Dose-dependent biphasic effects of arsenic disulfide on differentiation and apoptosis of HL-60 cells

Xiao-Mei Hu1,3, Bo Yuan2,#, Song Min-Min3, Kenji Onda1, Sachiko Tanaka1, Hiroo Toyoda2, Ai-Xiang Zhou3, Kentaro Sugiyama1 and Toshihiko Hirano1,*

1Department of Clinical Pharmacology, 2Department of Clinical Molecular Genetics, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan. 3National Therapeutic Center of Hematology of Traditional Chinese Medicine, XiYuan Hospital, China Academy of Chinese Medical Sciences, Beijing, 100091, P.R. China

ABSTRACT
We investigated the dose-dependent biphasic effects of arsenic disulfide (As$_2$S$_2$) on the differentiation and apoptosis of HL-60 cells. Cellular reactive oxygen species, glutathione, p38 mitogen-activated protein kinase (MAPK), as well as cell differentiation and apoptosis in HL-60 cells were assessed by flow cytometric analysis. The mean percentage of CD11b-positive cells induced by 8 μM As$_2$S$_2$ was more than 3 times higher than that induced by 16 μM As$_2$S$_2$. Eight μM As$_2$S$_2$ did not induce cell apoptosis, while 16 μM As$_2$S$_2$ induced apoptosis and decreased cell viability. Both 8 and 16 μM As$_2$S$_2$ increased cellular reactive oxygen species. Treatment with 16 μM As$_2$S$_2$ decreased the cellular glutathione levels at 1 h and 3 h after the exposure. The mitochondrial membrane potential depletions were observed in both 8 μM As$_2$S$_2$-induced differentiation and 16 μM As$_2$S$_2$-induced apoptosis. p38 MAPK inhibition enhanced As$_2$S$_2$-induced differentiation, but had little influence on As$_2$S$_2$-induced apoptosis. A moderate oxidative stress induced by 8 μM As$_2$S$_2$ can promote As$_2$S$_2$-induced differentiation, whereas more severe oxidative stress caused by glutathione depletion by 16 μM As$_2$S$_2$ reduced mitochondrial membrane potential, resulting in differentiation attenuation and apoptosis enhancement. p38 MAPK activation resulting from oxidative stress seems to be implicated in negative regulation of cell differentiation, rather than apoptosis, in As$_2$S$_2$-treated HL-60 cells.

KEYWORDS: arsenic disulfide, cell differentiation, HL-60 cells, mitochondrial membrane potential, p38 MAPK, reactive oxygen species

ABBREVIATIONS
As$_2$S$_2$, arsenic disulfide; MAPK, p38 mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PML/RAR-α, promyelocytic leukemia/retinoic acid receptor protein

INTRODUCTION
Notable success has been reported in the treatment of acute promyelocytic leukemia with arsenic trioxide (As$_2$O$_3$) [1, 2]. The clinical use of arsenic disulfide (As$_2$S$_2$)-containing formulae to treat myeloid leukemia in China started as early as the beginning of the medical use of As$_2$O$_3$ [3-5]. Qinghuang Powder containing QingDai (Natural Indigo) and Xiong huang (Red Orpiment) in the formula was used to treat myeloid leukemia early from 1960s in our institute in China [4]. It was found that the therapeutic efficacy of Qinghuang Powder could be enhanced by increasing the content of Xiong huang, which contains > 90%
As$_2$S$_2$, without strong adverse effects during administration even for more than 10 years [5, 6]. These experiences suggested that Qinghuang Powder is effective in the treatment of myeloid leukemia, and its therapeutic efficacy could be attributed to As$_2$S$_2$, the most important component of Xiong huang.

It has been suggested that As$_2$O$_3$ is able to induce both apoptosis and differentiation in leukemia cells depending on the dosage [7]. Studies carried out on a large number of leukemic cell lines have shown that this compound mainly exerts an apoptotic effect [8-11]. The action of As$_2$O$_3$-induced myeloid differentiation in acute promyelocytic leukemia model has been suggested to induce promyelocytic leukemia/retinoic acid receptor protein (PML/RAR-α) degradation [12, 13]. Recently, many results indicated that reactive oxygen species, intracellular glutathione, and p38 mitogen-activated protein kinase (MAPK) play roles in As$_2$O$_3$-induced apoptosis [14-20].

While knowledge on the action of As$_2$O$_3$ is thus increasing, similar aspects of As$_2$S$_2$ have not been sufficiently documented, although associations of reactive oxygen species and p38 MAPK pathways with As$_2$S$_2$-induced differentiation were reported in HL-60 cells [2, 21]. We suggested in our previous study that oxidative stress and oxidative stress-related activation of p38 MAPK have important roles in As$_2$S$_2$-induced differentiation and apoptosis of HL-60 cells [22]. Our previous data also suggested that moderate levels of oxidative stress induced by As$_2$S$_2$ positively contribute to HL-60 cell differentiation, and there might be dose-dependent biphasic effects of As$_2$S$_2$ on the cell differentiation and apoptosis [22]. Thus, in the present study, we further examined the role of p38 MAPK activation, reactive oxygen species generation, and intracellular glutathione levels in myeloid differentiation and apoptosis of HL-60 cells, induced by different concentrations of As$_2$S$_2$.

**MATERIALS AND METHODS**

**Reagents**

RPMI-1640 medium and fetal bovine serum were purchased from Gibco BRL, Grand Island, NY, USA. Cell proliferation kits I and II were obtained from Roche Diagnostics, Indianapolis, Ind, USA. As$_2$S$_2$, Rhodamine 123, 2′,7′-dichlorodihydrofluorescein diacetate, and ortho-phthaldialdehyde were purchased from Sigma Aldrich, St. Louis, Mo, USA. Phycoerythrin-conjugated anti-human CD11b (Clone D12) and mouse IgG1 isotype control were obtained from DakoCytomation, Denmark. p38 MAPK inhibitor SB203580 was purchased from Calbiochem, San Diego, CA, USA. Phycoerythrin-conjugated mouse anti-p38 MAPK (pT180/pY182) and the Annexin V-fluorescein isothiocyanate apoptosis detection kit I were obtained from BD PharMingen, Tokyo, Japan. 10%-Formaldehyde neutral buffer was a product of Nacalai Tesque, Tokyo, Japan. As$_2$S$_2$ was dissolved in 1N NaOH, and HCl was used to adjust the pH to 7.35-7.45. The solution was passed through a 0.20 μm membrane, and the stock solutions were made using phosphate-buffered saline at a concentration of 5 mM, which was diluted with phosphate-buffered saline to working concentrations before use. 2′,7′-dichlorodihydrofluorescein diacetate and Rhodamine 123 were dissolved in dimethylsulfoxide at 5 mM and 1 mg/ml, respectively, and stored at -20 °C. The ortho-phthaldialdehyde solution was freshly prepared in methanol at 10 mg/ml before use. SB203580 was dissolved in dimethylsulfoxide at a concentration of 20 mM, and stored at -20 °C until use.

**Cell culture**

HL-60 cells were obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA. The cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in continuous logarithmic growth between 2 x 10$^5$ and 1 x 10$^6$ cells/ml at 37 °C in a humidified 5% CO$_2$ incubator.

**Cell proliferation assay**

The leukemia cells were washed and resuspended in the above medium to 5 x 10$^5$ cells/ml. Then, 196 μl of the cell suspension was placed in each well of a 96-well flat-bottomed plate. Four μl of phosphate-buffered saline containing As$_2$S$_2$ was added to yield final concentrations of 0.5, 2, 8, and 16 μM. Four μl of phosphate-buffered saline was added to the control wells. The cells were incubated for 48 h in an atmosphere containing
5% CO₂ at 37 °C in a humidified chamber. After the incubation period, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) labeling reagent (final concentration 0.5 mg/ml) was added to each well and the plate was placed on a microshaker for 10 s, after which the cells were further incubated for 4 h in a humidified atmosphere. Subsequently, 100 μl of solubilizing solution was added to each well and mixed, after which the plate was left overnight in an incubator in humidified atmosphere. The spectrophotometric absorbance of the samples was measured on a microplate reader (LS-PLATE manager 2001, Japan) at 570 nm.

Assessment of myeloid differentiation
After HL-60 cells (1×10⁶/ml) were treated with the indicated concentrations of As₂S₂, the cells were washed twice and resuspended in 100 μl phosphate-buffered saline containing 2% fetal bovine serum. Then 10 μl phycoerythrin-conjugated CD11b antibody solution was added, and the cells were incubated in the dark at 4 °C for 30 min. After the incubation, the cells were resuspended in 400 μl phosphate-buffered saline containing 1% fetal bovine serum, and subsequently the cells were analyzed by a FACS Calibur™ flow cytometry (BD Biosciences, Mountain View, CA, USA). Isotypic mouse IgG₁ was used to set threshold parameters. A total of 30,000 non-gated cells were analyzed. For the assessment of differentiation induced by H₂O₂ combined with As₂S₂, cells were pre-incubated with H₂O₂ for 1 h before treatment with As₂S₂ for 48 h. For the assessment of effects of SB203580 on As₂S₂-induced differentiation, cells were incubated with As₂S₂ combined with SB203580 for 72 h, using 0.05% dimethylsulfoxide as vehicle in the control wells.

Apoptosis and viability assays
After HL-60 cells (1×10⁶/ml) were treated with As₂S₂, the cells were washed twice in cold phosphate-buffered saline, and resuspended in binding buffer at a density of 1 × 10⁶ cells/ml. Then, 1 × 10⁵ cells were stained with 5 μl Annexin V-fluorescein isothiocyanate and 5 μl propidium iodide solution for 15 min at 4 °C in the dark. Then, 400 μl of binding buffer was added, and the cells were analyzed by flow cytometer within 1 h of staining. A total of 30,000 non-gated cells were analyzed. Cell viability was determined by propidium iodide exclusion assay. Apoptotic cells were identified by Annexin V-staining. For the assessment of apoptosis-inducing effect of H₂O₂ combined with As₂S₂, cells were pre-incubated with H₂O₂ for 1 h before treatment with As₂S₂ for 72 h. For the assessment of the effects of SB203580 on As₂S₂-induced apoptosis, cells were incubated with As₂S₂ combined with SB203580 for 72 h, using 0.05% dimethylsulfoxide as vehicle in the control wells.

Measurement of cellular reactive oxygen species
Cellular reactive oxygen species were measured using 2',7'-dichlorodihydrofluorescein diacetate. Viable cells can deacetylate the agent to 2',7'-dichlorodihydrofluorescein, which is not fluorescent but can react with H₂O₂ to produce highly fluorescent dichlorofluorescein. The cytofluorometric measurement of dichlorofluorescein provides an index of cellular reactive oxygen species level. At the end of treatment with As₂S₂, the cells in each well were collected, washed twice in phosphate-buffered saline and incubated in 1 ml phosphate-buffered saline containing 10 μM 2',7'-dichlorodihydrofluorescein diacetate at 37 °C for 30 min. The cells were then washed and resuspended in 0.5 ml phosphate-buffered saline. The mean fluorescence intensity of dichlorofluorescein green fluorescence from 10,000 cells was detected by flow cytometry. The amount of reactive oxygen species in the treated cells as compared to that in control cells was represented by relative mean fluorescence intensity, which is defined as: sample mean fluorescence intensity/control mean fluorescence intensity (%).

Determination of cellular glutathione concentration
The temporal change in cellular glutathione amounts was evaluated using ortho-phthaldialdehyde, which can react with glutathione to yield a highly fluorescent product. Cultured cells were washed twice by phosphate-buffered saline and then suspended in 900 μl phosphate-buffered saline. Then, 100 μl of 10 mg/ml ortho-phthaldialdehyde dissolved in methanol was added, and the cells were incubated at 37 °C for 30 min. After being washed by phosphate-buffered saline, the cells were resuspended in 500 μl phosphate-buffered saline and kept on ice. Mean fluorescence
intensity thus produced from 10,000 cells was measured by flow cytometry. The cellular glutathione amounts were represented by relative mean fluorescence intensity.

**Analysis of activated p38 MAPK**

Activated p38 MAPK was detected by flow cytometry using phycoerythrin-labeled antibody specific to phosphorylated (activated) p38 MAPK. Cells (1 × 10⁶/ml) treated with As₂S₂ were collected by centrifugation and washed with phosphate-buffered saline, and fixed for 10 min at 37 °C in phosphate-buffered saline containing 2% formaldehyde neutral buffer. The cells were centrifuged and resuspended in 90% methanol, and then incubated for 30 min on ice. The cells were washed twice and resuspended with 100 µl phosphate-buffered saline. Subsequently, phycoerythrin-labeled antibody against the phosphorylated p38 MAPK was added, and the cells were incubated for 1 h at room temperature. The cells were resuspended in 400 µl phosphate-buffered saline containing 1% formaldehyde neutral buffer, and analyzed by flow cytometry.

**Determination of mitochondrial membrane potential**

The mitochondrial membrane potential was assessed using the cationic lipophilic dye Rhodamine 123. Cells (1 × 10⁶/ml) treated with As₂S₂ were collected and washed twice in phosphate-buffered saline, and incubated in 1 ml phosphate-buffered saline containing 10 µg/ml Rhodamine 123 at 37 °C for 30 min. The cells were washed and resuspended in 500 µl phosphate-buffered saline. Subsequently, 30,000 cells were analyzed with flow cytometry, and mitochondrial membrane potential is represented by relative mean fluorescence intensity.

**Statistics**

Data were shown as the mean ± standard deviation of three independent experiments. Differences were estimated by one-way ANOVA. A value of p < 0.05 was considered to be significant.

**RESULTS**

**Effects of As₂S₂ on cell proliferation, differentiation and apoptosis**

HL-60 cells were continuously treated with As₂S₂ at concentrations of 8 and 16 µM for 72 h, and cell proliferation was measured in an MTT assay. Eight µM As₂S₂ did not affect, while 16 µM As₂S₂ significantly inhibited, proliferation of HL-60 cells (p < 0.05) (Fig. 1).

Differentiated cells were detected by cell-surface expression of CD11b. After treatment of cells with As₂S₂ at concentrations of 8 and 16 µM for 48 h, the percentage of CD11b-positive cells significantly increased to 17 and 5.5 times of control, respectively (Fig. 2) (p < 0.05), and the mean percentage of CD11b-positive cells induced by 8 µM was more than 3 times higher than that induced by 16 µM As₂S₂ (Fig. 2) (p < 0.05). The degrees of CD11b expression peaked at 48 h in cells treated with both 8 and 16 µM As₂S₂ for 24-72 h (Fig. 2).

After treatment for 24 to 72 h, 16 µM As₂S₂ significantly induced apoptosis and decreased cell viability in a time-dependent manner (p < 0.05) (Fig. 3A, B) whereas As₂S₂ at 8 µM conversely decreased apoptosis and rather increased cell viability compared to the control (Fig. 3A, B).

**Cellular reactive oxygen species generated by As₂S₂ treatment**

After incubation of cells for 0.5 to 24 h in the presence of 8 or 16 µM As₂S₂, a notable amount of cellular reactive oxygen species was observed within a few hours. The amounts of cellular reactive oxygen species rapidly increased to more than 120% of control at 0.5 h and sustained to 3 h (Fig. 4A). Of note, the levels of cellular reactive oxygen species generated by treatment with 16 µM As₂S₂ were significantly higher than those generated by treatment with 8 µM As₂S₂ at 3 h (Fig. 4B).

**Contribution of oxidative stress to As₂S₂-induced cell differentiation and apoptosis**

The effects of H₂O₂ on As₂S₂-induced differentiation were assessed by measuring percentages of CD11b-positive cells after treatment with As₂S₂ and H₂O₂ alone or in combination of these agents for 48 h. H₂O₂ alone at 20 µM significantly promoted myeloid cell differentiation, as compared to control (Fig. 5A) (p < 0.05). Treatment of cells with 8 and 16 µM As₂S₂ increased the percentages of CD11b-positive cells (Fig. 5A), as already described in Fig. 2, whereas preincubation of the cells with 20 µM H₂O₂ for 1 h attenuated the differentiation-inducing activity of As₂S₂ (Fig. 5A) (p < 0.05).
significantly increased the percentages of apoptotic cells, as compared to control (Fig. 5B) (p<0.05). When cells were pre-incubated with 20 μM H2O2 for 1 h, followed by combined treatment with 8 or 16 μM As2S2, the percentages
Fig. 4. Generation of cellular reactive oxygen species in As$_2$S$_2$-treated HL-60 cells. (A) Changes in amounts of reactive oxygen species in cells after treatment with 8 or 16 μM As$_2$S$_2$ for 0.5 to 24 h. The amounts of reactive oxygen species were presented as the relative mean fluorescence intensity, which were defined as: (sample mean fluorescence intensity/control mean fluorescence intensity) × 100%. (B) The amounts of reactive oxygen species in cells after treatment with 8 or 16 μM As$_2$S$_2$ for 3 h. The data are the mean fluorescence intensity related to dichlorofluorescein produced in cells by reactive oxygen species. Data are the mean ± standard deviation of 3 independent experiments. *p < 0.05 as compared with control (0 μM As$_2$S$_2$); #p < 0.05 as compared to cells treated with 8 μM.

Fig. 5. Effects of hydrogen peroxide (H$_2$O$_2$) on As$_2$S$_2$-induced differentiation (A) and apoptosis (B) in HL-60 cells. Cells were treated with 20 μM H$_2$O$_2$, 8 μM As$_2$S$_2$ (A8), 16 μM As$_2$S$_2$ (A16), or pre-incubated with 20 μM H$_2$O$_2$ for 1 h followed by treatment with H$_2$O$_2$ plus A8, as well as H$_2$O$_2$ plus A16. After the treatment for 48 h (differentiation) or 72 h (apoptosis), the cells were stained with anti-CD11b antibody or Annexin-V, and then analyzed with flow cytometry. Data are the mean ± standard deviation of 3 independent experiments. Means were compared by 1-way ANOVA. *p < 0.05 as compared with control (without H$_2$O$_2$ and As$_2$S$_2$); #p < 0.05 as compared to cells treated with As$_2$S$_2$ alone.
of Annexin V-positive cells significantly increased (Fig. 5B) \(p < 0.05\).

**Cellular glutathione level**

Cellular glutathione amounts in cells treated with 8 or 16 \(\mu\)M AsS\(_2\) for 0.5-24 h transiently increased to 110% of control at 0.5 h, then rapidly declined and dropped to the minimum at 3 h (Fig. 6A). At this incubation time, the cellular glutathione amounts in cells treated with 16 \(\mu\)M AsS\(_2\) decreased to 66% of control \(p < 0.05\), which were less than those in cells treated with 8 \(\mu\)M AsS\(_2\) (88% of control, \(p < 0.05\)) (Fig. 6B) \(p < 0.05\). These changes in cellular glutathione amounts after treatment with AsS\(_2\) appeared to be negatively correlated with the changes in the amounts of reactive oxygen species, as shown in Fig. 4.

**Activation of p38 MAPK**

After incubation of cells with AsS\(_2\) at concentrations of 8 and 16 \(\mu\)M for 1 to 72 h, the cellular amounts of phosphorylated (activated) p38 MAPK gradually increased from 6 to 72 h, and rose to 128% and 145%, respectively, at 72 h with respect to the control (Fig. 7A). Of note, the amounts of the activated p38 MAPK weakly decreased to 82% of control in cells treated with 8 \(\mu\)M AsS\(_2\) for 1 h, whereas they significantly increased to 125% of control in cells treated with 16 \(\mu\)M (Fig. 7B) \(p < 0.05\).

**Loss of mitochondrial membrane potential**

The relative values of mitochondrial membrane potential significantly declined to 65% and 53% of the control in cells treated with 8 and 16 \(\mu\)M AsS\(_2\), respectively, for 48 h (Fig. 8A) \(p < 0.05\). Furthermore, the loss of mitochondrial membrane potential in cells treated with 16 \(\mu\)M AsS\(_2\) was more apparent than that in cells treated with 8 \(\mu\)M AsS\(_2\) at 48 h (Fig. 8B) \(p < 0.05\). Similar to our previous report [22], our experimental results also demonstrated that the mitochondrial membrane potential decreased to the minimum in cells treated with 8 \(\mu\)M AsS\(_2\) for 48 h (Fig. 8A), and conversely, the level of CD11b-positive cells increased to the maximum (Fig. 2). In contrast to these observations using 8 \(\mu\)M AsS\(_2\), the mitochondrial membrane potential decreased to the lowest level (25% of the control) in cells treated with 16 \(\mu\)M for 72 h (Fig. 8A), accompanied with the highest percentage level of apoptotic cells (Fig. 8C), which was also shown in Fig. 3A.
Effects of p38 MAPK inhibition on As$_2$S$_2$-induced apoptosis

As shown in Fig. 2, when the cells were treated with 8 and 16 μM As$_2$S$_2$ for 24-72 h, the ratio of CD11b-positive cells increased to the maximum at 48 h, while the ratio significantly decreased at 72 h. At this time, the amounts of activated p38 MAPK rose to the maximum (Fig. 7A). Treatment with a combination of 8 μM As$_2$S$_2$ and 10 μM SB203580, a specific p38 MAPK inhibitor, for 72 h was found to increase the CD11b-positive cell ratio compared to treatment with each alone, as shown in Fig. 9A, the observation of which was consistent with our previous data [22]. When the cells were treated with 16 μM As$_2$S$_2$ for 24-72 h, the percentages of apoptotic cells gradually increased as shown in Fig. 3A and Fig. 8C, and the increase in the amount of activated p38 MAPK was simultaneously observed. Then, the effects of p38 MAPK activation on apoptosis induced by 16 μM As$_2$S$_2$ were assessed. Almost no difference was observed between the combination treatment of 16 μM As$_2$S$_2$/10 μM SB203580 and the treatment with 16 μM As$_2$S$_2$ alone (Fig. 9B).

DISCUSSION

In the present study we showed, a dose-dependent biphasic effects of arsenic disulfide on the differentiation and apoptosis of HL-60 cells. As$_2$O$_3$ is known to affect multiple cellular functions via different molecular targets, such as PML-RARalpha fusion protein, mitochondria and MAPK. [7]. In details, As$_2$O$_3$ tends to promote differentiation at low concentrations while it induces apoptosis at higher concentrations in leukemic cells in vitro and in vivo. Studies carried out on a large number of leukemic cell lines have shown that this compound mainly exerts an apoptotic effect [8-11]. The exploration of the mechanism underlying As$_2$O$_3$-induced myeloid differentiation in acute promyelocytic leukemia was mainly focused on PML/RAR-α protein degradation [12, 13]. Differentiated cells induced by treatment with As$_4$S$_4$ have been observed in HL-60 cells [2, 18]. In these studies, 2 to 8 μM of As$_4$S$_4$ induced cell differentiation as assessed by CD11b expression, without incurring apoptosis. In our present study, 8 and 16 μM As$_2$S$_2$ significantly induced HL-60 cell differentiation by 17 and 5.5 times that of the control, respectively.
Reactive oxygen species play critical roles in cell proliferation, differentiation and apoptosis by eliciting a variety of cellular responses. Low concentrations of reactive oxygen species promote cell survival and proliferation; however, once the reactive oxygen species exceed the elimination capacity of antioxidants including glutathione, the cells suffer the so-called “oxidative stress”.

(Fig. 2), and the percentage of CD11b-positive cells in cells treated with 8 μM As2S2 was more than 3 times higher than that in cells treated with 16 μM As2S2 (Fig. 2). Interestingly, 8 μM As2S2 attenuated cell apoptosis and increased the viability of HL-60 cells, whereas 16 μM As2S2 significantly promoted cell apoptosis and decreased cell viability in a time-dependent manner (Fig. 3).

Fig. 8. Loss of mitochondrial membrane potential related to As2S2-induced differentiation and apoptosis in HL-60 cells. (A) Changes in mitochondrial membrane potential in cells treated with As2S2 at concentrations of 8 and 16 μM for 24 to 72 h, which were defined as: (sample mean fluorescence intensity/control mean fluorescence intensity) × 100%. (B) Loss of mitochondrial membrane potential in cells treated with 8 and 16 μM As2S2 for 48 h. Data are the mean ± standard deviation of 3 independent experiments. *p < 0.05 as compared with control (0 μM As2S2). #p < 0.05 as compared to cells treated with 8 μM As2S2. (C) Changes in mitochondrial membrane potential, the expression level of CD11b expression, and the amounts of activated p38 MAPK are presented as the relative mean fluorescence intensity. Reactive oxygen species play critical roles in cell proliferation, differentiation and apoptosis by eliciting a variety of cellular responses. Low concentrations of reactive oxygen species promote cell survival and proliferation; however, once the reactive oxygen species exceed the elimination capacity of antioxidants including glutathione, the cells suffer the so-called “oxidative stress”,
leading to severe dysfunction and cell death [23]. The amounts of cellular reactive oxygen species generated by 8 μM As$_2$S$_2$ was significantly lower than that generated by 16 μM As$_2$S$_2$ (Fig. 4). Whereas, when a high level of oxidative stress was additionally provided by H$_2$O$_2$, As$_2$S$_2$-induced differentiation was attenuated and apoptosis was enhanced in HL-60 cells (Fig. 5). These results suggested that a moderate oxidative stress promotes cell proliferation and differentiation, while more severe oxidative stress induces apoptosis and decreases viability in HL-60 cells.

Glutathione is the main non-protein antioxidant in cells, and provides electrons for enzymes such as glutathione peroxidase, which reduce H$_2$O$_2$ to H$_2$O. Glutathione is also known to protect cells from toxic insult by detoxifying toxic metabolites of drugs and reactive oxygen species [24, 25]. A previous work revealed that intracellular glutathione levels were regulated in a complex fashion during the maturation of HL-60 cells [26], in which a small transient increase in glutathione levels was observed during cell differentiation induction triggered by 1α,25-dihydroxyvitamine D$_3$ or dimethyl sulfoxide. They further demonstrated that transient glutathione depletion as a result of using low doses of glutathione-depleting agents enhanced the differentiation
process [26]. In the present study, when HL-60 cells were treated with As$_2$S$_2$ at concentrations of 8 and 16 μM, cellular glutathione amount transiently increased in response to reactive oxygen species generation (Fig. 6A). Furthermore, as the cellular reactive oxygen species rose to the maximum level, cellular glutathione amount simultaneously depleted to the minimum (Fig. 6A). The degrees of reactive oxygen species generation associated with glutathione reduction in cells treated with low concentration (8 μM) of As$_2$S$_2$, which induced cell differentiation, were lower than those in cells treated with high concentration (16 μM) of As$_2$S$_2$, which induced apoptosis. Oxidative stimuli are known to increase expression of the rate limiting enzyme in glutathione synthesis and hence elevate glutathione levels [27]. The initial increase in the amount of reactive oxygen species may result in a transiently high level of glutathione. However, severe oxidative stress caused by the addition of H$_2$O$_2$ or the treatment with high concentration of As$_2$S$_2$ (16 μM) exceeded the elimination capacity of antioxidants such as glutathione, which ultimately attenuated differentiation and induced apoptosis in HL-60 cells (Fig. 3 and Fig. 5).

Many studies have reported mitochondrial membrane potential-dependent apoptosis induced by arsenic [14, 17, 18]. In the present work, loss of mitochondrial membrane potential was also observed in the apoptosis induced by high concentration (16 μM) of As$_2$S$_2$ in HL-60 cells. On the other hand, much less has been revealed about the role of mitochondrial membrane potential in cell differentiation. Recently, the decrease of mitochondrial membrane potential was reported in As$_4$S$_4$-induced differentiation of HL-60 cells [28], in which it was shown that the decreased mitochondrial membrane potential in 6 μM As$_4$S$_4$-induced cell differentiation was mild and did not lead to apoptosis. In the present study, loss of mitochondrial membrane potential was observed in cells treated with 8 μM As$_2$S$_2$, a concentration which induces cell differentiation. Whereas, severe and irreversible loss of mitochondrial membrane potential was observed in cells treated with 16 μM As$_2$S$_2$, a concentration which induces cell apoptosis. The mitochondria can be viewed as a central regulator of the decision between cell survival and death [29]. The Bcl-2 family proteins play important roles in the regulation of apoptosis by targeting the mitochondria to exert their proapoptotic or antiapoptotic effects. There were evidences that the antiapoptotic function of Bcl-2 is dependent on its phosphorylation status rather than its expression level [17, 30]. A detailed analysis of relationship between the loss of mitochondrial membrane potential and the expression levels of Bcl-2 family proteins is currently underway.

p38 MAPK acts as a sensor of moderate oxidative stress, and is activated in a reactive oxygen species-dependent manner [19]. It was considered that both the extent and duration of activation of p38 MAPK contribute to the determination of cell fate, such as survival, differentiation and apoptosis [31]. Interestingly, p38 MAPK has previously been shown to mediate both proapoptotic/growth inhibitory [32] and antiapoptotic/progrowth [33] signals in different systems, apparently depending on the stimulus and cell type involved. We showed that p38 MAPK inhibitor (SB203580) enhanced the CD11b expression level (Fig. 9A). A recent paper has demonstrated that inhibition of p38 MAPK enhances differentiation induced by As$_2$O$_3$ in NB4 cells, suggesting that p38 MAPK signaling cascade is activated in a negative regulatory feedback manner to attenuate the anti-leukemic effects of As$_2$O$_3$ [14]. Our present study demonstrated that the activation of p38 MAPK was observed, followed by the accumulation of intracellular reactive oxygen species. Interestingly, expression levels of p38 MAPK decreased after treatment with 8 μM but increased with 16 μM treatment of As$_2$S$_2$ for 1 h (Fig. 7A), suggesting that much stronger stimuli was produced by 16 μM of As$_2$S$_2$ during the short period of time. Although an increase of apoptotic cells was accompanied by the activation of p38 MAPK (Fig. 8C), almost no alteration in the number of apoptotic cells was observed after treatment with As$_2$S$_2$ in combination with p38 MAPK inhibitor (Fig. 9B). Taking these observations into account, we suggest that the activation of p38 MAPK resulting from oxidative stress is implicated in the negative regulation of cell differentiation, rather than apoptosis induction, in HL-60 cells.
CONCLUSIONS
Our results demonstrated that As$_2$S$_2$ induces both differentiation and apoptosis depending on its dose in HL-60 cells. The results also suggest that moderate level of cellular reactive oxygen species is implicated in 8 μM As$_2$S$_2$-induced differentiation, while severe oxidative stress induced by 16 μM As$_2$S$_2$ leads to cell apoptosis. The generation of reactive oxygen species is suggested to be a contributing factor to the transient increase in glutathione, and it further involves in differentiation and apoptosis induction. We also suggest that activation of p38 MAPK was implicated in As$_2$S$_2$-induced differentiation rather than apoptosis induction in the HL-60 cells. Our present observations concerning biphasic actions of As$_2$S$_2$ related to dose imply the rationale and future directions of As$_2$S$_2$ as a potential anticancer drug candidate.

ACKNOWLEDGMENTS
This work was supported in part by grants from Japan-China Medical Association to Bo Yuan and Xiao-Mei Hu.

CONFLICT OF INTEREST STATEMENT
The authors declare that they have no conflicts of interest to disclose.

REFERENCES