Arsenic sulfide, the main component of realgar, a traditional Chinese medicine, induces apoptosis of gastric cancer cells in vitro and in vivo

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Background: Arsenic sulfide (As₄S₄), the main component of realgar, a traditional Chinese medicine, has shown antitumor efficacy in several tumor types, especially for acute promyelocytic leukemia. In this study, we aimed to explore the efficacy and mechanism of As₄S₄ in gastric cancer.

Methods: The effect of As₄S₄ on cell proliferation and apoptosis of gastric cancer cells was investigated by MTT assay, 4′,6-diamidino-2-phenylindole (DAPI) staining, and annexin V–fluorescein isothiocyanate/propidium iodide staining using gastric cancer cell lines AGS (harboring wild-type p53) and MGC803 (harboring mutant p53) in vitro. The expression of apoptosis-related proteins was measured by Western blotting, real-time polymerase chain reaction, and immunohistochemistry analysis. Mouse xenograft models were established by inoculation with MGC803 cells, and the morphology and the proportion of apoptotic cells in tumor tissues were detected by hematoxylin and eosin staining and TdT-mediated dUTP nick end labeling (TUNEL) assay, respectively.

Results: As₄S₄ inhibited the proliferation and induced apoptosis of AGS and MGC803 cells in a time- and dose-dependent manner. As₄S₄ upregulated the expression of Bax and MDM2 while downregulated the expression of Bcl-2. The expression of p53 increased significantly in the AGS cells but did not readily increase in the MGC803 cells, which harbored mutant p53. Pifithrin-α, a p53 inhibitor, blocked the modulation of As₄S₄ on AGS cells, but not on MGC803 cells. Using xenograft as a model, we showed that As₄S₄ suppressed tumor growth and induced apoptosis in vivo and that the expression of p53 increased accordingly.

Conclusion: As₄S₄ is a potent cytotoxic agent for gastric cancer cells, as it induced apoptosis both in vitro and in vivo through a p53-dependent pathway. Our data indicate that As₄S₄ may have therapeutic potential in gastric cancer.

Keywords: As₄S₄, p53, realgar, antitumor, xenograft

Introduction

Gastric cancer is the fourth most common malignant tumor worldwide.¹ According to “Cancer Statistics, 2014”,² approximately 22,220 new gastric cases are diagnosed annually, resulting in 10,990 deaths in the United States. In Asia, gastric cancer is the third most common cancer after breast and lung cancer, and the second most common cause of cancer death after lung cancer. There are more than 677,000 cases of gastric cancer annually in the developing countries, and one-half of the world total occurs in Eastern Asia, mainly in the People’s Republic of China.³,⁴ Although radical surgery for patients diagnosed at early stages can prolong overall survival, the high recurrence rate is still a major problem. Even though first-line chemotherapies have been proven to prolong overall survival and improve quality of life compared with supportive care, the 5-year survival rate in patients with advanced gastric cancer who...
receive palliative chemotherapy is barely 5% to 10%. Therefore, a new strategy for the treatment of gastric cancer is urgently needed.

Arsenic compounds have been used for more than 2,400 years as traditional Chinese medicines and have attracted much research attention in recent years. There are three main types of mineral arsenical: arsenolite (mainly As₂O₃, arsenic trioxide), realgar (mainly As₂S₃, tetrarsenic tetrasulfide), and orpiment (mainly As₂S₅, arsenic trisulfide). As₂O₃ has had excellent therapeutic impact in the treatment of acute promyelocytic leukemia (APL). Recently, As₂S₃, the main component of realgar, has gained more focus due to its advantages of oral administration, relative safety, and ample resources. As₂S₃ has antitumor activities in several cancers, especially APL, in vitro and in vivo, and the antitumor activities are correlated with its ability to inhibit cell proliferation and induce apoptosis.

p53 is a critical gatekeeper against oncogenesis and malignant cell proliferation. Mutations in the p53 gene are the most common genetic abnormality, and approximately 50% of human cancers contain p53 mutation. Wild-type p53 gene transfer enhances cytotoxicity of anticancer drugs in human cancer cells in vitro and in vivo. The crucial tumor suppressor activity of p53 involves both transcription-dependent and -independent mechanisms. Several studies have found that the state of p53 plays an important role in the process of drug-induced apoptosis of tumor cells.

In previous studies, we explored the anticancer effect and mechanism of As₂S₃ on a series of solid tumor cell lines, such as MKN45 cells (gastric cancer), HepG2 cells (hepatocellular carcinoma), A375 cells (malignant melanoma), and 8898 cells (pancreatic carcinoma), and showed that As₂S₃ possessed potent antitumor activities in solid tumors and induced apoptosis. To further investigate the cytotoxic effect and the molecular mechanism of As₂S₃ in gastric cancer and whether or not p53 is important in mediating the effect of As₂S₃, we selected the wild-type p53 containing AGS cells and the mutant p53 containing MGC803 cells. We found that As₂S₃ exerted potent antiapoptotic and cytotoxic effects in both cell lines; however, the effect of arsenic on the AGS cells was much more pronounced than on the MGC803 cells, indicating that p53 played a critical role in the process of As₂S₃ induced apoptosis of gastric cancer cells. Using p53 inhibitor pifithrin-α, we found that the cytotoxic effect was blocked only in the AGS cells, but not in MGC803. These findings provide evidence that p53 is a critical factor in mediating cytotoxic effects of As₂S₃ in gastric cancer cells.

Materials and methods

Chemicals, solutions, and antibodies

Highly purified realgar supplied by the Shanghai Institute of Hematology (Shanghai, People’s Republic of China) was prepared from mined natural realgar. The purity of As₂S₃ in our realgar preparation was greater than 98.0%, confirmed by repeated X-ray powder diffraction analyses (in collaboration with the Research Center at the Xi’an Institute of Geology and Mineral Resources [Xi’an, People’s Republic of China]). These results were compatible with pure As₂S₃ standards and excluded the potential for trace amounts of arsenic trioxide and other arsenic compounds that could influence the results. The high-purity realgar was dissolved in Dulbecco’s phosphate-buffered saline (DPBS) (Thermo Fisher Scientific, Waltham, MA, USA) and sterilized by filtration. The content of As in DPBS solution was determined by inductively coupled plasma atomic emission spectrometry at the Instrumental Analysis Center of Shanghai Jiao Tong University (Shanghai, People’s Republic of China). An As₂S₃ stock solution of 277.2496 µM was stored at 4°C. According to the blood arsenic levels from As₂S₃-treated patients, the stock solution was appropriately diluted between 100 and 1,000 times in DPBS to obtain a working solution. Anti-p53, anti-Bcl-2, and anti-Bax antibodies were purchased from Cell Signaling Technology (CST, Beverly, MA, USA). Anti-MDM2 antibody, pifithrin-α, MTT, and sodium dodecyl sulfate (SDS) powder were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). β-actin antibody was purchased from Proteintech Group, Inc (Wuhan, Hubei, People’s Republic of China). MTT powder was dissolved in DPBS at the concentration of 5 mg/mL. SDS powder was dissolved in water at the concentration of 10%.

Cells and cell culture

The human gastric adenocarcinoma cell line AGS (harboring wild-type p53) and MGC803 (harboring mutant p53) were obtained from Cell Bank, Chinese Academy of Sciences (Shanghai, People’s Republic of China). The human gastric epithelial cell line (also known as GES-1) was purchased from Beijing ComWin Biotech Co. Ltd. (Beijing, People’s Republic of China). AGS cells were cultured in DMEM/F-12 1:1 medium (Thermo Fisher Scientific), and MGC803 and GES-1 cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific). All media were supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin (Thermo Fisher Scientific). All cells were cultured at 37°C under an atmosphere of 95% air and 5% CO₂.
Cell viability assay
The effect of As$_4$S$_3$ on AGS, MGC803, and GES-1 cell proliferation was measured using MTT assay. The MTT assay was performed as previously described, with minor modifications according to the manufacturer’s protocol. Cells were seeded in 96-well plates at a density of $5 \times 10^4$ cells/well and incubated overnight, then treated with different concentrations of As$_4$S$_3$ for 24, 48, or 72 hours, respectively. At the end of the exposure period, $10 \mu$L MTT solutions were added to each well and cells were incubated at 37°C for 4 hours. Following the 4 hours’ incubation, $150 \mu$L SDS solutions (10% concentration) were added to each well to dissolve the formazan at 37°C overnight. Absorbance of each sample was measured at 570 nm. Data were analyzed based on three independent experiments.

4′,6-diamidino-2-phenylindole staining
4′,6-diamidino-2-phenylindole (DAPI) staining was used to observe the morphology changes of the nuclei of gastric cancer cells treated with As$_4$S$_3$. Cells were seeded at $5 \times 10^4$ cells/well in 24-well plates and incubated overnight, then treated with 1.25 µM of As$_4$S$_3$ for 0, 24, or 48 hours, respectively. Then, the supernatant was aspirated, and DAPI (Beyotime Institute of Biotechnology, Haimen, Jiangsu, People’s Republic of China) was added to each well for 5 minutes. DAPI-stained cell morphology was observed under a fluorescence microscope (DMI 3000B; Leica Microsystems, Wetzlar, Germany).

Annexin V–fluorescein isothiocyanate/propidium iodide staining
Annexin V–fluorescein isothiocyanate (FITC)/propidium iodide (PI) double staining was used to quantify the apoptotic rate of human gastric cancer cells treated with As$_4$S$_3$. Cells were seeded at $1 \times 10^4$ cells/dish in 10 cm dishes and incubated overnight, then treated with different concentrations of As$_4$S$_3$ (0 µM, 1.25 µM, 2.5 µM) for 24 hours. The cells were then harvested and stained with annexin V–FITC/PI (Vazyme Biotech Co. Ltd., Nanjing, Jiangsu, People’s Republic of China) following the manufacturer’s instructions. After incubation for 30 minutes at 4°C, the cells were analyzed using flow cytometry (FACS Canto; BD Biosciences, Franklin Lakes, NJ, USA).

Western blotting analysis
Total protein from the cells or the tumor specimens of animals was extracted using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology). Protein concentrations were analyzed using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology) according to the manufacturer’s protocol. The Western blotting analysis was performed as previously described with minor modifications according to the manufacturer’s protocol. Equal amounts of proteins (60 µg) were loaded, separated by SDS polyacrylamide gel electrophoresis gels, and transferred onto the polyvinylidene fluoride membrane (Millipore, Darmstadt, Germany). The membrane was incubated with specific primary antibodies (1:1,000) at 4°C overnight after blocking nonspecific binding sites with 5% nonfat milk. After washing thrice in Tris-buffered saline and Tween 20 for 10 minutes each time, membranes were incubated with goat anti-mouse or anti-rat antibody labeled with horseradish peroxidase (Beyotime Institute of Biotechnology) for 1 hour at room temperature. Proteins were detected with an enhanced chemiluminescence system using the Beyo ECL Plus kit (Beyotime Institute of Biotechnology) and were semi-quantified using Image J software.

Real-time polymerase chain reaction analysis
Total RNA was extracted using TRIzol (Takara, Japan), and reverse transcription and polymerase chain reaction (PCR) amplification were carried out with a reverse transcriptase kit (Takara) according to the manufacturer’s protocol. The primer sequences for targeted genes as well as GAPDH are listed in Table 1. Real-time PCR was performed using the ABI PRISM 7500 System (Thermo Fisher Scientific). The comparative cycle threshold (CT) ($\Delta \Delta$CT) method was used to determine the relative concentration.

Animals and tumor models
Twenty-eight 4-week-old male BALB/C-nu/nu mice weighing 17–20 g were purchased from Shanghai Laboratory Animal Research Center (Shanghai, People’s Republic of China) and were semi-quantified using Image J software.

Table 1 Primer sequences for real-time polymerase chain reaction

<table>
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<th>Gene</th>
<th>Primer sequence (5′-3′)</th>
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<td>Bax</td>
<td>F: aagctgagctgctcccag R: caagtagtaaagggcgaac</td>
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<tr>
<td>Bcl-2</td>
<td>F: gttgtagctcctgctcgc R: gcacccctctcaatgttgg</td>
</tr>
<tr>
<td>p53</td>
<td>F: gcctaaaatggagaggggga R: atccctttctttctctct</td>
</tr>
<tr>
<td>MDM2</td>
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</tr>
<tr>
<td>GAPDH</td>
<td>F: aagctgagctgctcccag R: atggtggaagccgccccag</td>
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</table>

Abbreviations: F, forward; R, reverse.
China. Mice were acclimatized for 1 week at the Animal Laboratory Centre of Tongji University, Shanghai, People’s Republic of China before any interventions were initiated. Xenograft tumor models were established by implanting MGC803 gastric cancer cells (5×10^6 cells/mouse in 200 μL RPMI 1640 medium) subcutaneously. The mice were kept under pathogen-free conditions in a laminar flow cabinet air filter at a constant temperature of 22°C±2°C, 55%±5% relative humidity, and a 12-hour dark/light cycle. Food and water were allowed ad libitum. All experiments were performed in accordance with the guidelines of the laboratory animal handling protocols of Tongji University.

**In vivo treatment**

At day 7 after tumor inoculation, when established tumors of 0.2 to 0.3 cm³ in diameter were detectable, drug administration was begun. The animals were randomly divided into four groups consisting of seven animals each: 1) blank control group (normal saline [NS] 20 mL/kg); 2) positive control group (cyclophosphamide [CTX], 25 mg/kg in 0.4 mL); 3) low-dose group (As₄S₄, 1 mg/kg in 0.4 mL); and 4) high-dose group (As₄S₄, 2 mg/kg in 0.4 mL). The method of injection was a once-a-day intraperitoneal injection for 3 weeks. The weight of the mice and tumor size were measured every other day. The mice were sacrificed after treatment for 3 weeks.

**Evaluation of tumor growth**

Tumor volumes were calculated according to the following formula:

\[(\text{length} \times \text{width}^2)/2.\]  

(1)

The therapeutic efficacy of the xenografts were assessed using the tumor growth inhibition (TGI), which was calculated according to the following formula:

\[(1-W/W_c) \times 100\%\]  

(2)

where \(W\) is the mean tumor weight of the treated group and \(W_c\) is the mean tumor weight of the control group.

**Hematoxylin and eosin staining**

The tumor tissues from mice were fixed with 10% buffered formalin for 24 hours, and then embedded in paraffinum, sectioned at a thickness of 3 mm. Tissue slides were stained with Ehrlich hematoxylin and eosin (Beyotime Institute of Biotechnology) followed by dehydration in graded alcohol. Slides were observed and analyzed using a light microscope.

**TdT-mediated dUTP nick end labeling assay**

Detection of apoptosis was performed using a TdT-mediated dUTP nick end labeling (TUNEL) assay kit, which was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Tissue slides were treated with proteinase K (20 μg/mL) for 15 minutes at room temperature, and then rinsed three times with phosphate-buffered saline (PBS) for 3 minutes each time. Slides were placed in PBS which containing 3% H₂O₂, incubated for 10 minutes to block endogenous peroxidase activity. After rinsing in PBS, tissues were incubated in TdT reaction buffer for 10 minutes. The slides were then incubated with TdT reaction mixture for 1 hour at 37°C, blocked with stop/wash buffer for 10 minutes, and incubated with streptavidin–horseradish peroxidase in PBS for 30 minutes at room temperature to detect the labeled DNA fragments. Finally, slides were incubated with DAB, and counterstained with hematoxylin.

**Immunohistochemistry**

The expression of p53 was analyzed in paraffin-embedded sections obtained from mouse tumors. The slides were deparaffinized, rehydrated, and antigen was retrieved. After blocking nonspecific proteins with bovine serum albumin, slides were incubated with mouse polyclonal antibodies against p53 (1:500; CST) overnight at 4°C. Horseradish peroxidase-labeled anti-mouse IgG and DAB (BOSTER, Wuhan, Hubei, People’s Republic of China) were incubated to visualize the p53 antibody at room temperature. Five separate fields were randomly selected in each slide and the average proportion of p53-positive cells were assessed in each field.

**Statistical analysis**

Statistical analysis was performed using SPSS software (v 13.0; SPSS Inc, Chicago, IL, USA). All data were expressed as mean ± standard deviation (SD). Data were analyzed using one-way analysis of variance, followed by either the least significant difference procedure (if variance was equal) or the Games–Howell procedure (if variance was unequal). A two-sided \(P<0.05\) was considered statistically significant.

**Results**

The cytotoxicity of As₄S₄ against different cell lines

AGS, MGC803, and GES-1 cells were treated with different concentrations of As₄S₄ (0.00, 0.31, 0.62, 1.25, 2.50, 5.00,
10.00 µM) for 24 hours. The half-maximal inhibitory concentration values (24 hours) of As$_4$S$_4$ for AGS, MGC803, and GES-1 cells were 2.69, 3.26, and 6.27 µM, respectively (Figure 1A), indicating that the wild-type p53-containing AGS cells were more sensitive to As$_4$S$_4$ than the mutant p53-containing MGC803 cells. Furthermore, these gastric cancer cells were more sensitive to As$_4$S$_4$ than the human gastric epithelium cells. Then, gastric cancer cells were treated with different concentrations of As$_4$S$_4$ (0.00, 0.31, 0.62, 1.25, 2.50, 5.00, 10.00 µM) for 24, 48, or 72 hours, respectively. Results showed that As$_4$S$_4$ inhibited the proliferation of gastric cancer cells in a time- and dose-dependent manner between 0.31 µM and 10.00 µM concentrations (Figure 1B and C).

**Apoptosis of gastric cancer cells treated with As$_4$S$_4$**

To assess the morphological change induced by As$_4$S$_4$, the gastric cancer cells were stained with DAPI and observed under a fluorescence microscope. After being treated with As$_4$S$_4$ (1.25 µM) for 24 hours, AGS and MGC803 cells revealed nuclear chromatin condensation to the edge, which is similar to the morphological changes observed in cells undergoing apoptosis, while the control cells exhibited intact and normal nuclei in morphology. Furthermore, AGS and MGC803 cells treated for 48 hours appeared to be in advanced phases of degradation, with disassembled and fragmented nuclei (Figure 2A).

**Quantification of apoptosis rate using annexin V–FITC/PI staining**

The apoptotic cells were assessed by flow cytometry using annexin V–FITC/PI staining. After treatment of AGS cells for 24 hours, 1.25 µM As$_4$S$_4$ resulted in approximately 6.73% of the cells had reached the early apoptotic phase (annexin V-positive, PI-negative staining) ($P<0.01$). With an increased concentration of As$_4$S$_4$, ie, 2.50 µM, 23.10% of the cells entered the early apoptotic phase ($P<0.001$) (Figure 2B). For MGC803 cells, the exposure to 2.50 µM As$_4$S$_4$ for 24 hours resulted in 11.48% of the cells entering the early apoptotic phase ($P<0.001$). As$_4$S$_4$ induced a greater apoptosis ratio in AGS cells than that induced in MGC803 cells (Figure 2B). Data represented the mean ± SD of three independent experiments.

**Figure 1** Curves of cell viability in gastric cancer cells treated with As$_4$S$_4$.

**Notes:** (A) The cytotoxicity of As$_4$S$_4$ against different cell lines. The comparisons of cytotoxicity of As$_4$S$_4$ against AGS, MGC803, and GES-1 cells when treated for 24 hours. The half-maximal inhibitory concentration values of As$_4$S$_4$ against AGS, MGC803, and GES-1 cells were 2.69, 3.26, and 6.27 µM, respectively. (B) Dose- and time-dependent curves of cell viability in AGS cells treated with As$_4$S$_4$. (C) Dose- and time-dependent curves of cell viability in MGC803 cells treated with As$_4$S$_4$. Data represent the mean ± standard deviation of three independent experiments.
A

AGS

0 hours 24 hours 48 hours

MGC803

B

AGS cells

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<th>As4S4 (µM)</th>
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<td>Apoptosis ratio (%)</td>
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MGC803 cells

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<tr>
<td>Apoptosis ratio (%)</td>
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<td>4.17%</td>
<td>11.48%</td>
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Figure 2 Arsenic sulfide-induced apoptosis of gastric cancer cells.

Notes: (A) DAPI staining of AGS and MGC803 cells treated with 1.25 µM As4S4 for 0, 24, and 48 hours. (B) Apoptosis analysis by annexin V–FITC/PI double staining of (a) AGS and (b) MGC803 cells treated with As4S4 (0 µM, 1.25 µM, 2.5 µM) for 24 hours. Apoptosis ratios represent the percent of cells in the early apoptosis stage (PI-negative/annexin V–FITC-positive). Data represent the mean ± standard deviation of three independent experiments. **P<0.01; ***P<0.001.

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; PI, propidium iodide; Q, quadrant.

Changes in expression of the apoptotic and antiapoptotic proteins induced by As4S4

In the AGS cells, Western blotting analysis showed that As4S4-induced apoptosis and reduction in cell viability was correlated with upregulation of p53 protein levels (Figure 3). However, real-time PCR analysis showed no significant changes in the p53 mRNA levels (Figure 4). This suggests that As4S4 increased the protein stability of p53, rather than affecting the expression of its RNA level. The proapoptotic protein Bax is one of the critical downstream mediators in p53 signaling pathways. Both the mRNA and the protein level of Bax were increased, as shown in Figures 3 and 4, while the protein level of Bcl-2 was significantly reduced when treated with
As$_4$S$_4$ (Figure 3). The expression of MDM2 was upregulated and peaked at 24 hours after the treatment with As$_4$S$_4$. This is consistent with p53 protein degradation accelerating 24 hours after the treatment with As$_4$S$_4$ and its level dropping to below the control level after 48 hours. In the p53 mutant-containing MGC803 cells, the upregulation of Bax protein was still observed, but at a much lower level, while downregulation of Bcl-2 protein was equally apparent compared to the AGS cells. The expression of mutant p53 was elevated at 48 hours, however, increased expression of MDM2 was observed at 6 hours and peaked at 48 hours (Figure 3).

As$_4$S$_4$ induce p53 transcription-dependent apoptosis in AGS cells

Pifithrin-α was used to determine the role of the p53 transcription-dependent pathway in the growth inhibition and apoptosis induction induced by As$_4$S$_4$. Pifithrin-α is a small molecule that binds to the DNA-binding domain of p53, thereby inhibiting its transcriptional activities. AGS and MGC803 cells were pretreated with 30 µM pifithrin-α (a concentration that does not cause significant cytotoxicity in these cells) for 30 minutes prior to addition of As$_4$S$_4$. MTT and Western blotting analysis showed that pretreatment with pifithrin-α suppressed As$_4$S$_4$-induced cytotoxicity within a certain range (~1.25 µM) (Figure 5A) and partially restored the protein levels of p53, MDM2, Bax, and Bcl-2 in the wild-type p53-containing AGS cells (Figure 5B and C), but did not show a protective effect on the mutant p53-containing MGC803 cells. Pifithrin-α alone did not modify p53, Bax, Bcl-2, or MDM2 expressions in the AGS cells compared with untreated control cells. These data indicate that p53 transcription-dependent pathway plays an important role
in As$_4$S$_4$-induced growth inhibition and apoptosis in the AGS cells.

As$_4$S$_4$ inhibited the growth of gastric cancer in vivo

In order to determine the effect of As$_4$S$_4$ in gastric cancer, we conducted experiments in vivo. For the in vivo study, there were no significant differences in the tumor volumes among the four groups before the start of the drug treatment ($P>0.05$). After treatment for 3 weeks, we found that the tumor size and weight in the groups treated with As$_4$S$_4$ were significantly reduced compared with the blank control group ($P<0.05$, Figure 6A–C). Interestingly, we found that As$_4$S$_4$ at a low dose (1 mg/kg) was more effective than at higher doses in tumor suppression (Figure 6C). TGI in the positive control group (CTX, 25 mg/kg), low-dose group (As$_4$S$_4$, 1 mg/kg), and high-dose group (As$_4$S$_4$, 2 mg/kg) was 52.81%, 38.01%, and 26.79%, respectively. The test for heterogeneity of tumor weights in each group was not significant ($P>0.05$). While conducting the experiment, we found that the toxic effects of As$_4$S$_4$ in mice were not apparent, as the differences in body weights between each group were not significant (Figure 6D). No unexpected death of mice was observed throughout the experiment.

As$_4$S$_4$ changed the histological structure of tumor in xenograft mice

The histological changes of the tumors were observed under a light microscope after hematoxylin and eosin staining. The tumor cells in the control group were densely packed, while the tumor cells were much less dense in the As$_4$S$_4$-treated group. This significant reduction of the number of tumor cells was accompanied by nuclear condensation and reduction of the nuclear-cytoplasmic ratio. In the As$_4$S$_4$ treatment groups, we observed significantly increased necrotic cells as well as increased inflammatory cells surrounding the tumors (Figure 7A).
As$_4$S$_4$ induces apoptosis in xenograft mice

TUNEL assay has been designed to detect the double-stranded DNA fragmentation of apoptotic cells undergoing extensive DNA degradation during the late stages of apoptosis. As shown in Figure 7B and C, the percentage of TUNEL-positive cells (stained in yellow) in the group treated with As$_4$S$_4$ was higher than in the blank control group ($P<0.05$), which suggests that As$_4$S$_4$ induces apoptosis of gastric cancer cells and inhibits proliferation.
As$_4$S$_4$ regulates the expression of p53 in xenograft mice

We examined the effect of As$_4$S$_4$ on the expression of p53 in vivo using a xenograft model. As shown in Figure 7D and E, the expression of p53 was significantly increased after treatment with As$_4$S$_4$. By immunohistochemical staining of xenograft tumor tissues, we found that the percentage of p53-positive tumor cells in the As$_4$S$_4$-treated mice (26.22%±3.14%) was much higher than in the control group (1.43%±1.37%) (P<0.001) (Figure 7F and G). This result is consistent with that of the Western blotting.

Discussion

As$_4$S$_4$, an arsenic compound, has shown antitumor activities in malignancies, especially in APL, with limited toxicities. The molecular mechanism of its antitumor activity is related to its ability to induce apoptosis and the redistribution of PML-RARα protein in the leukemic cells.

Much less is known about As$_4$S$_4$ in gastric cancer. In our study, As$_4$S$_4$ inhibited the proliferation of both AGS and MGC803 cells while showing minor toxicity to normal human gastric mucosa tissues. As$_4$S$_4$ reduced the viability of the gastric cancer cells in a time- and dose-dependent manner, and the wild-type p53-containing AGS cells was more sensitive to As$_4$S$_4$ than the mutant p53-containing MGC803 cells. These data suggest that p53 is a critical protein in As$_4$S$_4$-induced cytotoxicity in gastric cancer cells. Therefore, we investigated further the relationship between the antitumor activities of As$_4$S$_4$ in gastric cancer cells and the changes of p53 expression.

As a tumor suppressor gene, p53 functions as a transcription factor and stimulates the expression of many apoptotic effectors, such as PUMA, NOXA, BID, Bax, and...
Figure 7 Effect of As$_4$S$_4$ on the structural changes, cell apoptosis, and expression of p53 protein in xenograft mice.

Notes: (A) Detection of structural changes by HE staining under a light microscope at a magnification of 400x. (B) Detection of apoptotic cells by TUNEL under a light microscope at a magnification of 200x. (C) The percentage of TUNEL-positive cells. (D) Total protein from the tumor was extracted, and the expression of p53 protein was detected by Western blotting. (E) Integrated density of p53 protein, shown relative to the control. (F) The expression of p53 in tumor tissues was detected by immunohistochemical assay under a light microscope at a magnification of 200x. (G) Quantification of immunohistochemical assay is represented as percentage of positively stained cells. Data shown are mean ± standard deviation. *P < 0.05; **P < 0.001.

Abbreviations: CTX, cyclophosphamide; HE, hematoxylin and eosin; NS, normal saline; TUNEL, TdT-mediated dUTP nick end labeling.
p53 can also transcriptionally repress the expression of antiapoptotic proteins, such as Bcl-2, Bcl-XL, and survivin. In our study, apoptosis induction was confirmed by DAPI and annexin V–FITC/PI staining. Gastric cancer cells treated with As$_{2}$S$_{4}$ exhibited condensed and fragmented nuclei, which is a hallmark of apoptosis. Furthermore, quantitative analysis of the apoptosis ratio indicated that the percentage of cells in early apoptosis stage was increased after treatment with As$_{2}$S$_{4}$. These findings indicate that cytotoxicity induced by As$_{2}$S$_{4}$ involves the induction of apoptosis.

As$_{2}$S$_{4}$ treatment remarkably increased the p53 expression levels in a time-dependent pattern in the AGS cells. As expected, the increased p53 protein level was correlated with the upregulation of its downstream target gene Bax and the downregulation of Bel-2. Such an effect likely forms the basis for the induction of apoptosis of AGS cells. These findings are consistent with the previous results reported by Guo et al wherein As$_{2}$S$_{4}$ induced the apoptosis of SGC7901 cells by regulating the expression of Bcl-2 and Bax. Importantly, we found that the p53-specific transcriptional inhibitor pifithrin-α could partially reverse the effect of As$_{2}$S$_{4}$ on the AGS cell proliferation. Moreover, blocking the activation of p53 by pifithrin-α could partially restore the protein levels of p53, Bax, and Bel-2. Interestingly, in the annexin V–FITC/PI staining, we found that exposure to concentrations up to 2.50 μM resulted in more early apoptotic cells, while pifithrin-α only showed a protective effect within a certain dose range of As$_{2}$S$_{4}$ (≤1.25 μM). This low-dose protective effect may indicate that other apoptotic pathways also play a role in the apoptosis induced by the higher concentration of As$_{2}$S$_{4}$ (≥2.5 μM) in AGS cells. Additionally, the protective effect that occurred at this low-dose level of As$_{2}$S$_{4}$ may suggest a competitive binding mechanism, by which other factors that competitively bind to p53 may occupy the pifithrin-α binding sites and prevent the effects of pifithrin-α.

It has been reported that wild-type p53 protein is kept at a low level in cells by the proteasome degradation pathway under unstressed conditions, while mutant p53 protein usually accumulates to a high level in malignant cells. However, we found that the level of the mutant p53 in MGC803 cells was low in our study, and that treatment with As$_{2}$S$_{4}$ did not readily influence the expression of the mutant p53 in MGC803 cells, as there was no significant change in the levels of p53 protein until 48 hours. The mechanism of this delayed upregulation of p53 protein is unclear, and needs to be further explored. In our study, As$_{2}$S$_{4}$ was found to trigger apoptosis through the upregulation of Bax/Bcl-2 protein ratio, independent of p53 expression in the MGC803 cells. Furthermore, pifithrin-α did not show a protective effect in the MGC803 cells. These results indicate that short-duration treatment with As$_{2}$S$_{4}$ stimulated apoptosis in MGC803 cells, likely by increasing the Bax/Bcl-2 ratio and bypassing p53.

Real-time PCR analysis showed that AGS cells treated with As$_{2}$S$_{4}$ showed no significant changes in the p53 mRNA levels when compared to the untreated control group. Thus, how As$_{2}$S$_{4}$ upregulated p53 protein is still not completely clear. We postulate that As$_{2}$S$_{4}$ upregulates p53 protein through downregulating the expression of the MDM2 protein levels. MDM2, an E3 ubiquitin ligase, is a critical feedback regulator of p53. The transcription of the MDM2 is induced by the p53 protein activation after DNA damage, and then the MDM2 protein binds to p53 and blocks its activities and promotes its degradation. High levels of MDM2 protein promote polyubiquitination and subsequent proteasome-dependent degradation of p53.

In the present study, p53 and MDM2 protein levels were upregulated as early as 6 hours after treatment with 1.25 μM As$_{2}$S$_{4}$ in AGS cells. With the upregulation of MDM2 protein levels, p53 degradation started to accelerate at 24 hours (Figure 3). These findings concealed the feedback loop between p53 and MDM2 proteins. The transcription of the MDM2 was induced by the p53 protein activation after treatment with As$_{2}$S$_{4}$, and then the MDM2 protein promoted the degradation of p53 protein. But how As$_{2}$S$_{4}$ upregulated the p53 protein remains unclear. Due to the critical inhibitory effect of MDM2 on p53, targeting the interaction of MDM2 with p53 is a potential cancer therapeutic strategy. Therefore, combination treatment with As$_{2}$S$_{4}$ and small molecular-based approaches which target the p53–MDM2 axis may enhance the cytotoxicity of As$_{2}$S$_{4}$ in AGS cells.

In this study, we also assessed the effects of As$_{2}$S$_{4}$ in inducing apoptosis in gastric cancer in the xenograft nude mice. In the selection of tumor cells, we chose MGC803 cells rather than AGS cells because we found that AGS cells did not form tumor structures under the subcutaneous tumor model we established. By calculating the TGI and using immunohistochemical staining for TUNEL, we found that As$_{2}$S$_{4}$ induced apoptosis and inhibited proliferation in tumor tissues. In addition, the results of Western blotting and immunohistochemistry in vivo showed that As$_{2}$S$_{4}$ upregulated the expression of p53 in xenograft mice, which further confirms the inference that As$_{2}$S$_{4}$ induces apoptosis in a p53-dependent manner. Interestingly, there were a higher TGI and more
apoptotic cells in the low-dose group (As$_4$S$_3$, 1 mg/kg) than in the high-dose group (As$_4$S$_4$, 2 mg/kg), which suggests that the capabilities of As$_4$S$_3$ to induce apoptosis and inhibit proliferation were associated with the drug dose, however, the exact relationship still needs to be further elucidated.

**Conclusion**

The present study indicates that As$_4$S$_3$ can effectively inhibit gastric cancer cell proliferation by inducing apoptosis both in vitro and in vivo through a p53-dependent pathway. Our understanding of the mechanism by which As$_4$S$_3$ activates p53 remains incomplete and needs to be further explored. Our data demonstrate that As$_4$S$_3$ is a potent cytotoxic agent for gastric cancer cells and may have therapeutic potential.

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**Disclosure**

The authors report no conflicts of interest in this work.

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