Effects of arsenic sulfide (As$_2$S$_2$) on B and T lymphoma cell lines and possible underlying mechanisms

XIANGLU LI, XINYU LIU, LING WANG, XIAO LV, PEIPEI LI, KANG LU and XIN WANG

Department of Hematology, Provincial Hospital Affiliated to Shandong University, Jinan, Shandong, 250021, P.R. China

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Abstract. Lymphoma is a hematological malignancy that originates from lymph nodes and lymphoid tissues and is divided into Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL), based on its histopathological characteristics. The aim of this study was to investigate the effects of arsenic sulfide (As$_2$S$_2$), the main ingredient of realgar, on the proliferation and apoptosis of the Raji B-cell lymphoma and Jurkat T-cell lymphoma lines, comparing the sensitivity between the two cell lines and investigating the possible underlying mechanisms. The two lymphoma cell lines are more sensitive to As$_2$S$_2$ for different time periods. The cell proliferation was detected using the Cell Counting kit-8 (CCK-8). Apoptosis was assessed via flow cytometry. Expression levels of the apoptosis-associated genes (Homo sapiens Bcl-2-associated X protein (BAX), Homo sapiens B-cell CLL/lymphoma 2 (Bcl-2), Homo sapiens Bcl-2-like protein 1 (BCL2L1, Bcl-xL), Homo sapiens v-myc myelocytomatosis viral oncogene homolog (avian) (MYC, c-Myc) and Homo sapiens pim-1 oncogene (PIM)] were measured via the reverse transcription polymerase chain reaction (RT-PCR) method. The results demonstrated that As$_2$S$_2$ inhibited proliferation and induced apoptosis in the two lymphoma cell lines in a time- and concentration-dependent manner, with the Raji cells being more sensitive to As$_2$S$_2$ compared to Jurkat cells. As$_2$S$_2$ may alter the expression levels of different apoptosis-associated genes, with the alterations of the mRNA expression levels being different between Raji and Jurkat cells. These findings indicated that As$_2$S$_2$ may inhibit the proliferation and promote the apoptosis of non-Hodgkin lymphoma (NHL) cell lines and that B-cell lymphoma cell lines are more sensitive compared to T-cell lymphoma cell lines. The possible underlying mechanism is that As$_2$S$_2$ alters the expression levels of the apoptosis-associated genes and activates apoptosis-associated signaling pathways.

Introduction

Lymphoma is a hematological malignancy that originates from lymph nodes and lymphoid tissues and is divided into Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL), based on its histopathological characteristics. HL may have a better prognosis compared to NHL due to the use of combination chemotherapy. Among all NHLs, Burkitt and T-cell lymphoma are the most highly aggressive types and have a poor prognosis due to their rapid infiltration. Currently, R-CHOP or other combination chemotherapy do not achieve satisfactory outcomes and other effective therapeutic options are required.

Arsenic sulfide (As$_2$S$_2$) is the main ingredient of realgar, a traditional Chinese medicine, which may be orally administered. Since the identification of its promising therapeutic effects on acute promyelocytic leukemia (APL) in the 1990s, other studies based on the compound realgar natural indigo tablet were sequentially conducted. Results of those studies demonstrated that the therapeutic effects of this compound were reliable and equivalent to those of retinoic acid, the first-line drug for the treatment of APL. Additionally, it is safe to use this compound since, as opposed to arsenic trioxide (As$_2$O$_3$), it may be orally administered and the main adverse effects, such as gastrointestinal reactions and hepatic dysfunction, are highly manageable. Although a few studies on the effectiveness of the As$_2$O$_3$ on blood disorders are currently available, the number of studies on the effectiveness of the As$_2$S$_2$ on NHLs is limited.

In the present study, the effects of As$_2$S$_2$ on the proliferation and apoptosis on Raji and Jurkat NHL cell lines were measured, their sensitivities compared and the apoptosis-associated genes quantitatively measured to investigate the underlying mechanisms and provide a theoretical basis for the treatment of NHLs.

Materials and methods

Cell lines, reagents and drugs. The Raji human Burkitt lymphoma cell line and the Jurkat human T-lymphoma cell line were provided by our laboratory and cultured in RPMI-1640, supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), in a humidified atmosphere with 5% CO$_2$ at 37°C. We previously investigated other NHL
cell lines and made certain observations (12-15). Additional studies were also conducted on various NHL cell lines, such as MEC-1 and Ly-1, at that time. As$_S$$_2$ (Alfa Aesar, Ward Hill, MA, USA) was dissolved in 0.1 M sodium hydroxide to create a stock solution of 1 mM (16). Stock solutions were diluted in RPMI-1640 medium to achieve the final concentrations (16).

**Drug concentration settings.** The stock solutions were diluted to achieve the concentrations of 0.1, 1, 2, 3, 4, 5 and 6 µM As$_S$$_2$ on Raji cells and 1, 10, 20, 30, 40 and 50 µM As$_S$$_2$ on Jurkat cells. Based on the results of our preliminary experiments, cells were collected after 24, 48 and 72 h of treatment.

**Cell counting kit-8 (CCK-8) method.** Raji and Jurkat cells (1x10$^5$ cells/ml in 96-well microplates) were incubated with different concentrations of As$_S$$_2$ (0.1, 1, 2, 3, 4, 5 and 6 µM for Raji cells and 1, 10, 20, 30, 40 and 50 µM for Jurkat cells) for different time periods (24, 48 and 72 h). The control group was incubated only with RPMI-1640, supplemented with 10% fetal bovine serum. The CCK-8 assay was performed following the instructions of the CCK-8 kit (Biyuntian, Jiangsu, China). Cell proliferation was measured in terms of optical absorbance per well by a microplate reader at a wavelength of 450 nm. The growth inhibition rate and the 50% growth-inhibitory concentration ($C_{50}$) value of As$_S$$_2$ were calculated using SPSS 17.0 software. The growth inhibition rate (IR) was calculated as: IR (%) = ($A_{\text{control}} - A_{\text{experiment}}$) / $A_{\text{control}}$ x 100%.

**Flow cytometry.** A cytometric analysis (FACSCanto II flow cytometry; BD Biosciences, Franklin Lakes, NJ, USA) was used to evaluate cell apoptosis under different concentrations of As$_S$$_2$ (1, 2, 3, 4 and 5 µM for Raji cells and 10, 20, 30, 40 and 50 µM for Jurkat cells) as well as for different time periods (24, 48 and 72 h). The protocol was applied according to the instructions of the Annexin V-FITC Apoptosis Detection kit (Kaiji Bio Co., Nanjing, China). In the present study, the FITC*PE* and FITC*PE* cells were considered as apoptotic. The apoptotic rate was calculated as: [FITC*PE* cells + FITC*PE* cells] / total cells assessed. Subsequent to the calculation of all the apoptotic rates, the rates were compared with those of the control group to obtain a relative apoptosis rate ratio.

$qPCR$. The half maximal (50%) inhibitory concentration (IC) of As$_S$$_2$ ($IC_{50}$) on Raji and Jurkat cells was calculated, as described below. The $IC_{50}$ value of As$_S$$_2$ on Raji cells at 48 h was 2.942 µM, whereas that on Jurkat cells was 39.2 µM. Total RNA was isolated from Raji and Jurkat cell lines treated with or without As$_S$$_2$ (0.5 and 2.942 µM for Raji cells and 2.942 and 39.2 µM for Jurkat cells, respectively) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The quality of the isolated RNA was assessed using a UV spectrometer and the cDNA was synthesized with 1 µg of total RNA (Takara Bio Inc., Shiga, Japan). The amplification mix consisted of: i) SYBR Premix Ex Taq™, 10.0 µl; ii) Primer F (10 µM), 0.4 µl; iii) Primer R (10 µM), 0.4 µl; iv) DNA template, 4.0 µl; and v) dH$_2$O, 7.2 µl. Amplification was performed following the two-step standard amplification procedures on the LightCycler 480 quantitative PCR instrument, using $\beta$-actin as an internal reference. The primer sequences are listed in Table I. The calibrator-normalized ratio was calculated using the formula: RQ=$2^{-\Delta \Delta Ct}$, where $\Delta Ct$ = ($Ct_{\text{targeted gene}} - Ct_{\beta$-actin}$)sample - ($Ct_{\text{targeted gene}} - Ct_{\beta$-actin}$)calibration sample.\footnote{Statistical analysis.} Statistical analysis was performed using SPSS 17.0 software. Data were presented as means ± standard deviation. Comparisons between groups were performed with the Student's t-test. P<0.01 was considered to indicate a statistically significant difference.

**Results**

As$_S$$_2$ inhibits the proliferation of NHL cells, with Raji being more sensitive compared to Jurkat cells. To evaluate the inhibitory effects of As$_S$$_2$ on the growth of NHL cells, Raji and Jurkat cells were treated with different concentrations of As$_S$$_2$ (0.1, 1, 2, 3, 4, 5 and 6 µM for Raji and 1, 10, 20, 30, 40 and 50 µM for Jurkat cells) for 24, 48 and 72 h. IR and $IC_{50}$ were measured via the CCK-8 method. The results demonstrated that the growth of Raji and Jurkat cells was inhibited by As$_S$$_2$ in a time- and dose-dependent manner (Fig. 1). In addition, Raji cells were more sensitive compared to Jurkat cells. The IR for Raji cells reached 47.64% when treated with 3 µM As$_S$$_2$ for 24 h and reached a peak after 72 h (89.45%), whereas the IR for Jurkat cells only reached 48.11% when treated with 40 µM As$_S$$_2$ for 24 h, with a peak inhibitory rate of 75.69% with 40 µM As$_S$$_2$ for 72 h.

As$_S$$_2$ induces the apoptosis of NHL cells, with Raji cells being more sensitive compared to Jurkat cells. To investigate whether As$_S$$_2$ is able to induce apoptosis in the NHL cell lines, the Raji and Jurkat cells were treated with different concentrations of As$_S$$_2$ (1, 2, 3, 4 and 5 µM for Raji and 10, 20, 30, 40 and 50 µM for Jurkat cells) for 24, 48 and 72 h. FACSCanto II flow cytometry was used to measure cell apoptosis according to the instructions of the Annexin

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
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<tbody>
<tr>
<td>BAX</td>
<td>F: CCCCCAGGTCTTTTCCGAG R: CAGCCGTCATGTTCTGAT</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>F: ATGTGTGATGGAGCAGCTCAA R: ACGTGTCCCAAAAGGCATTCC</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>F: TCAGAGCTTGGAGCAGTGTAG R: AAGGCTCTAGGTGGTCATTC</td>
</tr>
<tr>
<td>c-Myc</td>
<td>F: GGCTTCTGGACAAAGGTCTA R: AGTTGTGTCTGTGTTGGAGA</td>
</tr>
<tr>
<td>PIM</td>
<td>F: GAGAAGGGACCGGATTTCCGAC R: CATGACAGGACCCCTAAATGACG</td>
</tr>
<tr>
<td>$\beta$-actin</td>
<td>F: TGGGCTTGGACATCCCGAAAG R: CTGGAAGGTGGCACACCGAGG</td>
</tr>
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**Table I. Primers used in reverse transcription polymerase chain reaction.**
V-FITC Apoptosis Detection kit. The results demonstrated that As$_2$S$_2$ induced apoptosis of the NHL cells in a time- and dose-dependent manner. In addition, As$_2$S$_2$ induced apoptosis in Raji cells at smaller doses and after a shorter treatment time. The apoptotic rate reached 52.50% when cells were treated with 4 µM of As$_2$S$_2$ for 48 h, whereas to achieve a similar apoptotic rate, Jurkat cells required treatment with 30 µM of As$_2$S$_2$ for 72 h (Tables II and III).
As$_2$S$_2$ alters the expression levels of apoptosis-related genes. Results of this study indicated that the expression levels of apoptosis-related genes were altered by As$_2$S$_2$ and the changes observed in Raji cells differed from those in Jurkat cells. The results (Fig. 2) demonstrated the following: i) in Raji cells, there was no significant change in Homo sapiens Bcl-2-associated X protein (BAX) expression (P>0.01) under low concentrations (0.5 µM) of As$_2$S$_2$ for 48 h, whereas it increased by 1.61-fold when treated with a higher concentration (IC$_{50}$ dose, 2.942 µM) (P<0.01). The expression of BAX in Jurkat cells was reduced to a lower level (P<0.01); ii) The expression variation trend of the Homo sapiens B-cell CLL/lymphoma 2 (Bcl-2) was similar in the two NHL cell lines: it increased with the increases in the concentration of As$_2$S$_2$ (P<0.01); iii) The expression of Homo sapiens Bcl-2-like 1 (BCL2L1; Bcl-xL) was initially increased, followed by a decrease in Raji cells, whereas in Jurkat cells it exhibited a decreasing tendency (P<0.01); iv) The expression variation trend of Homo sapiens v-myc myelocytomatosis viral oncogene homolog (avian) (MYC; c-Myc) was similar in the two cell lines: it was initially raised by a few folds compared to the control group (P<0.01) and then decreased to levels similar to those in the control group (P>0.01). v) As regards Homo sapiens pim-1 oncogene (PIM), there were no significant changes in Raji cells (P>0.01), whereas a significant decrease was observed in the Jurkat cell line (P<0.01). Changes in the expression levels of the apoptosis-associated genes under the effect of lower As$_2$S$_2$ concentrations were more distinct compared to those under higher concentrations.

Discussion

As$_2$S$_2$ has been attracting attention due to the merits of its oral administration and lower toxicity. Following a literature review, it was noted that the number of studies available on the effects of As$_2$S$_2$ on NHLs, particularly with regard to the comparison between B- and T-cell lymphomas, is limited. Our study aimed to elucidate the mechanism underlying the effects of As$_2$S$_2$ on NHL cells. Our findings indicated that, under certain concentration ranges, As$_2$S$_2$ may inhibit the proliferation of the Raji and Jurkat cell lines in a time- and dose-dependent manner. As$_2$S$_2$ may also induce apoptosis in the Raji and Jurkat cell lines in a time- and dose-dependent manner. The statistics mentioned above suggest that the key mechanisms underlying the effect of As$_2$S$_2$ on NHL cells are proliferation inhibition and apoptosis induction. The expression levels of the apoptosis-associated genes are also altered, leading to changes in certain signaling pathways.

In the present study, As$_2$S$_2$ distinctively inhibited the proliferation of Raji cells (IR, 47.64%) at a lower concentration (3 µM) and shorter acting time (24 h), whereas to achieve a similar inhibitory rate in Jurkat cells (48.11%), a significantly higher concentration of As$_2$S$_2$ (40 µM) was required for at least 24 h. A similar phenomenon was observed in the apoptosis rates using flow cytometry. A lower concentration and shorter acting time of As$_2$S$_2$ (4 µM for 48 h) achieved a high apoptosis rate in Raji cells (52.50%), whereas to achieve a similar result in Jurkat cells (53.93%), a higher concentration and longer acting time of As$_2$S$_2$ (30 µM for 72 h) was required. Therefore, Raji cells are more sensitive to As$_2$S$_2$ compared to Jurkat cells and may exhibit higher inhibitory and apoptotic rates following treatment with lower concentrations of As$_2$S$_2$ for shorter time periods. These results suggest that B-lymphoma cells were more sensitive to arsenic compared to T-lymphoma cells, as previously reported (4).

The significant difference between tumor and normal cells is that tumor cells have the ability to undergo uncontrollable proliferation, differentiation and apoptosis. The majority of chemotherapeutic drugs, including As$_2$S$_2$, are aimed at inhibiting the proliferation and inducing the apoptosis of these
uncontrollable cells. In the present study, apoptosis-related genes were investigated to elucidate the mechanisms underlying the effects of As$_2$S$_3$ on Raji and Jurkat cells. BAX, Bcl-2, Bcl-xL, c-Myc and PIM are important genes that are able to control the apoptotic processes of tumor cells. Among these, BAX, Bcl-2 and Bcl-xL are members of the Bcl-2 family and are involved in the mitochondrial apoptotic pathway as apoptotic inducers or inhibitors (17). The protein encoded by c-Myc is a multifunctional nuclear phosphoprotein that plays an important role in cell cycle progression, cellular apoptosis and transformation. The protein encoded by PIM plays a vital role in signal transduction in blood cells, contributing to cell proliferation and survival, thus providing a selective advantage in tumorigenesis. These two proteins may significantly affect the cell cycle and are highly expressed in malignant cells. Since c-Myc is an early response gene necessary for G1- to S-phase cell cycle transition, it may promote cell proliferation, regulate cell apoptosis, competition and differentiation, form heterodimers with Max and directly bind DNA at the E-box sequences to induce transcription and inhibit the interaction of c-Myc/Max with MIZ-1 at DNA initiator elements, exerting a dual effect on malignant cells (18). The PIM protein belongs to the Ser/Thr protein kinase family, is involved in multiple signal transduction pathways and is highly expressed in several types of tumors, including lymphoma (19).

In this study, the expression variation trends of BAX and Bcl-xL in Raji and Jurkat cells following treatment with As$_2$S$_3$ were in accordance with previous studies (20-22), with the difference that BAX in Jurkat cells was reduced to a lower level compared to the control group (P<0.01). Kang et al (23) conducted a study on human cervical cancer cells and reported that the translocation of BAX and the phosphorylation of Bcl-2 were associated with cell apoptosis and the increased level of mitochondrial BAX coexisted with no or minimal change in the total amount of BAX. Furthermore, BAX translocation presented as an increase in mitochondrial BAX, with no or minimal change in the total intracellular BAX. In addition, the composition of BAX may differ between the two cell lines originating from different ancestors. Consequently, BAX may exhibit a functional variability between cell lines. Furthermore, findings of that study (23) offered an explanation for our findings, which demonstrated that the expression levels of the Bcl-2 gene were increased following treatment with As$_2$S$_3$, which was inconsistent with previous studies (22,24-26): the Bcl-2 mRNA was high whereas the Bcl-2 protein levels were low due to intracellular Bcl-2 phosphorylation.

Despite the fact that there was no obvious variation in Raji cells following treatment with As$_2$S$_3$ (P>0.01), the expression variation trend of PIM was in accordance with that of Bcl-xL, as previously reported (27). According to previous findings (28), exposure to low doses (2 μM) of inorganic arsenic for long periods may increase the expression level of c-Myc in the J6B cell line and inorganic arsenic may cause changes in the cell cycle and increase the intracellular c-Myc protein levels (29). In addition, the effects of higher doses of As$_2$S$_3$ did not differ significantly from those of lower doses, the possible reason being that the toxicity of the drug itself affected the proliferation and apoptosis of tumor cells, i.e., the IC$_{50}$ dose was too high a burden for the cells to assess the mRNA levels.

In summary, As$_2$S$_3$ may inhibit cell proliferation and induce apoptosis in B- and T-lymphoma cell lines through altering the expression levels of apoptosis-related genes and activating or inhibiting specific signaling pathways within those cells. Furthermore, B-lymphoma cells are more sensitive to As$_2$S$_3$. These results may provide a foundation for the clinical treatment of NHLs. However, additional investigations are required to elucidate the interactions between the signaling pathways.

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References