Action Mechanisms of Arsenic Compounds in Leukemia Cells

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ABBREVIATIONS

AA	ascorbic acid
AML	acute myeloid leukaemia
APL	acute promyelocytic leukemia
As_2O_3	arsenic trioxide
As_2S_2	arsenic disulfide
BSO	L-buthionine-[S,R]-sulfoxide
DCFH-DA	2',7'-dichlorofluorescein diacetate
DNR	daunorubicin
FBS	fetal bovine serum
GSH	glutathione
H_2O_2	hydrogen peroxide
MFI	mean fluorescence intensity
MAPKs	mitogen activated protein kinases
MMP	mitochondrial membrane potential
MDR	multidrug resistance
MDS	myelodysplastic syndromes
NAC	N-acetylcysteine
NBT	nitroblue tetrazolium
P-gp	P-glycoprotein
PBS	phosphate-buffered saline
PI	propidium iodide
QHP	Qinghuang Powder
ROS	reactive oxygen species
R- PE	r-phycoerythrin
Rh123	rhodamine 123
SD	standard deviation

INTRODUCTION

Arsenic is a natural substance that has been used medicinally for over 2400 years [130]. In 1878, potassium arsenite was reported to have an anti-leukemic effect and was used for this purpose in the late 19th and early 20th centuries until it was replaced by busulfan in the 1950s [32, 34, 111]. In the modern era, interest in arsenic as a chemotherapy was rekindled after it was identified as an active ingredient in traditional medicines in China [24, 54, 68]. Arsenic drugs have been generally used for the treatment of malignant hematologic diseases [54, 114]. Although arsenic has been known as a poison, but it has been generally well tolerated as a therapeutic entity.

As_2O_3 has been confirmed to be an effective treatment for acute promyelocytic leukemia (APL) both in patients with newly diagnosed APL and in those with refractory and relapsed APL [42, 43, 91, 109, 112, 115, 142]. The inhibitory effects of As_2O_3 have been shown not only in parental APL NB_4 cells [14], but also in retinoic acid-resistant APL cells [9]. It has been reported that the effects of As_2O_3 are not confined to APL cells but can also be observed in various other cell lines of myeloid [125] and lymphoid origin [108, 143, 144] and in drug resistant sublines [31, 97]. The mechanisms of action of As_2O_3 in APL and other malignancies are thought to involve inhibition of growth and induction of apoptosis [1, 9, 14, 24, 143, 144].

MOLT-4, a human T-lymphoblastoid leukemia cell line, has been used extensively for studies of leukemia cell biology and antileukemia therapy [4, 25]. We have established a daunorubicin resistant MOLT-4 subline (MOLT-4/DNR) by exposing the parental MOLT-4 cells stepwise to increasing concentrations of DNR over 3 months [77]. These resistant MOLT-4/DNR cells have been shown to overexpress functional P-glycoprotein (P-gp) and MDR1 mRNA [77]. P-gp, a product of the multidrug resistance (MDR) gene, is a transmembrane efflux pump for different lipophilic compounds, including many anticancer drugs and fluorescent dyes [11, 12]. Intracellular drug accumulation in cells expressing functional P-gp has been found to be decreased as a result of drug efflux [26]. Most of the drugs excreted via this efflux pump are hydrophobic organic compounds, and As_2O_3 may not be excluded from drug-resistant cell lines expressing functional P-gp. However, whether As_2O_3 affects the growth of lymphocytic leukemia cells expressing functional P-gp, or the expression and/or function of P-gp, is unknown. Some data indicate that As_2O_3 is not sensitive to the drug efflux pump mechanisms of resistance [26, 142].

Thus, in Chapter 1, the effects of As_2O_3 on the growth of and apoptosis in parental MOLT-4 and resistant MOLT-4/DNR cells were investigated. These suppressive effects of As_2O_3 were also related to P-gp expression or function in MOLT-4/DNR cells.

Some reports suggest that sensitivity to As_2O_3 correlates with intracellular glutathione (GSH) levels in cancer cells [138]. The GSH content modulates the growth-inhibitory and apoptosis-inducing effects of arsenicals [18, 60, 92, 93, 105]. Cells expressing higher levels of GSH or GSH-associated enzymes are less sensitive to As_2O_3 than cells expressing lower levels of these molecules [60, 72]. Arsenic

resistant cells are also reported to contain higher levels of GSH [66, 72, 78]. Moreover, cells with increased GSH levels can be sensitized to As_2O_3 by agents that deplete intracellular GSH [18, 66]. The cytotoxic effects of As_2O_3 may be influenced by modulators of GSH [31, 33, 67, 89], especially in drug-resistant cell lines [28, 100]. A decrease in cellular GSH level acts as a potent early activator of apoptosis signaling [3]. Depletion of GSH, especially mitochondrial GSH, is believed to induce the loss of mitochondrial membrane potential (MMP) [84]. Mitochondrial permeability transition resulting from intracellular thiol depletion is known to be a critical event in apoptosis [75, 136]. Mitochondria undergoes major changes in membrane integrity before classical signs of apoptosis become manifest, and these changes lead to disruption of the MMP [116]. Mitochondrial depolarization precedes caspase-3 activation and apoptosis [107], and apoptotic cells express high caspase-3 activity [56, 147]. Caspase-3 is considered to be a primary executioner of apoptosis [10]. Caspase-3 activation has been causally related to the release of mitochondrial cytochrome c in the cytoplasm as a result of the collapse of the MMP [76]. As_2O_3 is known to act at several points in apoptosis induced through mitochondrial pathways [19], which includes the forming of reversible bonds with thiol groups [118] and the depletion of GSH [18, 66, 93, 138], loss of MMP [58], and activation of caspase-3 [28, 58, 59, 83, 96].

From these points of view, the apoptosis-inducing effects of As_2O_3 in the presence of GSH modulators in MOLT-4 and MOLT-4/DNR cells were examined in Chapter 2.

It has been recognized that benefit and risk of arsenic are strictly dependent on the individual chemical forms of arsenic instead of arsenic content alone [17]. Although As_2O_3 has been confirmed to be an effective treatment for APL [42, 127], which acts through a variety of mechanisms involving numerous signal transduction pathways, and its cellular effects were mediated through apoptosis induction, growth inhibition and differentiation induction [6, 64, 88]. However, serious adverse drug reaction induced by As_2O_3 was occasionally reported, which deters some physicians from using it clinically [27].

Arsenic disulfide (As_2S_2), the most important component of *Xiong huang* which contains > 90% As_2S_2 based on the quality standard in *Chinese Pharmacopeia* in 2010 edition, was a candidate for its good therapeutic reputation and perceived low toxicity in traditional medicines, and the drug may become another research focus following As_2O_3. Actually, the clinical use of arsenic-containing formulae to treat malignant hematologic diseases in China occurred as early as the beginning of medical use of As_2O_3 [54]. Especially, the famous traditional arsenic-containing formula, Qinghuang Powder (QHP)**,** which includes *Qing Dai* (Indigo naturalis) and *Xiong huang* in the formula, was reported to improve the clinical outcomes of hematologic malignancies in our clinical trials [49], and its therapeutic efficacy could be enhanced by increasing the *Xiong huang* content in the formula [145], which could be attributed to As_2S_2. Some patients treated with these regents have been reported to survive for 9-20 years with relatively good oral safety profiles [126]. These give As_2S_2 an advantage over As_2O_3 in maintenance treatment. Many results indicated that As_2O_3 induces apoptosis

in tumor cells by affecting the mitochondria and generation of reactive oxygen species [36, 40, 29, 63]. Mitogen activated protein kinases (MAPKs) are a family of enzymes that transduce signals from the cell membrane to the nucleus in response to a wide range of stimuli, and modulate several important biological functions including gene expression, mitosis, proliferation, motility, and apoptosis. Three major groups of MAPKs exist, the extracellular signal–regulated kinase, c-jun NH2-terminal kinase, and p38 MAPK families [98]. Roles of p38 MAPK in relation to reactive oxygen species (ROS) generation, GSH level and apoptosis induced by As_2O_3 have been demonstrated [40, 29, 63]. As_2S_2-mediated growth inhibition and apoptosis induction have been found in leukemia K562 cells [73], human ovarian and cervical cancer cells [132], and other cells [120]. However, the effects of As_2S_2 on HL-60 cells with a particular focus on proliferation, differentiation, oxidative stress associated with generation of reactive ROS and intracellular GSH depletion, and activation of p38 MAPK have not been addressed.

Thus, in Chapter 3, the effects of As_2S_2 on HL-60 cells were investigated by focusing on proliferation, differentiation, generation of ROS, intracellular GSH depletion, and activation of p38 MAPK.

Patients with AML from myelodysplastic syndrome (MDS/AML) have higher probabilities of multidrug resistance to chemotherapy, lower rates of complete remission, and the poor prognosis [71]. Univariate analyses showed that the hemoglobin level and cytogenetic abnormalities were factors that contributed to the overall survival [68]. The data have shown that MDS/AML patients tended to have complex type abnormalities including monosomy-7, which is considered to be an unfavorable risk subgroup [34, 68]. In our recent clinical trials, we have revealed that QHP is effective in the treatment of MDS, especially in patients with the erythroid improvement is significant, without serious adverse drug reaction observed even on long time [133-135]. Furthermore, a high responding rate (80.8%) was also observed in patients with blast cell-decrease and hematologic improvement having moderate- and high-risks, by increasing the daily dose of QHP [74]. These results indicated that QHP is effective in treating MDS or MDS/AML even in patients with the poor karyotype, and its therapeutic efficacy could be enhanced by increasing the *Xiong huang* content in the formula, which could be attributed to As_2S_2**.** F-36p cell line was established from a patient diagnosed as refractory anemia with excess blasts, the disease progressed to overt leukemia at approximately 5 months after the initial diagnosis [15]. The majority of the established cells had a homogeneous karyotype. F-36p cells can be induced to differentiate into the erythroid lineage in the presence of erythropoietin.

Thus, in Chapter 4, the effective mechanisms of As_2S_2 in the treatment of MDS or MDS/AML were studied using F-36p cell line.

Chapter 1
Arsenic trioxide induces apoptosis equally in T lymphoblastoid leukemia MOLT-4 cells and P-gp-expressing daunorubicin-resistant MOLT-4 cells

1.1 INTRODUCTION

As_2O_3 has recently been confirmed to be an effective treatment for APL both in patients with newly diagnosed APL and in those with refractory and relapsed APL [42, 43, 91, 109, 112, 115, 142]. The effects of As_2O_3 have been shown not only in parental APL NB_4 cells [14], but also in retinoic acid-resistant APL cells [9].

MOLT-4, a human T-lymphoblastoid leukemia cell line, has been used extensively for studies of leukemia cell biology and antileukemia therapy [4, 25]. A daunorubicin resistant MOLT-4 subline, MOLT-4/DNR, has been established by exposing the parental MOLT-4 cells stepwise to increasing concentrations of DNR over 3 months [77]. MOLT-4/DNR cells have been shown to overexpress functional P-gp and MDR1 mRNA [77].

In this Chapter, the effects of As_2O_3 on the growth of and apoptosis in parental MOLT-4 and resistant MOLT-4/DNR cells were investigated, and the possible mechanisms of growth suppression by As_2O_3 in these cell lines were discussed.

1.2 MATERIALS AND METHODS

1.2.1 Reagents

RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, N.Y.). Cell proliferation kits I and II (MTT) were purchased from Roche Diagnostics (Indianapolis, Ind.). Trypan blue, Rh123, DNR and As_2O_3 were obtained from Sigma Chemical Company (St. Louis, Mo.). DNR stock solutions were made at a concentration of 10 mmol/l with ethanol and diluted to working concentrations before use. As_2O_3 stock solutions were made at a concentration of 5 mmol/l with phosphate-buffered saline (PBS) and diluted to working concentrations before use. Mouse anti-human P-gp monoclonal antibody conjugated with Rphycoerythrin (R-PE) (monoclonal antibody 17F9) and R-PEconjugated mouse IgG2b isotype control monoclonal antibody were obtained from PharMingen (San Diego, Calif.). The annexin V-FITC apoptosis detection kit I was obtained from BD PharMingen.

1.2.2 Cell culture

MOLT-4 and MOLT-4/DNR cells were maintained in RPMI-1640 medium containing 10% FBS, 100 U/ml penicillin and 100 μg/mL streptomycin as described previously [37, 38]. The leukemia cells were washed and resuspended with the above medium to 5×10^5 cells/mL, then 196 μl of the cell suspension was placed in each well of a 96-well flat-bottomed plate. To the suspension in each well was added 4 μl PBS solution containing As_2O_3 and 4 μl ethanol solution containing DNR to yield final concentrations of 0.25, 0.5, 1, 2, 4, 5, 6, 8 and 16 μmol/l and 0.001, 0.01, 0.1, 1 and 10 μM, respectively; 4 μl PBS and 4 μl ethanol were added to the control wells,

respectively. The cells were incubated for 96 h in an atmosphere of air containing 5% CO_2 at 37 °C in a humidified chamber.

1.2.3 MTT assay

After the incubation period, 10 μl of 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 solubilization solution (Roche) was added to each well and the plate was placed on a microshaker for 10 s, after which the plate was left overnight in an incubator in a humidified atmosphere. The spectrophotometric absorbance of the samples was measured on a microplate reader (Corona MT P-32; Corona Company, Japan) at 570 nm. A dose response curve was plotted for each drug, and the concentrations that yielded a 50% inhibition of cell growth (IC_{50}) were calculated.

1.2.4 Cell viability assay

MOLT-4 and MOLT-4/DNR cell lines were cultured by initially seeding 2×10^5 cells/mL of fresh RPMI-1640 medium containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin in the presence of various concentrations of As_2O_3 for 24 to 144 h in an atmosphere of air containing 5% CO_2 at 37 °C in a humidified chamber. To avoid any possible effects of cell density on cell growth and survival, cells were maintained at less than 5×10^5 cells/mL by daily adjusting the cell concentration by adding fresh culture medium and the corresponding concentrations of As_2O_3 when necessary. The number or percentage of viable cells was determined by staining the cell populations with trypan blue. Before carrying out the experiments, four parts of 0.2% trypan blue (w/v in water) were mixed with one part of saline (4.25% NaCl w/v in water), and one part of the trypan blue saline solution was added to one part of the cell suspension. The cells were then loaded into a hemocytometer and the number of unstained (viable) cells and stained (dead) cells were counted separately within 3 min of staining with trypan blue. Actual cell numbers were calculated by multiplying the initial cell numbers by the times dilution.

1.2.5 Morphological changes in As_2O_3-treated MOLT-4 and MOLT-4/DNR cells

MOLT-4 and MOLT-4/DNR cells suspended at 5×10^5 cells/mL in fresh medium were cultured in the presence or absence of As_2O_3 for 24 to 144 h in an atmosphere of air containing 5% CO_2 at 37 °C in a humidified chamber. At the indicated times, pictures were taken under a microscope (Olympus Optical Company, Japan).

1.2.6 P-gp expression analysis

MOLT-4 and MOLT-4/DNR cells suspended at 1×10^6 cells/mL in fresh medium were cultured in the presence or absence of As_2O_3 for 3 to 24 h in an atmosphere of air containing 5% CO_2 at 37 °C in a humidified chamber. At the indicated times, the cells were collected and washed twice in washing buffer

(PBS/0.1% NaN_3, pH 7.2). Cells were resuspended in 50 μl buffer and then incubated with either 20 μl of monoclonal antibody 17F9 or 20 μl of the R-PEconjugated mouse IgG2b isotype control monoclonal antibody for 30 min in the dark. The cells were then centrifuged again in washing buffer (PBS/0.1% NaN_3, pH 7.2) at 1300 rpm for 5 min at 4 °C. The cells were resuspended in 400 μl staining buffer (PBS/ 0.1% NaN_3/1% FBS, pH 7.2) and P-gp expression was determined by flow cytometry (Becton Dickinson). The data were analyzed with Cell Quest software (Becton Dickinson).

1.2.7 P-gp efflux function

Cells (1×10^6) were collected and centrifuged at 1300 rpm for 5 min at 4 °C, resuspended in buffer containing 5 μg/mL of Rh 123, and left to stand for 10 min in an atmosphere of air containing 5% CO_2 at 37 °C. After washing, the cells were incubated in the presence or absence of As_2O_3 or inhibitor cyclosporine A for the indicated times in an atmosphere of air containing 5% CO_2 at 37 °C. After incubation, the cells were washed twice in washing buffer and resuspended in 400 μl staining buffer. The remaining intracellular Rh 123 fluorescence intensity was determined by a flow cytometry (Becton Dickinson). The data were analyzed with Cell Quest software (Becton Dickinson).

1.2.8 Apoptosis assays

MOLT-4 and MOLT-4/DNR cells (1×10^6/mL) were treated with the indicated concentrations of As_2O_3. After the indicated incubation times, cells were washed twice in cold PBS (pH 7.2) and resuspended in binding buffer at a density of 1×10^6 cells/mL, and then 1×10^5 cells were stained with 5 μl annexin V-FITC and 5 μl propidium iodide (Becton Dickinson) for 15 min at room temperature in the dark. Then 400 μl binding buffer was added to each tube, and the cells were analyzed by flow cytometry (Becton Dickinson) within 1 h of staining. A total of 30,000 non-gated cells were analyzed.

1.2.9 Statistics

Comparison of the data between two groups was carried out using Student's t-test. Comparison of the data among multiple (more than three) groups was carried out using the Bonferroni-Dun multiple comparison. A value of $p<0.05$ was considered significant.

1.3 RESULTS

1.3.1 Effects of As_2O_3 on parental MOLT-4 and resistant MOLT-4/ DNR cell growth

MOLT-4 and MOLT-4/DNR cells were continuously treated with As_2O_3 at concentrations in the range 0.25 to 16.0 μM for 96 h, and cell growth was measured in an MTT assay. The growth of MOLT-4 and MOLT-4/ DNR cells was suppressed by As_2O_3 treatment in a dose- dependent manner. The IC_{50} values in the parental and

subline cells were 5.1 μM and 5.0 μM, respectively (Fig. 1-1a).

At the same time, the viability of MOLT-4 and MOLT-4/DNR cells similarly decreased when the cells were incubated in the presence of As_2O_3 at a concentration of 5 μM for 24 to 144 h in a time-dependent manner (Fig. 1-2a). Following treatment with As_2O_3 for 4 days at concentrations of 1 to 16 μM, the viability of cells of both lines decreased to a similar extent in a dose-dependent manner (Fig. 1-2b).

The effects of DNR on the growth of MOLT-4 and MOLT-4/DNR cells were examined after 96 h in culture. The IC_{50} value of DNR in MOLT-4 cells assessed by the MTT assay was 0.1 μM, while in MOLT-4/ DNR cells the value was 12 times higher (1.2 μM) (Fig. 1-1b). Thus, DNR was effective against parental MOLT-4 cells, but less effective against MOLT-4/DNR cells, as expected.

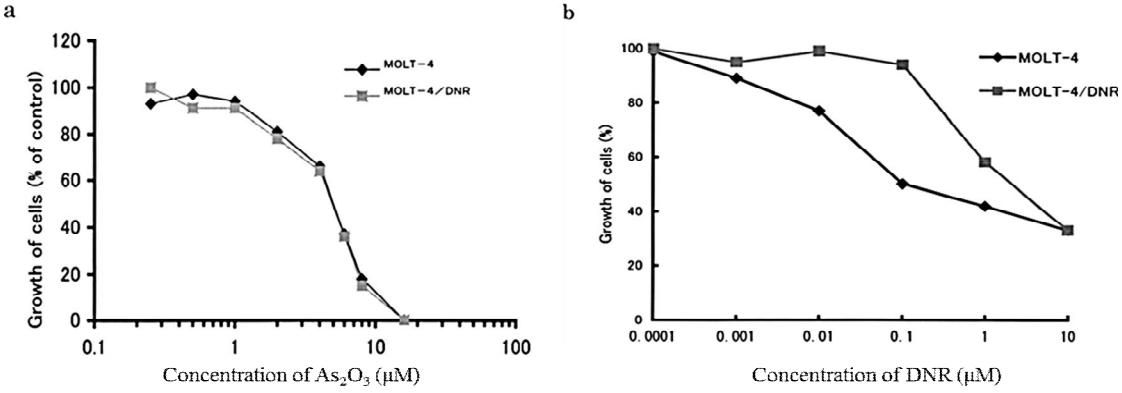


Fig. 1-1 Comparative effects of As_2O_3 (a) and DNR (b) on in vitro growth of MOLT-4 and MOLT-4/DNR cells. Cells were treated with different concentrations of As_2O_3 and DNR for 4 days. Cell growth was determined by an MTT assay. Values are the means of three independent experiments.

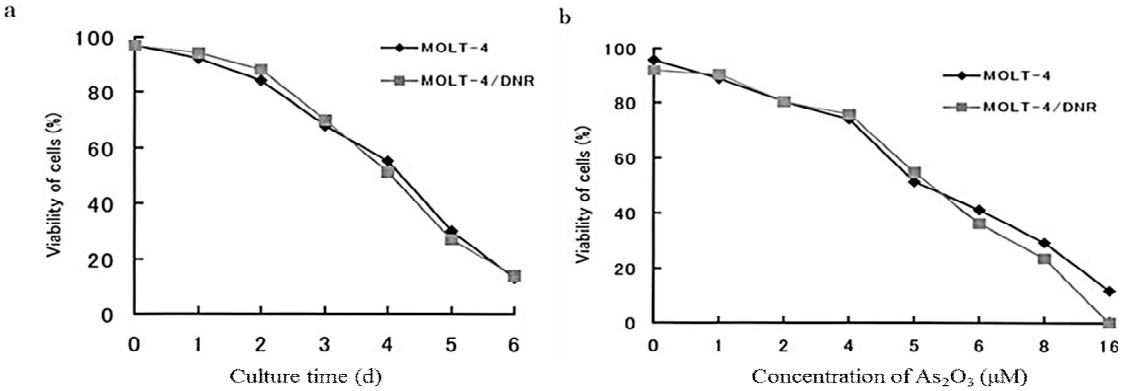


Fig. 1-2 Comparison of viabilities of cells treated with As_2O_3 as a function of culture days (a) and concentration of As_2O_3 (b) between MOLT-4 and MOLT-4/DNR cells. a: cells treated with 5 μM of As_2O_3, **b**: cells treated with the agent for 4 days. Cell viability was determined by a dye exclusion test. Values are the means of three independent experiments

1.3.2 Additive effect of As_2O_3 with DNR on MOLT-4/DNR cell growth

As described above, MOLT-4/DNR cells were less sensitive to DNR alone at concentrations < 0.1 μM, but when MOLT-4/DNR cells were cultured in the presence

of DNR combined with As_2O_3 at 2 μM, their growth was inhibited (Fig. 1-3a). The suppressive effects of As_2O_3 and DNR appeared to be additive, and depended on the dose of DNR (Fig. 1-3a).

As described above, the effects of As_2O_3 on the growth of MOLT-4/ DNR cells were observed at As_2O_3 concentrations > 2 μM (Fig. 1-1a, Fig. 1-2b). However, when the cells were treated with As_2O_3 at concentrations in the range 0.25 to 2 μM combined with 0.1 μM DNR, the effect of As_2O_3 on cell growth was additively increased (Fig. 1-3b).

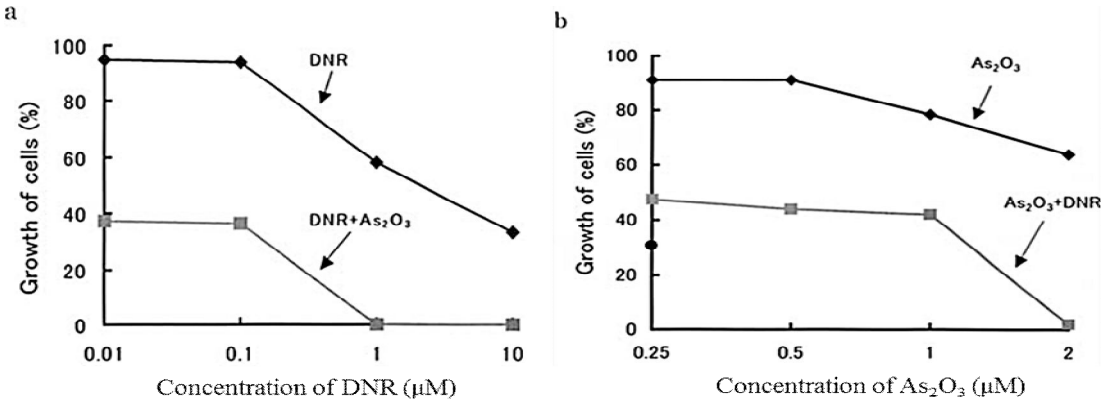


Fig. 1-3 Additive effect of DNR and As_2O_3 on the growth of MOLT-4/DNR cells. a: DNR concentrations varied, while As_2O_3 concentration was kept to be constant at 2 μM. **b:** As_2O_3 concentrations varied, while DNR concentration was kept to be constant at 0.1 μM DNR. Cells were treated for 4 days. Cell growth was determined by an MTT assay. Values are the means of three independent experiments

1.3.3 As_2O_3 did not change the P-gp expression and function of MOLT-4/ DNR cells

Staining of cells with FITC-conjugated mouse anti-human MDR monoclonal antibody revealed that only 2.5% of MOLT-4 cells expressed P-gp, whereas 93.7% of MOLT-4/DNR cells expressed P-gp (Fig.1- 4a). To evaluate the P-gp efflux function, 5μg/mL Rh123 as a P-gp substrate and 100ng/mL cyclosporine A as an MDR modulator were used in the P-gp efflux function experiments. MOLT-4/DNR cells incubated for 3 h in the absence of cyclosporine A showed a rapid decrease in intercellular Rh123 levels from 87.1 ± 0.6% to 53.3 ± 8.8% ($p<0.01$). The efflux was inhibited by cyclosporine A, and the intracellular Rh123 levels increased again to 76.6±1.2% ($p<0.05$) in the presence of 100 ng/mL cyclosporine A. In contrast, parental MOLT-4 cells still retained the dye (from 80.4 ± 3.6% to 80.8 ± 7.8%). This result showed that MOLT-4/DNR cells possess a high level of P-gp efflux activity (Fig. 1-4b).

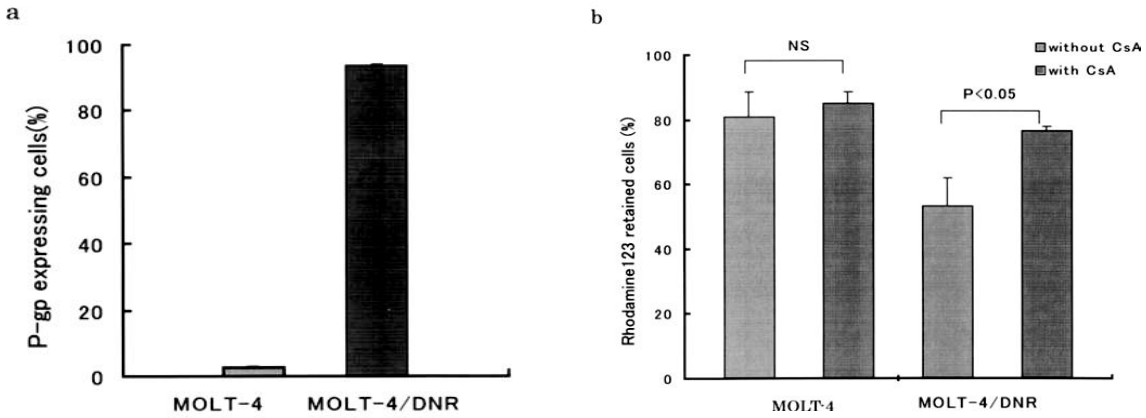


Fig. 1-4 Comparison of P-gp expression (a) and function (b) between MOLT-4 and MOLT-4/DNR cells. b; Effects of P-gp inhibitor cyclosporine A at 100 ng/mL on the P-gp expression and efflux function of MOLT-4 and MOLT-4/DNR cells. P-gp expression and function were determined by flow cytometry as described in Materials and methods. Values are the means ± SD of three independent experiments.

When MOLT-4/DNR cells were cultured in the presence of As_2O_3 at a concentration of 5 μM for 3 to 24 h, the percentages of P-gp expression did not change from those in the absence (0 h) of As_2O_3 (Fig. 1-5). When MOLT-4/DNR cells were incubated with As_2O_3 for 3, 6 or 24 h at concentrations of 1 to 8 μM, the percentage of cells expressing P-gp also did not change as compared to control (data not shown).

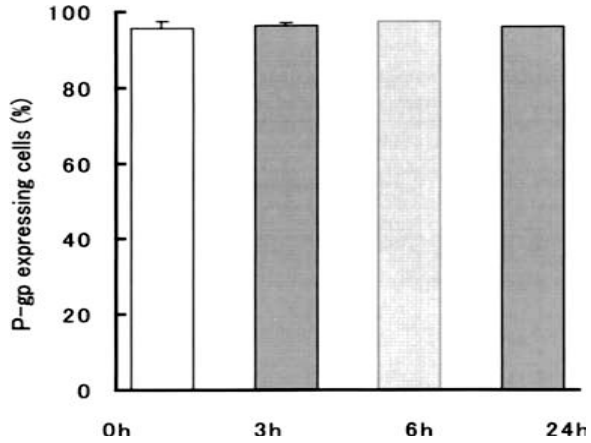


Fig. 1-5 Effect of As_2O_3 on P-gp expression of MOLT-4/DNR cells. Cells were incubated in the presence of 5 μM As_2O_3 for 3, 6 and 24 h, and the percentages of cells expressing P-gp determined by flow cytometry. Values are the means ± SD of three independent experiments.

In MOLT-4/DNR cells, intracellular Rh123 accumulation was significantly enhanced in the presence of cyclosporine A for 3 h at a concentration of 100 ng/mL ($p<0.05$, Fig. 1-6), but was not significantly changed in MOLT-4/DNR cells cultured in the presence of As_2O_3 at 5 μM ($p>0.05$. Fig. 1-6).

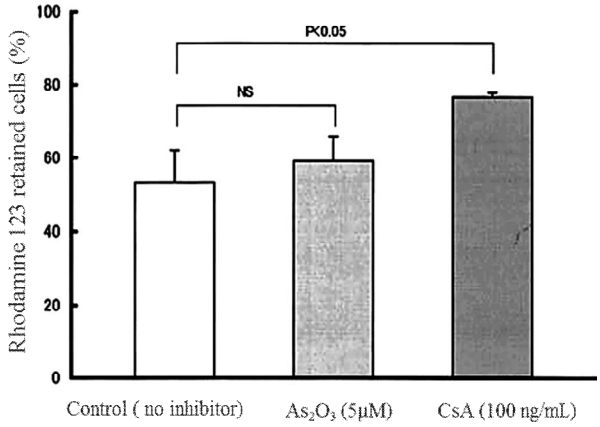


Fig. 1-6 Comparison of As_2O_3 and cyclosporine A **in inhibiting P-gp efflux function.** P-gp efflux function was determined by flow cytometry as described in Materials and Methods. Values are the means ± SD of three independent experiments.

1.3.4 As_2O_3 induces apoptosis in cells of both the MOLT-4 and MOLT-4/DNR cell line

MOLT-4 and MOLT-4/DNR cells showed a similar round morphology after culture for 96 h (Fig. 1-7a, b), but after treatment with various concentrations of As_2O_3 for 96 h, cells of both lines exhibited the typical morphological characteristics of apoptosis including chromatin condensation and fragmentation of nuclei (Fig. 1-7c, d). Numerous apoptotic bodies, which are membrane-enclosed vesicles that have budded off cytoplasmic extensions, were also observed in these cells (Fig.1- 7c, d).

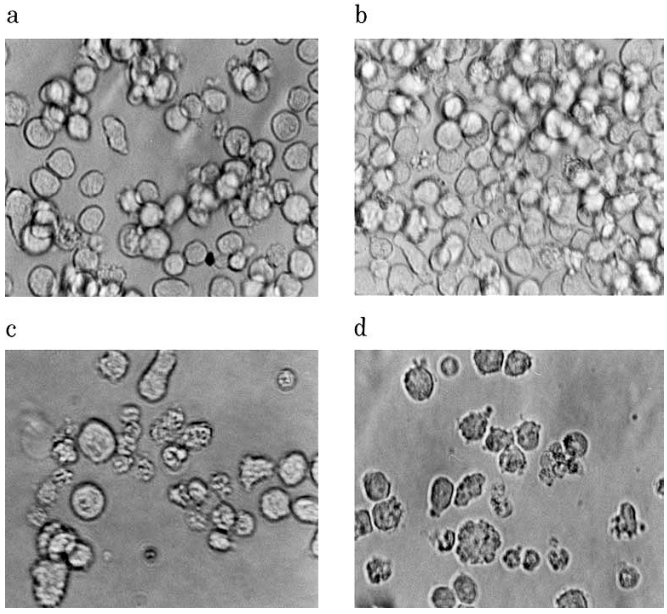


Fig. 1-7 Cell morphology after treatment with 5 μM As_2O_3 for 4 days. a; untreated MOLT-4 cells, **b;** untreated MOLT-4/DNR cells, **c;** As_2O_3-treated MOLT-4 cells, **d;** As_2O_3-treated MOLT-4/DNR cells

In MOLT-4 and MOLT-4/DNR cells cultured in the presence of As_2O_3 for 4 days at concentrations of 1 to 8 μM, the percentage of apoptotic cells as determined by annexin V increased in a dose-dependent manner (Fig. 1-8a), and when these cells were incubated with As_2O_3 at a concentration of 5 μM for 3 to 144 h, the percentage of apoptotic cells increased in a time-dependent manner (Fig. 1-8b).

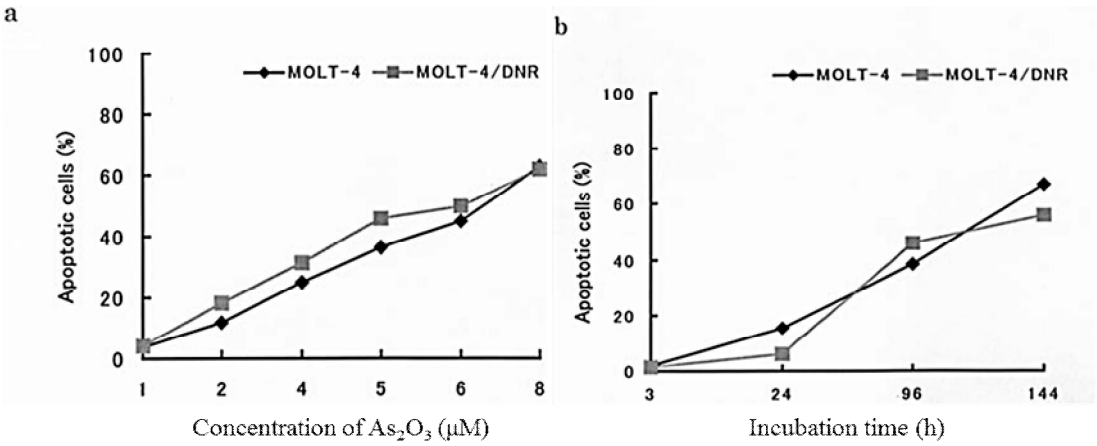


Fig. 1-8 Induction of apoptosis by As_2O_3 in MOLT-4 and MOLT-4/ DNR cells. a; Percent apoptotic cells as a function of As_2O_3 concentration after culture for 4 days, **b;** Percent apoptotic cells as a function of incubation time in the presence of 5 μM As_2O_3. Values are the means of three independent experiments.

1.4 DISCUSSION

The results described above showed that As_2O_3 inhibited growth and induced apoptosis equally in MOLT-4 cells and MOLT-4/DNR cells. The results also suggested that the effects of As_2O_3 on growth of MOLT-4/DNR cells were not due to suppression of P-gp expression or function in these cells.

The MOLT-4/DNR cell line was derived in our laboratory from the parental MOLT-4 cell line by exposing the cells stepwise to increasing concentrations of DNR [77], and the cell line was shown to be persistently resistant to DNR. The IC_{50} value of DNR in MOLT-4/DNR cells was 12 times higher than that in parental MOLT-4 cells as assessed by the MTT assay. The resistance of MOLT-4/DNR cells to DNR has been reported to be closely correlated with the expression of functional P-gp [77].

MDR is recognized as one of the most common causes of failure of chemotherapy in the treatment of cancer patients [45, 46, 47]. P-gp is an ATP-binding cassette transporter, which hydrolyses ATP and expels cytotoxic drugs from mammalian cells [102]. The fluorescent dye Rh123 has been found to be transported by P-gp [22, 39, 90], and the flow cytometric measurement of cellular Rh123 uptake/efflux is an efficient tool to assess the functional activity of P-gp in tumor samples [79, 80]. In this work, we revealed that 94% of MOLT-4/DNR cells express P-gp, and the percentage of P-gp expression in MOLT-4/DNR cells was significantly higher than that in MOLT-4 cells (less than 3%). Furthermore, MOLT-4/DNR cells exhibited significantly higher P-gp efflux activity than parental MOLT-4 cells. However, both parental MOLT-4 and resistant MOLT-4/DNR cell lines were highly sensitive to As_2O_3. As_2O_3 similarly inhibited growth and induced apoptosis in these two cell lines. These effects of As_2O_3 were time- and dose- dependent. These results suggest that P-gp in MOLT-4/DNR cells is not involved in the detoxification of As_2O_3.

Indeed, the expression of P-gp in resistant MOLT-4/ DNR cells did not revert in the presence of As_2O_3 at concentrations of 1 to 8 μM, which were efficiently cytotoxic

for the cell line. The efflux function of P-gp in MOLT-4/DNR cells also did not change in the presence of various concentrations of As_2O_3. These observations confirm our conclusion that As_2O_3 exhibited cytotoxicity without influencing functional P-gp in the resistant MOLT-4/DNR cells.

Arsenic is a natural substance that has been used medicinally for over 2400 years [130]. Recent clinical studies in China have shown that As_2O_3 is an effective and relatively safe drug in the treatment of APL [42, 43, 109, 142]. As_2O_3 can inhibit growth and trigger apoptosis in cells of the APL cell line NB4 [13, 14]. In particular, As_2O_3 is also effective in APL patients who are resistant to all-trans-retinoic acid (ATRA) and conventional chemotherapy [9, 13]. In some in vitro studies, the growth and survival of ATRA-resistant APL subline cells have been found to be inhibited effectively by As_2O_3 [9,14]. In addition, the suppressive effect of As_2O_3 is not specific for APL cells, but can be observed in various cell lines of myeloid [125], lymphoid [108, 143, 144] or T-cell origin [23, 57]. Among multiple myeloma cells, the P-gp-positive cell line 8226/Dox40 undergoes apoptosis in response to As_2O_3 in vitro, in a similar manner to the drug-sensitive cell line 8226/S [31]. Otherwise, As_2O_3-induced apoptosis in HL-60/VCR and HL-60/ADR cells is not significantly different from that in HL-60/neo cells [97]. Thus, previous results suggest that As_2O_3 is not a substrate for the mdr1 gene-encoded P-gp. In this study, we demonstrated that As_2O_3 is not sensitive to drug efflux pump mechanisms of resistance.

Studies on clinical pharmacokinetics of As_2O_3 have shown that the peak level of plasma As_2O_3 concentration under a general treatment schedule is 5.5 to 7.3 μM [109]. In the present study, the concentrations of As_2O_3 inducing apoptosis in vitro were from 1 to 8 μM in a dose- and time-dependent manner in both MOLT-4 and MOLT-4/DNR cells. A remarkably effective concentration of As_2O_3 inducing apoptosis in these cell lines was 5 μM, which is not over the peak level in clinical trials.

Previous studies based on experiments with the APL cell line NB4 have indicated that As_2O_3 causes apoptosis directly through down- regulation of bcl-2 [13]. On the other hand, the GSH redox system is known to modulate the growth-inhibitory effect of arsenicals [18, 92]. It was found that the sensitivity to As_2O_3-induced apoptosis is inversely related to the intracellular GSH content and that pharmacological modulation of intracellular GSH contents influences sensitivity to As_2O_3 [18]. Thus, the effects of As_2O_3 in inhibiting growth and inducing apoptosis in the parental MOLT-4 and the resistant MOLT-4/ DNR cells may possibly be related to the regulation of suppressor gene (s) particularly bcl-2 and the GSH redox system.

Since As_2O_3 showed no effect on P-gp expression and function in MOLT-4/DNR cells, the additive efficacy of As_2O_3 with DNR possibly results from the apoptosis-inducing effect of As_2O_3. This apoptosis- inducing effect of As_2O_3 is possibly caused by regulation of bcl-2 and/or depletion of GSH from the leukemic cells [13, 18, 92]. DNR is known to block DNA replication and RNA synthesis, and therefore, the apoptosis-inducing effect of As_2O_3 is possibly additive to (or synergistic with) the pharmacological action of DNR against the growth of MOLT-4/DNR cells. While P-gp may, in part, be involved in the efflux of DNR out of the cells in the

presence of As_2O_3 in MOLT- 4/DNR cells, a combination of these drugs may work additively through different growth-suppressing mechanisms. This additive effect can cause a considerable shift in the dose-response curve of each agent. These findings may imply potential clinical uses of DNR in combination with As_2O_3 for the treatment of patients with T-lymphoblastoid leukemia exhibiting DNR resistance.

1.5 CONCLUSION

The data in this chapter demonstrated that As_2O_3 is effective in suppressing growth and inducing apoptosis in both parental MOLT-4 cell line and its DNR-resistant MOLT-4/DNR cell line expressing functional P-gp. These suppressive effects of As_2O_3 are suggested not to be caused by inhibition of P-gp expression or function in MOLT-4/DNR cells.

Chapter 2

Arsenic trioxide induces apoptosis in cells of MOLT-4 and its daunorubicin-resistant cell line via depletion of intracellular glutathione, disruption of mitochondrial membrane potential and activation of caspase-3

2.1 INTRODUCTION

In response to the observations that patients with chemorefractory APL still respond to As_2O_3 therapy [42, 109, 112] and in vitro studies of the effects of As_2O_3 on drugresistant APL cell lines [30], investigations have been initiated to evaluate the therapeutic potential of As_2O_3 in several malignant diseases [108, 143, 144]. Most of the reports suggest that As_2O_3 suppresses growth and induces apoptosis in malignant cells including drug-resistant cells [13, 30, 108, 143, 144].

As shown in Chapter 1, As_2O_3 is effective in growth suppression and apoptosis induction in both MOLT-4 and MOLT-4/DNR cells.

Then, in this Chapter, the apoptosis- inducing mechanisms of As_2O_3 in MOLT-4 and MOLT-4/DNR cell lines were investigated from the viewpoint of modification of cellular GSH levels, MMP, and caspase-3.

2.2 MATERIALS AND METHODS

2.2.1 Reagents

Ascorbic acid (AA), N-acetylcysteine (NAC), and L-buthionine-[S,R]-sulfoxide (BSO) were obtained from Sigma Chemical Company (St. Louis, Mo.). All of the agents were dissolved in PBS and diluted to a working concentration before use. A GSH assay kit was obtained from Cayman Chemical Company (Ann Arbor, Mich.). Metaphosphoric acid and triethanolamine were purchased from Aldrich Chemical Company (Milwaukee, Wis.). A PhiPhiLux-G1D2 kit was from MBL (OncoImmunin, Gaithersburg, Md.). Propidium iodide (PI) was from BD PharMingen.

2.2.2 Cell culture

Culture of MOLT-4 and MOLT-4/DNR cells was carried out as described in Chapter 1. The leukemia cells were washed and resuspended with the above medium to 5×10^5 cells/mL, then 196 μl cell suspension was placed in each well of a 96-well flat-bottom plate. PBS solutions (4 μl) containing As_2O_3, AA, BSO, and NAC alone or in combination with As_2O_3 with AA, BSO or NAC were added to yield the final indicated concentrations (see Results). PBS (4 μl) was added to the control wells.

2.2.3 MTT assay

After the incubation period, 20 μl of the MTT labeling reagent was added to each well to yield a final concentration of 0.5 mg/mL. Other assay procedures are same as those described in Chapter 1.

2.2.4 Viability of cells

MOLT-4 and MOLT-4/DNR cell lines were cultured by seeding 5×10^5 cells/mL

of fresh medium in the presence or absence of As_2O_3 alone or in combination with As_2O_3 with AA, BSO and NAC for 24-96 h in an atmosphere comprising 5% CO_2/air at 37 ^oC in a humidified chamber. The number or percentage of viable cells was determined by staining cell populations with trypan blue, as described in Chapter 1.

2.2.5 Measurement of intracellular GSH

Cells (1×10^7) were treated for 48 h with various agents and collected by centrifugation at 1300 g for 10 min at 4 ^oC. The cells were resuspended in 500 µl of 50 mM cold MES buffer, and then homogenized with a Handy pestle (Toyobo Company, Osaka, Japan) or sonicated with a Handy sonic (Tomy Seiko Company, Tokyo, Japan). After centrifugation at 10,000 g for 15 min at 4 ^oC, the supernatant was removed and stored on ice. The supernatant was deproteinated with 5% MPA at room temperature for 5 min and centrifuged at 3000 g for 4 min. Subsequently, 50 µl of 4 M TEAM reagent per ml of the supernatant was added, and the solution was mixed immediately with a vortex mixer. The standard and samples were combined with fresh assay cocktail according to the manufacturer's instructions. Samples were incubated in the dark on an orbital shaker before measurement. The absorbance in the wells was measured at 415 nm using a microplate reader. The total GSH levels were determined by the End Point method.

2.2.6 Evaluation of MMP

MOLT-4 and MOLT-4/DNR cells (1×10^6/mL) were treated with As_2O_3 alone or in combination with AA, BSO, or NAC. The cells were washed twice in a cold PBS (pH 7.2) and then incubated with 10 µg/mL Rh123 for 15 min. The cells were then washed twice in cold PBS, and were analyzed by flow cytometry. Living cells concentrate Rh123 in the mitochondria, while the MMP in cells undergoing apoptosis is disrupted and the mitochondria in such cells release Rh123 [39]. Therefore, the disruption of MMP and subsequent cell apoptosis can be detected by the decrease in the Rh123 fluorescence intensity in the cells. A total of 30,000 non-gated cells were analyzed using a FACSCalibur analyzer (Becton Dickinson, San Jose, Calif.) to obtain dot data. These data were further analyzed using CellQuest Software (Becton Dickinson).

2.2.7 Assessment of caspase-3 activity

Cells (1×10^6/mL) were treated with As_2O_3 alone or in combination with AA, BSO, or NAC. After incubation, an aliquot of cells was placed into a 1.5-ml microcentrifuge tube, and the cells were then centrifuged and the entire culture medium was removed. Subsequently, 50 µl of a substrate solution containing 10 µM caspase-3 substrate was added to the cell pellet, and the suspension was mixed by flicking the tubes with the fingertip. The substrate molecule contains a peptide homodoubly labeled with a fluorophore. The cleaved substrate has specific fluorescence peak characteristics (λ_{ex} 505 nm and λ_{em} 530 nm), which can be detected by flow cytometry. After incubation of the tubes in 5% CO_2/air at 37 ^oC in a humidified chamber for 60 min, the cells were washed once by adding 1 ml ice-cold

flow cytometry dilution buffer. The cells were resuspended in 1 ml fresh dilution buffer for analysis by flow cytometry. All samples were analyzed within 6 min of the end of the 37 °C incubation.

2.2.8 Apoptosis assays

After collecting data for caspase-3 activity, 5 μl 50 μg/mL PI was added and samples were reanalyzed flow cytometrically according to the manufacturer's instructions. The data were reanalyzed within 5 min of PI addition, and the caspase+/PI-) cells were considered apoptotic cells.

2.2.9 Morphological changes of cells

MOLT-4 and MOLT-4/DNR cells (5×10^5/mL) were cultured in the absence or presence of As_2O_3 alone or in combination with AA, BSO, or NAC for 72 h in an atmosphere comprising 5% CO_2/air at 37 °C in a humidified chamber. Pictures were taken under a microscope (Olympus Optical Company, Tokyo, Japan).

2.2.10 Statistics

Student's t-test was used to compare the data between two groups, and the Bonferroni/Dun multiple comparison test was used to compare the data among multiple (more than three) groups. A value of $p<0.05$was considered to be significant.

2. 3 RESULTS

2.3.1 Effects of BSO, AA, or NAC on the growth-inhibitory action of As_2O_3 in MOLT-4 and MOLT-4/DNR cells

After 72 h of treatment, the growth-inhibitory effects of 5 μM As_2O_3 on MOLT-4 and MOLT-4/DNR cells were significantly enhanced by 100 μM BSO and 125 μM AA (Fig. 2-1). BSO is known to be a selective inhibitor of γ-glutamylcysteine synthetase, the rate-limiting enzyme in GSH synthesis [5]. The IC_{50} values of As_2O_3 on the cell growth of MOLT-4 and MOLT-4/DNR cells were 4.8 and 5.3 μM, respectively (Table 2-1). Thus, BSO and AA increased sensitivity to As_2O_3 treatment. The IC_{50} values of As_2O_3 decreased significantly to less than 2 μM in the presence of 100 μM BSO ($p<0.001$). The IC_{50} values of As_2O_3 in both cell lines were also decreased significantly ($p<0.01$) by the addition of AA (Table 2-1). Whereas, 100 μM BSO and 125 μM AA alone did not affect the growth of either MOLT-4 or MOLT-4/DNR cells (the IC_{50} values of both BSO and AA were higher than 500 μM). In contrast, NAC efficiently protected both MOLT-4 and MOLT-4/DNR cells from the cytotoxicity of As_2O_3, when the two cell lines were incubated with 5 μM As_2O_3 in combination with 10 mM NAC for 72 h (Fig. 2-1). Thus, NAC made the cells of both the MOLT-4 and MOLT-4/DNR unresponsive to the growth-inhibitory effect of As_2O_3. The IC_{50} values of As_2O_3 in the presence of NAC in these cell lines were determined to be higher than 8 μM (Table 2-1).

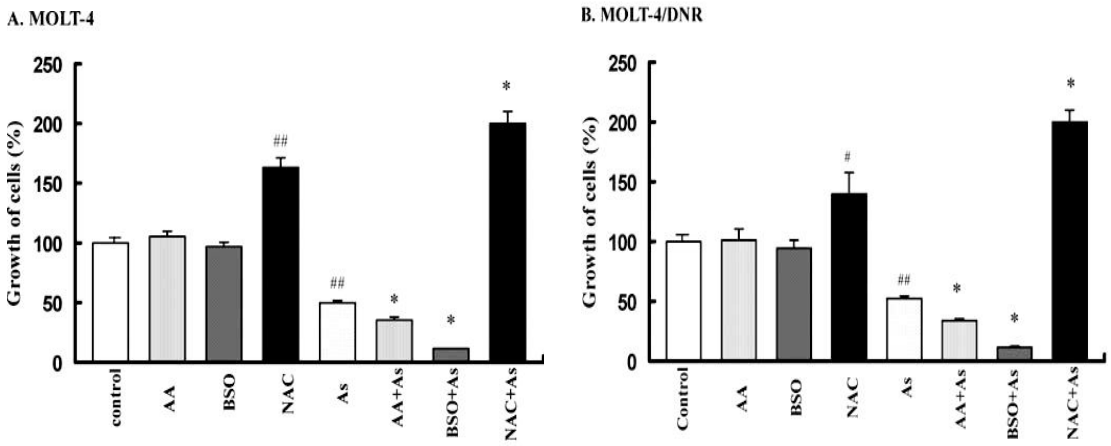


Fig. 2-1 Modulation of As_2O_3-mediated growth inhibition of MOLT-4 (A) and MOLT-4/DNR (B) cells by BSO, AA, and NAC. Cells were treated with 5 μM of As_2O_3 in the absence or presence of 100 μM BSO, 125 μM AA, and 10 mM NAC for 72 h. Cell growth was determined by an MTT assay. Values are the means ± SD of three independent experiments. $^{\#}p<0.05$, $^{\#\#}p<0.001$ vs control; *p<0.01 vs As_2O_3 alone.

Table 2-1. IC_{50} values (μM) of As_2O_3 alone or in combination with BSO, AA, and NAC on MOLT-4 and MOLT-4/DNR cell growth.

Treatment	MOLT-4	MOLT-4/DNR
	IC_{50}	
As_2O_3 alone	4.8 ± 0.2	5.3 ± 0.3
As_2O_3+AA (125 μM)	3.5 ± 0.1**	4.3 ± 0.1*
As_2O_3+BSO (100 μM)	1.2 ± 0.0**	1.1 ± 0.0**
As_2O_3+NAC (10 mM)	>8	>8

Cells were treated with 100 μM BSO, 125 μM AA, and 10 mM NAC in presence or absence of serial concentrations of As_2O_3 for 72 h, and cell growth was determined by an MTT assay. Values are the means ± SD of three independent experiments. *p<0.01, **p<0.001 vs As_2O_3 alone.

Similarly, the viability of MOLT-4 and MOLT-4/DNR cells was significantly decreased following incubation for 72 h with 5 μM As_2O_3 plus 100 μM BSO or 125 μM AA, as compared to cells treated with As_2O_3 alone (P<0.001, Fig. 2-2). In contrast, 10 mM NAC markedly increased the viability of these cells under the same culture conditions (p<0.001, Fig. 2-2).

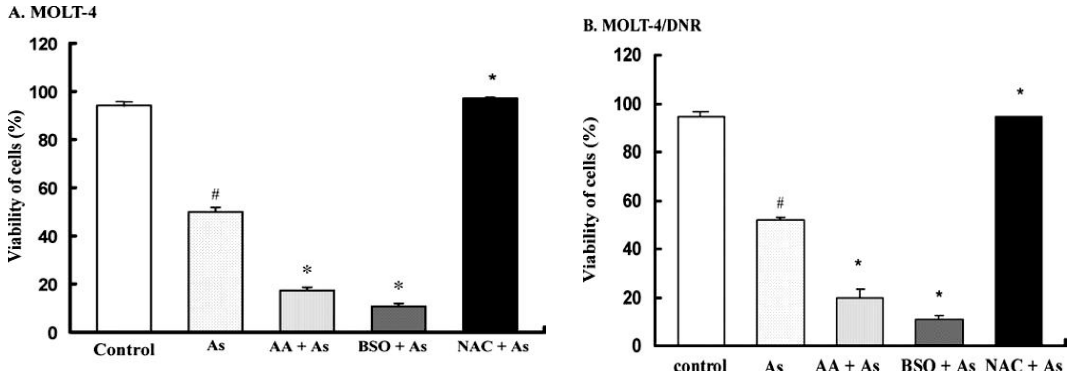


Fig. 2-2 Modulation of As_2O_3-mediated reduction in viability of MOLT-4 (A) and MOLT-4/DNR (B) cells by BSO, AA, and NAC. Cells were incubated in the absence or presence of 5 μM As_2O_3 in the absence or presence of 100 μM BSO, 125 μM AA, or 10 mM NAC for 72 h. Cell viability was determined by a dye exclusion test. Values are the means ± SD of three independent experiments. # $p<0.001$ vs control; *$p<0.001$ vs As_2O_3 alone.

2.3.2 Intracellular GSH content modulated by BSO, AA, or NAC in MOLT-4 and MOLT-4/DNR cells

The GSH contents of MOLT-4 and MOLT-4/DNR cells were 105.0 ± 1.6 and 105.6 ± 2.4 μM/10^7 cells, respectively, when the two cell lines were incubated in the absence of the modulating agents for 48 h. Thus, the intracellular GSH contents of MOLT-4 and MOLT-4/DNR cells were at the same level.

MOLT-4 and MOLT-4/DNR cells were incubated in the absence or presence of 100 μM BSO, 125 μM AA or 10 mM NAC for 48 h. These treatments resulted in significant changes in GSH content (Fig. 2-3). BSO decreased the GSH content to a greater extent ($p<0.01$), and AA also significantly decreased GSH level ($p<0.05$) in both MOLT-4 (Fig. 2-3A) and MOLT-4/DNR (Fig. 2-3B) cells. Whereas, NAC significantly increased the GSH content ($p<0.05$) in these cells.

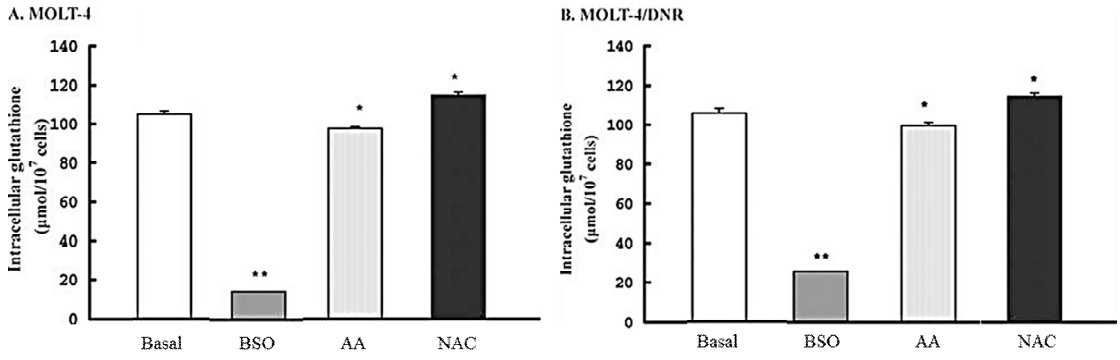


Fig. 2-3 Modulation of intracellular GSH levels by BSO, AA, and NAC in MOLT-4 (A) and MOLT-4/DNR (B) cells. Cells were incubated in the absence or presence of 100 μM BSO, 125 μM AA, or 10 mM NAC for 48 h. GSH levels were measured using a GSH assay kit as described in Materials and Methods. Values are the means ± SD of three independent experiments carried out in triplicate. *$p<0.05$, **$p<0.01$ vs basal level.

2.3.3 Disruption of MMP in MOLT-4 and MOLT-4/DNR cells

The disruption of MMP by As_2O_3 and its modulation by AA, BSO or NAC are presented in Figs. 2-4 and 2-5. In each histogram, the percentage of cells with decreased MMP is indicated. The disruption of MMP in MOLT-4 (Figs. 2-4A and 2-5A) and MOLT-4/DNR (Figs. 2-4B and 2-5B) cells was significantly higher ($p<0.01$) after 72 h treatment with 5 μM As_2O_3 than in untreated cells. This As_2O_3-induced disruption of MMP was markedly enhanced by AA ($p<0.01$) and BSO ($p<0.001$) in both MOLT-4 (Fig. 2-5A) and MOLT-4/DNR (Fig. 2-5B) cells (Figs. 2-4Ac,d, Bc,d). In particular, BSO elevated this ability almost completely (Fig. 2-5), while the As_2O_3-induced disruption of MMP was blocked by NAC in these cells (Figs. 2-4Ad,e, Bd,e, and Fig. 2-5). In contrast, 125 μM AA, 100 μM BSO and 10 mM NAC treatment alone did not influence the MMP of these cells (data not shown).

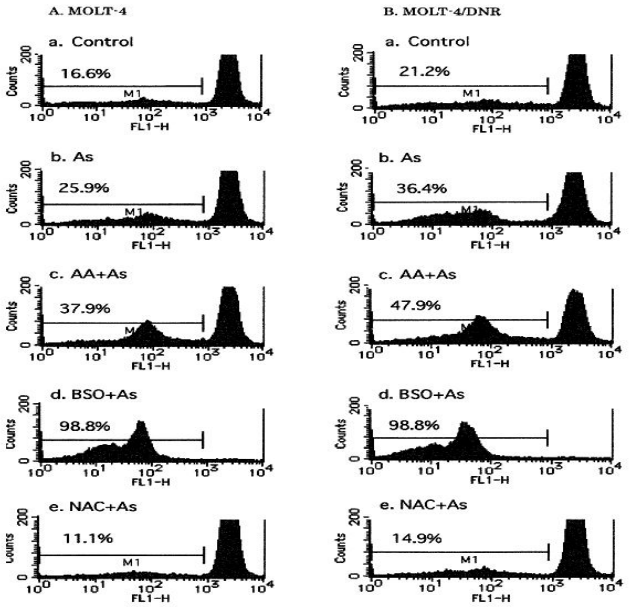


Fig. 2-4 Typical histograms showing the disruption of MMP in MOLT-4 cells (A) and MOLT-4/DNR cells (B) incubated for 72 h in the absence (a) or presence of 5 μM As_2O_3 alone (b) or with a combination of 5 μM As_2O_3 and 125 μM AA (c), 100 μM BSO (d), or 10 mM NAC (e). The percentage given in each histogram is the percentage of cells with decreased MMP.

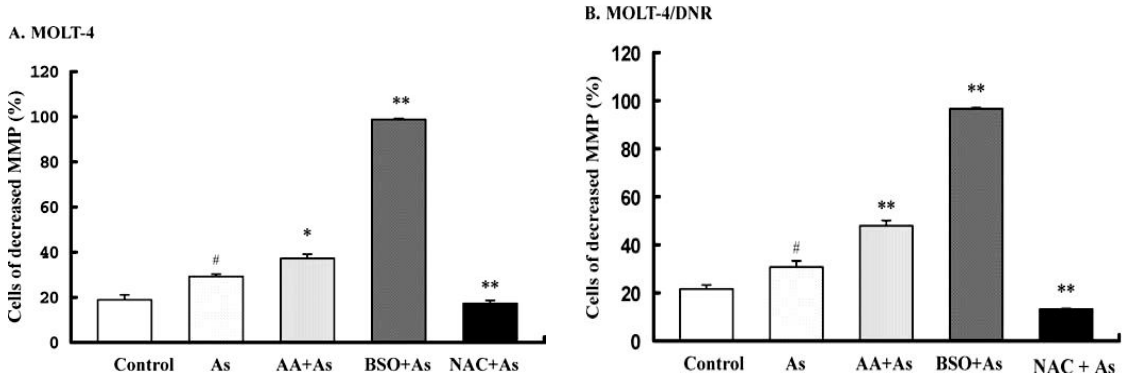


Fig. 2-5 MMP disruption in MOLT-4 cells (A) and MOLT-4/DNR cells (B) treated with 5 μM As_2O_3 alone or with a combination of 5 μMAs_2O_3 and 125 μM AA, 100 μM BSO, or 10 mMNAC for 72 h. Values are the means ± SD of three independent experiments. # $p<0.01$ vs control; *$p<0.01$, **$p<0.001$ vs As_2O_3 alone.

2.3.4 Activation of caspase-3 in MOLT-4 and MOLT-4/DNR cells

The activation of caspase-3 by As_2O_3 and its modulation by AA, BSO or NAC is presented in Figs. 2-6 and 2-7. In each histogram in Fig. 2-6, the percentage of cells expressing relatively high activity of caspase-3 is indicated. After 72 h of treatment, 5 μM As_2O_3 significantly induced caspase-3 activity in MOLT-4 ($p<0.01$) (Fig. 2-7A) and MOLT-4/DNR ($p<0.01$) (Fig. 2-7B) cells (Fig. 2-6). This activation of caspase-3 was markedly potentiated by AA ($p<0.001$) and BSO ($p<0.001$) in both MOLT-4 (Fig. 2-7A) and MOLT-4/DNR (Fig. 2-7B) cells (Figs. 2-6Ac,d, Bc,d). In particular, BSO elevated the levels of caspase-3 activity to a greater extent, whereas the enzyme activation by As_2O_3 was completely attenuated by NAC in both MOLT-4 ($p<0.01$) (Fig. 2-7A) and MOLT-4/DNR ($p<0.001$) (Fig. 2-7B) cells (Figs. 2-6Ae, Be). In contrast, 125 μM AA, 100 μM BSO and 10 mM NAC treatment alone did not modulate the caspase-3 activity of these cells (data not shown).

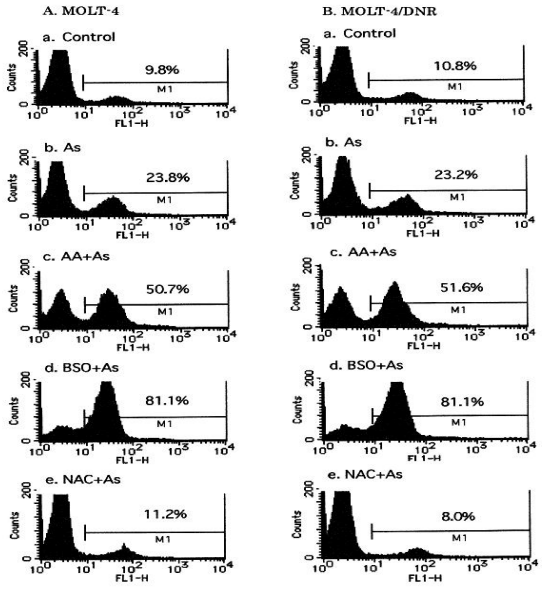


Fig. 2-6 Typical histograms showing the changes in percentage of MOLT-4 cells (A) and MOLT-4/DNR cells (B) exhibiting caspase-3 activity following treatment for 72 h in the absence (a) or presence of 5 μM As_2O_3 alone (b) or with a combination of 5 μM As_2O_3 and 125 μM AA (c), 100 μM BSO (d), or 10 mM NAC (e). The percentage given in each histogram is the percentage of cells with caspase-3 activity.

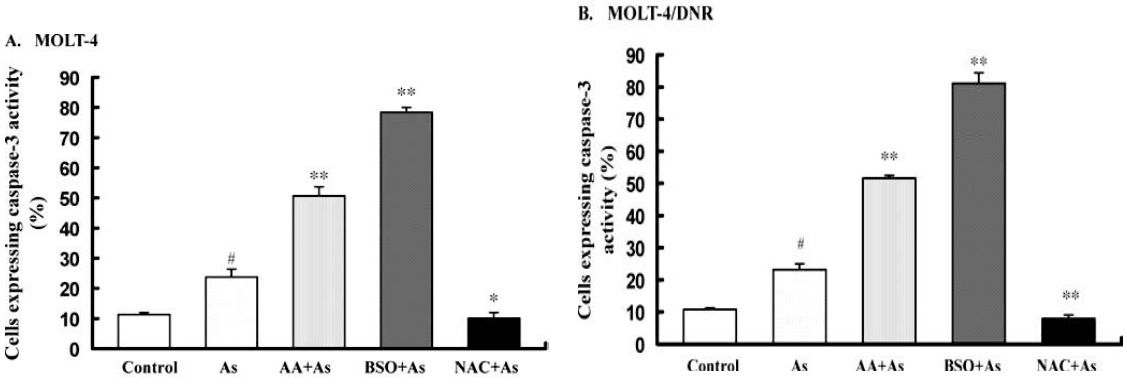


Fig. 2-7 Changes in caspase-3 activity after treating MOLT-4 cells (A) and MOLT-4/DNR cells (B) for 72 h with 5 μM As_2O_3 alone or with a combination of 5 μM As_2O_3 and 125 μM AA, 100 μM BSO, or 10 mM NAC. Values are the means ± SD of three independent experiments. # $p<0.05$ vs control; *$p<0.01$, **$p<0.001$ vs As_2O_3 alone.

2.3.5 Modulation of As_2O_3-induced apoptosis by AA, BSO, or NAC in MOLT-4 and MOLT-4/DNR cells

As_2O_3-induced apoptosis and its modulation by AA, BSO or NAC are presented in Figs. 2-8 and 2-9. The dots in the upper left quadrant are those of caspase+/PI- cells indicating apoptotic cells. In each dot plot the percentage of apoptotic cells is indicated. In these experiments, the cells were treated in the absence or presence of 5 μM As_2O_3 alone or in combination with 100 μM BSO, 125 μM AA or 10 mM NAC. 5 μM As_2O_3 treatment alone induced apoptosis in both MOLT-4 ($p<0.01$) (Fig. 2-9A) and MOLT-4/DNR cells ($p<0.001$) (Fig. 2-9B) as compared to the control cells (Figs. 2-8Aa,b, Ba,b). BSO and AA significantly ($P<0.001$) increased the percentage of apoptotic cells induced by As_2O_3 in both MOLT-4 (Fig. 2-9A) and MOLT-4/DNR cells (Figs. 2-9B, 2-8Ac,d, and 2-8Bc,d), whereas NAC markedly decreased the percentage of apoptotic cells induced by As_2O_3 in both MOLT-4 ($p<0.001$, Fig. 2-9A) and MOLT-4/DNR ($p<0.01$) (Fig. 2-9B) cells (Fig. 2-8Ae, Be). Cells treated with 100 μM BSO, 125 μM AA or 10 mM NAC alone did not show increase in the percentage of apoptotic cells (data not shown).

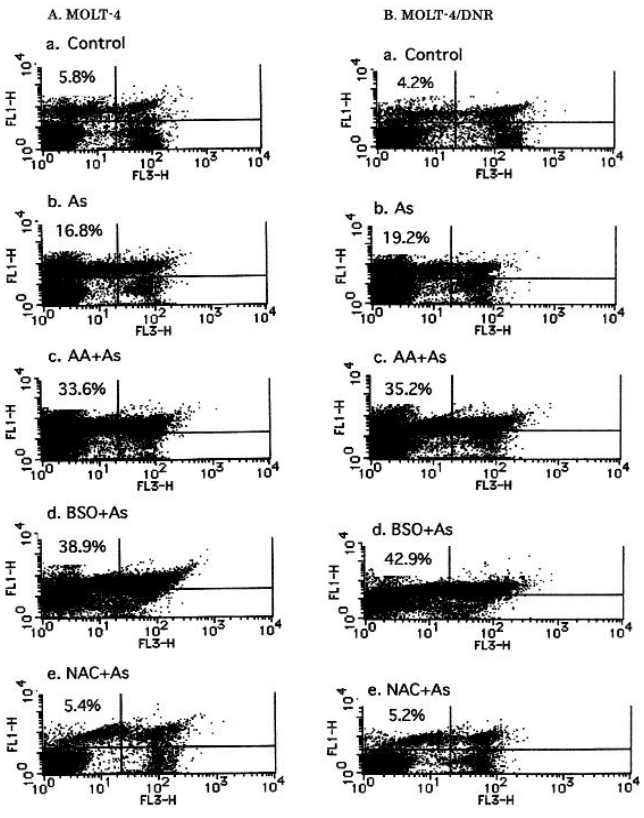


Fig. 2-8 Typical dot plots showing apoptotic MOLT-4 cells (A) and MOLT-4/DNR cells (B) following incubation for 72 h in the absence (a) or presence of 5 μM As_2O_3 alone (b) or with a combination of 5 μM As_2O_3 and 125μM AA (c), 100 μM BSO (d), or 10 mM NAC (e). The percentage given in each dot plot is the percentage of apoptotic cells. The dots in the upper left quadrant are caspase+/ PI- cells indicating apoptotic cells (abscissa PI-related intensity, ordinate caspase activity-related intensity).

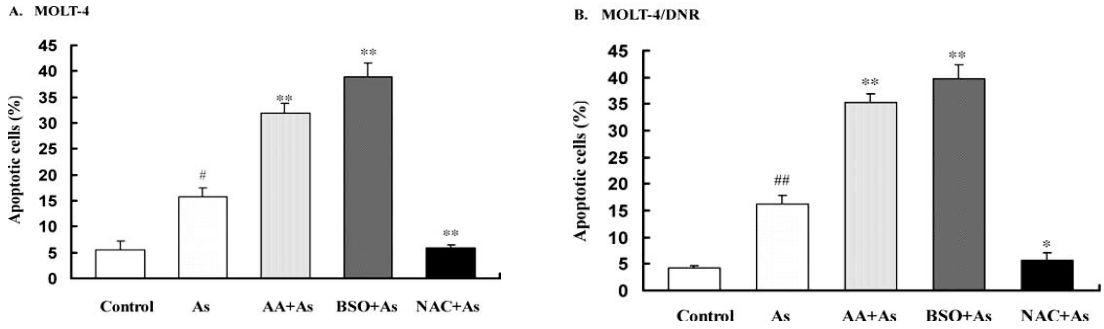


Fig. 2-9 As_2O_3-induced apoptosis and its modulation by BSO, AA, or NAC in MOLT-4 cells (A) and MOLT-4/DNR cells (B) treated with 5 μM As_2O_3 alone or with a combination of 5 μM As_2O_3 with 125 μM AA, 100 μM BSO, or 10 mM NAC for 72 h. Values are the means ± SD of three independent experiments. # $p<0.01$, ## $p<0.001$ vs control; *$p<0.01$, **$p<0.001$ vs As_2O_3 alone.

BSO and AA potentiated the apoptotic morphology induced by As_2O_3 in MOLT-4 (Figs. 2-10Ac, d) and MOLT-4/DNR (Figs. 2-10Bc, d) cells. Whereas, NAC markedly restored a normal morphology to MOLT-4 (Fig. 2-10Ae) and MOLT-4/DNR (Fig. 2-10Be) cells treated with As_2O_3, in comparison to those treated with As_2O_3 alone (Fig. 2-10Ab, Bb).

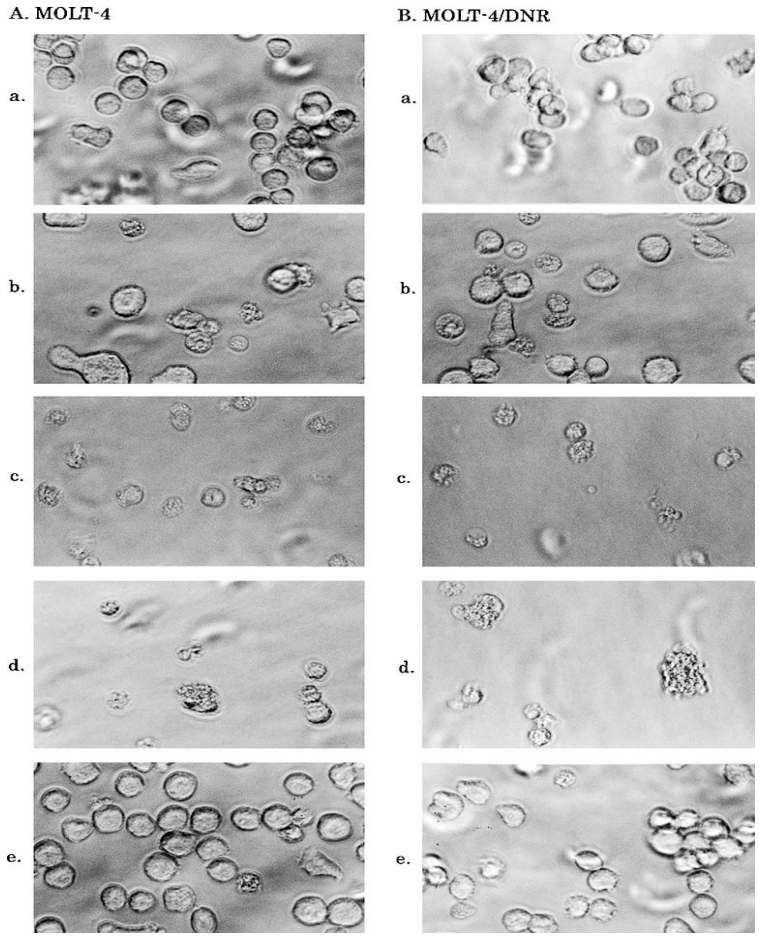


Fig. 2-10 Morphology of MOLT-4 cells (A) and MOLT-4/DNR cells (B) after treatment with 5 μM As_2O_3 alone or with combination of 5 μM As_2O_3 and 100 μM BSO, 125 μM AA, or 10 mM NAC for 72 h. a; untreated cells, **b;** cells treated with 5 μM As_2O_3 alone, **c;** cells treated with 5 μM As_2O_3 combined with 125 μM AA, **d;** cells treated with 5 μM As_2O_3 combined with 100 μM BSO, **e;** cells treated with 5 μM As_2O_3 combined with 10 mM NAC.

2.4 DISCUSSION

2.4.1 GSH content had a decisive effect on As_2O_3-mediated cytotoxicity

The data in Chapter 1 showed that the effects of As_2O_3 on the growth of MOLT-4/DNR cells are the same as its effects on parental MOLT-4 cells, and the actions of As_2O_3 do not influence their P-gp expression or function [44]. The data in this chapter indicated that the intracellular GSH levels in parental MOLT-4 and MOLT-4/DNR cells are almost the same. A reduction in GSH levels following incubation with BSO and AA enhanced the As_2O_3-mediated inhibition of cell growth, disruption of MMP, activation of caspase-3 and apoptosis in MOLT-4 cells and MOLT-4/DNR cells. Whereas, NAC elevated the intracellular GSH levels and protected the cells from the cytotoxicity of As_2O_3. These observations suggested that the sensitivity of both MOLT-4 and MOLT-4/DNR cells to As_2O_3 is closely correlated with intracellular GSH levels.

GSH is a low molecular weight, non-protein, sulfhydryl compound and its role in the protection of cells from oxidative injury has been documented in a study that demonstrated the enhancement of damage by depletion of cellular GSH [92]. GSH exerts antioxidant effects and it can conjugate with and thereby inactivate molecules that generate free radicals. Trivalent arsenic has been reported to form a complex with GSH, forming a transient $As(GS)_3$ molecule [118], which is easily excreted by the MRP2/cMOAT transporter [62]. Thus, GSH maintains an optimum cellular redox potential, and depletion, physical efflux from the cell, or intracellular redistribution of GSH are associated with the onset of apoptosis [16].

BSO is a selective inhibitor of γ -glutamylcysteine synthetase, the rate-limiting enzyme in the synthesis of GSH. Cells incubated with BSO are depleted of GSH as a result of the inhibition of enzymatic GSH production [92]. AA has been shown to have pro-oxidant properties [6, 103]. Auto-oxidation of AA to dehydroascorbate results in the production of H_2O_2 [106, 119]. Dehydroascorbate is then rapidly reduced back to AA by glutaredoxin in a GSH-dependent manner. This reduction of dehydroascorbate to AA results in a decrease in intracellular GSH [122, 129]. Clinically relevant doses of AA act as oxidizing agents decreasing the GSH content of the cells, and synergizes with the growth-inhibitory and apoptosis-inducing effects of As_2O_3. This potentiating effect of AA is due to its capacity to undergo auto-oxidation resulting in the formation of H_2O_2, which enhances the effects of As_2O_3 [31]. Indeed, it has been found that AA significantly synergizes with As_2O_3 in treating patients with AML [5]. NAC is an antioxidant since it donates a cysteine to the de novo synthesis of GSH. As_2O_3 has been reported to bind to vicinal thiol groups [128], while NAC contains two such thiol groups [31] and gives GSH. Taking these observations into consideration, the decrease in GSH levels following incubation with BSO and AA could abate As_2O_3 exclusion, thereby causing an intracellular accumulation of As_2O_3 and enhancing its cytotoxicity. In contrast, the increase in intracellular GSH levels following incubation with NAC could enhance As_2O_3 exclusion, and thereby attenuate the toxic effects of As_2O_3.

2.4.2 As_2O_3-induced apoptosis via depletion of GSH, loss of MMP and activation of caspase-3

Intracellular GSH depletion results in morphological and functional changes of mitochondria [110]. It has been found that the changes of mitochondria after treatment with As_2O_3 can be divided into three stages [110]. In the early stage, mitochondria appear to undergo an adaptive proliferation. In the middle stage, a degenerative change can be observed. In the late stage, the mitochondria swell, and this change is followed by damage to the outer membrane and cell death which exhibits apoptotic changes to the nucleus. Mitochondria are known to play a major role in apoptosis triggered by many stimuli [20]. In the first stage of apoptosis, signal transduction cascades or damage pathways are activated. Subsequently, the mitochondrial membrane function is lost, and then proteins released from mitochondria cause the activation of catabolic proteases and nucleases [106]. Caspases are cysteine proteases that mediate apoptosis by proteolysis of specific substrates [65]. Caspase-3 is considered to be a primary executioner of apoptosis [107]. The data in this Chapter showed that the disruption of MMP and activation of caspase-3 are caused by As_2O_3, and are enhanced by AA and BSO, while blocked by NAC, in both MOLT-4 and MOLT-4/DNR cells. The extent of MMP disruption and caspase-3 activation are closely associated with intracellular GSH levels and occur in a time-dependent manner.

Recent reports suggest that sensitivity to As_2O_3 correlates with intracellular GSH levels in cancer cells [138]. The GSH content modulates the growth-inhibitory and apoptosis-inducing effects of arsenicals [18, 60, 92, 93, 105]. Cells expressing higher levels of GSH or GSH-associated enzymes are less sensitive to As_2O_3 than cells expressing lower levels of these molecules [60, 72]. Arsenic- resistant cells are also reported to contain higher levels of GSH [66, 72, 78]. Moreover, cells with increased GSH levels can be sensitized to As_2O_3 by agents that deplete intracellular GSH [18, 66]. The cytotoxic effects of As_2O_3 may be influenced by modulators of GSH [31, 33, 67, 89], especially in drug-resistant cell lines [28, 100].

A decrease in GSH level in cells acts as a potent early activator of apoptosis signaling [3]. Depletion of GSH, especially mitochondrial GSH, is believed to induce the loss of MMP [84]. In my present study, the GSH content significantly decreased in both MOLT-4 (Fig. 2-3A) and MOLT-4/DNR (Fig. 2-3B) cells incubated with BSO and AA for 48 h. As_2O_3-induced disruption of MMP was markedly enhanced by AA and BSO in both MOLT-4 (Fig. 2-5A) and MOLT-4/DNR (Fig. 2-5B) cells (Figs. 2-4Ac,d, Bc,d) incubated with BSO and AA for 72 h. The percentage of apoptotic cells induced by As_2O_3 significantly increased in both MOLT-4 (Fig. 2-9A) and MOLT-4/DNR cells (Figs. 2-9B, 2-8Ac,d, and 2-8Bc,d) incubated with BSO and AA for 72 h, Whereas, NAC significantly increased the GSH content, and the As_2O_3-induced disruption of MMP and apoptosis were blocked by NAC in these cells.

Mitochondrial permeability transition resulting from intracellular thiol depletion is known to be a critical event in apoptosis [75, 136]. Mitochondria undergo major changes in membrane integrity before classical signs of apoptosis become manifest, and these changes lead to disruption of the MMP [116]. These alterations in

mitochondrial activity can be distinguished and monitored by the fluorescent dye Rh123 on flow cytometry [106, 119], which specifically stains mitochondria depending on the MMP [70, 94, 101]. Mitochondrial depolarization precedes caspase-3 activation and apoptosis [107], and apoptotic cells express high caspase-3 activity [56, 147]. Caspase-3 is considered to be a primary executioner of apoptosis [10, 65]. Caspase-3 activation has been causally related to the release of mitochondrial cytochrome c in the cytoplasm as a result of the collapse of the MMP [76].

As_2O_3 is suggested to act at several points in apoptosis induced through mitochondrial pathways [19], which includes the forming of reversible bonds with thiol groups [118] and the depletion of GSH [18, 66, 93, 138], loss of MMP [58], and activation of caspase-3 [28, 58, 59, 83, 96]. From these points of view, the apoptosis inducing effect of As_2O_3 in the presence of GSH modulators in MOLT-4 and MOLT-4/DNR cells was investigated in this Chapter.

2.5 CONCLUSION

In this chapter, it can be concluded that As_2O_3 induces apoptosis in MOLT-4 and MOLT-4/DNR cells via the depletion of intracellular GSH, and subsequent MMP disruption and caspase-3 activation, based on the following observations:

(1) MOLT-4 cells and MOLT-4/DNR cells were similarly sensitive to the apoptosis-inducing effect of As_2O_3.

(2) BSO and AA rendered these cells more sensitive to As_2O_3, whereas NAC reduced this sensitivity.

(3) BSO and AA decreased, but NAC increased, the intracellular GSH contents of both MOLT-4 and MOLT-4/DNR cells.

(4) Decreasing GSH with BSO or AA potentiated As_2O_3- mediated growth inhibition, disruption of MMP, activation of caspase-3 and apoptosis of cells.

Chapter 3
Involvement of oxidative stress associated with glutathione depletion and p38 MAPK activation in arsenic disulfide-induced differentiation in HL-60 cells

3.1 INTRODUCTION

As_2S_2, the most important component of *Xiong huang* (contains > 90% As_2S_2 based on the quality standard in *Chinese Pharmacopeia* in 2010 edition), was a candidate for its good therapeutic reputation and perceived low toxicity, and the drug has become another research focus following As_2O_3 [131]. As_2S_2 was found as effective as As_2O_3 in clinical trials, with relatively good oral safety profiles even on chronic administration [131, 49]. These may give As_2S_2 an advantage over As_2O_3 in maintenance treatment.

As_2S_2-mediated growth inhibition and apoptosis induction have been found in leukemia K562 cells [73], human ovarian and cervical cancer cells [132], and other cells [120]. However, the effects of As_2S_2 on HL-60 cells with a particular focus on proliferation, differentiation, oxidative stress associated with generation of reactive ROS and intracellular GSH depletion, and activation of p38 MAPK have not been addressed.

3.2 MATERIALS AND METHODS

3.2.1 Reagents

As_2S_2, phorbol 12-myristate 13-acetate, nitroblue tetrazolium (NBT), 2',7'-dichlorofluorescein diacetate (DCFH-DA), and ortho-phthaldialdehyde were purchased from Sigma Aldrich (St. Louis, Mo., USA). SB203580 was purchased from Calbiochem (San Diego, CA, USA). R-PE conjugated anti-human CD11b (Clone D12) and mouse IgG1 isotype control were obtained from DakoCytomation (DakoCytomation Denmark A/S). PE Mouse Anti-p38 MAPK (pT180/pY182) and the AnnexinV-FITC apoptosis detection kit I were obtained from BD PharMingen. Hydrogen peroxide (H_2O_2) and methanol_were purchased from Wako (Wako Pure Chemical Industries, Ltd, Japan). 10% - Formaldehyde Neutral Bufferwas product of Nacarai Tesque (Japan). As_2S_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 filter (Advantec, Japan), and the stock solution was made by PBS at a concentration of 5mM, which was diluted by PBS to working concentrations before use. DCFH-DA and Rhodamine 123 were dissolved in DMSO 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.

3.2.2 Cell culture

HL-60 cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). The cells were maintained in RPMI-1640 medium containing 10% FBS, 100U/mL penicillin and 100μg/mL streptomycin in continuous logarithmic growth between 2 - 10 × 10^5 cells/mL at 37 °C in a humidified 5% CO_2

incubator.

3.2.3 MTT assay

The leukemia cells were washed and resuspended with the above medium to 5×10^5 cells/mL, and then 196 µl of the cell suspension was placed in each well of a 96-well flat-bottomed plate. To the suspension in each well, 4 µl PBS solution containing As_2S_2 was added to yield final concentrations of 0.5, 2, 8, and 16µM, respectively; 4 µl PBS was added to the control wells. The cells were incubated for 48h in an atmosphere containing 5% CO_2 at 37 ^oC in a humidified chamber. After the incubation period, 20 µl of MTT labeling reagent (final concentration 0.5 mg/mL) was added to each well and the plate was placed on a microshaker for 10s, after which the cells were further incubated for 4 h in a humidified atmosphere. Subsequently, 100 µl of solubilization solution (Roche) was added to each well and the plate was placed on a microshaker for 10s, after which the plate was left overnight in an incubator in a humidified atmosphere. The spectrophotometric absorbance of the samples was measured on a microplate reader (LS-PLATE manager 2001, Japan) at 570 nm. A dose response curve was plotted.

3.2.4 Assessment of cell differentiation

HL-60 cells (1×10^6/mL) were treated with the indicated concentrations of As_2S_2. After the indicated incubation times, cells were washed twice by PBS containing 2% FBS and resuspended in 100 µl PBS containing 2% FBS. Then, 10 µl PE-conjugated CD11b antibody was added, and cells were incubated in the dark at 4 °C for 30 min. After the incubation, cells were resuspended in 400 µl PBS containing 1% FNB, and subsequently the cells were analyzed by Becton Dickinson flow cytometry. Isotypic mouse IgG1 was used to set threshold parameters. A total of 30,000 non-gated cells were analyzed. In case of the assessment of synergistic differentiation induced by H_2O_2 combined with As_2S_2, cells were pre-incubated with indicated concentration of H_2O_2 for 1 h before treatment with As_2S_2 for 48h. When assessment of effects of SB203580 on As_2S_2-induced differentiation, cells were incubated with indicated concentrations of As_2S_2 alone or combined with SB203580 for 72h, 0.05% DMSO was used as control.

The As_2S_2-induced HL-60 cell differentiation was observed by morphological changes, which were determined by flow cytometry after treatment of As_2S_2 for 48h. Light scattering signal measured in the forward is known as forward scattering (forward scatter, FSC) in flow cytometry analyses, which reflects the size of the cell volume.

The As_2S_2-induced differentiation of HL-60 cells to neutrophil-like cells was also assessed by NBT reduction assay [82, 123]. After HL-60 cells (1×10^6/mL) were treated with the indicated concentrations of As_2S_2 for 72 h, cells were washed by PBS, and then cells were suspended in 1 ml serum free RPMI-1640 medium containing 4 mg/ml of NBT and 2 µg/mL of phorbol 12-myristate 13-acetate. After incubation at 37 °C for 1 h, 200 µl of 6 M HCl was added and the cells chilled on ice for 30 min. Then the samples were centrifuged at 1,000 g for 10 min, and the formazan deposits

were dissolved with 600 μl DMSO. The absorbance was measured at 560 nm.

3.2.5 Apoptosis and viability assays

After HL-60 cells (1×10^6/mL) were treated with the indicated concentrations of As_2S_2 for the indicated incubation times, the cells were washed twice by cold PBS (pH 7.2) and resuspended in binding buffer at a density of 1×10^6 cells/mL. Then 1×10^5 cells were stained with 5 μl Annexin V-FITC and 5 μl PI for 15 min at 4 ° C in the dark. Then 400 μl binding buffer was added to each tube, and the cells were analyzed by Becton Dickinson flow cytometry within 1 h of staining. A total of 3×10^4 non-gated cells were analyzed. Cell viability was determined by PI exclusion. Cell apoptosis was identified by positive Annexin V-staining.

3.2.6 Cellular ROS production measurement

Cellular ROS production was measured by flow cytometry using DCFH-DA [36, 40, 63, 123]. Viable cells can deacetylate DCFH-DA to DCFH. The latter is not fluorescent but can react with H_2O_2 to produce fluorescent DCF. The cytofluorimetric measurement of DCF provides an index of cellular ROS level [123]. At the end of treatment by As_2S_2, cells in each well were collected, washed twice in PBS and incubated in 1 ml PBS containing 10 μM DCFH-DA at 37 °C for 30 min. Cells were then washed in PBS and resuspended in 0.5 ml PBS. The mean fluorescence intensity (MFI) of DCF green fluorescence from 1×10^4 cells was detected by Becton Dickinson flow cytometry. The ROS level in the treated cells against that of the control is represented by relative MFI.

3.2.7 Determination of level of cellular GSH

The temporal change in cellular GSH was evaluated using ortho-phthaldialdehyde [123], which can react with GSH to yield a highly fluorescent product. At the indicated times of cells incubated with As_2S_2, cells were washed twice by PBS and then suspended in 900 μl PBS. Then, 100μl of 10 mg/mL ortho-phthaldialdehyde in methanol was added, and cells were incubated at 37 °C for 30 min. After being washed by PBS, cells were resuspended in 500 μl PBS and kept on ice. MFI thus produced from 1×10^4 cells was measured by Becton Dickinson flow cytometry. The levels of cellular GSH were represented by relative MFI.

3.2.8 Analysis for p38 MAPK activation

Activation of p38 MAPK was detected by flow cytometry [81]. Cells (1×10^6/mL) were exposed to different concentrations of As_2S_2 for indicated times. The cells were collected by centrifugation and washed by PBS, and then fixed for 10 minutes at 37 °C in PBS containing 2% FNB. Cells were centrifuged and resuspended in 90% methanol, and then incubated for 30 minutes on ice. The cells were washed twice by PBS and resuspended in PBS 100 μl. Subsequently, PE-labeled anti phosphorylated p38 MAPK antibody was added and incubated for 1 h at room temperature. Cells were resuspended in 400 μl PBS containing 1% FNB and analyzed with Becton Dickinson flow cytometry.

3.2.9 Western blot analysis

Protein samples were separated on a SDS–PAGE, followed by transferring to a nitrocellulose membrane as described previously [140]. Protein bands were detected using the following primary antibodies: mouse anti-human β-actin (1:5000 dilution, Sigma–Aldrich, MO, USA); rabbit anti-human phospho-p38 MAPK (Thr180/Tyr182) (1:1000 dilution), and rabbit anti-human p38 MAPK (1:1000 dilution) (Cell Signaling Technology, MA, USA). Blotted protein bands were detected with respective horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescenceWestern blot analysis system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

3.2.10 Determination of MMP

MMP was assessed using the cationic lipophilic dye R123 [48, 36, 123, 137]. Cells (1×10^6/mL) were treated with different concentrations of As_2S_2 for indicated times. At the end of incubation, cells were collected and washed twice in PBS and incubated in 1 ml PBS containing 10 μg/ mL Rh123 at 37 °C for 30 min. Cells were washed with PBS and resuspended in 500 μl PBS. 3×10^4 cells were analyzed with Becton-Dickinson flow cytometry, and MMP is represented by relative MFI.

3.2.11 Statistics

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

3.3 RESULTS

3.3.1 Effects of As_2S_2 on cell growth and differentiation

After treatment with As_2S_2 at concentrations in the range of 0.5 to 16.0 μM for 72 h, cell growth was measured in an MTT assay. As_2S_2 at 16 μM inhibited growth of HL-60 cells, while the agent at 0.5, 2, and 8 μM did not inhibit the proliferation (Fig. 3-1).

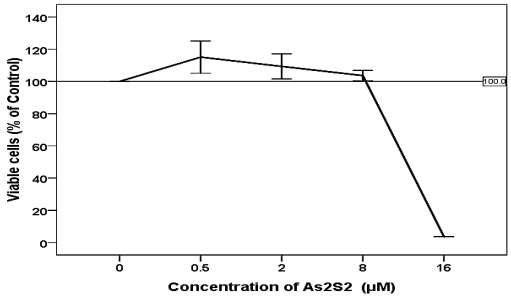
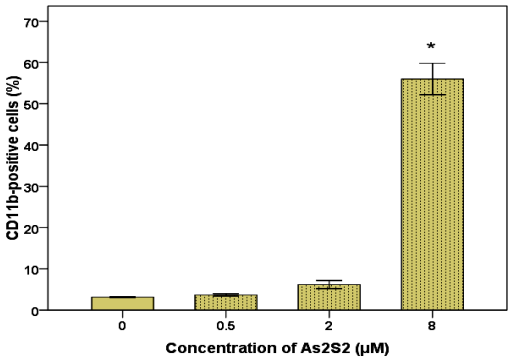


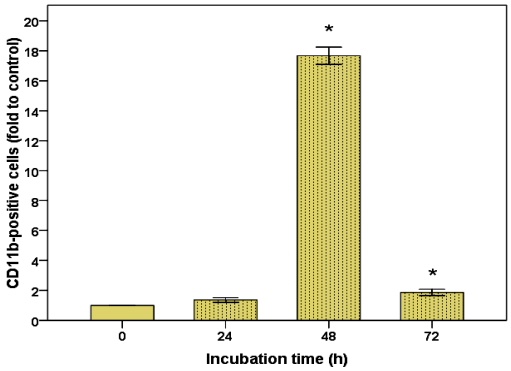
Fig. 3-1 Effects of As_2S_2 on in vitro growth of HL-60 cells. Cells were treated with serial concentrations of As_2S_2 for 72 h. Cell growth was determined by an MTT assay. Data are the mean ± SD of 3 independent experiments.

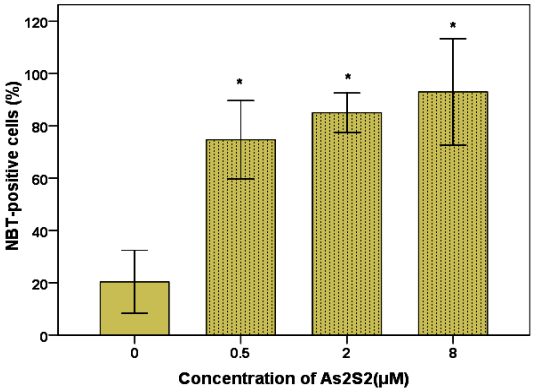
CD11b, which is widely used in differentiation studies of leukemia cells [82, 123, 137], is a kind of cell surface antigen that is characteristic when myeloid precursors differentiate into mature monocytic cells. Then, differentiated cells after treatment

with As_2S_2 were detected by CD11b expression as well as by cell-size characterization at 48 h, followed by examining NBT reduction ability at 72 h. The cells treated with As_2S_2 at concentrations of 0.5, 2, and 8 μM for 48h increase the CD11b-positive cells tenuously in the presence of 0.5 and 2 μM, and significantly to 56% in the presence of 8 μM As_2S_2 (Fig. 3-2A) ($p<0.05$). When the cells were treated with As_2S_2 at a concentration of 8 μM for 24-72 h, the degree of cell differentiation peaked by 17 times of control at 48 h (Fig. 3-2B) ($p<0.05$). The NBT-positive cells significantly increased to approximately 3 times of control after incubation with As_2S_2 at concentrations of 0.5, 2, and 8 μM for 72 h (Fig. 3-2C) ($p<0.05$). Sizes of the cells changed to be larger than the untreated cells, which could be observed by FSC on the X-axis (Fig. 3-2D). Histograms showed the changes of CD11b expression of cells following treatment of As_2S_2 at concentrations of 0.5, 2, and 8 μM for 48 h (Figs. 3-2Ea-c), and at a concentration of 8 μM for 24 to 72 h (Fig. 3-2F).

B







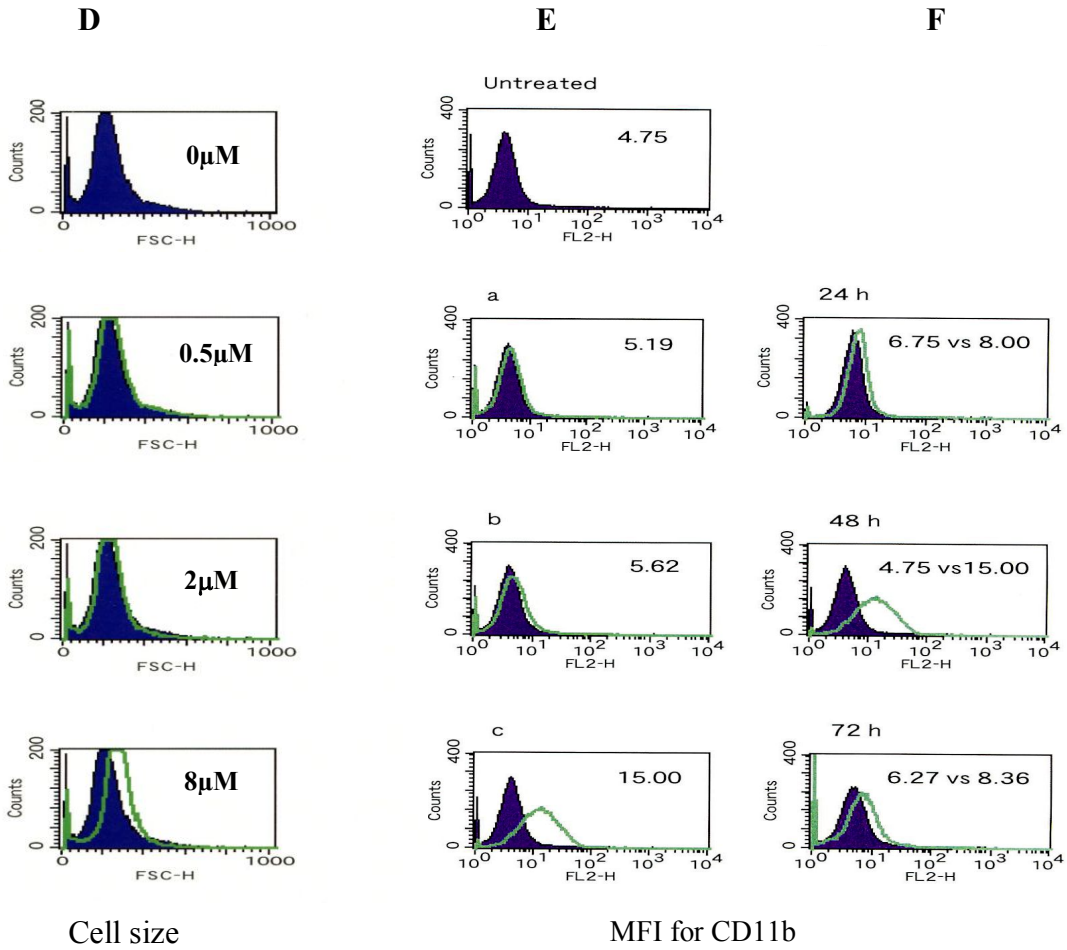


Fig. 3-2. As_2S_2-induced differentiation in HL-60 cells. CD11b-positive cells treated with As_2S_2 at concentrations of 0.5, 2, and 8 μM for 48 h (A) or cells treated with 8 μM As_2S_2 for 24 to 72 h (B). NBT reduction positivity in HL-60 cells following treatment with As_2S_2 at concentrations of 0.5, 2, and 8 μM for 72 h (C). Data are the mean ± SD of 3 independent experiments. *$p<0.05$ as compared with control group. (D) HL-60 cells treated with 0.5, 2, or 8 μM As_2S_2 were analyzed with flow cytometry, and the data were presented by histograms plotted by FSC (abscissa), which reflects cell size. Histograms showed the changes of CD11b expression in HL-60 cells, as assessed by MFI, following treatment with As_2S_2 at concentrations of 0.5 (a), 2 (b), and 8 (c) μM, respectively, for 48 h (E), and those in cells following treatment with 8 μM As_2S_2 for 24 to 72 h (F). Shaded or unshaded area in D-F represents the data of untreated- or treated- cells, respectively. Data are the MFI.

3.3.2 Apoptosis and viability of cells treated with As_2S_2

As_2S_2 did not induce apoptosis and inhibition of viability at concentrations of 0.5, 2, and 8 μM for 48 h or at a concentration of 8 μM for 24 to 72 h. In contrast, As_2S_2 decreased apoptosis and increased cell viability at concentrations of 2 and 8 μM for 48 h or at a concentration of 8 μM for 48 to 72 h compared with control, when apoptosis was identified by Annexin V-staining (Fig. 3-3A, B) and viability determined by a PI exclusion (Fig. 3-3C, D), respectively. Dot plot analysis of PI/Annexin-V stained cells showed the changes of dead cells (FL1) and apoptotic

cells (FL2) following treatment of As_2S_2 at concentrations of 0.5, 2, and 8 μM for 48 h, respectively (Fig. 3-3E).

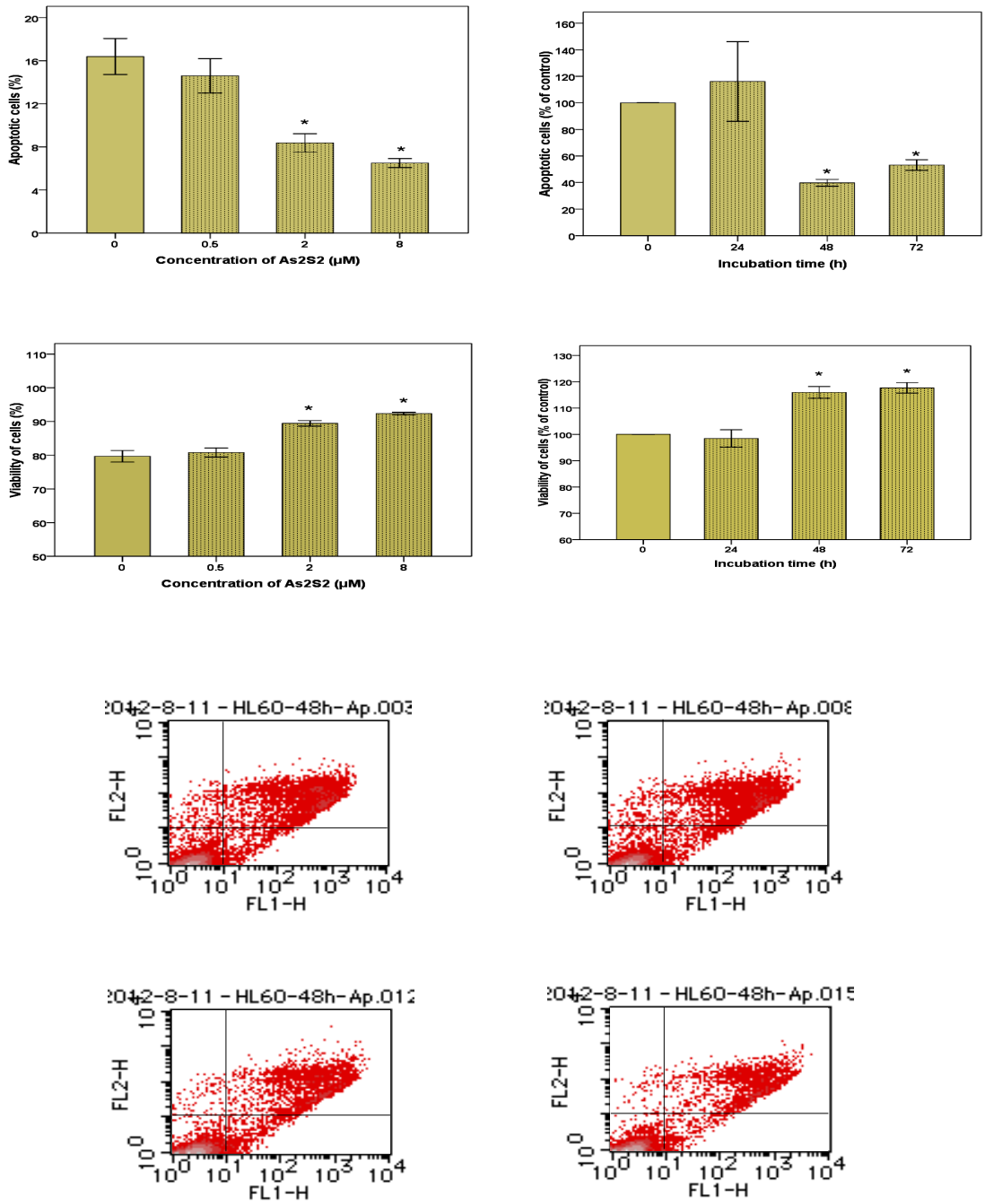


Fig. 3-3 Apoptosis and viability in As_2S_2-treated HL-60 cells. The percentages of apoptotic cells treated with 0.5, 2, and 8 μM As_2S_2 for 48 h (A), and those treated with 8 μM As_2S_2 for 24 to 72 h (B). The percentages of viability of cells treated with 0.5, 2, and 8 μM As_2S_2 for 48 h (C), and those treated with 8 μM As_2S_2 for 24 to 72 h (D). Data are the mean ± SD of 3 independent experiments. *$p<0.05$ as compared with control group. (E) Dot plot analysis showing dead cells stained by PI (FL1) and apoptotic cells stained by Annexin V (FL2) following treatment with As_2S_2 at concentrations of 0.5 (a), 2 (b), and 8 (c) μM, respectively, for 48 h.

3.3.3 Involvement of oxidative stress in the As_2S_2-induced cell differentiation

Cells incubation for 0.5 to 24 h in the presence of 0.5, 2, and 8 μM As_2S_2 generated the cellular ROS, and the levels of ROS peaked at 1 h notably at 8 μM (Fig. 3-4A). After incubation for 1 h, the cellular ROS rapidly increased to 109%, 123%, and 129% of control at the concentrations of 0.5, 2, and 8 μM, respectively (Fig. 3-4B). The changes of cellular ROS were shown in histograms with the agent concentrations of 0.5, 2, and 8 μM for 0.5 to 24 h (Fig. 3-4C).

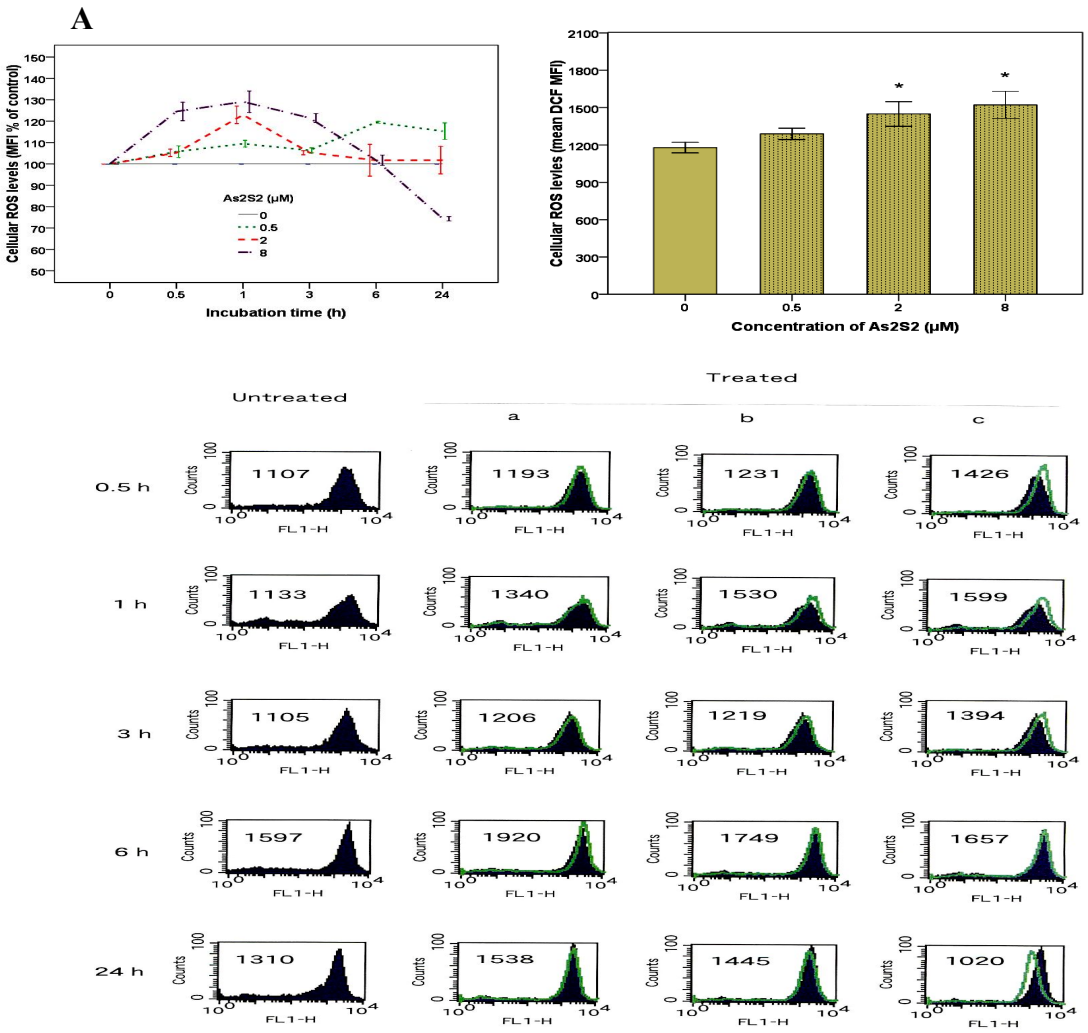


Fig. 3-4. Induction of cellular ROS by treatment with As_2S_2 at concentrations of 0.5, 2, and 8 μM in HL-60 cells. (A) Changes in levels of ROS in cells incubated with As_2S_2 for 0.5 to 24 h. ROS levels were presented as the relative MFI, which were defined as: (sample MFI/control MFI) ×100. (B) The levels of ROS in cells incubated with As_2S_2 for 1 h. The data are the MFI of DCF. Data are the mean ± SD of 3 independent experiments. * $p<0.05$ as compared with control group. (C) Histograms showing the changes of cellular ROS, as assessed by MFI, following treatment of cells with As_2S_2 at concentrations of 0.5 (a), 2 (b), and 8 (c) μM, respectively, for 0.5 to 24 h. Shaded or unshaded area represents the data of untreated- or treated- cells, respectively. Data are the MFI.

The effects of H_2O_2 on As_2S_2-induced cell differentiation were assessed by CD11b-positive cells in the presence of As_2S_2 and H_2O_2 alone or in combination. H_2O_2 alone at a concentration of 20 μM showed a significant effect to increase CD11b-positive cells as compared to control (Fig. 3-5A) ($p<0.05$). When cells were pre-incubated with 20 μM H_2O_2 for 1 h followed by incubation with As_2S_2 for 48 h, as compared to treatment with As_2S_2 alone, the number of CD11b-positive cells significantly increased in the presence of H_2O_2 and As_2S_2 in combination at concentrations of 20 and 0.5 or 2 μM, respectively (Fig. 3-5A). As_2S_2 at 0.5 and 2 μM generated a relative low level of cellular ROS (Fig. 3-4A). Treatment of cells with 8 μM As_2S_2 produced the highest concentration of ROS (Fig. 3-4A), as well as the highest percentage of CD11b-positive cells (Fig. 3-5A). Whereas, the combined treatment with H_2O_2 and 8 μM As_2S_2 decreased, rather than increased, the percentages of CD11b-positive cells, as compared to the treatment of cells with 8 μM As_2S_2 alone (Fig. 3-5A) ($p<0.05$). The changes of CD11b expression in cells treated with H_2O_2 and As_2S_2 in combination at As_2S_2 concentrations of 0.5, 2, and 8 μM for 48 h were shown in Fig. 3-5B as histograms.

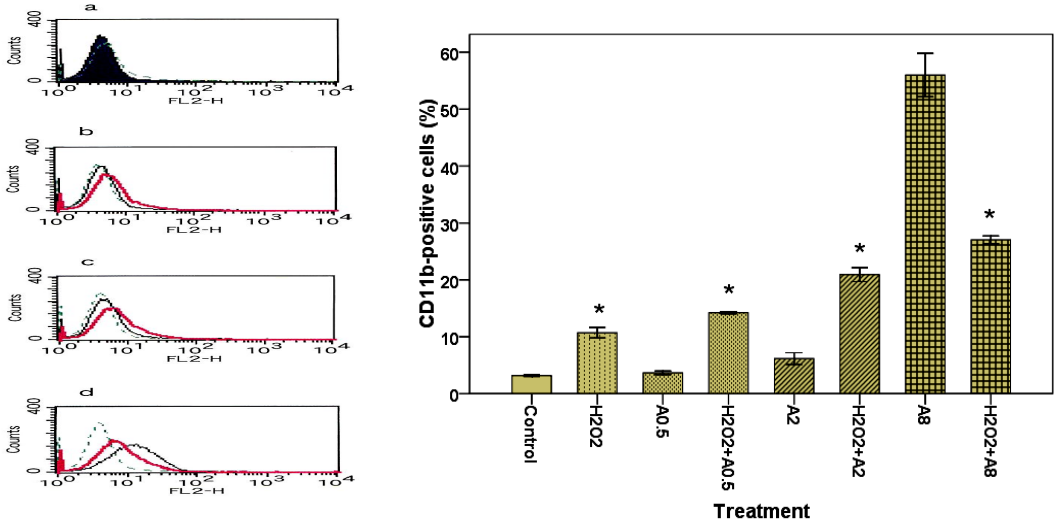
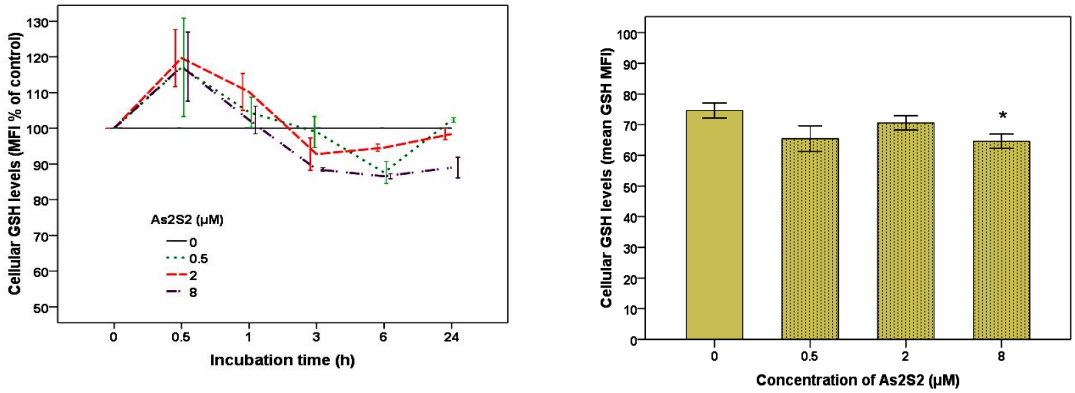


Fig. 3-5 The modifying effects of hydrogen peroxide (H_2O_2) on As_2S_2-induced differentiation in HL-60 cells. Cells were incubated with 20 μM H_2O_2 and As_2S_2 alone at concentrations of 0.5 (A0.5), 2 (A2), and 8 (A8) μM, respectively, or pre-incubated with 20 μM H_2O_2 for 1 h before treatment with As_2S_2 for 48 h (H_2O_2 + A). After treatment, the cells were stained for CD11b and then analyzed with flow cytometer. Data are the mean ± SD of 3 independent experiments. Means were compared by 1-way ANOVA. *$p<0.05$ as compared with control group. (B) Histograms showing the changes of CD11b expression in HL-60 cells, as assessed by MFI, treated with 20 μM H_2O_2 and As_2S_2 alone or in combination with As_2S_2 at concentrations of 0.5, 2, and 8 μM, respectively, for 48 h. a, Shaded or unshaded (open line) area are the data of untreated- or treated- cells with 20 μM H_2O_2. b-d, Open line represent the MFI in cells treated with H_2O_2 alone. Thin or thick solid line represent the MFI in cells treated with As_2S_2 at concentrations of 0.5 (b), 2 (c) and 8 (d) μM alone or combination with H_2O_2, respectively.

3.3.4 Cellular GSH level

Cells were treated with As_2S_2 at concentrations of 0.5, 2, and 8 μM for 0.5 to 24 h, and levels of cellular GSH were measured. Cellular GSH concentration increased to 110% of control at 0.5 h, and then the level rapidly declined and dropped to the minimum at 6 h (Fig. 3-6A). At this time, cells treated with 8 μM As_2S_2 showed a significant GSH depletion to 86% of control (Fig. 3-6B). As showed in Fig. 3-4A, after treatment of As_2S_2 for 0.5 to 24 h, cellular ROS level tenuously increased at 0.5 h, and then rose to the maximum at 1 h. The changes of cellular GSH level were shown in Fig. 3-6C as histograms.



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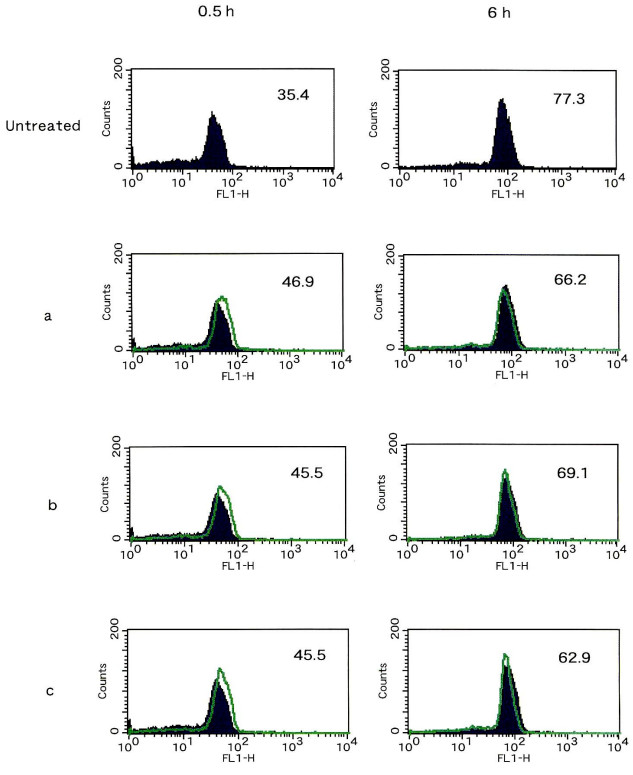
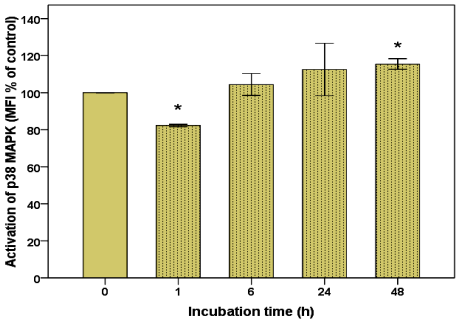


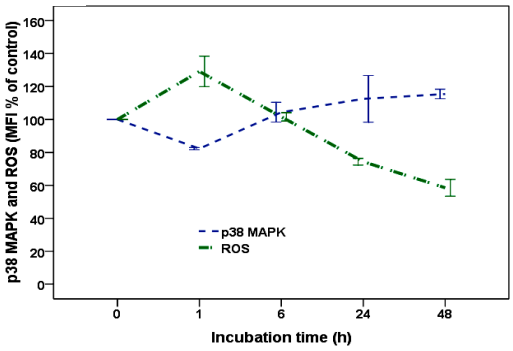
Fig. 3-6. Changes of cellular GSH by treatment with As_2S_2 at concentrations of 0.5, 2, and 8 μM in HL-60 cells. (A) Changes in levels of GSH in cells incubated with As_2S_2 for 0.5 to 24 h. GSH levels are presented as the relative MFI, which were defined as: (sample MFI/control MFI) × 100. (B) The levels of GSH in cells incubated with As_2S_2 for 6 h. The data are the MFI related from ortho-phthaldialdehyde-GSH. Data are the mean ± SD of 3 independent experiments. *$p<0.05$ as compared with control group. (C) Typical histograms showing the changes of cellular GSH, as assessed by MFI, following treatment with As_2S_2 at concentrations of 0.5 (a), 2 (b), and 8 (c) μM for 0.5 and 6 h, respectively. Shaded or unshaded area represents the data of untreated- or the treated-cells, respectively.

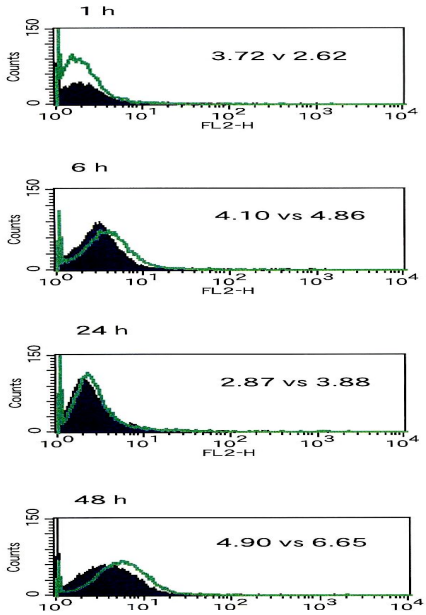
3.3.5 Activation of p38 MAPK

After treatment of cells with As_2S_2 at a concentration of 8 μM for 1 to 48 h, as compared to control, the level of p38 MAPK initially decreased to 82% at 1 h, and then gradually increased to 115% at 48 h (Fig. 3-7A). Conversely, the levels of cellular ROS initially increased to 130% at 1 h, and then gradually decreased to 75% at 48 h (Fig. 3-7B). The changes of p38 MAPK activation after treatment with As_2S_2 were also shown as histograms in Fig. 3-7C.

After treatment with 8 μM of As_2S_2 for 1, 6, 24 and 48 h, phosphorylation of p38 MAPK was investigated using western blot. As shown in Fig. 3-7D, endogenous p38 MAPK activation was constantly observed in untreated HL-60 cells (control groups) during the incubation period based on the detection of the similar expression level of phospho-p38 MAPK. Furthermore, compared to control groups, the expression level of phospho-p38 MAPK in the As_2S_2 treated cells appeared to increase at 24 h. However, in contrast to FACS data showing approximate 15% increase in the expression level of phospho-p38 MAPK at 48 h, its expression level seemed to decrease at this time point. It should be noted that there were no changes in the expression levels of total p38 MAPK expression observed in both untreated and treated cells.







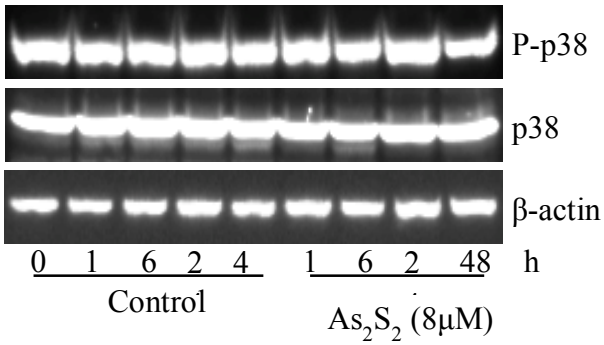


Fig. 3-7. Changes in p38 MAPK activity in HL-60 cells treated with As_2S_2. (A) p38 MAPK activation was detected by PE-labeled phosphorylated p38 MAPK antibody. Cells were treated with 8 μM As_2S_2 for 1 to 48 h. Data are the mean ± SD of 3 independent experiments. *$p<0.05$ as compared with control group. (B) Changes in levels of cellular p38 MAPK compared with ROS in cells treated with 8 μM As_2S_2 for 0.5 to 24 h. p38 MAPK and changes of ROS levels are presented as the relative MFI, both of which were defined as: (sample MFI/control MFI) × 100. (C) Typical histograms showing the changes of activation of p38 MAPK, as assessed by MFI, following treatment with As_2S_2 at a concentration of 8 μM for 1 to 48 h. Shaded or unshaded area represents the data of untreated- or the treated- cells, respectively. Data are the MFI. (D) Detection of phosphorylated p38 MAPK in As_2S_2-treated HL-60 cells. After treatment with 8 μM of As_2S_2 for 1, 6, 24 and 48 h, the expression profiles of phospho-p38 MAPK (active form of p38, P-p38), total p38 MAPK (p38) and β-actin proteins in HL-60 cells were analyzed as described in Materials and Methods.

3.3.6 Depletion of MMP

The relative value of MMP significantly declined to 65% of control in cells treated with 8 μM As_2S_2 for 48 h ($p<0.05$), whereas no notable changes were observed in cells treated with As_2S_2 at concentrations of 0.5 and 2 μM (Fig. 3-8A). Under treatment with 8 μM As_2S_2 for 24 to 72 h, cells showed a minor change in MMP at 24 h, and significant depletions of MMP were observed at 48 h and 72 h (Fig. 3-8B) ($p<0.05$). Noticeably, the minimum of MMP level was at 48 h, and at this time, the level of CD11b positive cells increased to the maximum (Fig. 3-8C). The depletions of MMP were also shown as histograms in Fig. 3-8D.

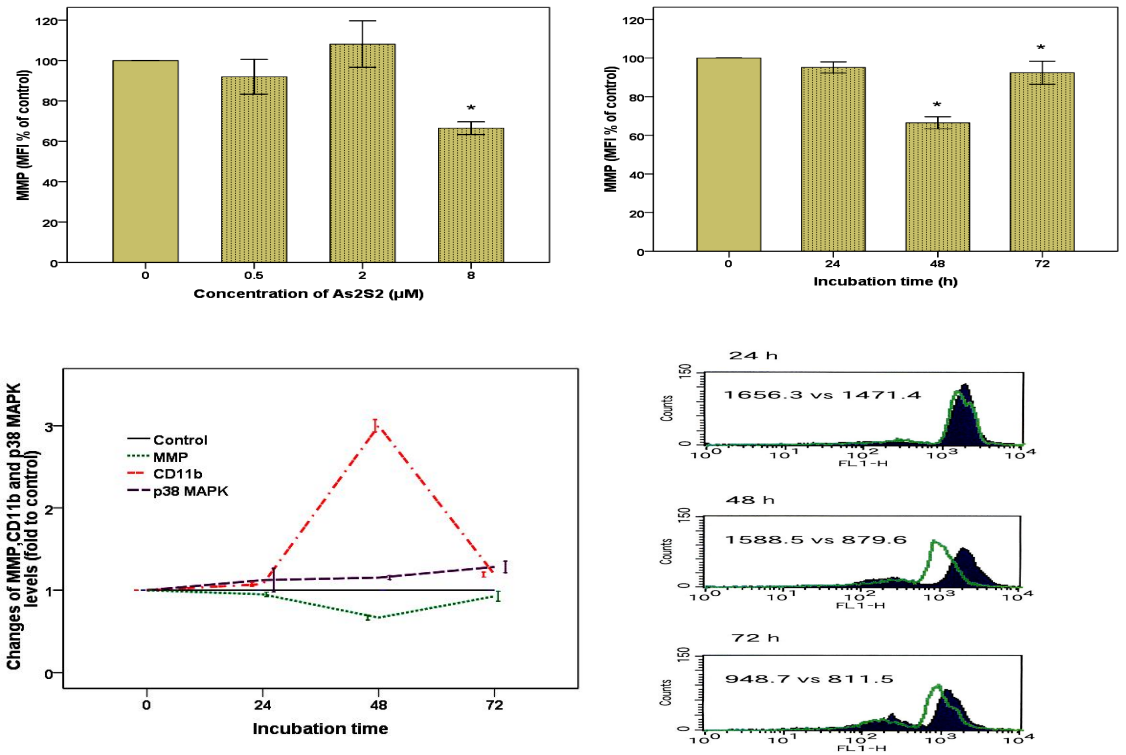
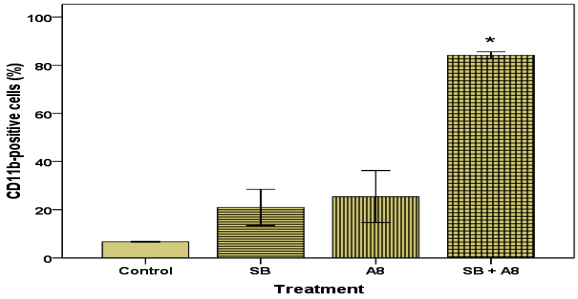


Fig. 3-8. Depletion of MMP related to As_2S_2-induced differentiation in HL-60 cells. (A) Changes in levels of MMP in cells incubated with As_2S_2 at concentrations of 0.5, 2 and 8 μM for 48 h. (B) Depletion of MMP in cells incubated with As_2S_2 at a concentration of 8 μM for 24 to 72 h. Data are the mean ± SD of 3 independent experiments. *$p<0.05$ as compared with control group. (C) Changes in levels of CD11b expression compared with MMP and p38 MAPK in cells incubated with 8 μM As_2S_2 for 24 to 72 h. CD11b expression, MMP and p38 MAPK levels are presented as the relative MFI, each of which was defined as: (sample MFI/control MFI) × 100. (D) Typical histograms showing the changes of MMP, as assessed by MFI, following treatment with As_2S_2 at a concentration of 8 μM for 24 to 72 h in HL-60 cells. Shaded or unshaded area represents the data of untreated- or treated- cells, respectively.

3.3.7 Effects of inhibition of p38 MAPK activation on As_2S_2-induced differentiation

As showed in Fig. 3-2B and Fig. 3-8C, when the cells were treated with As_2S_2 at a concentration of 8 μM for 24-72 h, the CD11b-positive cells increased to the maximum at 48 h, while that significantly decreased at 72 h, and at this time, the activation of p38 MAPK rose to the maximum. Then, the effects of activation of p38 MAPK on As_2S_2-induced differentiation were assessed. After treatment of cells with 8 μM As_2S_2 alone or combination with 10 μM SB203580, an inhibitor of p38 MAPK, for 72 h, the CD11b-positive cells significantly increased following treatment with combination as compared to that with As_2S_2 alone (Fig. 3-9A) ($p<0.05$). The changes of CD11b-positive cells treated with 8 μM As_2S_2 alone or combination with 10 μM SB203580 were also shown as histograms in Fig. 3- 9B.



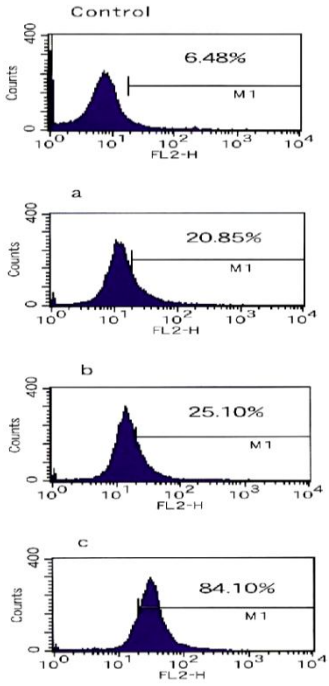


Fig. 3-9. The modified effects of inhibitor (SB203580) of p38 MAPK on As_2S_2-induced differentiation in HL-60 cells. Cells were incubated with 10 μM SB203580 (SB) and 8 μM As_2S_2 (A8) alone or in combination (SB + A8) for 72 h, respectively. After treatment, the cells were stained for CD11b and then analyzed with flow cytometer. Data are the mean ± SD of 3 independent experiments. Means were compared by 1-way ANOVA. *$p< 0.05$ as compared with 8 μM As_2S_2 (A8) alone. (B) Typical histograms showing the changes of CD11b-positive cells treated with 10 μM SB203580 (a), 8 μM As_2S_2 (b), and combination of 10 μM SB203580 with 8 μM As_2S_2 (c), respectively, for 72 h.

MFI for CD11b

3.4 DISCUSSION

In this Chapter, I showed that 16 μM of As_2S_2 induced remarkable proliferation inhibition in HL-60 cells, while 0.5, 2, and 8 μM of As_2S_2 did not produce inhibition of proliferation (Fig. 3-1). Contrarily, 2 and 8 μM As_2S_2 enhanced the viability and attenuated apoptosis in HL-60 cells following treatment for 48 h (Fig. 3-3A, B, C, and D). Similarly, a previous report has demonstrated that 8 μM As_4S_4 did not induce apoptosis in HL-60 cells following treatment for 48 h [123]. It is noteworthy that treatment with 5 μM As_2S_2 for 48 h induced apoptosis in K562, a human chronic myelogenous leukemia cell line [73]. Thus, the difference in differential response could be attributed to different cell types.

HL-60 is a leukemia cell line blocked at the level of promyelocytic differentiation. On the other hand, the cells can be induced to differentiate into neutrophils and other functional cells depending on the inducer molecules. The differentiation is accompanied by the increase in CD11b expression level and the acquisition of a number of functions such as NBT-reducing activity [21, 87]. It has been reported that arsenic compounds such as As_2O_3 and As_4S_4 induce differentiation in HL-60 cells, as evidenced by the increased CD11b expression level and acquisition of NBT reduction ability [123, 124, 141,]. Similarly, our experimental data demonstrated that the CD11b-positive cells significantly increased by 17 times at 48 h (Figs. 3-2A, B) and the NBT-positive cells increased at 72 h by 3 times (Fig. 3-2C), after treatment of cells with 8 μM As_2S_2, as compared to control. The differentiated cells also showed an increase in size (Fig. 3-2D). Whereas, the number of CD11b positive cells at 48 h culture with 8 μM As_2S_2 was reduced after 72 h. The underlying reason for the reduction after 72 h (Fig. 3-2B) can be explained by a negative regulatory feedback as a result of an activation of p38 MAPK signaling cascade. p38 MAPK is well known as a sensor of moderate oxidative stress, and p38 MAPK activation paralleled with the intracellular ROS accumulation (Fig. 3-7B). We observed that a decrease in CD11b expression level was accompanied by the p38 MAPK activation (Fig. 3-8C), suggesting that p38 MAPK activation resulted from oxidative stress is implicated in the negative regulation of cell differentiation. Furthermore, our experimental data showed that p38 MAPK inhibitor restored the CD11b expression level (Fig. 3-9), providing support for our proposal. Indeed, Giafis et al. has demonstrated that inhibition of p38 MAPK enhances As_2O_3 induced differentiation in NB4 cells [29].

Compared with experimental results from flow cytometry analysis, an increase in the expression level of phosphorylated p38 was observed after treatment with 8 μM As_2S_2 for 24 h, rather than 48 h. Conversely, the expression level of phosphorylated p38 seemed to decrease at 48 h. The discrepancy in the expression level of phosphorylated p38 between western blot and flow cytometry data might be attributed to different analysis techniques. Compared with western blot analysis, flow cytometry is considered to allow for screening single cell to offer the more precise expression level of phosphorylated p38. More importantly, western blot analysis also indicated the increase in the expression level of phosphorylated p38 in As_2S_2-treated HL-60 cells. Furthermore, we demonstrated that SB203580, p38 MAPK inhibitor,

significantly enhanced the CD11b expression induced by As_2S_2 treatment (Fig. 3-9). Taken together, these findings suggest that p38 MAPK is implicated in As_2S_2-mediated cellular responses including differentiation.

ROS play very critical roles in the determination of cell fate by eliciting a wide variety of cellular responses, such as proliferation, differentiation and apoptosis. Moderate levels of ROS promote cell survival and proliferation; however, once ROS exceed the capacity of the antioxidant system including GSH to remove them, the cells suffer so-called "oxidative stress", and are leading to severe dysfunctions or deaths [146]. We have demonstrated a transient increase in ROS levels in HL-60 cells treated with As_2S_2. A transient increase in the amount of intracellular GSH was observed 0.5 h after treatment with As_2S_2, followed by a substantial decrease in the GSH content after that. These results suggest that As_2S_2 induced oxidative stress in HL-60 cells through the depletion of GSH, although a transient increase in GSH content was observed as a result of response to increased ROS production.

Furthermore, as we indicated in this study, treatment with As_2S_2 ranging from 0.5 to 8μM for 24 to 48 h dramatically augmented the percentage of the CD11b-positive cells (Figs. 3-2A, B). Importantly, pretreatment with H_2O_2 significantly increased the percentage of the CD11b-positive cells induced by relatively lower concentrations of As_2S_2 (0.5 or 2 μM) when compared to each alone (Fig. 3-5A). On the other hand, compared to the treatment with 8 μM As_2S_2 alone, combination of 8 μM As_2S_2 and H_2O_2 significantly decreased the percentage of the CD11b-positive cells (Fig. 3-5A). Taken together, these results suggested that oxidative stress induced by As_2S_2 positively contribute to HL-60 cell differentiation, and that 8 μM As_2S_2 would be the more adequate concentration to achieve a high level of differentiation.

The association of oxidative stress with cell differentiation via the activation of MAPK signaling has been documented [121]. Both extent and duration of activation of MAPKs contribute to the determination of cell fate, such as survival, differentiation and apoptosis [86]. p38 MAPK is considered as a sensor of moderate oxidative stress, and this kinase was activated in a ROS-dependent manner [104]. Interestingly, p38 MAPK has previously been shown to mediate both proapoptotic/growth inhibitory [55] and antiapoptotic/progrowth [61] signals in different systems, apparently depending on the stimulus and cell type involved. A recent paper has demonstrated that inhibition of p38 MAPK enhances differentiation induced by As_2O_3 in NB4 cells, suggesting that p38 MAPK signaling cascade is activated in a negative regulatory feedback manner to control induction of mediated antileukemic effects [29]. Our study demonstrated that an activation of p38 MAPK was observed followed by the accumulation of intracellular ROS. Interestingly, the decrease in expression level of phospho-p38 MAPK at 1 h after treatment with As_2S_2 was also observed compared to 0-h point. This discrepancy may be explained that the stress resulted from medium change could contribute to relatively higher expression level at 0-h point. It is noteworthy that a decrease in CD11b expression level was accompanied by the activation of p38 MAPK (Fig. 3-8C), although the number of differentiated cells increased to the maximal level at 48 h post-treatment with As_2S_2 (Fig. 3-2B). Furthermore, an inhibitor (SB203580) of p38 MAPK restored the CD11b expression

level (Fig. 3-9). Taking these previous results and our observations into account, we suggested that activation of p38 MAPK resulted from oxidative stress seems to be implicated in the negative regulation of the differentiation.

We also surprisingly observed that 8 μM As_2S_2 did not induce apoptosis and decrease cell viability, and instead, attenuated apoptosis and enhanced cell viability, as compared to control (Figs. 3-3B, D). At the same time, a significant restoration of decreased MMP was observed at 72h post-treatment with 8 μM As_2S_2, although the decrease in MMP was confirmed at 48 h point. It is well known that the mitochondria can be viewed as a central regulator of the decision between cellular survival and death [8]. The Bcl-2 family proteins play important roles in the regulation of apoptosis by targeting to the mitochondria to exert their proapoptotic or antiapoptotic effects. There is evidence that the antiapoptotic function of Bcl-2 is dependent on its phosphorylation status rather than its expression level [63, 7]. Of note, a recent study demonstrated that p38 MAPKs are upstream regulators for the phosphorylation of Bcl-2 [63]. Furthermore, a previous report demonstrated that inhibition of p38 MAPK enhanced arsenic-induced Bcl-2 down-regulation, which suggested that p38 MAPK up-regulates Bcl-2 expression and generation of antiapoptotic responses [29]. Taken together, apoptosis suppression could be attributed to MMP restoration associated with Bcl-2 upregulation as a result of negative feedback control of p38 MAPK activation.

3.5 CONCLUSION

The following observations (1)-(4) obtained in this Chapter suggest that the activation of p38 MAPK resulted from oxidative stress implicated in the negative regulation of As_2S_2 induced differentiation in HL-60 cells.

(1) As_2S_2 induced cell differentiation based on the increment in CD11b expression, NBT-positive cells, and cell size.

(2) A transient increase in ROS level along with intracellular GSH level was observed after As_2S_2 treatment.

(3) p38 MAPK activation gradually increased after ROS generation and sustained during the As_2S_2 induced cell differentiation.

(4) Decreased CD11b expression was accompanied by p38 MAPK activation, and p38 MAPK inhibitor restored the CD11b expression.

Chaper 4

Arsenic disulfide induced apoptosis and concurrently promoted erythroid differentiation in cytokine-dependent MDS-progressed leukemia cell line F-36p with complex karyotype including monosomy 7

4.1 INTRODUCTION

MDS are a group of clonal disorders of haematopoietic stem cells characterised by ineffective haematopoiesis and a tendency to evolve into AML [50]. Chromosomal abnormalities are found in 40-60% of MDS patients, and karyotypes with monosomy 7 (-7) and complex abnormality were defined as the unfavorable cytogenetics for MDS progression to AML (MDS/AML [32]. It was reported that more than 60% of MDS/AML patients have abnormal karyotypes, including complex type abnormalities at the onset of AML [111]. MDS/AML patients with complex type abnormalities including -7 is considered to be an unfavorable risk subgroup [34, 68]. These results were also confirmed by our clinical investigations [51-53].

In our recent clinical trials, we have revealed that QHP is effective in the treatment of MDS, especially in patients with the erythroid improvement is significant, without serious adverse drug reaction observed even on long time [133-135]. Furthermore, a high responding rate (80.8%) was also observed, with blast cells decrease and hematologic improvement, in patients with moderate- and high-risk by increasing the daily dose of QHP [74]. It is a spring of our new observation that QHP was notably effective in treating two patients with acute erythroid leukemiaprogressed from MDS, although these two patients presented with the unfavorable cytogenetics of -7 and complex karyotype, respectively [35]. These results indicated that QHP is effective in treating MDS or MDS/AML even with the poor karyotype, and its therapeutic efficacy could be enhanced by increasing the *Xiong huang* content in the formula, which could be attributed to As_2S_2, the most important component of *Xiong huang*.

F-36p cell line was established from a patient diagnosed with refractory anemia with excess blasts, approximately 5 months after the initial diagnosis, and the disease progressed to overt leukemia [15]. This cell line requires granulocyte macrophage colony-stimulating factoror interleukin- 3 for continuous growth, and the cells die within several days when these cytokines are depleted. The majority of the established cells had a homogeneous karyotype: 43,Y, Xp+, -5, -7, -13, -16, -17, -19, -21,2q-, 9p+, 10q+, +4mar. This cell line has a multilineage phenotype, suggesting that the transformation event occurred in a multipotent stem cell. It is also evident that the F-36p cells can be induced to differentiate into the erythroid lineage in the presence of erythropoietin. Thus, F-36p is a desirable cell line for study on the effective mechanisms of As_2S_2 in the treatment of MDS or MDS/AML.

4.2 MATERIALS AND METHODS

4.2.1 Reagents

The reagents used in this Chapter are same as those described in Chapters 1-3.

4.2.2 Cells and cell culture

F-36P cellsestablished from a patient with MDS, were obtained from RIKEN Cell Bank (Tsukuba, Japan). F-36p cells were cultured in RPMI-1640 supplemented with 5% inactivated FBS and 5 ng/mL recombinant human interleukin- 3 (Kirin, Japan) at 37° C in a 5% CO_2 humidified atmosphere.

4.2.3 MTT assay

The assay was carried out according to the procedures as described in Chapter 3.

4.2.4 Apoptosis and viability assays

The assay was carried out according to the procedures as described in Chapter 3.

4.2.5 Assessment of erythroid differentiation

F-36p cells (1×10^6/mL) were treated with the indicated concentrations of As_2S_2. After incubation for 72 h, cells were washed twice by PBS containing 2% FBS and then resuspended with 100 µl PBS containing 2% FBS. Subsequently, 10 µl FITC-conjugated CD235a (Glycophorin) antibody was added, and cells were incubated in the dark at 4 ° C for 30 min. After the incubation, cells were resuspended in 400 µl PBS containing 1% FNB, and the cells were analyzed by Becton Dickinson flow cytometry. Isotypic mouse IgG1 was used to set threshold parameters. A total of 30,000 non-gated cells were analyzed.

4.2.6 Statistics

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

4.3 RESULTS

4.3.1 Cell proliferation inhibited by As_2S_2

F-36p cells were continuously treated with As_2S_2 at a concentration range of 0.25 to 32.0 µM for 72 h, and cell growth was measured in an MTT assay. The IC_{50} value of As_2S_2 on the F-36p cell growth was 6 µM. As_2S_2 produced inhibition of proliferation in F-36p cells in a dose-dependent manner (Fig. 4-1).

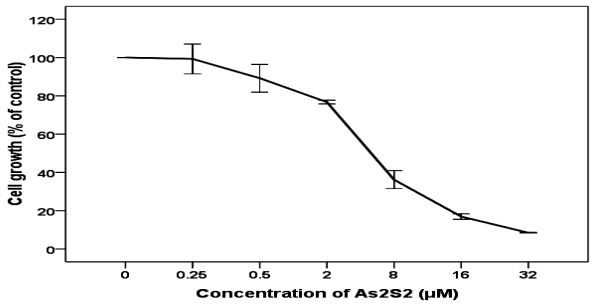


Fig. 4-1. Effects of As_2S_2 on proliferation of F-36p cells. Cells were treated with serial concentrations of As_2S_2 for 72 h. Cell proliferation was determined by an MTT assay. Data are the mean ± SD of 3 independent experiments.

4.3.2 Apoptotic cells induced by As_2S_2

Apoptotic cells were identified by annexin V-staining. After treatment with As_2S_2 at concentrations of 2, 8 and 16 μM for 72 h, the apoptotic cells significantly increased as compared to control ($p<0.05$), which were in a dose-dependent manner as analyzed by the multiple comparisons ($p<0.05$) (Fig. 4-2A). Histograms also showed the changes of apoptotic cells following treatment with As_2S_2 at concentrations of 2, 8 and 16 μM for 72 h (Fig. 4-2B).

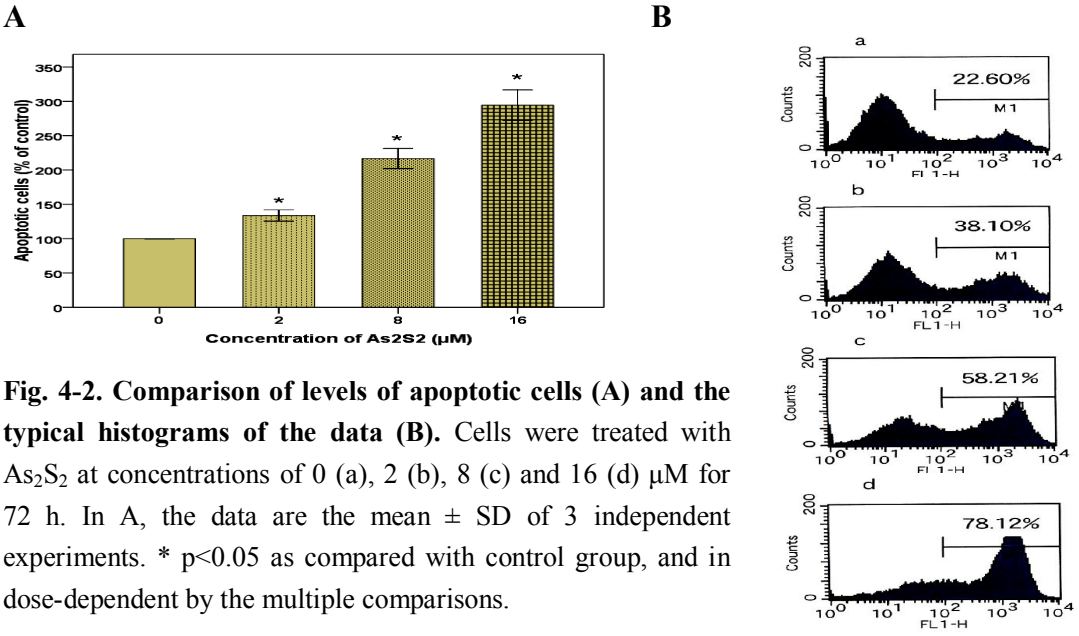


Fig. 4-2. Comparison of levels of apoptotic cells (A) and the typical histograms of the data (B). Cells were treated with As_2S_2 at concentrations of 0 (a), 2 (b), 8 (c) and 16 (d) μM for 72 h. In A, the data are the mean ± SD of 3 independent experiments. * $p<0.05$ as compared with control group, and in dose-dependent by the multiple comparisons.

4.3.3 Cell viability after treatment with As_2S_2

Cell viability was determined by a PI exclusion. After treatment with As_2S_2 at concentrations of 2, 8 and 16 μM for 72 h, the cell viability significantly decreased as compared to control ($p<0.05$), which were in a dose-dependent manner as analyzed by the multiple comparisons ($p<0.05$) (Fig. 4-3A). Dot plot analysis also showed the changes of inviable cells (Fig. 4-3B) following treatment of As_2S_2 at concentrations of 2, 8 and 16 μM for 72 h.

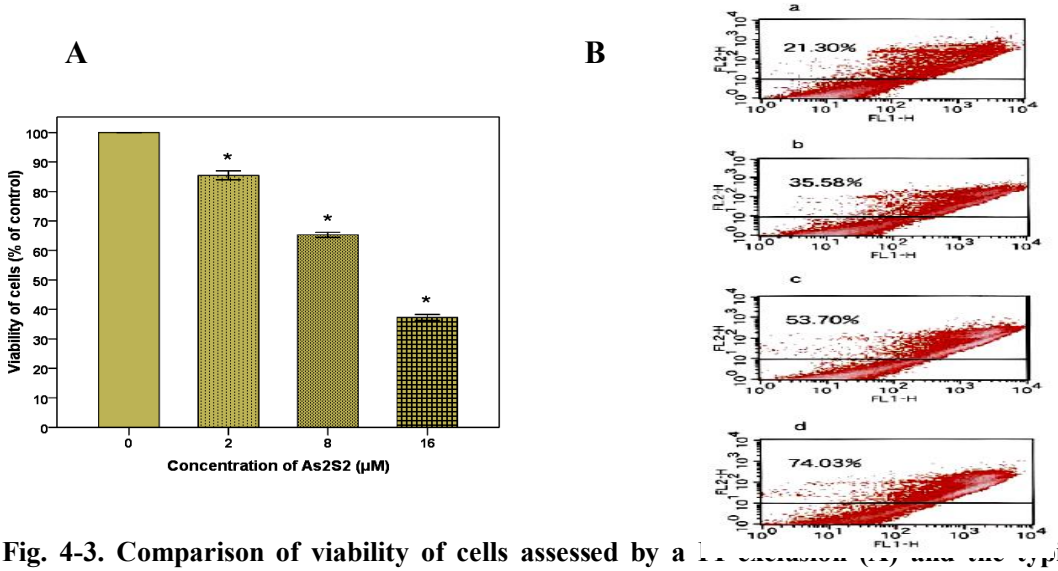


Fig. 4-3. Comparison of viability of cells assessed by a PI exclusion (A) and the typical

intensity dots of the data of inviable cells (B). Cells were treated with As_2S_2 at concentrations of 0 (a), 2 (b), 8 (c) and 16 (d) μM for 72 h. In A, the data are the mean ± SD of 3 independent experiments. *p<0.05 as compared with control group, and in dose-dependent by the multiple comparisons.

4.3.4 Erythroid differentiation of cells induced by As_2S_2

Erythroid differentiated cells were detected by CD235a (GpA) expression. After treatment of cells with As_2S_2 at concentrations of 2, 8 and 16 μM for 72 h, the CD235a-positive cells significantly increased as compared to control ($p<0.05$), which were in a dose-dependent manner as analyzed by the multiple comparisons ($p<0.05$) (Fig. 4-4A). Histograms showed the changes of CD235a expression of cells following treatment with As_2S_2 at concentrations of 2, 8 and 16 μM for 72 h (Fig. 4-4B).

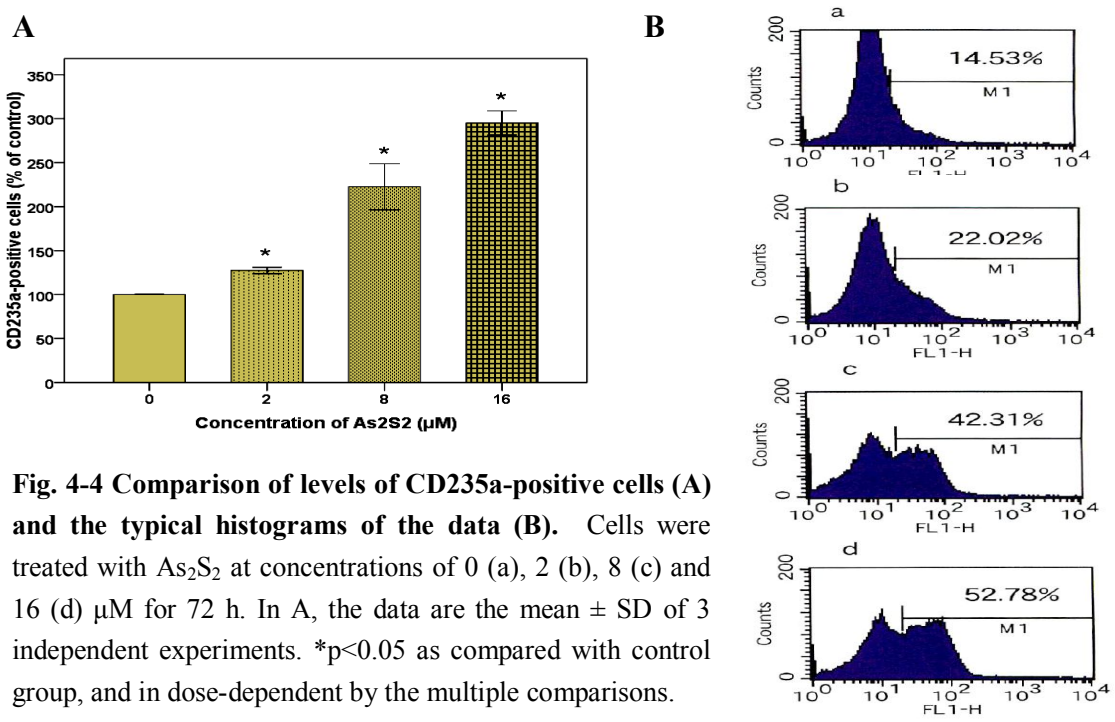


Fig. 4-4 Comparison of levels of CD235a-positive cells (A) and the typical histograms of the data (B). Cells were treated with As_2S_2 at concentrations of 0 (a), 2 (b), 8 (c) and 16 (d) μM for 72 h. In A, the data are the mean ± SD of 3 independent experiments. *p<0.05 as compared with control group, and in dose-dependent by the multiple comparisons.

4.4 DISCUSSION

MDS/AML patients have higher probabilities of multidrug resistance to chemotherapy, lower rates of complete remission, and the poor prognosis [71]. Univariate analyses showed that the hemoglobin level and cytogenetic abnormalities were factors that contributed to the overall survival [68]. The data have shown that MDS/AML patients tended to have complex type abnormalities including -7, which is considered to be an unfavorable risk subgroup [34, 68]. The response rate to aggressive chemotherapies such as AML induction regimens for these patients was 41-72% and median survival time was 9-14.5 months [41]. In a recent report of the 29 patients with MDS/AML, 21 patients had an abnormal karyotype. Among them, 13 had complex type abnormalities and/or -7, and the response rate in the patients with a normal karyotype was significantly better than in those with an abnormal karyotype (62.5% vs. 4.8%) [68].

In addition, patients with MDS or MDS/AML often have symptoms due to low haemoglobin levels [99]. Much of the effort has been devoted to the correction of anemia. Transfusion support is still the mainstay of the treatment of anemia, and which was the sole therapeutic option in many cases [2]. Recombinant human erythropoietin offer substantial possibilities of haematological improvement, while response was poor in these patients [113,117]. Hematopoietic stem cell transplantation is thought to be the only promising treatment for fundamental solution of the disease. However, it is generally restricted to younger adults who have an HLA-matched donor.

Our clinical trials have documented that QHP is effective in the treatment of MDS or MDS/AML patients by hematologic improvement and blast cells decrease, without serious adverse drug reaction observed even on long time [35, 74, 133, 134, 135]. Especially, the life spans of two MDS/AML patients were 8 and 2 years, respectively, which are longer than those of the other reports [34, 68], after treatment with QHP by increasing the *Xiong huang* content in the formula, although these patients presented with the unfavorable cytogenetics of -7 and complex karyotype, respectively [35]. *Xiong huang* contains > 90% As_2S_2 based on the quality standard in *Chinese Pharmacopeia* in 2010 edition. The therapeutic efficacy of QHP could be enhanced by increasing the *Xiong huang* content in the formula, which could be attributed to As_2S_2.

F-36p cell line was established from a patient with AML progressed from MDS [68]. Chromosomal study of the freshly obtained bone marrow cells indicated the presence of normal and abnormal clones. Thirteen of 18 mitotic figures showed normal karyotype (43, XY). Four showed essentially the same karyotype: -5**,** -7, -9, -17, -18, 19p+, and some markers, and additional random loss was also observed in each mitotic figure. The majority of the established cells had a homogeneous karyotype: 43,Y, Xp+, -5, -7, -13, -16, -17, -19, -21,2q-, 9p+, 10q+, +4mar. This cell line has a multilineage phenotype and can be induced to differentiate into the erythroid lineage in the presence of erythropoietin. F-36p cell line might provide a desirable cell model for the study of effective mechanisms of As_2S_2 in the treatment of MDS or MDS/AML. In the present study, As_2S_2 could inhibit proliferation and viability, induce apoptosis, and concurrently promote erythroid differentiation in F-36p cells, which were in a dose-dependent manner.

In the previous Chapters, the mechanisms of As_2O_3-induced apoptosis were well documented in leukemia cells, which were via depletion of intracellular glutathione, disruption of mitochondrial membrane potential and activation of caspase-3 [44, 48]. While there is little information of arsenic-induced apoptosis or the involvement of apoptosis in F-36p cells, the expression of P2 purinergic receptor genes was reported to be observed in these cells, and extracellular ATP could induce apoptosis and control cell cycle via P2 receptor genes [139]. F-36p cells can be induced to differentiate into the erythroid lineage in the presence of erythropoietin [68]. It was reported that STAT5, signal transducers and activators of transcription, appears to mediate transcriptional regulation of cyclin D1, thereby contributing to cytokine-dependent growth of F-36p cells [85]. The in vitro kinase assay demonstrated that the

erythropoietin-initiated signal transduction of erythroid differentiation can be occurred through Src, a prototype molecule of Src family PTKs, and phosphatidylinositol 3-kinase (PI3-kinase) in F-36p cells [95, 69]. The association of As_2S_2 inducing apoptosis and erythroid differentiation promotion with these molecules is needed to assess in future studies.

4.5 CONCLUSION

It can be concluded from the following observations (1)-(4) that As_2S_2 could inhibit proliferation and viability, induce apoptosis, and concurrently promote erythroid differentiation dose- dependently in F-36p cells. This, to our knowledge, is the first discription that As_2S_2 can inhibit proliferation and viability, induce apoptosis, and concurrently promote erythroid differentiation in cytokine-dependent MDS-progressed human leukemia cell line F-36p with complex karyotype including -7.

(1) As_2S_2 inhibited the proliferation of F-36p cells.

(2) The apoptotic cells significantly increased by As_2S_2 in a dose-dependent manner.

(3) The cell viability was significantly inhibited by As_2S_2 dose-dependently.

(4) Significant increase in CD235a-positive cells was concurrently observed by As_2S_2 dose-dependently.

GENERAL DISCUSSION AND CONCLUSIONS

MDR is recognized as one of the most common causes of failure of chemotherapy in the treatment of cancer patients [45-47]. P-gp is an ATP-binding cassettetransporter, which hydrolyses ATP and expels cytotoxic drugs from mammalian cells [102]. The data in Chapter 1 revealed that 94% of MOLT-4/DNR cells express P-gp. MOLT-4/ DNR cells exhibited significantly higher P-gp efflux activity than parental MOLT-4 cells. However, both parental MOLT-4 and resistant MOLT-4/DNR cell lines were highly sensitive to As_2O_3. As_2O_3 similarly inhibited growth and induced apoptosis in these two cell lines, and the effects of As_2O_3 were time- and dose-dependent, which suggested that P-gp in MOLT-4/DNR cells was not involved in the detoxification of As_2O_3. Furthermore, the data in Chapter 2 showed that As_2O_3 induces apoptosis in MOLT-4 and MOLT-4/DNR cells via the depletion of intracellular GSH, and subsequent MMP disruption and caspase-3 activation.

As_2S_2, the most important component of *Xiong huang* which contains > 90% As_2S_2 based on the quality standard in *Chinese Pharmacopeia* in 2010 edition, was a candidate for its good therapeutic reputation and perceived low toxicity in traditional medicines. The data in Chapter 3 demonstrated that As_2S_2 induced cell differentiation in HL-60 cells based on the increment in CD11b expression, NBT-positive cells, and cell size. A transient increase in ROS level along with intracellular GSH level was also observed. p38 MAPK activation gradually increased after ROS generation and sustained during the cell differentiation. Decreased CD11b expression was accompanied by p38 MAPK activation, and p38 MAPK inhibitor restored the CD11b expression. These results suggest that the activation of p38 MAPK resulted from oxidative stress seems to be implicated in the negative regulation of As_2S_2 induced differentiation in HL-60 cells.

F-36p cell line was established from a patient diagnosed with MDS progressed to overt leukemia, which is a desirable cell line for study on the effective mechanisms of As_2S_2 in the treatment of MDS or MDS/AML. This work shown in Chapter 4, to our knowledge, is the first discription that As_2S_2 can inhibit proliferation and viability, induce apoptosis, and concurrently promote erythroid differentiation in cytokine-dependent MDS-progressed human leukemia cell line F-36p with complex karyotype including -7. My investigation for the precise action mechanisms of As_2S_2 in vitro in human malignant cells thus imply the rationale and future directions of As_2S_2 as a potential anticancer drug candidate.

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