

Original Article

Anticancer efficacies of arsenic disulfide through apoptosis induction, cell cycle arrest, and pro-survival signal inhibition in human breast cancer cells

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Abstract: Arsenic disulfide, a major effective component of realgar, has been investigated for its anti-cancer potential and shown to have therapeutic efficacies in hematological and some solid tumors. However, its effect against breast cancer is rarely reported. In this study, we investigated the anti-cancer effects of As₂S₂ in human breast cancer cell lines MCF-7 and MDA-MB-231, and further elucidated its underlying mechanisms. As₂S₂ significantly inhibited cell viabilities, induced apoptosis, and led to cell cycle arrest in both cell lines with a dose- and time-dependent manner. As₂S₂ upregulated pro-apoptotic proteins like p53 and PARP in MCF-7 cells. Besides, As₂S₂ downregulated anti-apoptotic proteins like Bcl-2 and Mcl-1, as well as cell cycle-related proteins cyclin A2 and cyclin D1 in both cell lines. Of note, the expression level of cyclin B1 was downregulated in MCF-7 cells, whereas, upregulated in MDA-MB-231 cells. Moreover, As₂S₂ significantly inhibited the pro-survival signals in PI3K/Akt pathway in both cell lines. In conclusion, As₂S₂ inhibited cell viabilities, induced apoptosis and cell cycle arrest in both MCF-7 and MDA-MB-231 cell lines by regulating the expression of key proteins involved in related pathways. These results provide fundamental insights into the clinical application of As₂S₂ for treatment of patients with breast cancer.

Keywords: Arsenic disulfide, MCF-7, MDA-MB-231, cell viability, apoptosis, cell cycle

Introduction

Breast cancer is the most common malignancy among women, with an estimated 400,000 cancer death annually throughout the world [1, 2]. Current conventional therapies, including chemotherapy, surgery and radiation treatment, are insufficient to overcome drug side effects and drug resistance [3, 4]. Herein, the development of new therapeutic agents with effectiveness and mild side effects is critically needed for the treatment of breast cancer.

Arsenic compounds, the component of traditional Chinese medicine, have been used widely and successfully for the treatment of various diseases in China for thousands of years and also in the western world [5-7]. In particular, arsenic trioxide, one of the trivalent arsenics, has been approved as a standard agent for the treatment of relapsed and refracto-

ry acute promyelocytic leukemia (APL) [8, 9] for its significant reputation of therapeutic effects. However, adverse side effects including liver dysfunction, acute toxicity, and potential carcinogenicity limit its wide clinical applications [10, 11]. Compared with arsenic trioxide, arsenic disulfide (As₂S₂), as a less toxic arsenic compound, possesses the advantages of oral administration safety and abundant resources [12]. It has been demonstrated that As₂S₂ has a similar anti-tumor potential but with less adverse effects than other arsenic compounds [13]. Emerging evidence has revealed the anti-tumor effects of As₂S₂ in various malignancies, which included hematopoietic tumors and solid malignancies [14-18]. Evidence from animal experiments further confirmed the safety and efficacy of As₂S₂ administrated orally in normal mice or xenograft mice cancer model [19-22]. More importantly, many clinical studies demonstrated the safety and effectiveness of

the clinical application of As_2S_2 especially in respect to the oral administration of As_2S_2 [23-25] to patients with APL. Nevertheless, a limited number of studies have reported the potential antitumor activity of As_2S_2 in human breast cancer [26]. In addition, there is even more rare study investigating the underlying mechanisms of the effects of As_2S_2 against breast carcinoma.

Apoptosis induction and cell cycle arrest are the two important causes to inhibit cancer cell growth and proliferation [27]. Apoptosis is a genetically programmed cell death, which has been taken as a major mechanism of chemotherapy-induced cell death [28, 29]. There are two main established pathways that lead to apoptosis: the extrinsic (cell death receptor) pathway and the intrinsic (mitochondrial) pathway [29, 30]. The intrinsic pathway is mainly involved in the apoptosis induced by small molecule anti-cancer drugs in cancer cells [31]. Recent studies have shown that As_2S_2 could induce apoptosis in hematopoietic and some solid tumor cell lines [32-34]. However, the proapoptotic effect of As_2S_2 in breast cancer cells has been rarely reported. Cell cycle is a process characterized by a series of organized events, which involves a set of sequential phases (G_1 , S, G_2 and M phases) to promote cell proliferation. Previous studies suggest that the development of cancer can be considered as a dysregulation of cell cycle [35], with the dysfunctions implicated in cell cycle checkpoints in most human tumors [36]. Previous studies reported that As_2S_2 can inhibit cell proliferation by inducing certain cell-cycle phase arrest in some human solid tumor cell lines, such as osteosarcoma [18], hepatocellular carcinoma HepG2 [17], and melanoma [37] cells. Thus, we assumed that As_2S_2 might have effects in regulating cell cycle phases in breast cancer cells.

The present study aimed to investigate the effects of As_2S_2 on different breast cancer cell lines by focusing on apoptosis induction and cell cycle arrest, with various drug concentrations and different incubation time courses. Our results demonstrated that As_2S_2 exerted an inhibitory effect on cell viabilities of breast cancer cells, in both dose- and time-dependent manners, along with apoptosis induction and cell cycle arrest. Moreover, the results indicated the remarkable effect of As_2S_2 in regulating

cell survival-, apoptosis-, and cell cycle-related proteins.

Materials and methods

Reagents

Cell counting kit-8 (CCK-8) was purchased from DOJINDO Laboratories (Tokyo, Japan). Calcein-AM was purchased from Molecular Probes (Eugene, Oregon, USA). FITC Annexin V Apoptosis Detection Kit was obtained from BD Biosciences (San Diego, CA, USA). Arsenic disulfide, PI and RNase A solution were purchased from Sigma (St. Louis, MO, USA). ECLTM Western Blotting Analysis System and ECLTM Prime Western Blotting Detection Reagent were purchased from GE Healthcare (Buckinghamshire, UK). Pro-Survival B-cell lymphoma 2 (Bcl-2) Family Antibody Sampler Kit, rabbit anti-human p53, rabbit-anti human poly (ADP-ribose) polymerase (PARP), rabbit anti-human phosphatidylinositol 3-kinase (PI3K), rabbit anti-human Akt, mouse anti-human cyclin A2, mouse anti-human cyclin B1, and rabbit anti-human cyclin D1 were obtained from Cell Signaling (Danvers, MA, USA).

Cell line and cell culture

The human breast cancer cell lines MCF-7 and MDA-MB-231 were purchased from the American Type Culture Collection (Manassas, VA, USA). MCF-7 cells and MDA-MB-231 cells were cultured in MEM-alpha medium (Gibco, Grand Island, NY, USA) supplemented with penicillin, streptomycin, fetal bovine serum (FBS) (10% FBS for MCF-7 and 15% FBS for MDA-MB-231, respectively) (Sigma, St. Louis, MO, USA), and maintained as attached cells at 37°C in 5% carbon dioxide in a humidified atmosphere.

Cell culture assays and drug treatment

Both MCF-7 and MDA-MB-231 cells were seeded at a density of 10,000 cells per well in 500 µl of cell culture media into 48-well plates (IWAKI micro-plates), followed by overnight incubation. Reagents, including vehicle as controls (cell culture media) and As_2S_2 stock solutions (0.6 mM), were subsequently added into the corresponding wells to adjust the final drug concentrations of As_2S_2 to be 0-24 µM. Both MCF-7 and MDA-MB-231 cells were allow-

ed to grow for 24, 48 and 72 h in the presence of vehicle or As_2S_2 , respectively, followed by cytotoxicity assay.

Cytotoxicity assay

Cell cytotoxicity was analyzed by CCK-8 assay. For both cell lines, 1×10^4 cells per well were seeded into 48-well plates (IWAKI micro-plates), and As_2S_2 was subsequently added into the corresponding wells to adjust the final drug concentrations of 0-24 μM , respectively. Then, the plates were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 24, 48 and 72 h, respectively. After incubation, 25 μl CCK-8 reagent was added into each well, followed by additional incubation for 3 h at 37°C . The OD value of each well was measured by a micro-plate reader (Corona MT P-32; Corona Co., Hitachi, Ibaraki, Japan) at 570 nm. The cell viability rate was calculated according to the following formula:

$$\text{Cell viability rate} = (\text{OD sample value} - \text{OD blank value}) / (\text{OD control value} - \text{OD blank value}) \times 100\%.$$

Microscopy images

MCF-7 and MDA-MB-231 cells were seeded in a 96-well plate at the density of 5×10^3 cells per well in 100 μl culture medium, followed by the exposure to different concentrations of As_2S_2 (0, 4, 8, 12, and 16 μM), for 24, 48 and 72 h, respectively. Then the cells were stained for 15 min in the dark at 37°C with the specific live probe Calcein-AM, followed by images measured and analyzed by a fluorescence micro-plate reader and Harmony software (Operetta CLS, PerkinElmer, Japan).

Assessment of apoptosis

MCF-7 and MDA-MB-231 cells with the density of 4×10^5 cells per well were seeded in 6-well plates (IWAKI micro-plates) respectively, and treated with serial concentrations of As_2S_2 (0, 4, 8, 12 and 16 μM), and followed by an additional incubation for 24, 48 and 72 h, respectively. The apoptotic rates of both cell lines were detected by using FITC Annexin V Apoptosis Detection Kit. The staining procedure was performed according to the manufacturer's instructions. Approximate 1×10^4 cells were analyzed using a flow cytometer (BD Bio-

sciences, CA, US). The cells were subsequently assessed for total apoptotic cells composed by early apoptotic (Annexin V⁺/PI⁻) and late apoptotic (Annexin V⁺/PI⁺) cells.

Cell cycle analysis

Both MCF-7 and MDA-MB-231 cells were seeded at a density of 4×10^5 cells per well in 6-well plates (IWAKI micro-plates), followed by overnight incubation. Cells were treated with 0, 4, 8, 12 and 16 μM As_2S_2 for 24, 48 and 72 h, respectively. Then, the cells were harvested and washed with PBS, subsequently, fixed in 70% ethanol overnight at -20°C , and stained with PI and RNase A solution (5 $\mu\text{g}/\text{ml}$ PI, and 0.5 $\mu\text{g}/\mu\text{l}$ RNase A). The DNA content was determined by flow cytometry (BD Biosciences, CA, US), and data were analyzed by CellQuest analysis software. ModFit LTTM Ver.3.0 (Verity Software House, Topsham, ME, USA) was used to calculate the number of cells at each G_0/G_1 , S and G_2/M phase fraction.

Western blot analysis

Western blot was performed in order to evaluate the protein levels of Bcl-2, Mcl-1, p53, PARP, PI3K, Akt, cyclin A2, cyclin B1, and cyclin D1 in both MCF-7 and MDA-MB-231 cells. The total protein was extracted from both cell lines treated by As_2S_2 with various concentrations (0, 4, 8 and 16 μM) in different time courses (24, 48 and 72 h) respectively. Briefly, cell lysates were separated by SDS-PAGE and transferred into a polyvinylidene difluoride transfer membrane (Immobilon-P, Darmstadt, Germany). Membranes were blocked with 5% milk for 1 h. The membranes were washed by Tris buffered saline with Tween (TBST) and then incubated overnight at 4°C with anti-rabbit p53 specific antibody (1:1000, Cell Signaling, #9282), anti-rabbit PARP specific antibody (1:1000, Cell Signaling, #9542), anti-rabbit Bcl-2 specific antibody (1:1000, Cell Signaling, #4223), anti-rabbit Mcl-1 specific antibody (1:1000, Cell Signaling, #5453), anti-mouse cyclin A2 specific antibody (1:1000, Cell Signaling, #4656), anti-mouse cyclin B1 specific antibody (1:1000, Cell Signaling, #4135), anti-rabbit cyclin D1 specific antibody (1:1000, Cell Signaling, #2922), anti-rabbit PI3K p85 specific antibody (1:1000, Cell Signaling, #4228), and anti-rabbit Akt specific antibody (1:1000, Cell Signaling, #4691), respectively. Membr-

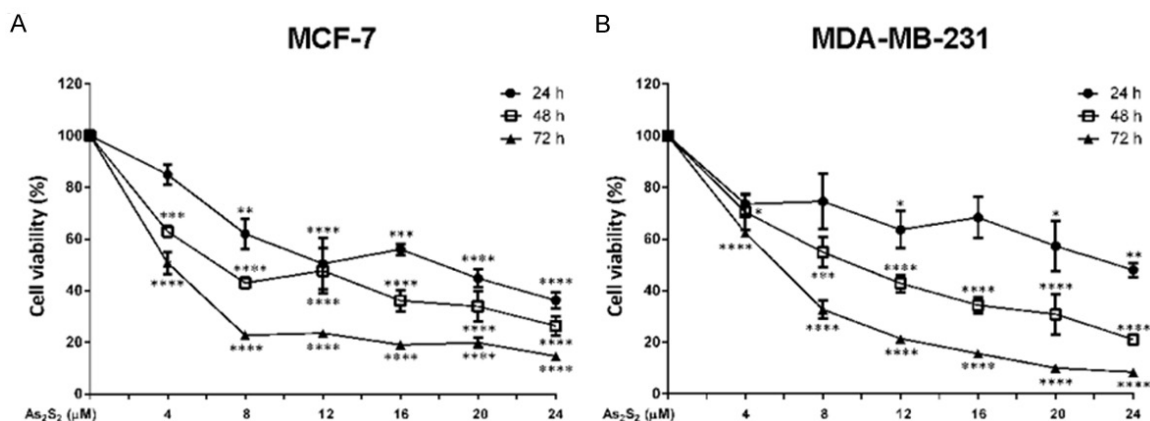


Figure 1. Effects of As₂S₂ on the viability of breast cancer cells. (A) MCF-7 and (B) MDA-MB-231 cells were treated with various concentrations of As₂S₂ (0, 4, 8, 12, 16, 20 and 24 μM) for 24 h (●), 48 h (□) and 72 h (▲), respectively, and the cell viability was assessed by CCK-8 assay procedures. All of the data were expressed as the mean ± SEM (n ≥ 3). Asterisks indicate significant differences between the control and the drug treatment groups (*P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001).

Table 1. IC₅₀ values of As₂S₂ in human breast cancer cell lines exposed for different time

	IC ₅₀ (μM as As ₂ S ₂)		
	24 h	48 h	72 h
MCF-7	15.27±0.49	8.13±1.11 ^{##}	3.69±0.47 ^{##,&}
MDA-MB-231	25.5±4.00	9.18±1.78 ^{##}	5.37±0.12 ^{##,\$}

Data are the means ± SEM from at least three independent experiments. ^{##}P < 0.01 vs. 24 h. [&]P < 0.05 vs. 48 h. ^{\$}P < 0.05 vs. MCF-7 with the same exposure time.

anes were also probed with anti-beta-actin (abcam, ab49900) at 1:3000 dilutions as the internal control. The membranes were incubated with respective primary antibodies listed above at 4°C overnight. Then, the membranes were incubated with anti-mouse or anti-rabbit specific polyclonal secondary antibodies for 1 h at room temperature, and followed by three-time washes with TBST. Signals were detected using ECL Western Blot detection kit in a luminescent image analyzer (Fujifilm, LAS-3000, Tokyo, Japan).

Statistical analysis

Data processing was carried out using software GraphPad Prism version 6.0 and results were presented as means ± SEM of at least three independent experiments. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons, and the Student's t-test for comparison of two groups. P

< 0.05 was considered to be statistically significant.

Results

As₂S₂ inhibits cell proliferation of breast cancer cells

To investigate the cytotoxicity of As₂S₂ against breast cancer cells, MCF-7 and MDA-MB-231 cells were exposed to serial concentrations of As₂S₂ from 0 to 24 μM for 24, 48 and 72 h, and the cell viability was evaluated by CCK-8 assay. As shown in **Figure 1**, As₂S₂ inhibited the cell proliferation of breast cancer cell lines MCF-7 and MDA-MB-231 both in dose- and time-dependent manner.

In MCF-7 cells, as shown in **Figure 1A**, a significant decrease in cell viability was observed in a dose- and time-dependent manner after treatment with different concentrations of As₂S₂. In detail, compared to the control group (0 μM As₂S₂), the cell viability significantly reduced to 84.95±3.81 (P = 0.3837), 62.93±2.17 (P = 0.0009) and 50.80±4.22% (P < 0.0001) after exposure to 4 μM As₂S₂ for 24, 48 and 72 h, respectively. Exposure of cells to 24 μM As₂S₂ for 24, 48 and 72 h significantly reduced the cell viability to 36.31±3.26 (P = 0.0001), 26.38±3.78 (P < 0.0001), and 14.68±1.27% (P < 0.0001), respectively.

In MDA-MB-231 cells, as shown in **Figure 1B**, a significant decrease in cell viability was also

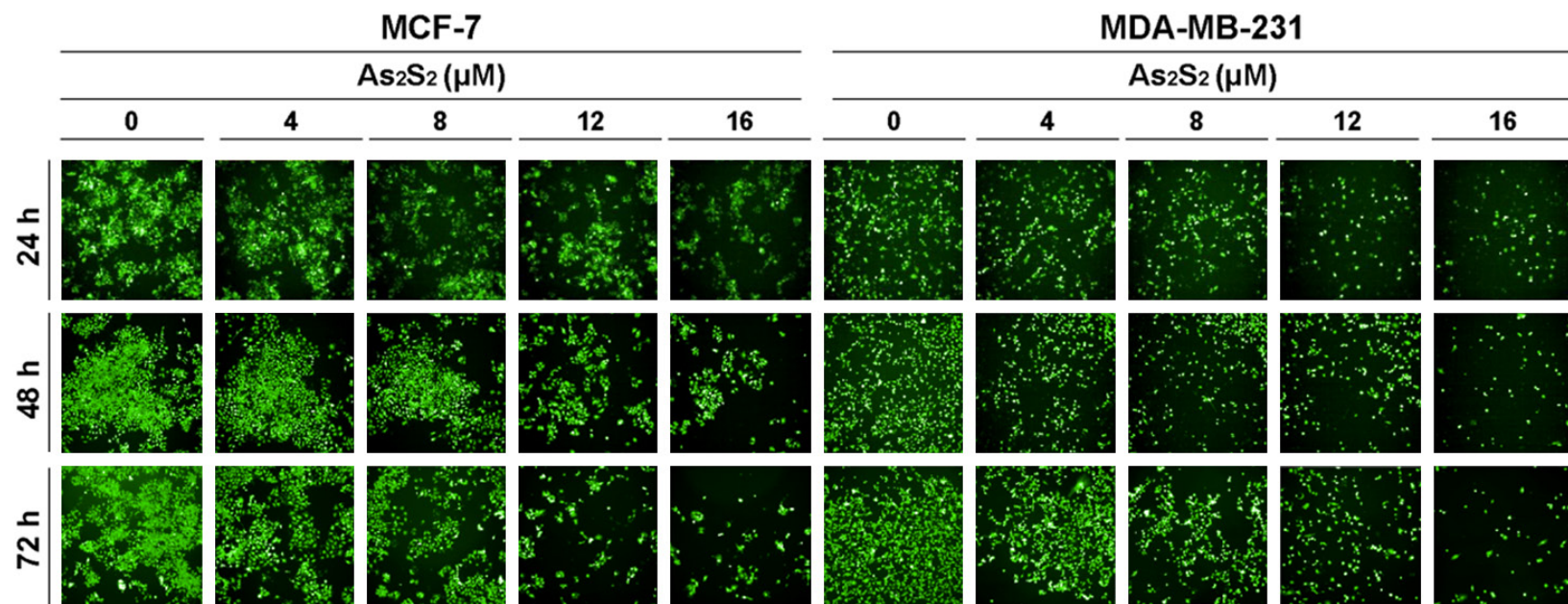


Figure 2. Assessment of cell viability by calcein-AM staining. MCF-7 and MDA-MB-231 cells were seeded at the density of 5,000 cells per well. Cells were treated with a serial concentrations of As₂S₂ (0, 4, 8, 12 and 16 μM) for 24, 48 and 72 h, respectively. Viable cells exposed to calcein-AM showed bright green fluorescence. Images were taken and analyzed by a fluorescence Micro-plate reader (Operetta CLS, PerkinElmer, Japan) with 10 × objective (original magnifications 100 ×).

observed in a dose- and time-dependent manner after treatment with different concentrations of As_2S_2 . In detail, compared to the control group (0 μM As_2S_2), cell viability significantly reduced to 73.57 ± 4.17 ($P = 0.1819$), 70.49 ± 6.80 ($P = 0.0102$), and $62.42 \pm 0.30\%$ ($P < 0.0001$) after exposure to 4 μM As_2S_2 for 24, 48 and 72 h, respectively. Exposure to 24 μM As_2S_2 for 24, 48 and 72 h further reduced cell viability to 48.03 ± 2.64 ($P = 0.0019$), 21.15 ± 1.52 ($P < 0.0001$) and $8.49 \pm 0.25\%$ ($P < 0.0001$).

The half-maximal inhibitory concentrations (IC_{50} values) of As_2S_2 on MCF-7 and MDA-MB-231 cells in different time courses were listed in **Table 1**. The mean of IC_{50} values of As_2S_2 in MDA-MB-231 cells were relatively higher than that in MCF-7 cells when treated with As_2S_2 for 24 and 48 h. A significant difference was further observed in the IC_{50} values between two cell lines after exposure to As_2S_2 for 72 h ($P = 0.03$).

The results indicated that As_2S_2 inhibits cell proliferation of breast cancer cells in dose- and time-dependent manners, and the MCF-7 cells were relatively more sensitive to As_2S_2 in comparison with MDA-MB-231 cells.

As_2S_2 affects cell morphology of breast cancer cells

To better understand the cell growth inhibition induced by As_2S_2 in breast cancer cells, the morphological features of MCF-7 and MDA-MB-231 cells were observed following As_2S_2 exposure in different time courses. The fluorescent images of treated and untreated cells were examined after staining the cells with a fluorescent dye Calcein-AM, which restricts to label living cells with green fluorescent [38, 39]. Consistent with cell viability assays (**Figure 1**), a similar dose- and time-dependent decrease in the cell density was observed in both cell lines (**Figure 2**).

As_2S_2 triggers cell cycle arrest in breast cancer cells

To investigate the correlation of cell growth inhibition and cell cycle arrest, MCF-7 and MDA-MB-231 cells were treated with serial concentrations of As_2S_2 (0, 4, 8, 12 and 16 μM) for

24, 48 and 72 h, respectively, and the cell cycle distribution was analyzed by flow cytometry.

In MCF-7 cells, treatment with As_2S_2 mainly induced cell cycle arrest in the G_0/G_1 and G_2/M phases.

As shown in **Figure 3A, 3B**, in MCF-7 cells after 24 h of As_2S_2 treatment, the percentage of cells at G_2/M phase significantly increased with As_2S_2 at concentrations of 12 μM ($P = 0.0021$) and 16 μM ($P = 0.0110$). Whereas, the percentage of MCF-7 cells at S phase significantly decreased after exposure to As_2S_2 at concentrations of 12 μM ($P = 0.0078$) and 16 μM ($P = 0.0125$) for 24 h.

As shown in **Figure 3A, 3C**, in MCF-7 cells after 48 h of As_2S_2 treatment, the percentage of cells at G_0/G_1 phase significantly increased with As_2S_2 at concentrations of 4 μM ($P < 0.0001$) and 8 μM ($P < 0.0001$). The percentage of MCF-7 cells at G_2/M phase significantly increased 48 h after treatment with As_2S_2 at concentrations of 8 μM ($P < 0.0001$), 12 μM ($P < 0.0001$) and 16 μM ($P < 0.0001$), respectively. In contrast, the percentage of MCF-7 cells at S phase significantly decreased after exposure to As_2S_2 for 48 h at concentrations ranging from 4 to 16 μM ($P < 0.0001$ respectively).

As shown in **Figure 3A, 3D**, in MCF-7 cells after 72 h of As_2S_2 treatment, the percentage of cells at G_0/G_1 phase significantly increased with As_2S_2 at concentrations of 4 and 8 μM , but significantly decreased at concentrations of 12 and 16 μM ($P < 0.0001$, respectively). The percentage of MCF-7 cells at G_2/M phase significantly increased with As_2S_2 at concentrations of 8 μM ($P = 0.0008$), 12 μM ($P < 0.0001$) and 16 μM ($P < 0.0001$) for 72 h. In contrast, the percentage of MCF-7 cells at S phase significantly decreased after exposure to As_2S_2 for 72 h at concentrations of 4 μM ($P < 0.0001$), 8 μM ($P < 0.0001$) and 12 μM ($P = 0.0006$) for 72 h, whereas significantly increased at 16 μM ($P = 0.0159$).

In MDA-MB-231 cells, treatment with As_2S_2 mainly induced cell cycle arrest in G_2/M and S phases in a dose- and time-dependent manner.

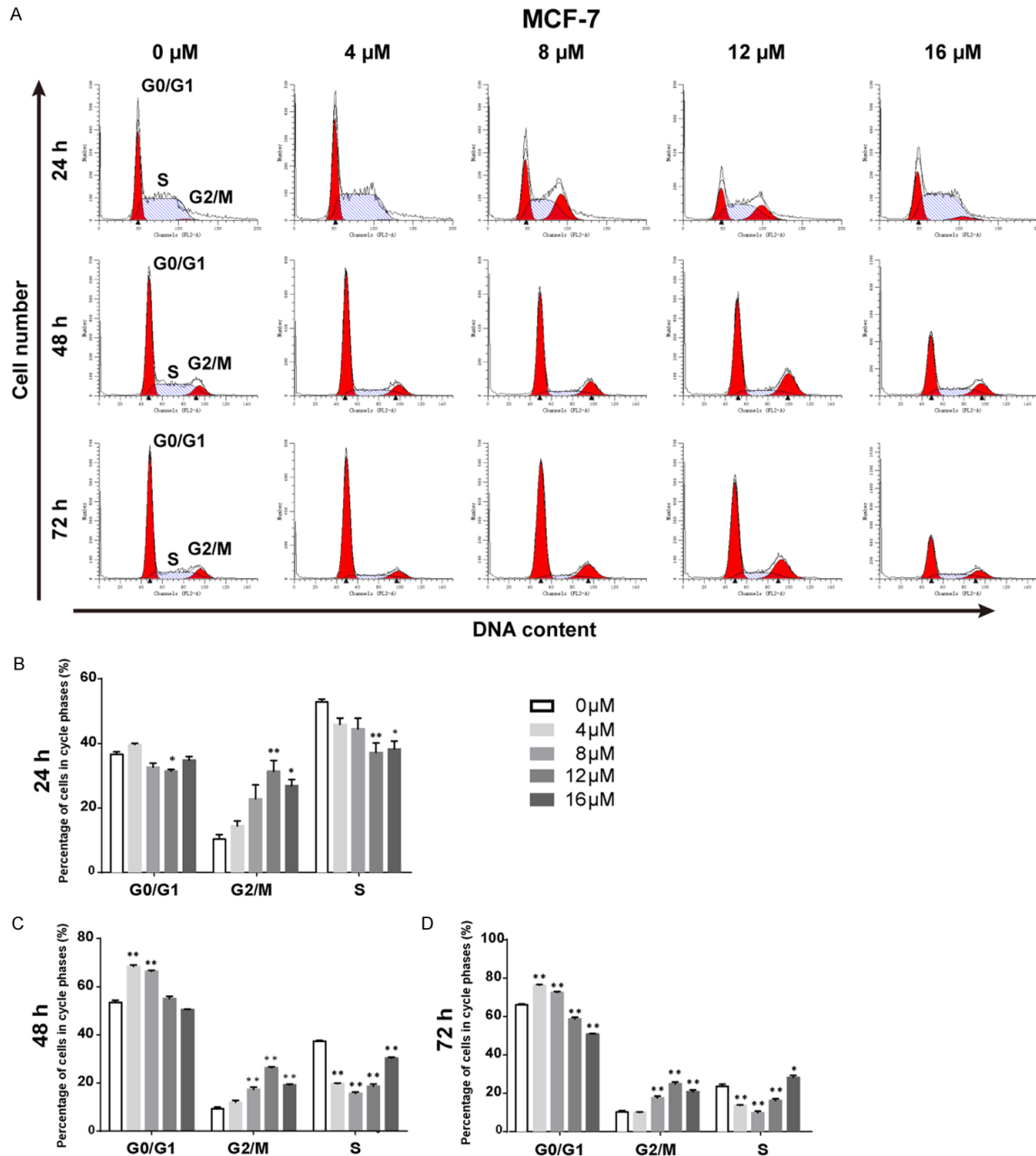


Figure 3. As_2S_2 triggers cell cycle arrest in MCF-7 cells. (A) MCF-7 cells were treated with various concentrations of As_2S_2 (0, 4, 8, 12 and 16 μM) for 24, 48 and 72 h. The peaks in the figure represent the G_0/G_1 , S, and G_2/M phases in the cell cycle, respectively. (B) The percentages of cell numbers in the cell cycle of MCF-7 cells after 24 h, (C) 48 h, and (D) 72 h of drug treatment. All data were expressed as the mean $\pm$ SEM ($n \geq 3$). Asterisks indicate significant differences between the control (0 μM) and the drug treatment groups (* $P < 0.05$, ** $P < 0.01$).

As shown in **Figure 4A, 4B**, in MDA-MB-231 cells after 24 h of As_2S_2 treatment, the percentage of cells at G_0/G_1 phase significantly decreased with As_2S_2 at concentrations of 8, 12, and 16 μM ($P < 0.0001$, respectively). The percentage of MDA-MB-231 cells at G_2/M phase significantly increased with As_2S_2 at con-

centrations ranging from 8 to 16 μM ($P < 0.0001$ respectively).

As shown in **Figure 4A, 4C**, in MDA-MB-231 cells after 48 h of As_2S_2 treatment, the percentage of cells at G_0/G_1 phase significantly decreased with As_2S_2 at concentrations rang-

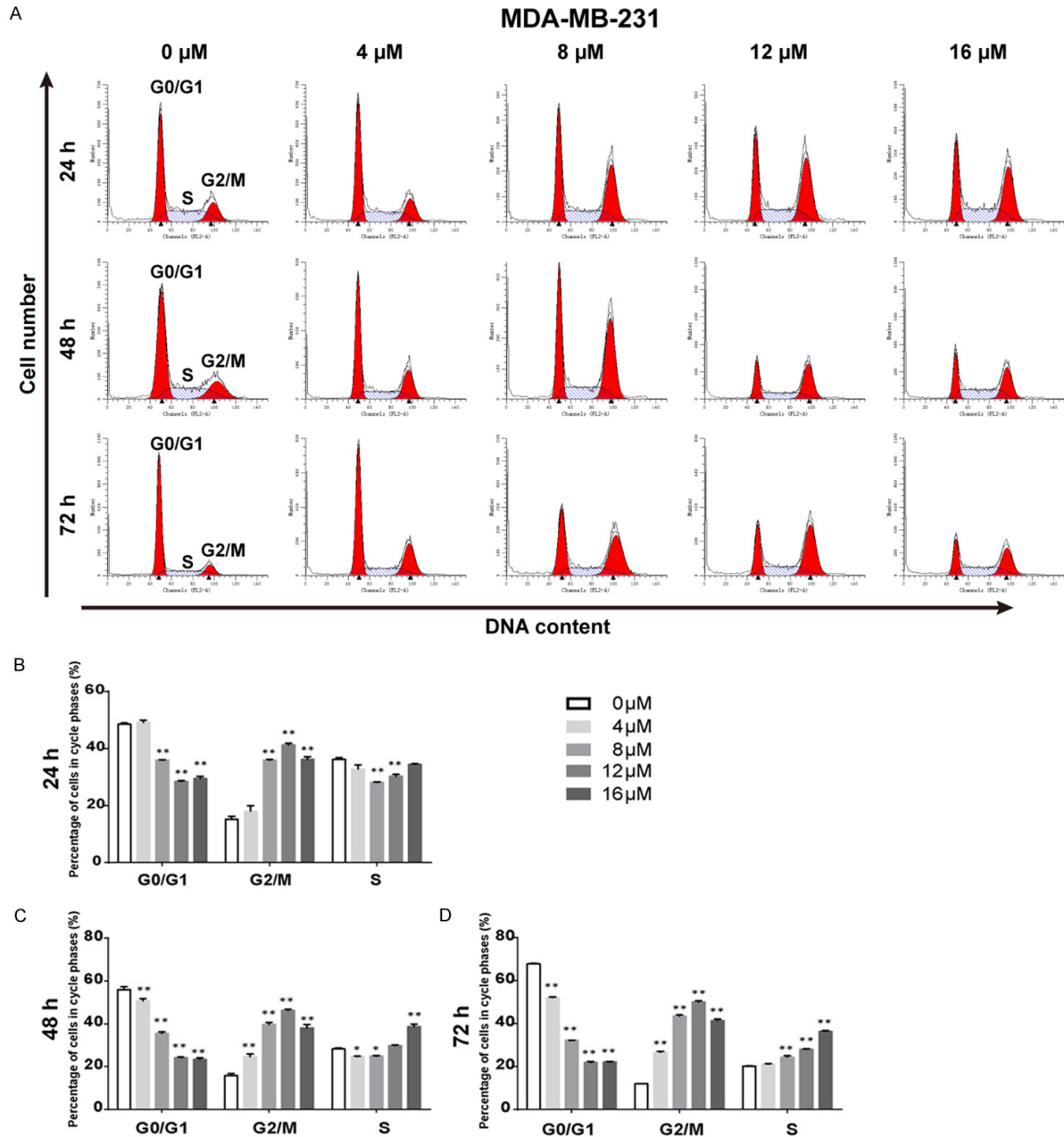


Figure 4. As₂S₂ triggers cell cycle arrest in MDA-MB-231 cells. (A) MDA-MB-231 cells were treated with serial concentrations of As₂S₂ (0, 4, 8, 12 and 16 μ M) for 24, 48 and 72 h. The peaks in the figure represent the G₀/G₁, S, and G₂/M phases in the cell cycle, respectively. (B) The percentages of cell numbers in the cell cycle of MDA-MB-231 cells after 24 h, (C) 48 h, and (D) 72 h of drug treatment. All data were expressed as the mean $\pm$ SEM ($n \geq 3$). Asterisks indicate significant differences between the control (0 μ M) and the drug treatment groups (* $P < 0.05$, ** $P < 0.01$).

ing from 4 to 16 μ M ($P = 0.0093$ at 4 μ M, $P < 0.0001$ at 8, 12, and 16 μ M, respectively). The percentage of cells at G₂/M phase significantly increased with As₂S₂ at concentrations ranging from 4 to 16 μ M ($P = 0.0020$ at 4 μ M, $P < 0.0001$ at 8, 12 and 16 μ M, respectively). The percentage of MDA-MB-231 cells at S phase

significantly decreased after exposure to As₂S₂ for 48 h at concentrations of 4 μ M ($P = 0.0157$) and 8 μ M ($P = 0.0216$), whereas significantly increased at 16 μ M ($P < 0.0001$).

As shown in **Figure 4A, 4D**, in MDA-MB-231 cells after 72 h of As₂S₂ treatment, the per-

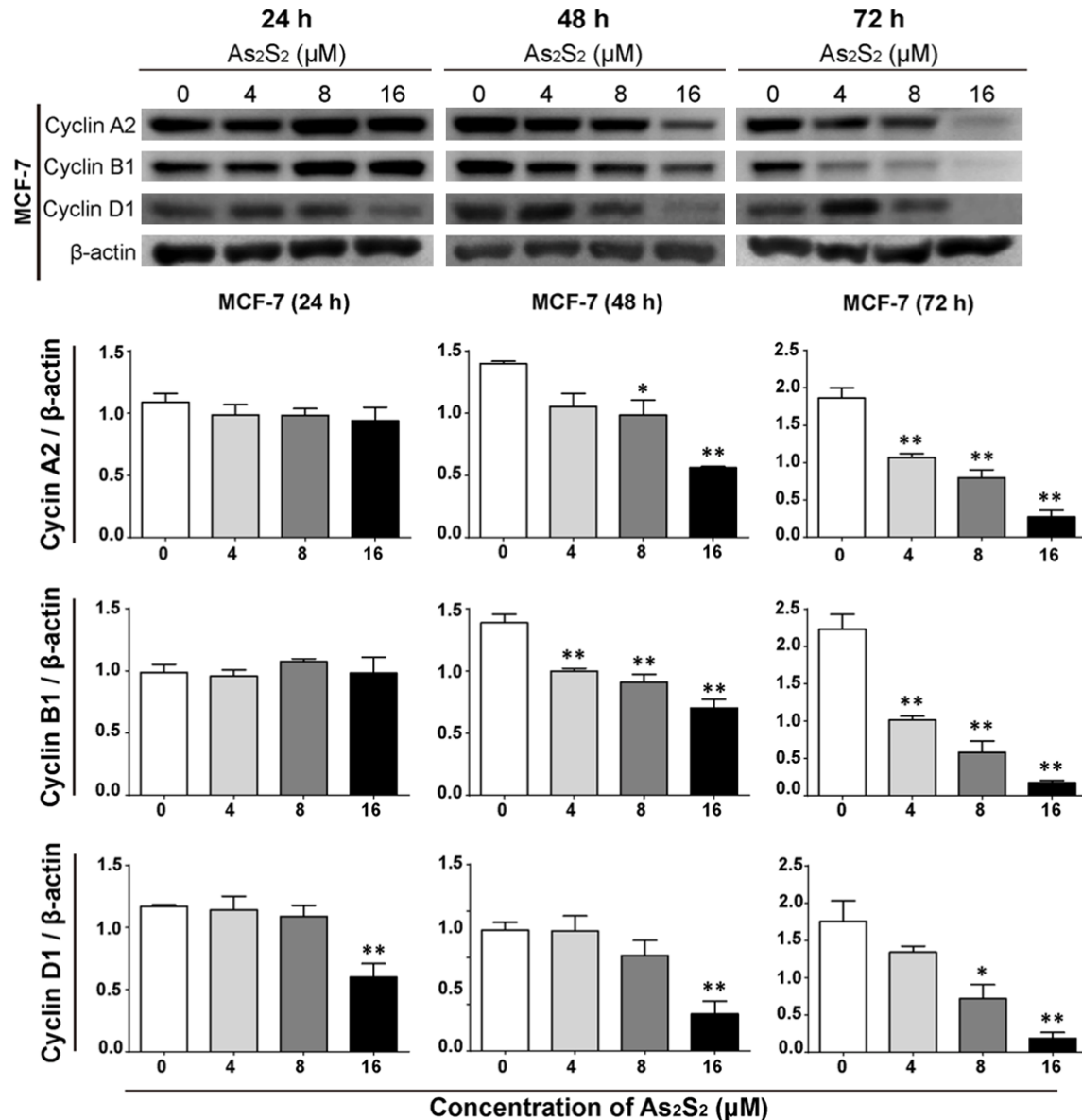


Figure 5. Effects of As₂S₂ on cell cycle regulators in MCF-7 cells. MCF-7 cells were cultured with various concentrations of As₂S₂ (0, 4, 8 and 16 μM) for 24, 48 and 72 h, and Western blot assays were carried out to examine the effects of As₂S₂ on the expressions of key proteins cyclin A2, cyclin B1 and cyclin D1 in MCF-7 cells after 24, 48 and 72 h of drug treatment. Protein β-actin was used as internal control. All images are representative of three independent analyses from three independent cellular preparations. Asterisks indicate significant differences between the control (0 μM) and the drug treatment groups (**P* < 0.05, ***P* < 0.01).

entage of cells at G₀/G₁ phase significantly decreased with As₂S₂ at concentrations ranging from 4 to 16 μM (*P* < 0.0001 respectively). The percentage of MDA-MB-231 cells at G₂/M phase significantly increased with As₂S₂ at concentrations ranging from 4 to 16 μM (*P* < 0.0001 respectively) for 72 h. The percentage of MDA-MB-231 cells at S phase significantly increased with As₂S₂ at concentrations ranging from 8 to 16 μM (*P* = 0.0001 at 8 μM,

P < 0.0001 at 12 and 16 μM, respectively) for 72 h.

As₂S₂ regulates cell cycle-related proteins

Cell cycle progression is controlled by cyclins and cyclin-dependent kinases (CDKs) which become the rational targets for drug treatment to be blocked in order to induce cell death [40, 41]. To elucidate the underlying mechanisms,

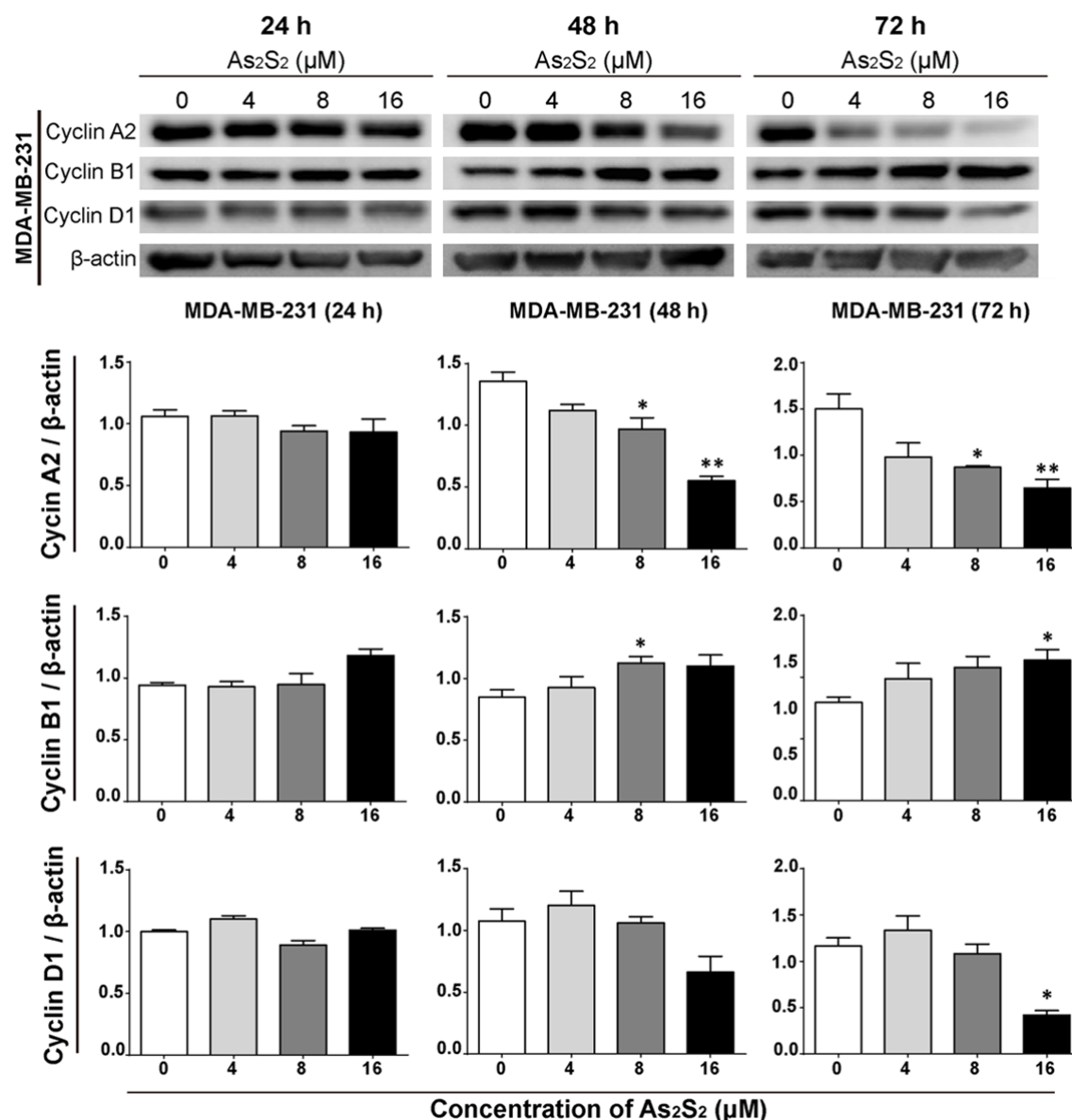


Figure 6. Effects of As_2S_2 on cell cycle regulators in MDA-MB-231 cells. MDA-MB-231 cells were cultured with serial concentrations of As_2S_2 (0, 4, 8 and 16 μM) for 24, 48 and 72 h. Western blot assays were carried out to examine the effects of As_2S_2 on the expressions of key proteins cyclin A2, cyclin B1 and cyclin D1 in MDA-MB-231 cells after 24, 48 and 72 h of drug treatment. Protein β -actin was used as internal control. All images are representative of three independent analyses from three independent cellular preparations. Asterisks indicate significant differences between the control (0 μM) and the drug treatment groups (* $P < 0.05$, ** $P < 0.01$).

we measured the expression of selected cell-cycle related proteins so as to correlate the cell cycle arrests in different phases with actual alterations of these protein levels. As shown in **Figures 5 and 6**, in both MCF-7 and MDA-MB-231 cells, As_2S_2 regulated cell cycle-related proteins, such as cyclin A2, cyclin B1, and cyclin D1 in dose- and time-dependent manners.

Compared to control, the expression levels of cyclin A2 in MCF-7 cells and MDA-MB-231 sig-

nificantly decreased with As_2S_2 at concentrations of 8 μM ($P = 0.0273$ in MCF-7, $P = 0.0137$ in MDA-MB-231) and 16 μM ($P = 0.0003$ in MCF-7, $P = 0.0001$ in MDA-MB-231) after 48 h of As_2S_2 treatment. After exposure to As_2S_2 for 72 h, the statistically significant decrease in the amount of cyclin A2 expression started to occur in MCF-7 cells with As_2S_2 at concentrations of 4 μM ($P = 0.0021$) and in MDA-MB-231 cells with As_2S_2 at concentrations of 8 μM ($P = 0.0241$). Compared to con-

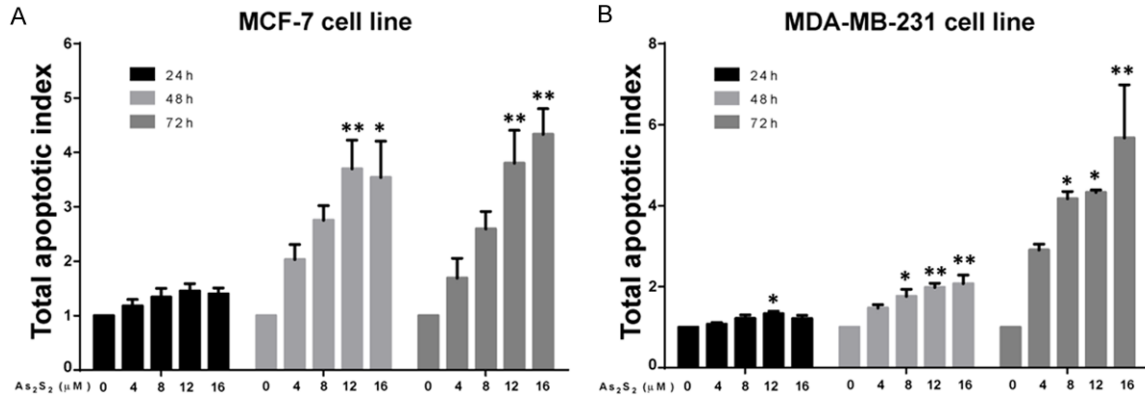


Figure 7. As₂S₂ induces apoptosis in breast cancer cells. (A) MCF-7 and (B) MDA-MB-231 cells were treated with different concentrations of As₂S₂ (0, 4, 8, 12 and 16 μM) for 24, 48 and 72 h, followed by staining with Annexin V/PI, and then analyzed by flow cytometry. The cells were assessed for total apoptotic cells composed by early apoptotic (Annexin V⁺/PI⁻) and late apoptotic (Annexin V⁺/PI⁺) cells. The apoptotic index was defined as the ratio of total apoptotic cell percentages between As₂S₂ treatment groups (with concentrations of As₂S₂ as 4, 8, 12 and 16 μM) and the control (As₂S₂ as 0 μM). All data were expressed as the mean ± SEM (n ≥ 3). Asterisks indicate significant differences between the control and the drug treatment groups (*P < 0.05, **P < 0.01).

Table 2. Apoptotic indices following exposure to As₂S₂ in human breast cancer cell lines

		Apoptotic Index (folds of control)				
Breast cancer cell lines	Exposure time	As ₂ S ₂ concentrations (μM)				
		0	4	8	12	16
MCF-7	24 h	1	1.18±0.12	1.34±0.16	1.45±0.13	1.40±0.11
	48 h	1	2.03±0.28	2.76±0.27	3.70±0.53**	3.54±0.67*
	72 h	1	1.69±0.36	2.60±0.32	3.80±0.61**	4.33±0.47**
MDA-MB-231	24 h	1	1.08±0.04	1.22±0.09	1.33±0.06*	1.21±0.08
	48 h	1	1.48±0.08	1.77±0.17*	1.99±0.10**	2.08±0.21**
	72 h	1	2.91±0.15	4.17±0.18*	4.33±0.06*	5.68±1.31**

Data are the means ± SEM from at least three independent experiments. *P < 0.05, **P < 0.01 vs. control (As₂S₂ 0 μM).

trol, the expression levels of cyclin D1 in MCF-7 cell significantly decreased by 16 μM of As₂S₂ at all of the three time courses (P = 0.0086 in 24 h, P = 0.0084 in 48 h, and P = 0.0010 in 72 h). In contrast, the significant downregulation of cyclin D1 expression in MDA-MB-231 cells merely occurred after 72 h of As₂S₂ treatment (16 μM, P = 0.0049). These results are congruent with G₂/M and S phases arrest caused by As₂S₂ in both breast cancer cell lines.

Interestingly, the protein levels of cyclin B1 significantly decreased in MCF-7 cells after 48 h and 72 h of As₂S₂ treatments with final concentrations of 4 μM (P = 0.0072 in 48 h, P = 0.0006 in 72 h), 8 μM (P = 0.0020 in 48 h, P < 0.0001 in 72 h) and 16 μM (P = 0.0002 in 48 h, P < 0.0001 in 72 h), whereas the pro-

tein levels of cyclin B1 significantly increased in MDA-MB-231 cells after 72 h of As₂S₂ treatment (16 μM, P = 0.0227). These data suggested that As₂S₂ induced G₂/M arrests by inhibiting cyclin B1 expression in MCF-7 cells, whereas by activating cyclin B1 levels in MDA-MB-231 cells, respectively.

As₂S₂ induces apoptosis in breast cancer cells

Apoptotic dysfunction is central to tumorigenesis and tumor progression which are in part characterized by imbalance between cell growth and programmed cell death. Numerous drug treatments act through inducing apoptosis in cancerous cells [42, 43]. To confirm whether the inhibition of cell proliferation in breast cancer cells induced by As₂S₂ was attributed to apoptotic induction, we performed

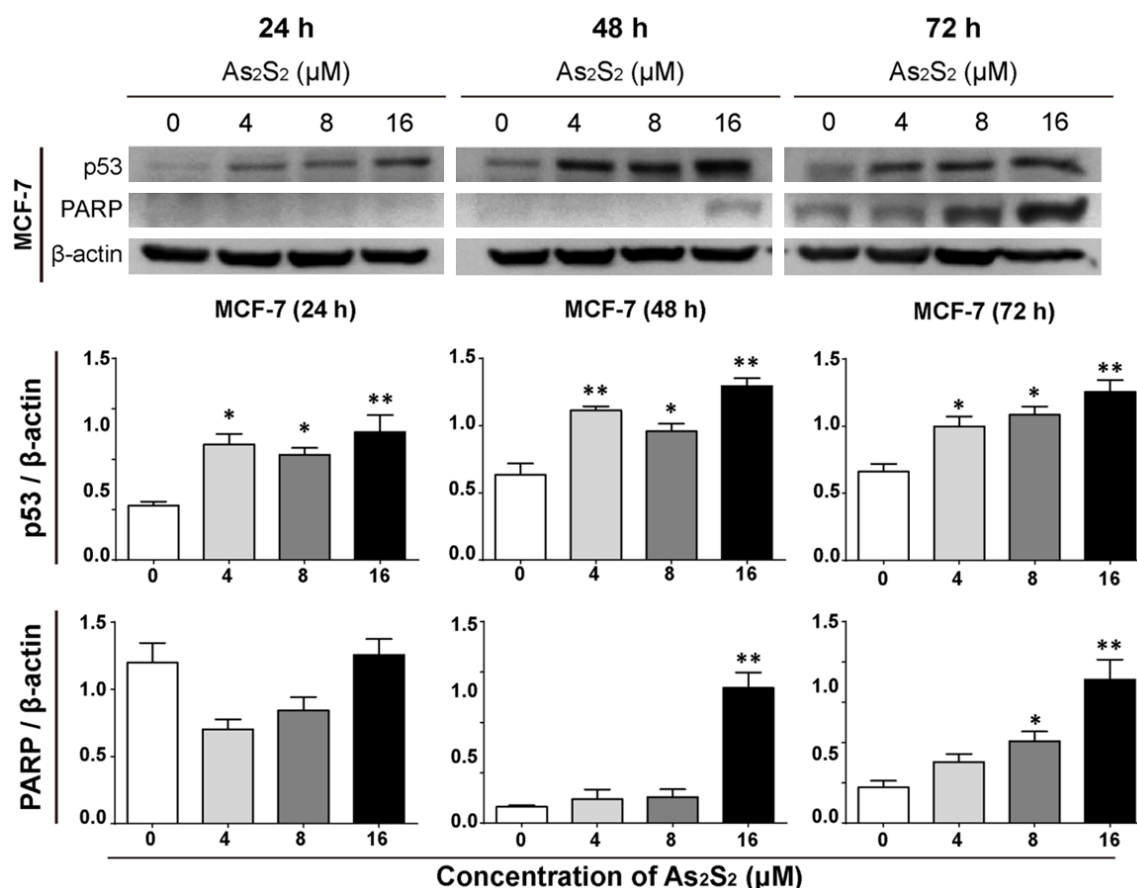


Figure 8. Effects of As_2S_2 on the expression levels of pro-apoptotic proteins in MCF-7 cells. MCF-7 cells were treated with different concentrations of As_2S_2 (0, 4, 8 and 16 μM) for 24, 48 and 72 h. Western blot assays were carried out to examine the effects of As_2S_2 on the expressions of pro-apoptotic proteins p53 and PARP in MCF-7 cells after 24, 48 and 72 h of drug treatment. Protein β -actin was used as internal control. All images are representative of three independent analyses from three independent cellular preparations. Asterisks indicate significant differences between the control (0 μM) and the drug treatment groups (* $P < 0.05$, ** $P < 0.01$).

Annexin V/PI double staining assay followed by flow cytometry analysis. The apoptotic index was defined as the ratio of total apoptotic cell percentages between As_2S_2 treatment groups (with concentrations of As_2S_2 as 4, 8, 12 and 16 μM) and the control (with the concentration of As_2S_2 as 0 μM).

As shown in **Figure 7A** and **Table 2**, the total apoptotic indices in MCF-7 cells treated with 4, 8, 12 and 16 μM of As_2S_2 increased in a dose- and time-dependent manner after 24, 48 and 72 h drug treatments. After exposure to As_2S_2 for 24 h, the total apoptotic indices elevated to a certain degree by As_2S_2 at 4-16 μM , though the difference was not statistically significant. In contrast, significant increases of apoptotic indices were observed in the cells after treat-

ment with 12 and 16 μM As_2S_2 for 48 and 72 h, respectively ($P < 0.05$).

In case of MDA-MB-231 cells (**Figure 7B** and **Table 2**), after 24 h exposure to As_2S_2 , the total apoptotic index significantly increased by 1.33 ± 0.06 ($P = 0.0257$) folds in the presence of 12 μM As_2S_2 , when compared with control (1.00). Statistically significant increases of apoptotic indices began to appear 48 and 72 h after the exposure of cells to 8 μM of As_2S_2 , with significant increase by 1.77 ± 0.17 ($P = 0.0159$) and 4.17 ± 0.18 ($P = 0.0234$) folds, respectively, when compared with control (1.00).

Taken together, the results indicated that apoptotic indices of both MCF-7 and MDA-MB-231 cells increased in a dose- and time-depend-

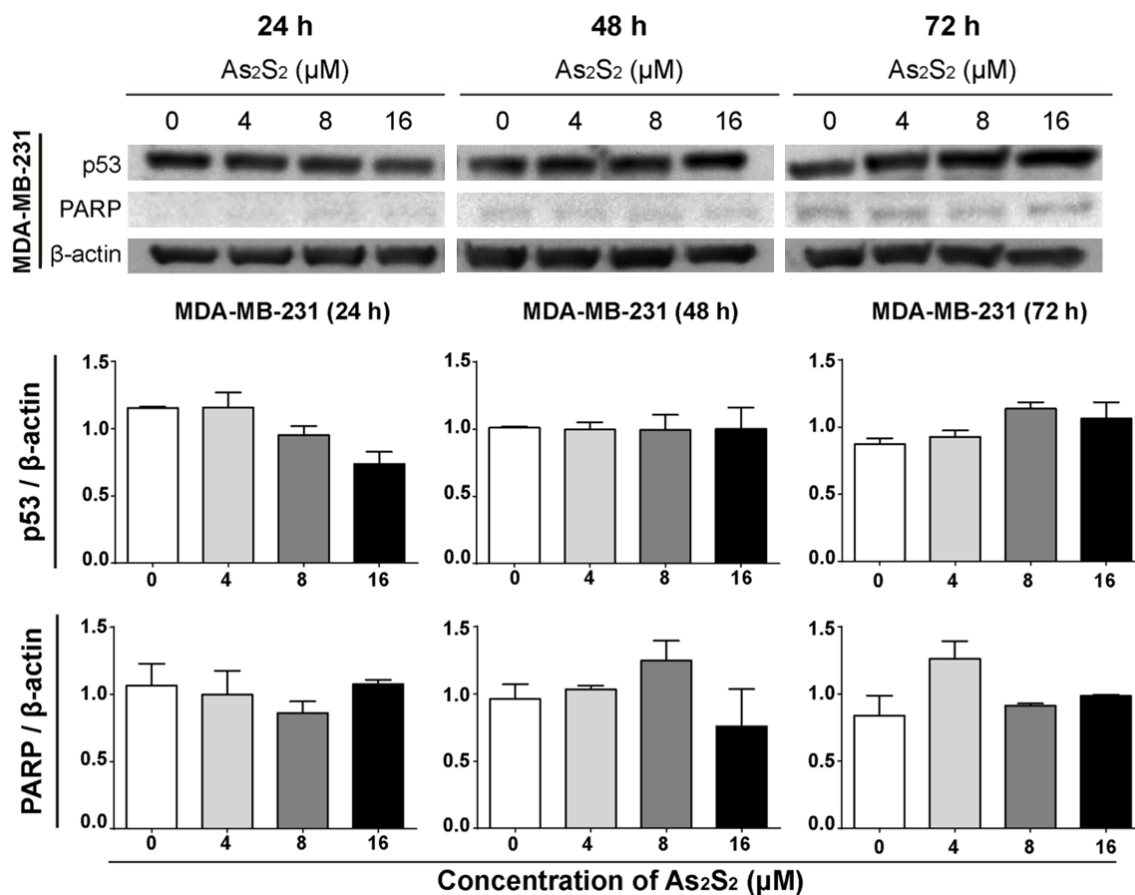


Figure 9. Effects of As_2S_2 on the expression levels of pro-apoptotic proteins in MDA-MB-231 cells. MDA-MB-231 cells were treated with different concentrations of As_2S_2 (0, 4, 8 and 16 μM) for 24, 48 and 72 h. Western blot assays were carried out to examine the effects of As_2S_2 on the expressions of pro-apoptotic proteins p53 and PARP in MDA-MB-231 cells after 24, 48 and 72 h of drug treatment. Protein β -actin was used as internal control. All images are representative of three independent analyses from three independent cellular preparations.

ent manner after exposure to As_2S_2 for 24, 48 and 72 h.

As_2S_2 regulates apoptosis-related proteins

To further delineate and validate the apoptotic induction by As_2S_2 in breast cancer cells, the expression of apoptosis-related proteins were explored by western blot analysis.

As shown in **Figure 8**, in case of MCF-7 cells with wild-type p53, an increase in pro-apoptotic p53 and cleavage PARP expressions were observed after treatment by As_2S_2 in dose- and time-dependent manners. Compared to control, the expression levels of p53 in MCF-7 cells significantly increased by As_2S_2 (with concentrations of 4, 8 and 16 μM) at all of three time courses (with 4 μM , $P = 0.0167$ in 24 h,

$P = 0.0024$ in 48 h, and $P = 0.0392$ in 72 h). The statistically significant increases of cleavage PARP expression started to occur in MCF-7 cells with As_2S_2 at concentrations of 8 μM ($P = 0.0155$) and 16 μM ($P = 0.0001$) after 48 h and 72 h of As_2S_2 treatments, respectively. By contrast, in case of MDA-MB-231 cells with mutated p53, the expressions of p53 neither changed by any concentrations of As_2S_2 nor altered by any incubation time course in the presence of As_2S_2 (**Figure 9**). In addition, the expressions of cleavage PARP were little altered by As_2S_2 treatment in MDA-MB-231 cells as well (**Figure 9**).

As shown in **Figures 10** and **11**, the protein expressions of anti-apoptotic markers Bcl-2 and Mcl-1 were inhibited by As_2S_2 in both MCF-7 and MDA-MB-231 cells, in dose- and time-

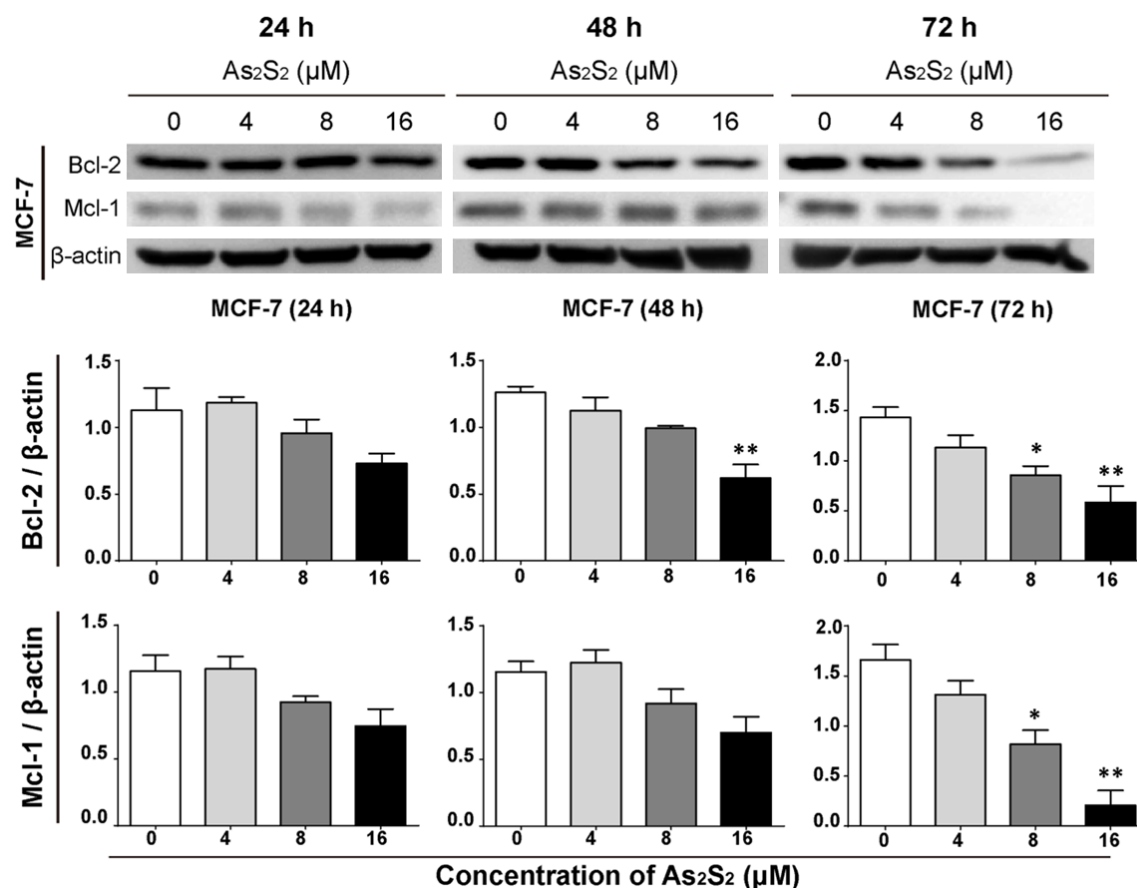


Figure 10. Effects of As₂S₂ on the expression levels of anti-apoptotic proteins in MCF-7 cells. MCF-7 cells were treated with different concentrations of As₂S₂ (0, 4, 8 and 16 μM) for 24, 48 and 72 h. Western blot assays were carried out to examine the effects of As₂S₂ on the expressions of anti-apoptotic proteins Bcl-2 and Mcl-1 in MCF-7 cells after 24, 48 and 72 h of drug treatment. Protein β-actin was used as internal control. All images are representative of three independent analyses from three independent cellular preparations. Asterisks indicate significant differences between the control (0 μM) and the drug treatment groups (**P* < 0.05, ***P* < 0.01).

dependent manners. Significant decreases of Bcl-2 expression were observed in MCF-7 cells after treatment with 16 μM of As₂S₂ for 48 h (*P* = 0.0014), as well as 8 μM (*P* = 0.0424) and 16 μM (*P* = 0.0054) of As₂S₂ for 72 h, respectively (**Figure 10**). Compared to control, the expression levels of Mcl-1 in MCF-7 cells significantly decreased by As₂S₂ with concentrations of 8 μM (*P* = 0.0147) and 16 μM (*P* = 0.0005) after 72 h of treatment (**Figure 10**). In case of MDA-MB-231, significant decreases of Bcl-2 (*P* = 0.0045) and Mcl-1 (*P* = 0.0487) expressions were observed after treatment with 16 μM of As₂S₂ for 72 h (**Figure 11**).

As₂S₂ inhibits cell survival-related proteins

To further investigate the molecular mechanism underlying the inhibitory effects of As₂S₂

on cell survival and growth in breast cancer cells, we evaluated the effect of As₂S₂ on pro-survival PI3K/Akt pathway. Abundant evidence demonstrates the essential role of this cell survival pathway in cell growth, proliferation, and survival [44, 45]. The protein expressions of PI3K and Akt were detected in breast cancer cells by western blot analysis.

As shown in **Figures 12** and **13**, in both MCF-7 and MDA-MB-231 cells, the protein expressions of PI3K and Akt were effectively down-regulated by As₂S₂ in dose- and time-dependent manners. Compared to control, the expression levels of PI3K in MCF-7 (**Figure 12**) and MDA-MB-231 (**Figure 13**) cells significantly decreased by As₂S₂ at all of three time courses (*P* < 0.05). Significant decreases of Akt expression were observed in both MCF-7 (FI-

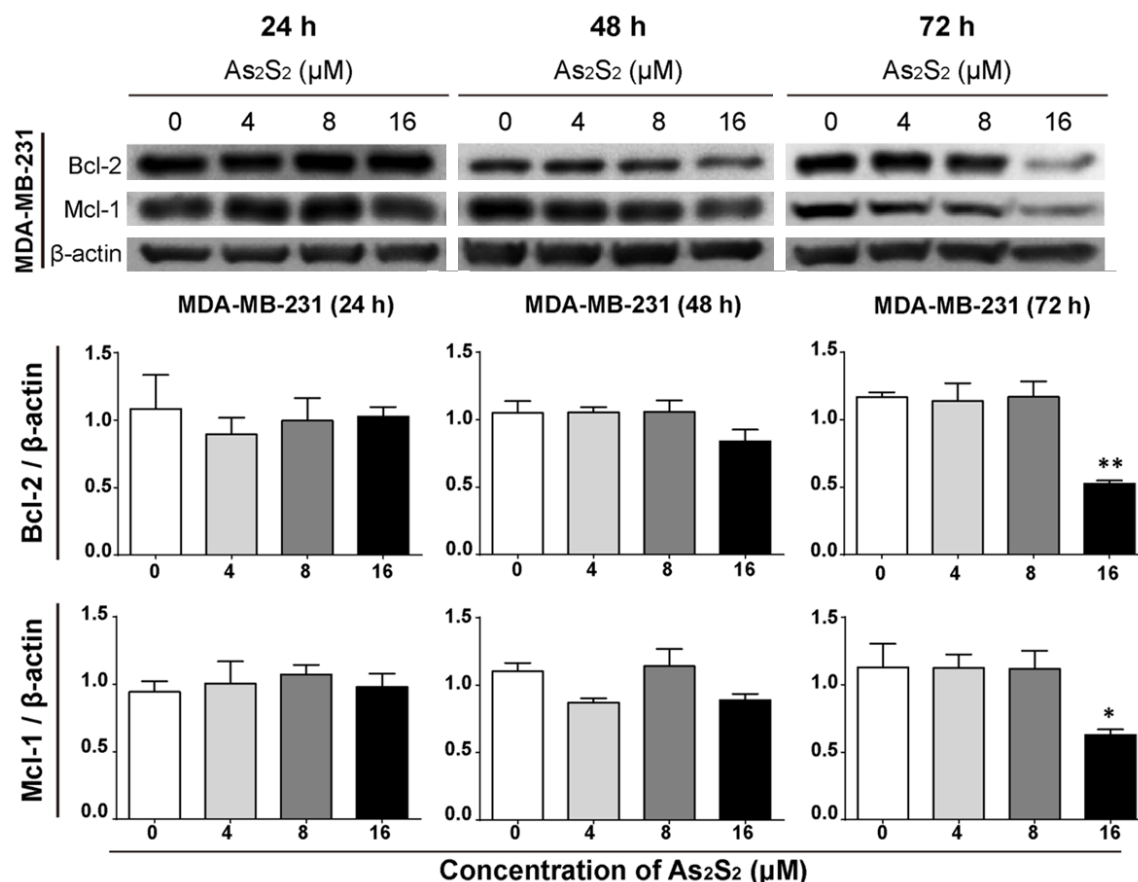


Figure 11. Effects of As₂S₂ on the expression levels of anti-apoptotic proteins in MDA-MB-231 cells. MDA-MB-231 cells were treated with different concentrations of As₂S₂ (0, 4, 8 and 16 μM) for 24, 48 and 72 h. Western blot assays were carried out to examine the effects of As₂S₂ on the expressions of anti-apoptotic proteins Bcl-2 and Mcl-1 in MDA-MB-231 cells after 24, 48 and 72 h of drug treatment. Protein β-actin was used as internal control. All images are representative of three independent analyses from three independent cellular preparations. Asterisks indicate significant differences between the control (0 μM) and the drug treatment groups (**P* < 0.05, ***P* < 0.01).

Figure 12) and MDA-MB-231 (Figure 13) cells after treatment with 16 μM of As₂S₂ for 48 h (*P* = 0.0006 in MCF-7, *P* = 0.0181 in MDA-MB-231) and 72 h (*P* = 0.0424 in MCF-7, *P* = 0.0281 in MDA-MB-231), respectively.

Collectively, the results indicated that the blockade of PI3K/Akt signals by As₂S₂ in breast cancer cells may contribute to the induction of apoptosis as well as the inhibition of cell viability following As₂S₂ exposure.

Discussion

Numerous studies on breast carcinomas are based on in vivo and in vitro researches focusing on breast cancer cell lines [38, 46]. In this study, we took two typically distinctive subtypes of breast cancer cell lines as the re-

search subjects, which can represent different categories of breast cancer disease, namely MCF-7 and MDA-MB-231 cell lines. MCF-7 is relatively a non-aggressive breast cancer cell line, characterized by estrogen-dependent, higher differentiated, ER-positive, and weakly invasive features. By contrast, MDA-MB-231 is an aggressive breast cancer cell line, characterized by estrogen-independent, undifferentiated, ER-negative, and highly invasive features [47-49].

In this study, we have provided the first evidence that As₂S₂ exerts potent antitumor effects on both MCF-7 and MDA-MB-231 cell lines. The cytotoxicity of As₂S₂ against human breast cancer cells was measured by assessing different parameters. The results demonstrated that As₂S₂ inhibited the cell viabilities

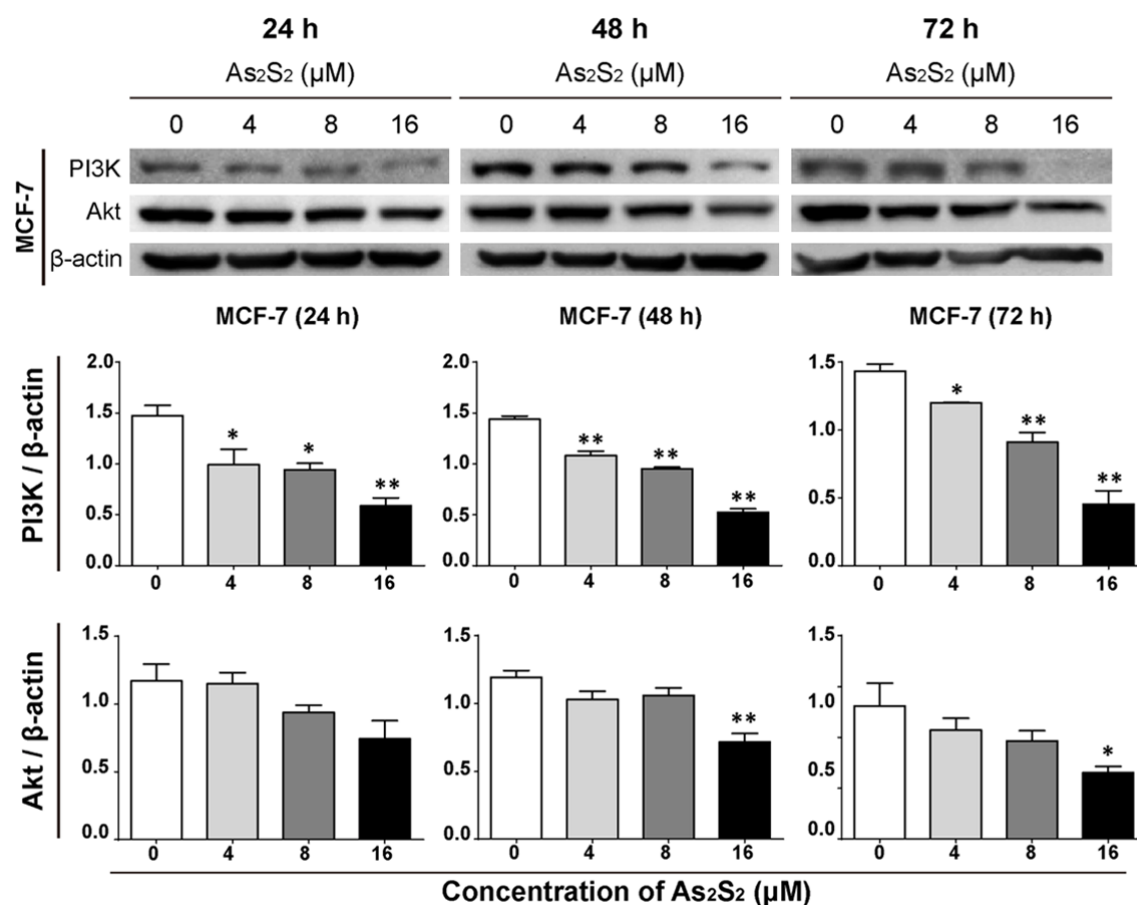


Figure 12. Effects of As_2S_2 on the expression levels of pro-survival proteins in MCF-7 cells. MCF-7 cells were cultured with various concentrations of As_2S_2 (0, 4, 8 and 16 μM) for 24, 48 and 72 h. Western blot assays were carried out to examine the effects of As_2S_2 on the expressions of key proteins PI3K and Akt in MCF-7 cells after 24, 48 and 72 h of drug treatment. Protein β -actin was used as internal control. All images are representative of three independent analyses from three independent cellular preparations. Asterisks indicate significant differences between the control (0 μM) and the drug treatment groups (* P < 0.05, ** P < 0.01).

by inducing cell cycle arrest and cell apoptosis in both MCF-7 and MDA-MB-231 cells. These antitumor effects of As_2S_2 were correlated to the regulation of signals that related to cell cycle progression, apoptosis and cell survival pathway. Our results also showed that As_2S_2 triggered cell cycle arrest in MCF-7 and MDA-MB-231 cells via regulating the protein expressions of cyclin A2, B1 and D1. Furthermore, activation of pro-apoptotic signals such as p53 and PARP, and the inhibition of anti-apoptotic signals such as Bcl-2 and Mcl-1 were connected to the induction of apoptosis in the breast cancer cells. In addition, the blockage of the pro-survival PI3K/Akt pathway also contributed to the antitumor effect of As_2S_2 in MCF-7 and MDA-MB-231 cell lines. To our knowledge, the results herein showed for the

first time the anti-cancer effects of As_2S_2 on different types of breast cancer cells, which especially involve cell cycle arrest and the programmed cell death pathway such as apoptosis induction, as well as the related underlying molecular mechanisms.

Since uncontrolled cell proliferation and the loss of regulated cell death are two major characteristics of cancer cells, accumulating anti-tumor strategies are emerging to develop therapies related to cell growth suppression by activating cell death pathways [29, 50-51]. Previous studies have reported significant inhibitory effects of As_2S_2 on cell viability and proliferation in a number of cancer cell lines representing different cancer diseases including gastric, hepatocellular, melanoma, pancreatic,

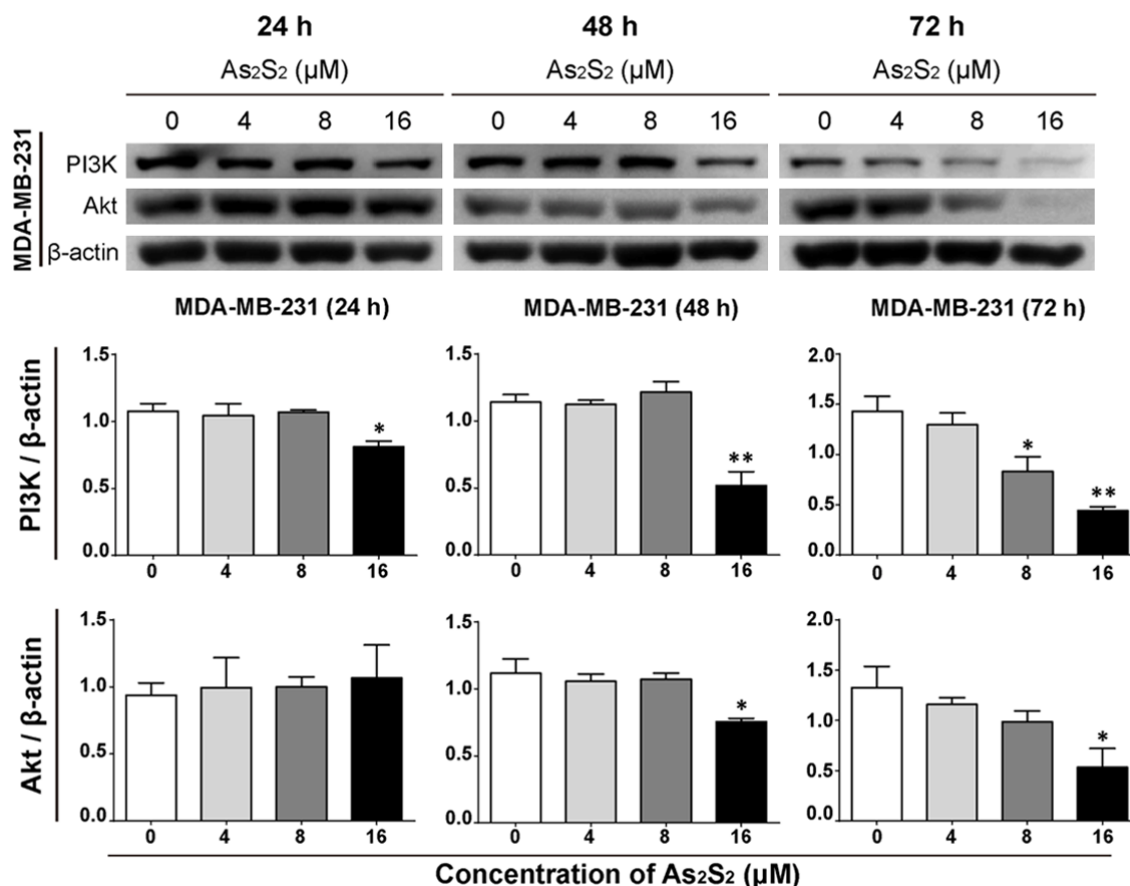


Figure 13. Effects of As_2S_2 on the expression levels of pro-survival proteins in MDA-MB-231 cells. MDA-MB-231 cells were cultured with serial concentrations of As_2S_2 (0, 4, 8 and 16 μ M) for 24, 48 and 72 h. Western blot assays were carried out to examine the effects of As_2S_2 on the expressions of key proteins PI3K and Akt in MDA-MB-231 cells after 24, 48 and 72 h of drug treatment. Protein β -actin was used as internal control. All images are representative of three independent analyses from three independent cellular preparations. Asterisks indicate significant differences between the control (0 μ M) and the drug treatment groups (* P < 0.05, ** P < 0.01).

cervical and ovarian carcinoma [12, 27, 33, 52], with little effect on normal control cells [12, 33, 53]. In the present study, we focused on the anti-tumor effects of As_2S_2 on human breast cancer cell lines, which has been rarely reported before. The results from WST assay showed a potent inhibitory action on cell viabilities exerted by As_2S_2 in both MCF-7 and MDA-MB-231 cells in dose- and time-dependent manners (Figure 1A, 1B). The viabilities of MCF-7 and MDA-MB-231 cells were confirmed and visualized by calcein staining test after exposure to As_2S_2 with different final concentrations (0, 4, 8, 12 and 16 μ M) and in different time courses (24, 48 and 72 h after drug exposure) (Figure 2). The morphology results have shown that live cell numbers decreased by As_2S_2 treatment in both breast cancer cell lines in dose- and time-dependent manners,

which is congruent with the cell viability results detected by WST assay. Moreover, the IC_{50} values of As_2S_2 in each cell line in different time courses exhibited culture time-dependent efficacy of As_2S_2 with the highest effect at 72 h after the treatment (Table 1). Furthermore, our results demonstrated that MCF-7 cells are more sensitive to As_2S_2 than MDA-MB-231 cells.

Abundant studies indicate that tumor cell response to chemotherapies is mainly through several cell death mechanisms, which include cell cycle arrest and apoptosis [54, 55]. Cell cycle is a series of organized events that allow cells to grow and divide into two daughter cells [35, 56]. This process consists of four sequential phases, namely G_1 , S, G_2 and M (mitosis) phases. Since cell cycle dysfunction

often contributes to tumorigenesis, it turns into one of the essential targets for current anticancer therapies to exert their actions by regulating cell cycle progression [36]. In the present study, we found that As₂S₂ treatment arrested the cell cycle progression at G₂/M phase in both two breast cancer cell lines with dose- and time-dependent manners. In addition to the G₂/M phase-arrest effect, As₂S₂ also blocked G₀/G₁ phase in MCF-7 cells, whereas the agent led to S phase arrest in MDA-MB-231 cells. The different cell cycle arrest observed in MCF-7 and MDA-MB-231 cells at different phases could be ascribed to the biological variation between the two cell lines. More importantly, the blockage of cell cycle progression by As₂S₂ treatment may facilitate the induction of apoptosis [57]. Apoptosis is the most common and well-orchestrated form of programmed cell death, which is essential for many biological processes [55]. Dysregulation of apoptosis can result in numerous pathological conditions, including different types of cancer [58]. Therefore, the induction of apoptosis became one of the principles to develop novel chemotherapeutic agents. In the present study, we found that As₂S₂ induced apoptosis in both MCF-7 and MDA-MB-231 breast cancer cell lines in both dose- and time-dependent manners. These results are consistent with the inhibited cell growth and cell cycle arrest triggered by As₂S₂ in both MCF-7 and MDA-MB-231 cells, suggesting the promising anti-cancer characteristics of As₂S₂ against breast cancer.

The molecular mechanisms underlying the anticancer actions of As₂S₂ in breast cancer cells in the present study involved various signals and pathways, including the activation of apoptosis pathways, regulation of specific proteins in cell cycle, as well as the suppression of pro-survival pathway. In terms of regulating apoptosis related proteins, we found that As₂S₂ activated the pro-apoptotic signals such as p53 and PARP in MCF-7 cells in dose- and time-dependent manners (**Figure 8**), while the agent showed little effect in MDA-MB-231 cells (**Figure 9**). The tumor suppressor p53 plays essential role in the prevention of human cancer through either cell cycle regulation or apoptosis induction [59, 60]. It is found to be mutated in 50-55% of human cancers [61]. In this study, different genetic status of p53 in

MCF-7 (with wide-type gene) and MDA-MB-231 (with mutant gene) cells may attribute to the discrepancy in p53 protein expression activated by As₂S₂ between these two different cell lines. Cleavage of PARP is a hallmark of apoptosis, which is commonly observed during apoptosis and mediated by the activation of caspases [62]. Our results showed that As₂S₂ promoted the expression of cleaved PARP in MCF-7 cells other than MDA-MB-231 cells, suggesting possible caspase-dependent apoptotic induction in MCF-7 cells by As₂S₂ treatment. Furthermore, the different status of these two key pro-apoptotic indicators after treatment by As₂S₂ may be partially responsible for the different sensitivities of MCF-7 and MDA-MB-231 cells to As₂S₂. Bcl-2 protein family has a critical role in regulating the intrinsic pathway of apoptosis [63]. The anti-apoptotic members in Bcl-2 family, such as Bcl-2 and Mcl-1 undergo down-regulation during apoptosis induction [31]. In this research, we found that As₂S₂ inhibited Bcl-2 and Mcl-1 expressions in both MCF-7 and MDA-MB-231 cells in dose- and time-dependent manners, suggesting that these proteins were related to As₂S₂ induced apoptosis in both cell lines (**Figures 10 and 11**). Cell cycle progression is regulated by protein kinase complexes, which consists of cyclins and CDKs [36]. Different cyclins are required at different phases of the cell cycle. In detail, cyclin D regulates the progression from G₁ phase to S phase, while cyclin B and cyclin A regulate the progression from G₂ to M phase [36, 64]. The results of the present study indicated that the treatment of the cells by As₂S₂ decreased the protein expression of cyclin D1 and cyclin A2 in the two breast cancer cell lines, which is in agreement with the cell cycle arrests induced by As₂S₂ in S and G₂/M phases. In addition, the protein expression of cyclin B1 was down-regulated by As₂S₂ in MCF-7 cells, whereas up-regulated in MDA-MB-231 cells. Both inhibition and activation of cyclin B1 could lead to cell cycle arrest in G₂/M phase [65, 66]. The opposite regulatory effect of As₂S₂ on cyclin B1 amounts may correspond to the biological variation between the two different breast cancer cell lines. PI3K/Akt signaling pathway implicates in tumorigenesis by regulating cell growth, cell cycle progression, cell apoptosis, and cell metabolism [44, 45]. Disruption of PI3K/Akt signals contributes to inhibiting cell survival, restraining tumor growth, and promoting

pro-apoptotic activities. Our results demonstrated that As₂S₂ treatments suppressed the PI3K/Akt signals in the two breast cancer cell lines with similar dose- and time-dependent manners. These observations suggest the possible inhibitory function of As₂S₂ to control cancer cell survival signals, which acts in accordance with the regulation of apoptotic signals as well as cell cycle related proteins.

Taken together, this study provides the first evidence that As₂S₂ has the ability to exert its anticancer effects against different human breast cancer cell lines, by inhibiting cell proliferation, blocking cell cycle progression, and inducing apoptosis. Such anticancer functions by As₂S₂ are suggested to be correlated with molecular alterations involving different cellular signaling pathways. The present research provides mechanistic bases for therapeutic potential of As₂S₂ in the application of treating breast cancer. However, the present study is confined to elucidate the antitumor effect of As₂S₂ against breast cancer in vitro. Further studies will be performed to investigate its therapeutic potentials in animal models of breast cancer.

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Disclosure of conflict of interest

None.

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