). Values are expressed as means ± SD. *P<0.05, statistically significant difference compared with the untreated control, and **P<0.01, compared with the untreated control. As$_2$S$_2$, arsenic disulfide; DLBCL, diffuse large B cell lymphoma; CCK-8, Cell Counting kit-8.

**As$_2$S$_2$ induces the apoptosis of DLBCL cells.** To determine the effect of As$_2$S$_2$-induced apoptosis in LY1 and LY8 cells, Annexin V-FITC/PI dual staining followed by flow cytometric analysis was assessed. As shown in Figs. 2 and 3, As$_2$S$_2$ induced apoptosis in LY1 and LY8 cells in a concentration- and time-dependent manner. Exposure to As$_2$S$_2$ at concentrations of 2-10 µM for 24 h led to a relatively low rate of apoptosis.
In contrast, exposure to As$_2$S$_2$ at the same concentrations for 72 h resulted in a more notable apoptosis. After treatment with various doses of As$_2$S$_2$, the maximal apoptosis rate was 22.06±6.54% in LY1 cells and 27.30±6.56% in LY8 cells, respectively, at 24 h. When the treatment time increased to 72 h, the maximal apoptosis rate was 58.94±0.79% in LY1 cells and 61.30±6.06% in LY8 cells. In contrast, not more than 5% of the untreated control DLBCL cells underwent apoptosis under the same conditions.

As$_2$S$_2$ increases the Bax/Bcl-2 ratio and upregulates caspase-3 expression at the mRNA level. To further investigate whether the As$_2$S$_2$-induced apoptosis is dependent on the mitochondrial-mediated apoptosis pathway, the effect of As$_2$S$_2$ on the mRNA levels of Bax, Bcl-2 and caspase-3 genes was measured by quantitative real-time PCR. Following treatment with 10 µM As$_2$S$_2$ for 48 h, the expression of Bax mRNA in LY1 cells was upregulated by ~2-fold while Bcl-2 mRNA expression was reduced by more than half (Fig. 4). Yet, no significant
difference in expression of Bcl-2 was observed between the \( \text{As}_2\text{S}_2 \)-treated LY8 cells and the untreated controls. In addition, the Bax/Bcl-2 ratio was significantly upregulated by ~3-fold in the LY1 and LY8 cells. The expression of caspase-3 was markedly upregulated in the LY1 and LY8 cells.

\( \text{As}_2\text{S}_2 \) increases the Bax/Bcl-2 ratio in comparison to a decrease in pro-caspase-3 expression in DLBCL cells at the protein level. We also investigated whether the protein expression levels of Bax, Bcl-2 and caspase-3 were altered after the \( \text{As}_2\text{S}_2 \) treatment. As shown in Fig. 5, after treatment with different doses of \( \text{As}_2\text{S}_2 \) (2 and 10 µM) for 48 h, the Bcl-2 expression was markedly downregulated whereas Bax expression was substantially upregulated in the LY1 and LY8 cells. In addition, the Bax/Bcl-2 ratio was significantly upregulated.

Western blotting also demonstrated that after exposure to 2 µM
As$_2$S$_2$, the levels of 21-kDa Bax were increased but Bax was not detectably cleaved. In contrast, after exposure to 10 µM As$_2$S$_2$, not only the levels of the 21-kDa Bax protein were increased but also Bax cleavage (18-kDa Bax) was substantial. Concomitantly, pro-caspase-3 expression was evidently downregulated.

**Discussion**

In the present study, we demonstrated that As$_2$S$_2$ significantly inhibited proliferation and induced apoptosis in the LY1 and LY8 cells. In addition, we also found that the mechanism by which As$_2$S$_2$ induced apoptosis was through the mitochondrial-mediated pathway which involved concomitant Bax cleavage. Therefore, this is the first study concerning the effects of As$_2$S$_2$ on DLBCL cells in vitro.

It is widely believed that apoptosis is initiated by two major pathways: the extrinsic pathway and the intrinsic pathway (26). The intrinsic pathway is characterized by mitochondrial dysfunction with release of caspase activators, followed by activation of caspase-9 and -3. Mitochondrial-induced apoptosis requires involvement of the Bcl-2 family (27), which
consists of anti-apoptotic proteins, multi-domain pro-apoptotic proteins and BH3-only pro-apoptotic proteins (28,29). It is known that Bax and Bcl-2 are representative members of the Bcl-2 family; Bax promotes apoptosis and Bcl-2 inhibits apoptosis. The apoptosis inducing-effect is more dependent on the ratio of Bax/Bcl-2 than on individual Bax and Bcl-2 quantity (30). Accordingly, the ratio of Bax/Bcl-2 is frequently used as an index of apoptosis (31). As expected, we observed that the ratio of Bax/Bcl-2 in the As$_{5}$S$_{2}$-treated cells was significantly increased when compared with the control cells, not only at the mRNA but also at the protein level which was consistent with a previous study (24).

Notably, western blot analysis of Bax protein expression showed that a smaller band of ~18 kDa was produced in addition to a 21-kDa band when DLBCL cells were exposed to As$_{5}$S$_{2}$ at a concentration of 10 µM. In contrast, 18-kDa Bax was not detected in the control cells and the As$_{5}$S$_{2}$-treated cells at a concentration of 2 µM. Staining with β-actin antibody confirmed that the observed discrimination in protein expression was not caused by a different protein quantity/lane. It is well known that 21-kDa Bax exists primarily in the cytosol and rapidly translocates to mitochondria upon the induction of apoptosis where 21-kDa Bax is cleaved into a 18-kDa fragment (18-kDa Bax). The reason for Bax cleavage may be explained by the fact that Bax underwent conformational change in the presence of cytotoxic As$_{5}$S$_{2}$. It has been reported that Bax cleavage occurs in lymphoma cell lines, myeloid leukemia cells, chronic lymphocyte leukemia and other solid tumor cell lines treated with various chemotherapeutic agents (32-51). Previous studies have demonstrated that 18-kDa Bax, a characteristic feature of Bax activation, behaves similar to the BH3-only protein and is a more potent inducer of apoptosis than 21-kDa Bax. Further research confirmed that interruption of Bax cleavage obviously reduces drug-induced apoptosis, while depression of 18-kDa Bax degradation significantly augments drug-mediated apoptosis (34,47,52). In addition, Bax cleavage is thought to be triggered by calpain-dependent protease, which has been reported to occur at different sites (38,47,53-55). Previous research also found that As$_{5}$O$_{3}$-induced cleavage of Bax was partially blocked by calpeptin in neuroblastoma cells, which demonstrates that calpain is involved in the process of Bax cleavage (56,57). Thus, we hypothesized that As$_{5}$S$_{2}$-induced cleavage of Bax in DLBCL cells may be calpain-dependent, which requires further investigation. Therefore, our findings suggest that Bax is critical for the initiation of apoptosis of As$_{5}$S$_{2}$-treated DLBCL cells. This is the first study to demonstrate the role of Bax in As$_{5}$S$_{2}$-induced apoptosis.

We further investigated the potential involvement of caspases in the induction of apoptosis by As$_{5}$S$_{2}$ in the LY1 and LY8 cells. Caspase activation plays a key role in the course of mitochondrial-mediated apoptosis (58). Firstly, release of cytochrome c in the cytoplasm activates caspase-9, which then activates caspase-3 in a cascade. The common terminal mediator of mitochondrial death cascade is activation of caspase-3 which is responsible for initiating the apoptotic program. In the present study, the upregulation of the ratio of Bax/Bcl-2 protein occurred in LY1 and LY8 cells after treatment with As$_{5}$S$_{2}$, which resulted in the activation of caspase-3. Moreover, our findings demonstrated the down-regulation of pro-caspase-3, which may indirectly indicate its cleavage and the increase in the level of the active form of caspase-3.

In conclusion, the search for novel and effective treatments for DLBCL remains a challenge. Our findings found that As$_{5}$S$_{2}$ inhibited the proliferation and induced the apoptosis of DLBCL cells in vitro through the mitochondrial pathway. In addition, Bax was critical for the initiation of apoptosis in As$_{5}$S$_{2}$-treated DLBCL cells. Thus, As$_{5}$S$_{2}$ may be useful as a potential therapeutic agent against DLBCL and further research is warranted.

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References


