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## Induction of K562 Cell Apoptosis by As<sub>4</sub>S<sub>4</sub> via Down-Regulating miR181

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Data Collection B  
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Data Interpretation D  
Manuscript Preparation E  
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**Background:** Chronic myelogenous leukemia (CML) has unsatisfactory treatment efficacy at present. As the major component of red orpiment, tetra-arsenic tetra-sulfide (As<sub>4</sub>S<sub>4</sub>) has been recently used in treating leukemia, but with unclear mechanism targeting CML. MicroRNA (miR) is a group of endogenous non-coding RNAs regulating pathogenesis. MiR181 has been shown to exert important roles in tumor progression. The relationship between miR181 and As<sub>4</sub>S<sub>4</sub> in inducing K562 cell apoptosis, however, is still unclear.

**Material/Methods:** CML cell line K562 was cultured *in vitro* in a control group and in groups receiving various dosages (20 μM and 40 μM) of As<sub>4</sub>S<sub>4</sub>. MTT assay was employed to detect the effect on K562 cell survival. MiR181 expression was quantified by real-time PCR. MTT assay and assay kit were used to determine K562 cell survival and caspase 3 expression. Cell apoptosis was assessed by flow cytometry. Bcl-2 expression was determined by real-time PCR and Western blotting.

**Results:** As<sub>4</sub>S<sub>4</sub> significantly suppressed proliferation of K562 cells (p<0.05) and decreased miR181 expression, and increased caspase3 activity compared to the control group. It can induce K562 cell apoptosis via remarkably down-regulating mRNA and protein expressions of Bcl-2 (p<0.05).

**Conclusions:** As<sub>4</sub>S<sub>4</sub> can facilitate K562 cell apoptosis via down-regulating miR181, inhibiting Bcl2 expression, and enhancing apoptotic protein caspase3 activity.

**MeSH Keywords:** **bcl-2-Associated X Protein • Caspase 14 • Caspase Inhibitors • Genes, bcl-2 • Tacrine**

**Full-text PDF:** <http://www.medscimonit.com/abstract/index/idArt/899214>



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## Background

Chronic myelogenous leukemia (CML) is the third most common type of leukemia [1,2], after acute granulocytic leukemia and acute lymphocytic leukemia. It is a malignant proliferation of granulocytic lineage of hematological stem cells [3,4]. CML is mainly caused by translocation of Abl and Bcr genes, which are located at chromosomes 9q34 and 22q11, respectively, thus forming the so-called Philadelphia (Ph) chromosome, which expresses BCR-ABL fusion protein. This protein has inherent tyrosine kinase activity for inducing tumorigenesis by anti-apoptosis and cell cycle disruption [5]. Currently, targeted drugs for BCR/ABL fusion protein combined with tyrosine kinase inhibitors are major approaches for treating CML. Currently available tyrosine kinase inhibitors such as imatinib are only effective during the chronic phase, but have poor efficacy in BCR/ABL fusion protein-positive disease during the acute phase, and is ineffective for CML stem cells during the latent stage [6,7]. Other common chemotherapy agents such as hydroxyurea and myleran lack tumor specificity, thus causing poor efficacy in CML [8]. Moreover, the long-term application of chemotherapy medicine or tyrosine kinase inhibitor (such as imatinib) also leads to the development of drug resistance in tumor cells, as well as more adverse effects. Xenograft bone marrow transplantation is an approach to curing CML, but is often restricted by donor, disease course, condition, and transplantation, plus high cost [9,10]. Therefore, it is of critical importance to develop novel treatment approaches for CML. As the major component of red orpiment, which is a traditional Chinese medicine, tetra-arsenic tetra-sulfide (As<sub>4</sub>S<sub>4</sub>) has been recently implicated in treating leukemia [11]. However, its treatment effects and mechanism targeting CML are unknown.

MicroRNA (miR) is a kind of small molecule RNA that participates in various biological functions via negative regulation and pairing for inhibiting downstream target protein expression, degrading mRNA, and regulating protein translation at the post-transcriptional level. miR has pluripotent functions for regulating body development and enhancing acclimation to the environment [12,13]. Among various endogenous non-coding RNAs regulating multiple diseases and physiological events, miR181 has been shown to be involved the progression of multiple tumors [14,15]. The role of As<sub>4</sub>S<sub>4</sub> in inducing CML tumor cell line K562 apoptosis via miR181 is unknown.

## Material and Methods

### Reagents and equipment

CML cell line K562 was purchased from the ATCC cell bank (USA). RPMI 1640 culture medium and penicillin/streptomycin were purchased from Hyclone (USA). As<sub>4</sub>S<sub>4</sub> was extracted

from red orpiment crystals and was dissolved in RPMI 1640 medium (160 mM stock). DMSO and MTT powder were purchased from Gibco (USA). Trypsin-EDTA buffer was obtained from Sigma (USA). PVDF membrane and caspase 3 activity kit were purchased from Pall Life Sciences (USA). Reagents for Western blotting were provided by Beyotime (China). ECL kits were purchased from Amersham Biosciences (USA). Rabbit anti-human Bcl2 monoclonal antibody and goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) were obtained from Cell Signaling (USA). Annexin V-FITC apoptotic assay kit, RNA extraction kit, reverse transcription kit, and microplate reader were obtained from BD (USA). MiR-181 mimic was purchased from Shanghai Genechem Co., Ltd. (Shanghai, China). DNA amplifier was purchased from PE (Gene Amp PCR System 2400). Other common reagents were purchased from Sangon (China).

### K562 cell culture and grouping

K562 cells in liquid nitrogen were resuscitated at 37°C until complete thawing. Cells were collected by 1000 rpm centrifugation for 3 min and were re-suspended in 1 ml fresh culture medium in a 5-ml culture flask containing 2 ml fresh culture medium in a 37°C humidified chamber with 5% CO<sub>2</sub> for 24~48 h. Cells were then seeded into a 6-well plate at 1×10<sup>5</sup> per ml using high-glucose RPMI-1640 medium containing 10% FBS and 100 U/ml penicillin and 100 µg/ml streptomycin in a 37°C humidified chamber with 5% CO<sub>2</sub>. Cells at log phase with 3~8 generations of passage were randomly divided into 3 groups for 48-h drug treatment: the control group, the low dosage (20 µM) group, and the high dosage (40 µM) As<sub>4</sub>S<sub>4</sub> group.

### MTT assay

K562 cells at log phase were digested, counted, and inoculated into a 96-well plate (3000 cells per well). Cells were randomly divided into 3 groups: control group, low dosage (20 µM) As<sub>4</sub>S<sub>4</sub> group, and high dosage (40 µM) As<sub>4</sub>S<sub>4</sub> group (N=5 each) for 48-h drug treatment. We added 5 g/L MTT solution into each group (20 µl each) for 4-h incubation. Supernatant was then removed, followed by the addition of 150 µl DMSO for 10-min vortexing until complete resolving of violet crystals. A microplate reader was used to detect absorbance (A) value at 570 nm wavelength of each well for calculating cell proliferation rate in triplicate.

### Real-time PCR

Trizol reagent was used to extract total RNA from K562 cells following the manual's instructions. DNA was then synthesized by reverse transcription using pre-designed primers (by Primer6.0, synthesized by Yingjun Biotech, China, see Table 1) for real-time PCR under the following conditions: (for GAPDH

**Table 1.** Primer sequences.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
GADPH	AGTACCAGTCTGTGGCTGG	TAATAGACCCGGATGTCTGGT
miR181	ATCTCTGTTTCTGAAGCTGG	TCACG CCCTCGCTGTGCACA
BCL-2	CCTCTGTCCACATAAG	GTACCCATTGGGGTAATT

and miR188), 55°C 1 min, followed by 35 cycles each containing 92°C 30 s, 58°C 45 s, and 72°C 35 s; (for Bcl-2), 55°C 1 min, followed by 35 cycles each containing 92°C 30 s, 57°C 50 s, and 72°C 35 s. Data were collected to calculate CT values of all samples using GAPDH as the reference based on fluorescent quantification on PCR equipment. Using CT values of the standard samples as the reference, a standard curve was plotted for semi-quantitative analysis by 2<sup>-ΔCt</sup> approach.

### Flow cytometry

Tumor cells were digested and inoculated in a 50-mL culture flask at 5×10<sup>5</sup>/mL and were randomly divided into 3 groups: control group; low dosage (20 μM) As<sub>4</sub>S<sub>4</sub> group, and high dosage (40 μM) As<sub>4</sub>S<sub>4</sub> group for 48-h drug treatment. To study the effect of As<sub>4</sub>S<sub>4</sub> on apoptosis in cells with miR-181 overexpression, K562 cells were seeded for 12 h to reach a confluence of 70–80% and then transfected by miR-181 mimic (sequence: 5'-ACGUUCGAAUCGGUUGGA-3') or negative control (5'-AUUCCGGUAGGCAUGAUG-3') using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following manufacturer's instructions. Then, cells were digested, inoculated in a 50-mL culture flask at a density of 5×10<sup>5</sup>/mL, and then randomly divided into another 3 groups: control group, miR-181 overexpression group, and As<sub>4</sub>S<sub>4</sub> (40 μM) group. After that, cells were then digested and collected in 1XPBS at 2×10<sup>6</sup> per ml. After 1000 rpm centrifugation and 5 min washing, 75% cold ethanol was used to fix cells, which were incubated at 4°C overnight. After discarding ethanol, cells were re-washed in 1×PBS by 1000 rpm centrifugation for 5 min. Cells were then re-suspended into 0.8 ml 1×PBS with 1% BSA. After adding 100 μg/ml PI dye (3.8% sodium citrate, pH7.0), 100 RNA enzyme (RNase A, 10 mg/ml) was added for 37°C dark incubation for 30 min. Flow cytometry was used to collect data for analysis in FCSEXPRESS 3.0 software.

### Caspase3 activity assay

The activity of caspase3 in all cells was detected by use of a test kit following manual instructions. In brief, trypsin was first used to digest cells, which were centrifuged at 600 g for 5 min to discard the supernatants. Cell lysis buffer was then added for ice incubation for 15 min. The mixture was centrifuged at 20 000 g for 5 min at 4°C. We added 2 mM Ac-DEVD-pNA to detect OD value at 405 nm wave length for calculating activity of caspase3.

### Western blotting

K562 cells were collected and lysed on ice for 15–30 min. Ultrasound was used to rupture cells (5 s ×4 times). The mixture was then centrifuged at 10 000 g for 15 min at 4°C to collect supernatant, in which protein was quantified. In Western blotting assay, proteins were separated by 10% SDS-PAGE and transferred to PVDF membrane by semidry method. Non-specific background was removed by 5% defatted milk powder for 2 h at room temperature. Bcl-2 monoclonal antibody (1:1000 dilution) was added for 4°C overnight incubation. On the next day, the membrane was washed in PBST and mixed with 1:2000 goat anti-rabbit secondary antibody for 30-min incubation. ECL chromogenic substrate was used to develop the membrane for 1 min, followed by X-ray exposure. Quantity One software was used to scan X-ray films and measuring optical density of each band (N=4 in each group).

### Statistical analysis

SPSS16.0 software was used to process all collected data, and measurement data are presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was employed to compare means across multiple groups. The LSD test was employed for paired comparison. Statistical significance was defined when p<0.05.

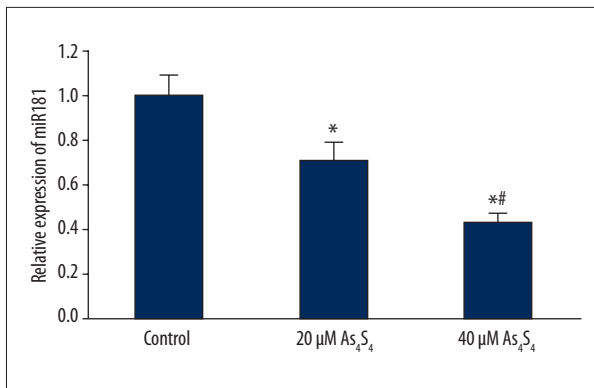
## Results

### Effects of As<sub>4</sub>S<sub>4</sub> on the expression level of miR181

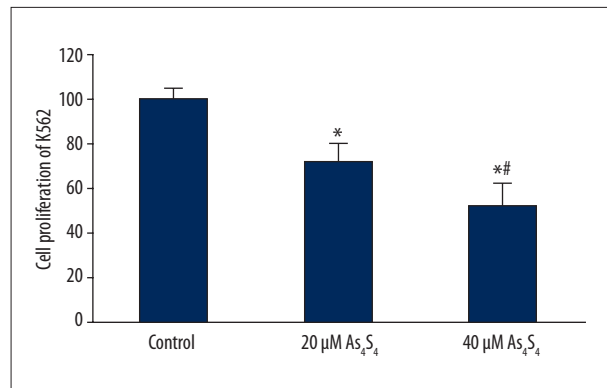
Real-time PCR was used to quantify miR181 expression levels under different concentrations of As<sub>4</sub>S<sub>4</sub>. Results showed significant suppression of miR181 expression in CML K562 cells after adding As<sub>4</sub>S<sub>4</sub> in a dose-dependent manner; 40 μM drug has more potent effects than 20 μM (p<0.05, Figure 1).

### As<sub>4</sub>S<sub>4</sub> and K562 cell proliferation

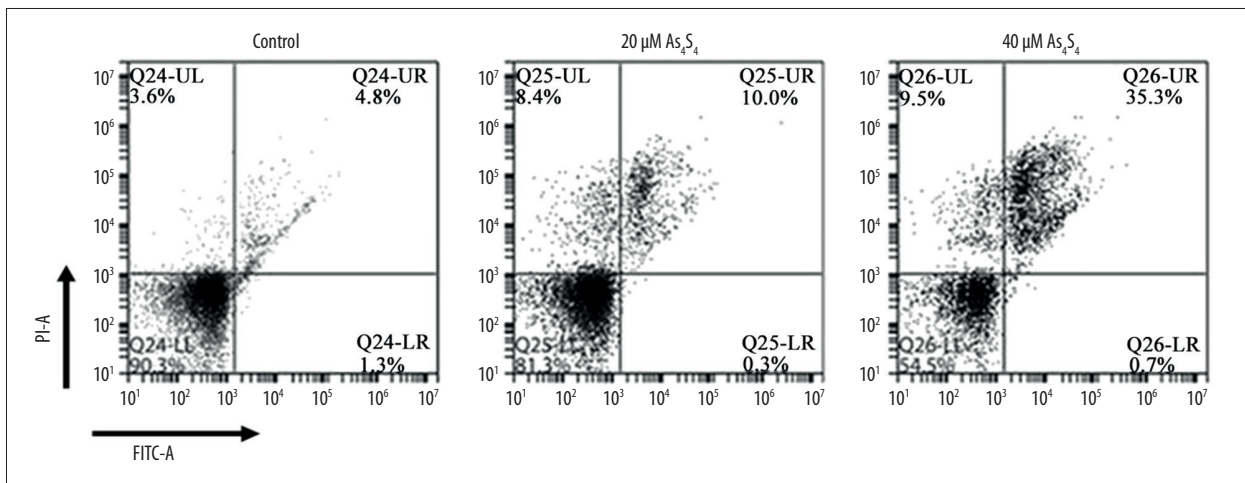
We used MTT assay to describe the proliferation of CML K562 cells after 48-h drug treatment. Results showed that As<sub>4</sub>S<sub>4</sub> could effectively inhibit cell proliferation in a dose-dependent manner; as 40 μM drug had more potent effects than 20 μM



**Figure 1.** miR181 expression in As<sub>4</sub>S<sub>4</sub>-treated K562 cells. \* p<0.05 compared to control group; # p<0.05 compared to 20 μM As<sub>4</sub>S<sub>4</sub> group.



**Figure 2.** Proliferation of As<sub>4</sub>S<sub>4</sub>-treated K562 cells. \* p<0.05 compared to control group; # p<0.05 compared to 20 μM As<sub>4</sub>S<sub>4</sub> group.



**Figure 3.** Effects of As<sub>4</sub>S<sub>4</sub> on apoptosis of CML K562 cells.

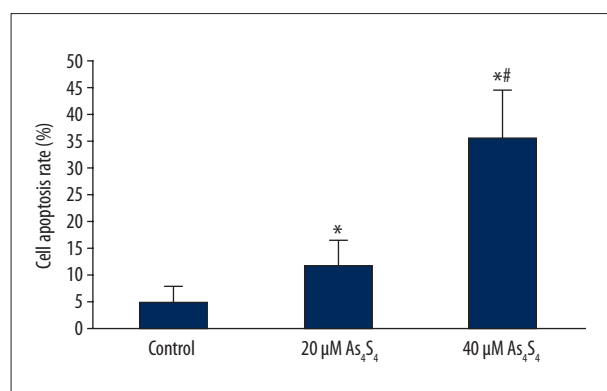
(p<0.05, Figure 2). These results suggested that As<sub>4</sub>S<sub>4</sub> could inhibit the proliferation of CML K562 cells after down-regulating miR181.

### As<sub>4</sub>S<sub>4</sub> regulates apoptosis of CML K562 cells

Flow cytometry was used to detect the effect of different concentrations of As<sub>4</sub>S<sub>4</sub> on the apoptosis of CML K562 cells. Results showed that As<sub>4</sub>S<sub>4</sub> could induce tumor cell apoptosis in a dose-dependent manner; 40 μM drug has more potent effects than 20 μM (p<0.05, Figures 3, 4). These results suggested that As<sub>4</sub>S<sub>4</sub> could induce the apoptosis of CML cells after down-regulating miR181.

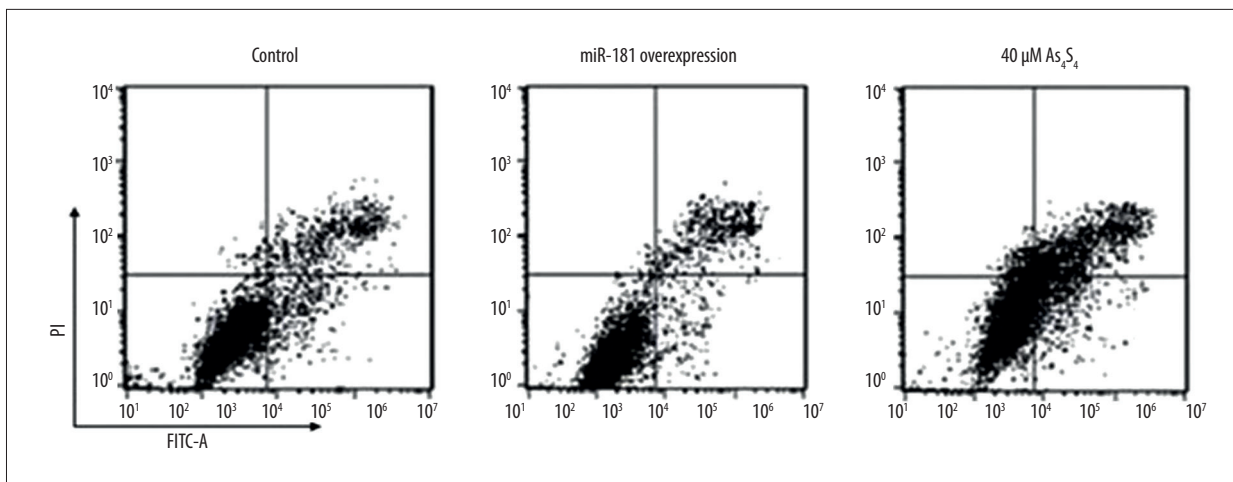
### Effects of As<sub>4</sub>S<sub>4</sub> on the apoptosis of K562 cells with miR-181 overexpression

K562 cells with miR-181 overexpression was established through transfection of miR-181 mimic and then treated by As<sub>4</sub>S<sub>4</sub> followed by measurement of cell apoptosis. As seen in

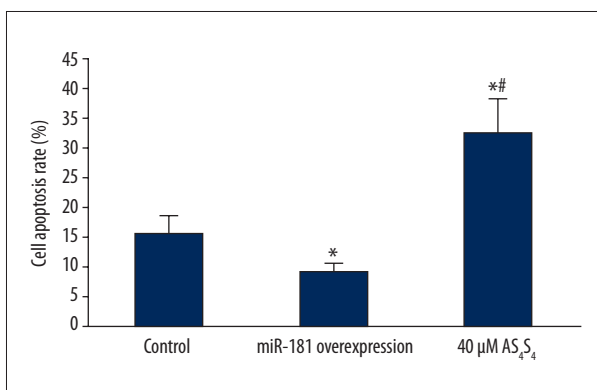


**Figure 4.** Analysis of K562 cell apoptosis by As<sub>4</sub>S<sub>4</sub>. \* p<0.05 compared to control group; # p<0.05 compared to 20 μM As<sub>4</sub>S<sub>4</sub> group.

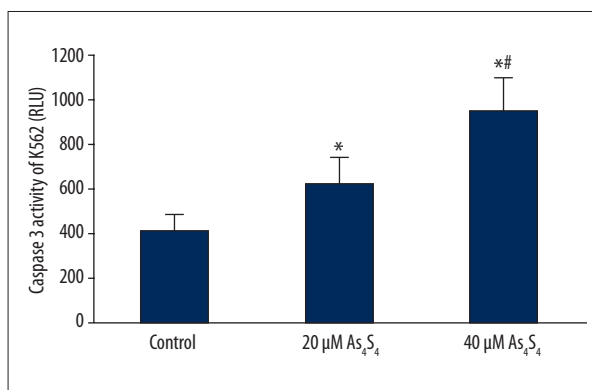
Figures 5 and 6, the apoptosis rate of cells with miR-181 overexpression was significantly lower compared to controls (p<0.05). The apoptosis rate in the miR-181 overexpression group was also significantly lower than in the As<sub>4</sub>S<sub>4</sub> group (p<0.05).



**Figure 5.** Effects of As<sub>4</sub>S<sub>4</sub> on apoptosis of CML K562 cell with miR-181 overexpression.



**Figure 6.** Analysis of apoptosis of K562 cell with miR-181 overexpression by As<sub>4</sub>S<sub>4</sub>. \* p<0.05 compared to control group; # p<0.05 compared to miR-181 overexpression group.



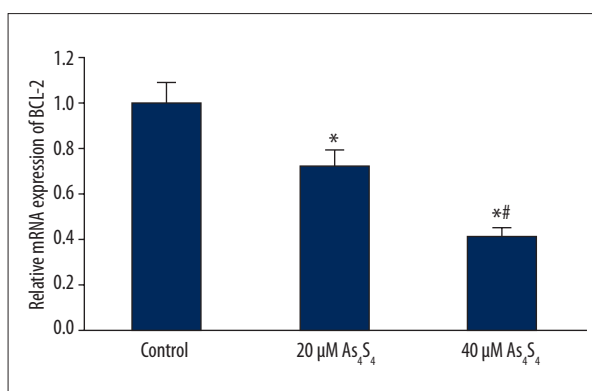
**Figure 7.** Caspase3 activity in CML K562 cells after treating with As<sub>4</sub>S<sub>4</sub>. \* p<0.05 compared to control group; # p<0.05 compared to 20 μM As<sub>4</sub>S<sub>4</sub> group.

### Caspase3 activity of CML K562 cells

Using a caspase3 activity assay kit to analyze the effect of different dosages of As<sub>4</sub>S<sub>4</sub> on the caspase3 activity, we found that As<sub>4</sub>S<sub>4</sub> remarkably increased the activity of caspase3 in K562 cells compared to the control group (p<0.05). We found that 40 μM drug has more potent effects than 20 μM (p<0.05, Figure 7). These results suggested that As<sub>4</sub>S<sub>4</sub> could enhance the activity of caspase3 after down-regulating miR181 for further inducing CML cell apoptosis.

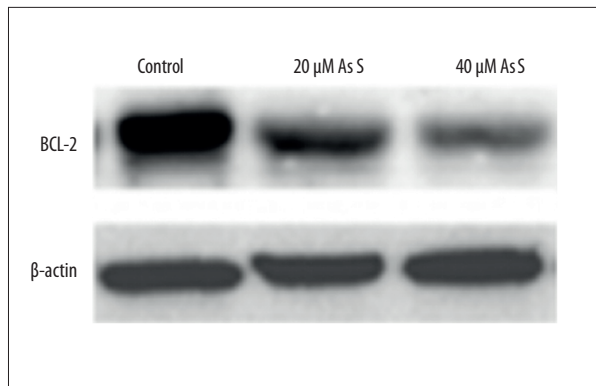
### Bcl-2 mRNA expression in K562 cells

Real-time PCR was employed to detect the mRNA expression of Bcl-2 gene in CML K562 cells after treatment with different concentrations of As<sub>4</sub>S<sub>4</sub>. Results showed that As<sub>4</sub>S<sub>4</sub> could remarkably inhibit the expression of Bcl-2 mRNA in K562 cells compared to controls (p<0.05). We found that 40 μM drug has more potent effects than 20 μM (p<0.05, Figure 8).



**Figure 8.** Effects of As<sub>4</sub>S<sub>4</sub> on the mRNA level of Bcl-2 in CML K562 cells. \* p<0.05 compared to control group; # p<0.05 compared to 20 μM As<sub>4</sub>S<sub>4</sub> group.





**Figure 9.** BCL-2 protein expression in CML K562 cells.

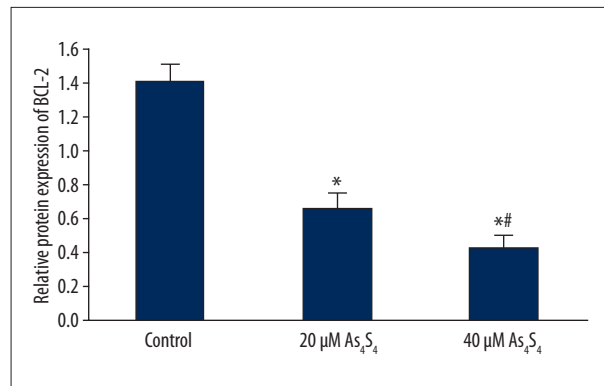
### Effects of As<sub>4</sub>S<sub>4</sub> on BCL-2 protein expression in CML K562 cells

Western blotting was employed to further reveal the protein expression level of BCL-2 in CML K562 cells treated with different concentrations of As<sub>4</sub>S<sub>4</sub>. Results showed that, similar to those of mRNA level, As<sub>4</sub>S<sub>4</sub> could remarkably inhibit the expression of BCL-2 protein in K562 cells compared to the control group ( $p < 0.05$ ). We found that 40  $\mu\text{M}$  drug has more potent effects than 20  $\mu\text{M}$  ( $p < 0.05$ , Figures 9, 10). These results suggest that As<sub>4</sub>S<sub>4</sub> can inhibit the expression of anti-apoptotic protein BCL-2 expression after down-regulating miR181 to induce tumor cell apoptosis.

## Discussion

The pathogenesis of CML has been illustrated as clinical symptoms and occurrence of Ph chromosome or Bcr/Abl fusion gene [5]. Clinically, CML can be divided into chronic, accelerating, and blastic phases. In chronic phase, CML has slow disease onset and late occurrence of symptoms. After diagnosis, however, patients are mostly in the accelerating phase, thus increasing the difficulty of treatment [7]. Although significant improvements have been obtained, CML is inherently insensitive to chemotherapy and other classical approaches, thus impeding the improvement of 5-year survival rate [6]. The development of a novel drug for CML can benefit the life quality of patients. As<sub>4</sub>S<sub>4</sub> is a paragenetic mineral of As<sub>2</sub>S<sub>3</sub>, which has been shown to be effective in treating tumors, including leukemia [16]. As<sub>4</sub>S<sub>4</sub> has also been reported to have potential roles in treating CML [14,17], but details of its mechanism are unclear.

At present, understanding of the miRNA-related regulatory mechanism involved in CML pathogenesis is incomplete. The role of tumor-related miRNA in diagnosis and treatment of CML, including the identification of key target miR along with Bcr/Abl fusion gene, has become a focus of CML pathogenesis research. The abnormal expression of miR can affect the



**Figure 10.** Analysis of BCL-2 protein expression after As<sub>4</sub>S<sub>4</sub> treatment. \*  $p < 0.05$  compared to control group; #  $p < 0.05$  compared to 20  $\mu\text{M}$  As<sub>4</sub>S<sub>4</sub> group.

regulation of multiple biological processes, including development, cell proliferation/apoptosis, cell growth/differentiation, and cell cycle modulation [18]. miR can regulate the post-translational level and can modulate certain signal molecules such as cell growth factor, transcriptional factor, and death gene expression, thus affecting cell death, proliferation, differentiation, development, and metabolism [19]. miR181 has been reported to be over-expressed in CML cells [20]; therefore, we investigated the regulator effect of As<sub>4</sub>S<sub>4</sub> on miR181 and its role in CML. Our results proved that in an *in vitro* K562 cell line of CML at different concentrations of As<sub>4</sub>S<sub>4</sub> could significantly down-regulate miR181 expression in K562 cells, further causing higher apoptotic rates of cells and inhibiting cell proliferation.

The abnormal proliferation is an important factor in tumor formation and progression. The rapid proliferation of tumor cells and reduced cell death directly contribute to the dysregulation of tumor progression, in which cell apoptosis is a crucial factor. As part of homeostasis regulation, cell apoptosis can retard the occurrence of tumors via inhibiting tumor overgrowth. When a cell initiates its apoptotic program, a related family can be activated and is the most potent regulatory factor inducing apoptosis. As<sub>4</sub>S<sub>4</sub> can induce tumor cell apoptosis via mediating caspase3 activity. When Bcl-2 is over-expressed, injured cell may not undergo programmed cell death, but rather transform into tumors under the direction of related genes, including those regulating cell proliferation and growth inhibition [21,22]. In addition, Bcl-2 was identified as a target of miR-181, suggesting miR-181 might affect cell apoptosis through regulation of Bcl-2 [20]. This study revealed the dose-dependent inhibition of Bcl-2 expression (both mRNA and protein) by As<sub>4</sub>S<sub>4</sub> after its down-regulation on miR181 expression. It can also potentiate the activity of caspase3, further inducing the apoptosis of CML.

## Conclusions

As<sub>4</sub>S<sub>4</sub> can inhibit Bcl-2 mRNA and protein expression via down-regulating miR181 expression, and increase caspase3 activity

for facilitating the apoptosis of CML cells and inhibiting disease progression. Our study provides potential drug candidates for a novel strategy for treating CML.

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