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ORIGINAL ARTICLE

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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

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Abstract *Purpose*: To demonstrate that arsenic trioxide (As_2O_3) induces apoptosis via a mitochondrial pathway in both parent T lymphoblastoid leukemia MOLT-4 cells and cells of its daunorubicin-resistant subline, MOLT-4/DNR, expressing functional P-gp. *Methods*: Cell growth was measured using an MTT assay. Cell viability was determined using a dye exclusion test. Intracellular glutathione (GSH) was measured using a glutathione assay kit. Mitochondrial membrane potential (MMP) was assessed by rhodamine 123 (Rh123) staining intensity on flow cytometry. Caspase-3 activity was evaluated using a commercially available assay kit on flow cytometry. The percentage of cells undergoing apoptosis was estimated in terms of caspase$^+$/PI$^-$ cells on flow cytometry after assessment for activation of caspase-3 by adding PI. *Results*: MOLT-4 cells and MOLT-4/DNR cells were similarly sensitive to the apoptosis-inducing effect of As_2O_3. Buthionine sulfoxide (BSO) and ascorbic acid (AA) rendered these cells more sensitive to As_2O_3, whereas N-acetylcysteine (NAC) reduced this sensitivity. BSO and AA decreased, but NAC increased, the intracellular GSH contents of both MOLT-4 and MOLT-4/DNR cells. Decreasing GSH with BSO potentiated As_2O_3-mediated growth inhibition, disruption of MMP, activation of caspase-3 and apoptosis of cells. Clinically relevant doses of AA enhanced the anticancer effects of As_2O_3 via the disruption of MMP, activation of caspase-3, and induction of apoptosis. In contrast, increase GSH levels with NAC attenuated all of these As_2O_3-mediated actions. *Conclusions*: The sensitivity of MOLT-4 and MOLT-4/DNR cells to As_2O_3 was associated with the intracellular GSH content. As_2O_3 induced apoptosis in parent MOLT-4 cells and MOLT-4/DNR cells expressing functional P-gp via depletion of intracellular GSH, and subsequent disruption of MMP and activation of caspase-3.

Keywords Arsenic trioxide · Apoptosis · Glutathione · Mitochondrial membrane potential · Caspase-3

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Introduction

In response to the observation that patients with chemorefractory acute promyelocytic leukemia (APL) still respond to arsenic trioxide (As_2O_3) therapy [14, 45, 47] and in vitro studies of the effects of As_2O_3 on drug-resistant APL cell lines [11], investigations have been initiated to evaluate the therapeutic potential of As_2O_3 in several malignant diseases [44, 56, 57]. Most of the reports suggest that As_2O_3 suppresses growth and induces apoptosis in malignant cells including drug-resistant cells [5, 11, 44, 56, 57].

Some data indicate that As_2O_3 is not sensitive to drug efflux pump mechanisms of resistance [12, 37]. We have established a cell line resistant to daunorubicin (DNR) from T lymphoblastoid leukemia MOLT-4 cells [28]. This resistant cell line, MOLT-4/DNR, has been revealed to overexpress functional P-glycoprotein (P-gp) [28]. Our previous study showed that As_2O_3 is effective in the suppression of growth of MOLT-4 and MOLT-4/DNR cells [15]. Moreover, we found that As_2O_3 induces apoptosis in both MOLT-4 and MOLT-4/DNR cells [15]. MOLT-4/DNR cells have been revealed to be more than ten times more resistant to DNR than the parent MOLT-4 cells, as assessed by an MTT assay [15]. As_2O_3 inhibits growth and induces apoptosis in MOLT-4/DNR

cells without having an influence on their P-gp expression and function [15].

Thus, in the present study, we examined the apoptosis-inducing mechanisms of As_2O_3 in MOLT-4 and MOLT-4/DNR cell lines from the viewpoint of modification of cellular GSH levels, mitochondrial membrane potential (MMP), and caspase-3.

Materials and methods

Reagents

RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, N.Y.). Cell proliferation kits I and II (MTT) were purchased from Roche Diagnostics (Indianapolis, Ind.). Ascorbic acid (AA), N-acetylcysteine (NAC), L-buthionine-[*S*,*R*]-sulfoxide (BSO), rhodamine 123 (Rh123) and As_2O_3 were obtained from Sigma Chemical Company (St. Louis, Mo.). All of the agents were dissolved in phosphate-buffered saline (PBS) and diluted to a working concentration before use. A GSH assay kit was obtained from Cayman Chemical Company (Ann Arbor, Mich.). Metaphosphoric acid (MPA) and triethanolamine (TEAM) 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.

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. 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. The cells were incubated for 72 h in an atmosphere comprising 5% CO_2/air at 37°C in a humidified chamber.

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 and the plate was mixed on a microshaker for 10 s. The cells were further incubated for 4 h in a humidified atmosphere. Subsequently, 100 μl solubilization solution (Roche) were added to each well, and the plate was mixed on a microshaker for 10 s and allowed to stand overnight in an incubator in a humidified atmosphere. The spectrophotometric absorbance of the sample was measured on a microplate reader (Corona MT P-32; Corona Company, Ibaragi, Japan) at 570 nm. A dose response curve was plotted for each drug, and the concentrations that yielded 50% inhibition of cell growth (IC_{50}) were calculated.

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°C in a humidified chamber. The number or percentage of viable cells was determined by staining cell populations with trypan blue. On the day of determination, four parts of 0.2% trypan blue (w/v in water) were mixed with one part of 5× saline (4.25% NaCl, w/v in water), then one part of the trypan blue saline solution was added to one part of the cell suspension. Subsequently, the cells were loaded into a hemocytometer, and the unstained (viable) cells and the stained (dead) cells were counted separately within 3 min of being stained with trypan blue.

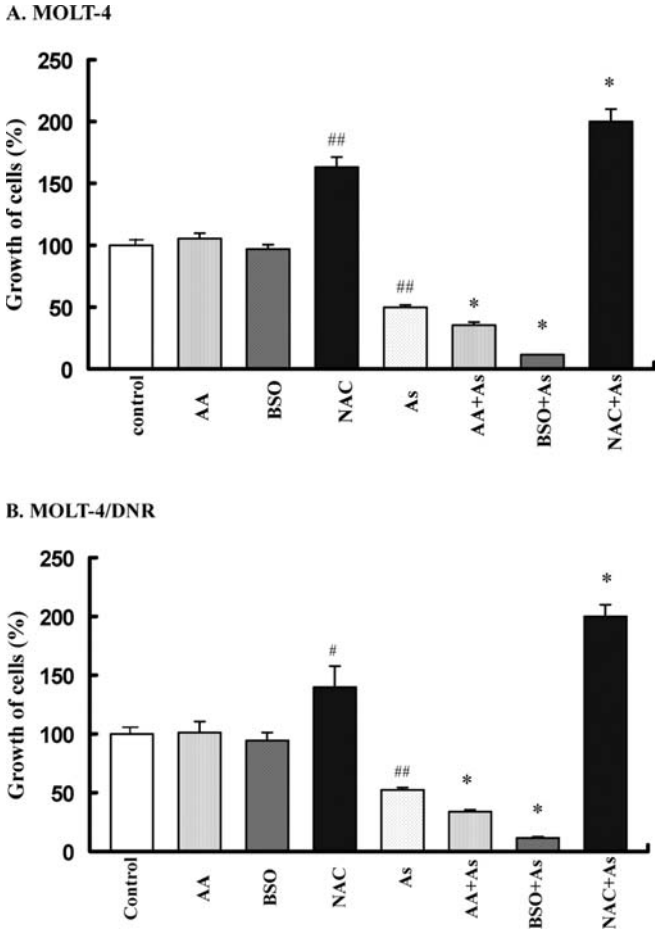

Fig. 1A, B Modulation of As_2O_3-mediated growth inhibition of MOLT-4 (**A**) and MOLT-4/DNR (**B**) cells by BSO, AA, and NAC. Cells were treated with 5 μmol/l of As_2O_3 in the absence or presence of 100 μmol/l BSO, 125 μmol/l AA, and 10 mmol/l NAC for 72 h. Cell growth was determined by an MTT assay. 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

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°C. The cells were resuspended in 500 μl 50 m*M* 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°C, 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 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

Table 1 IC_{50} values (μM) of As_2O_3 alone or in combination with BSO, AA, and NAC. Cells were treated with 100 μmol/l BSO, 125 μmol/l AA, and 10 mmol/l NAC for 72 h, and cell growth was determined by an MTT assay. Values are the means ± SD of three independent experiments

Treatment	MOLT-4	MOLT-4/DNR
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

*$P < 0.01$, **$P < 0.001$ vs As_2O_3 alone

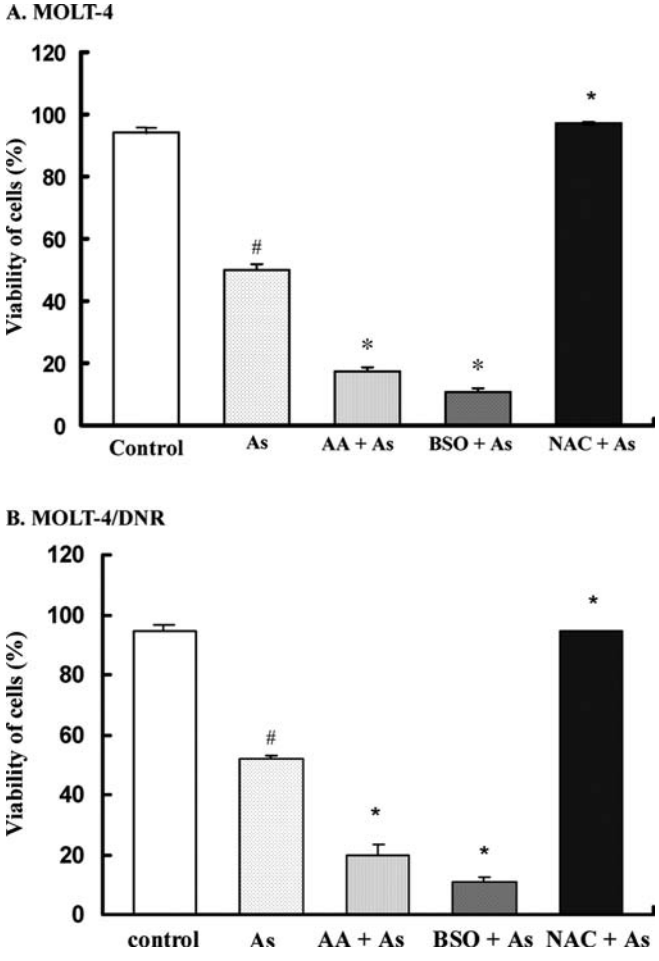

Fig. 2A, B Modulation of As_2O_3-mediated reduction in viability of MOLT-4 (**A**) and MOLT-4/DNR (**B**) cells by BSO, AA, and NAC. Cells were incubated in the absence or presence of 5 μmol/l As_2O_3 in the absence or presence of 100 μmol/l BSO, 125 μmol/l AA, or 10 mmol/l NAC for 72 h. Cell viability 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

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.

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

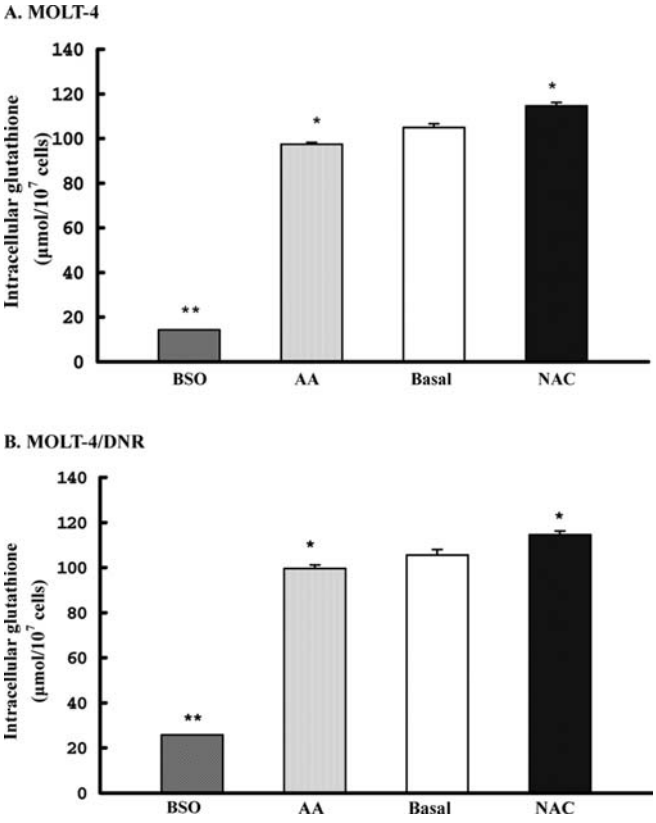

Fig. 3A, B Modulation of intracellular GSH levels by BSO, AA, and NAC in MOLT-4 (**A**) and MOLT-4/DNR (**B**) cells. Cells were incubated in the absence or presence of 100 μmol/l BSO, 125 μmol/l AA, or 10 mmol/l NAC for 48 h. GSH levels were measured using a GSH assay kit as described in Materials and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

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).

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°C 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 resus-

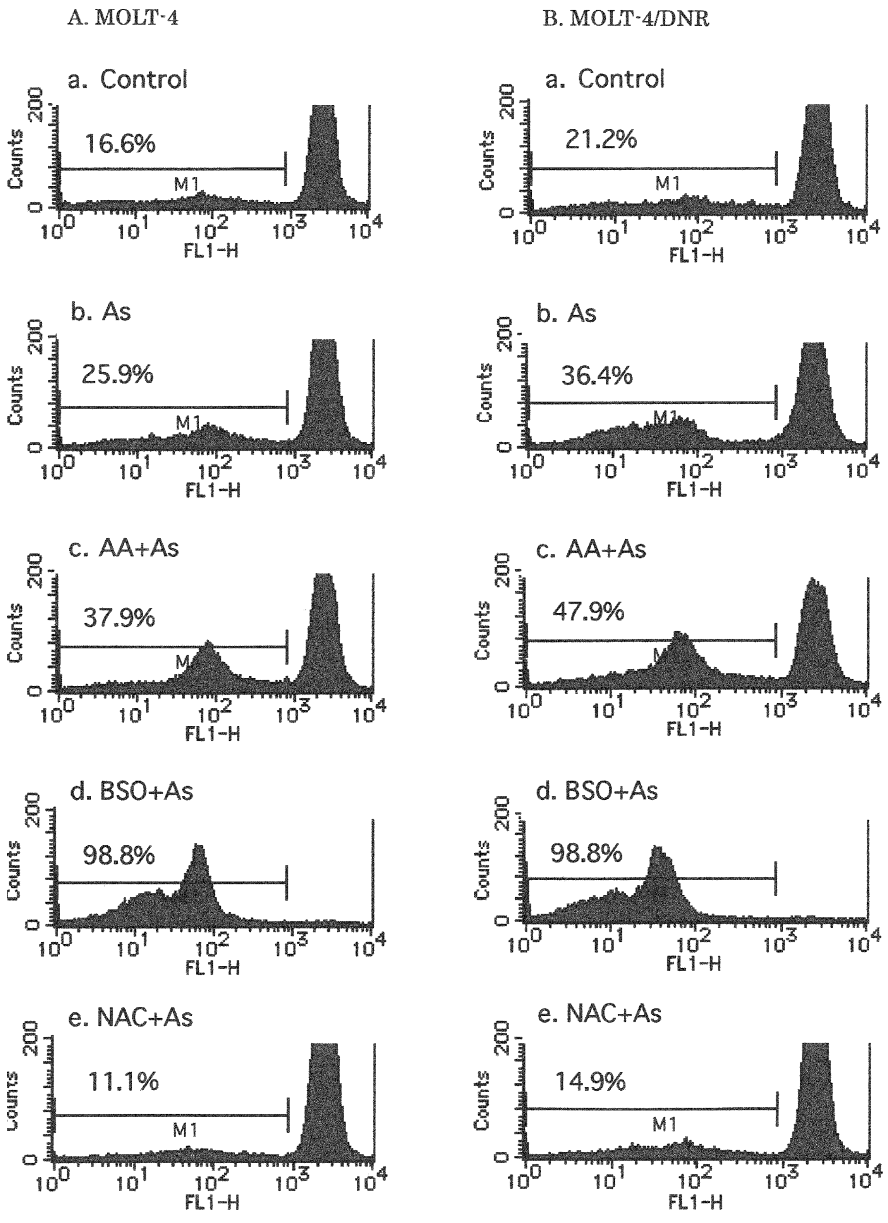

Fig. 4A, B Histograms showing the disruption of MMP in MOLT-4 cells (**A**) and MOLT-4/DNR cells (**B**) incubated for 72 h in the absence (*a*) or presence of 5 μmol/l As_2O_3 alone (*b*) or with a combination of 5 μmol/l As_2O_3 and 125 μmol/l AA (*c*), 100 μmol/l BSO (*d*), or 10 mmol/l NAC (*e*).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

pended 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.

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.

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).

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. In each case, *P* values less than 0.05 were considered to be significant.

Results

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 μmol/l As_2O_3 on MOLT-4 and MOLT-4/DNR cells were significantly enhanced by 100 μmol/l BSO and 125 μmol/l AA (Fig. 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 the MOLT-4 and MOLT-4/DNR cells were 4.8 μmol/l and 5.3 μmol/l, respectively (Table 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 μmol/l in the presence of 100 μmol/l 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 1). Whereas, 100 μmol/l BSO and 125 μmol/l AA alone did not affect the growth of either MOLT-4 or MOLT-4/DNR cells (the IC_{50} cells of both BSO and AA were higher than 500 μmol/l). 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 μmol/l As_2O_3 in combination with 10 mmol/l NAC for 72 h (Fig. 1). Thus, NAC made the cells of these two cell lines 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 μmol/l (Table 1).

The viability of MOLT-4 and MOLT-4/DNR cells was significantly decreased following incubation for 72 h with 5 μmol/l As_2O_3 plus 100 μmol/l BSO or 125 μmol/l AA, as compared to cells treated with As_2O_3 alone ($P < 0.001$, Fig. 2). In contrast, 10 mmol/l NAC markedly increased the viability of these cells under the same culture conditions ($P < 0.001$, Fig. 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 $\mu M/10^7$ cells, respectively, when the two cell lines were incubated in the absence of 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 μmol/l BSO, 125 μmol/l AA or 10 mmol/l NAC for 48 h. These treatments resulted in significant changes in GSH content (Fig. 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. 3A) and MOLT-4/DNR (Fig. 3B) cells. Whereas, NAC significantly increased the GSH content ($P < 0.05$) in these cells.

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 Fig. 4. In each histogram, the percentage of cells with decreased MMP is indicated. The disruption of MMP in MOLT-4 (Figs. 4A and 5A) and MOLT-4/DNR (Figs. 4B and 5B) cells was significantly higher ($P < 0.01$) after 72 h treatment with 5 μmol/l As_2O_3 than in untreated cells. This

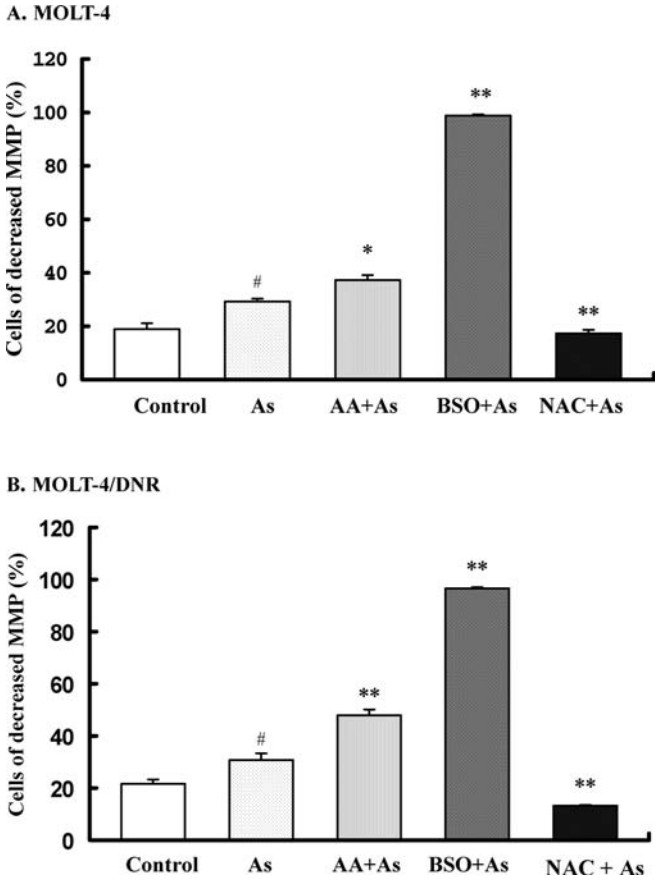

Fig. 5A, B MMP disruption in MOLT-4 cells (**A**) and MOLT-4/DNR cells (**B**) treated with 5 μmol/l As_2O_3 alone or with a combination of 5 μmol/l As_2O_3 with 125 μmol/l AA, 100 μmol/l BSO, or 10 mmol/l NAC for 72 h. Values are the means + SD of three independent experiments. $^{\#}P < 0.01$ vs control; $^{*}P < 0.01$, $^{**}P < 0.001$ vs As_2O_3 alone

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. 5A) and MOLT-4/DNR (Fig. 5B) cells (Fig. 4Ac,d, Bc,d). In particular, BSO elevated this ability almost completely (Fig. 5), while the As_2O_3-induced disruption of MMP was blocked by NAC in these cells (Fig. 4Ad,e, Bd,e, and Fig. 5). In contrast, 125 μmol/l AA, 100 μmol/l BSO and 10 mmol/l NAC treatment alone did not influence the MMP of these cells (data not shown).

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 Fig. 6. In each histogram the percentage of cells expressing relatively high activity of caspase-3 is indicated. After 72 h of treatment, 5 μmol/l As_2O_3 significantly induced caspase-3 activity in MOLT-4 ($P < 0.01$, Fig. 7A) and MOLT-4/DNR ($P < 0.01$, Fig. 7B) cells (Fig. 6). This activation of caspase-3 was markedly potentiated by AA

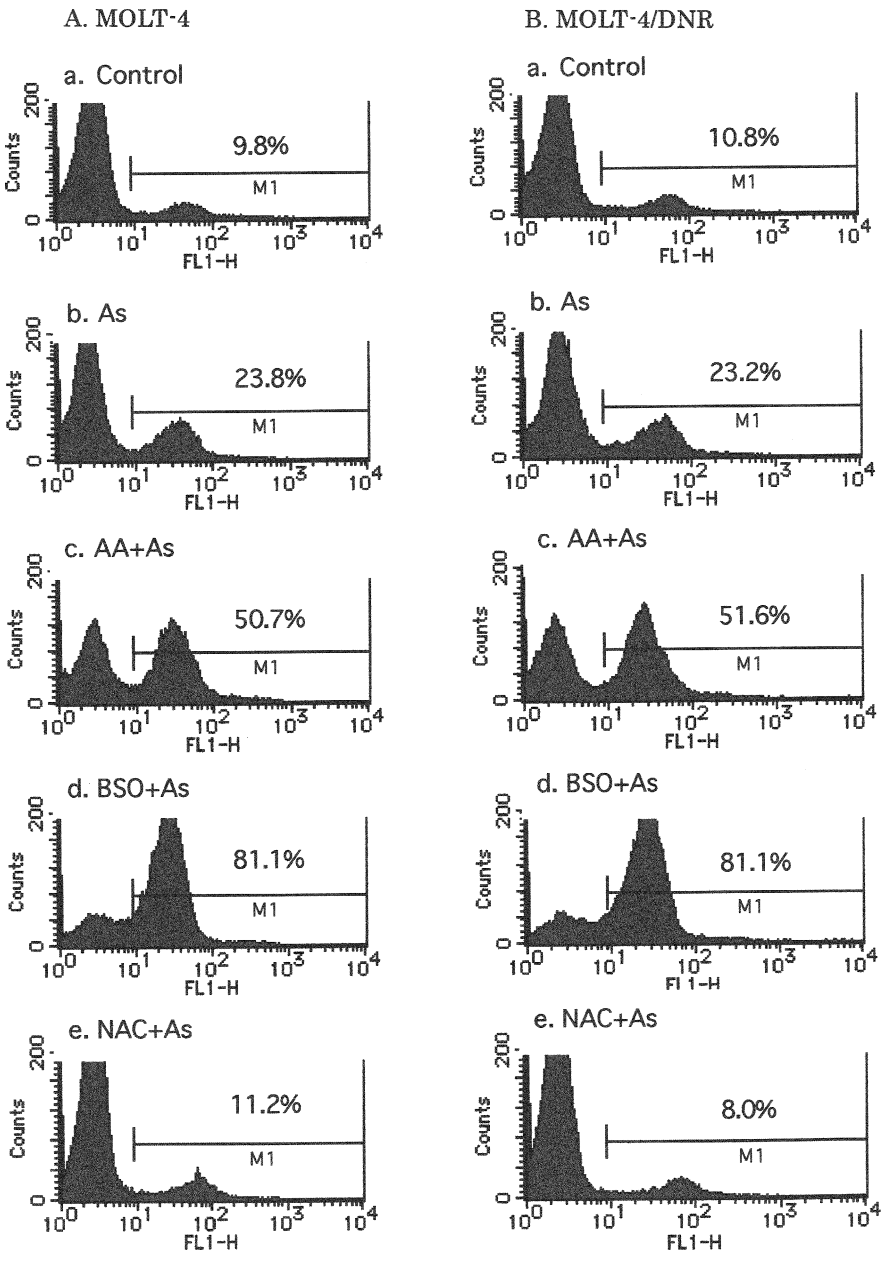

Fig. 6A, B Histograms showing the changes in percentage of MOLT-4 cells (**A**) and MOLT-4/DNR cells (**B**) exhibiting caspase-3 activity following treatment for 72 h in the absence (*a*) or presence of 5 μmol/l As_2O_3 alone (*b*) or with a combination of 5 μmol/l As_2O_3 with 125 μmol/l AA (*c*),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

($P < 0.001$) and BSO ($P < 0.001$) in both MOLT-4 (Fig. 7A) and MOLT-4/DNR (Fig. 7B) cells (Figs. 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. 7A) and MOLT-4/DNR ($P < 0.001$, Fig. 7B) cells (Fig. 6Ae, Be). In contrast, 125 μmol/l AA, 100 μmol/l BSO and 10 mmol/l NAC treatment alone did not stimulate the caspase-3 activity of these cells (data not shown).

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 Fig. 8. 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 μmol/l As_2O_3 alone or in combination with 100 μmol/l BSO, 125 μmol/l AA or 10 mmol/l NAC. 5 μmol/l As_2O_3 treatment alone induced apoptosis in both MOLT-4 ($P < 0.01$, Fig. 9A) and MOLT-4/DNR cells ($P < 0.001$, Fig. 9B) as compared to the control cells (Figs. 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. 9A) and MOLT-4/DNR cells (Figs. 9B, and 8Ac,d, Bc,d), whereas NAC markedly decreased the percentage of apoptotic cells induced by As_2O_3 in both MOLT-4 ($P < 0.001$, Fig. 9A) and MOLT-4/DNR ($P < 0.01$,

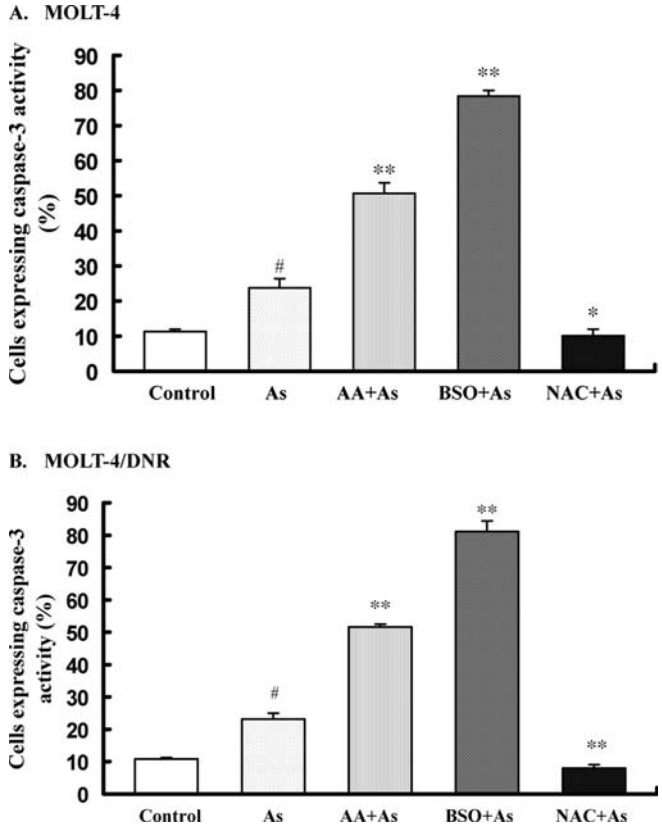

Fig. 7A, B Changes in caspase-3 activity after treating MOLT-4 cells (**A**) and MOLT-4/DNR cells (**B**) for 72 h with 5 μmol/l As_2O_3 alone or with a combination of 5 μmol/l As_2O_3 with 125 μmol/l AA, 100 μmol/l BSO, or 10 mmol/l NAC. Values are the means + SD of three independent experiments. $^{\#}P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

Fig. 9B) cells (Fig. 8Ae, Be). Cells treated with 100 μmol/l BSO, 125 μmol/l AA or 10 mmol/l NAC alone did not show an increase in the percentage of apoptotic cells (data not shown).

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

Discussion

GSH content had a decisive effect on As_2O_3-mediated cytotoxicity

Our previous work has shown 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 [15]. The findings of this study indicate 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 findings led us to conclude 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 [33]. 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 [49], which is easily excreted by the MRP2/cMOAT transporter [20]. 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 [6]. 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 [33].

AA has been shown to have pro-oxidant properties [3, 40]. Auto-oxidation of AA to dehydroascorbate results in the production of H_2O_2 [42, 50]. 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 [51, 53]. 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 [12]. Indeed, it has been found that AA significantly synergizes with As_2O_3 in treating patients with acute myeloid leukemia (AML) [2]. 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 [52], while NAC contains two such thiol groups [12] and gives GSH. Taking these observations into consideration, the decrease in GSH levels following incubation with BSO and AA could abate As_2O_3 efflux, 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 efflux, and thereby attenuate the toxic effects of As_2O_3.

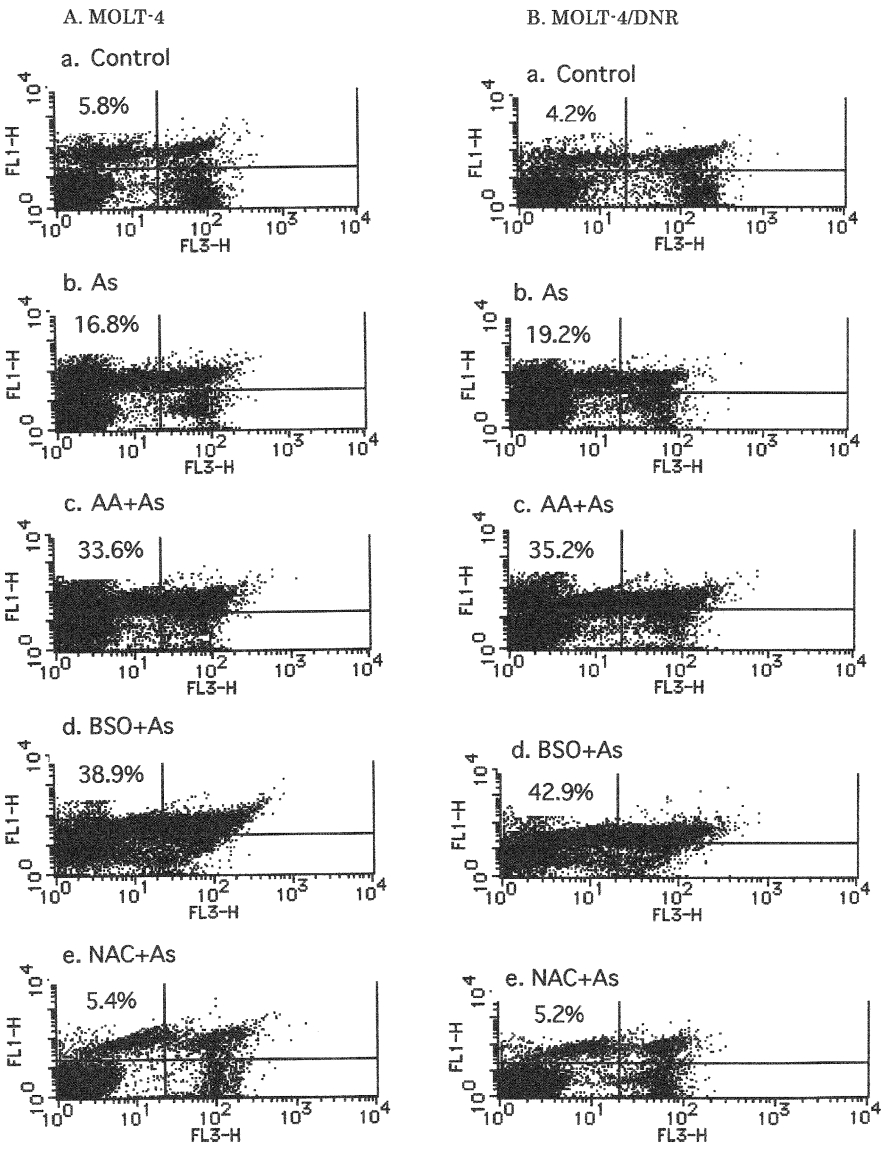

Fig. 8A, B Dot plots showing apoptotic MOLT-4 cells (**A**) and MOLT-4/DNR cells (**B**) following incubation for 72 h in the absence (*a*) or presence of 5 μmol/l As_2O_3 alone (*b*) or with a combination of 5 μmol/l As_2O_3 with 125 μmol/l AA (*c*), 100 μmol/l BSO (*d*), or 10 mmol/l NAC (*e*). The 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)

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 to mitochondria [46]. It has been found that the changes to mitochondria after treatment with As_2O_3 can be divided into three stages [46]. 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 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 [9]. 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 [42]. Caspases are cysteine proteases that mediate apoptosis by proteolysis of specific substrates [21]. Caspase-3 is considered a primary executioner of apoptosis [43].

In the present study, the As_2O_3-mediated growth inhibition in parental MOLT-4 and MOLT-4/DNR cells is similar to that observed in our previous work [15]. These suppressive effects of As_2O_3 can be potentiated by BSO, which inhibits synthesis of GSH, and by AA, which reduces GSH-dependent dehydroascorbate. In contrast, the effect of As_2O_3 was attenuated by NAC, which promotes synthesis of GSH in both MOLT-4 and MOLT-4/DNR cells. Furthermore, the disruption of MMP and activation of caspase-3 are not only caused by As_2O_3 itself, but are also enhanced by AA and BSO and 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. After 72 h of treatment with As_2O_3 alone or in combination with

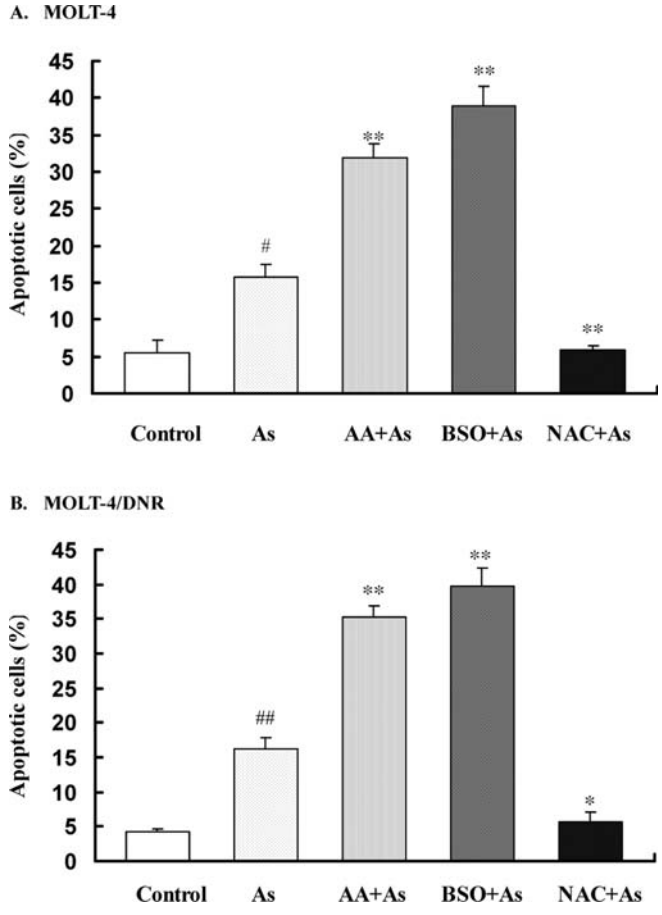

Fig. 9A, B As_2O_3-induced apoptosis and its modulation by BSO, AA, or NAC in MOLT-4 cells (**A**) and MOLT-4/DNR cells (**B**) treated with 5 μmol/l As_2O_3 alone or with a combination of 5 μmol/l As_2O_3 with 125 μmol/l AA, 100 μmol/l BSO, or 10 mmol/l NAC for 72 h. Values are the means + SD of three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

BSO or AA, the percentages of cells with decreased MMP and activated caspase-3 were higher than the percentage of cells that underwent apoptosis. These results suggest that MMP collapse and caspase-3 activation occur prior to apoptosis.

Recent reports suggest that sensitivity to As_2O_3 correlates with intracellular GSH levels in cancer cells [55]. The GSH content modulates the growth-inhibitory and apoptosis-inducing effects of arsenicals [7, 19, 33, 34, 41]. 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 [19, 25]. Arsenic-resistant cells are also reported to contain higher levels of GSH [22, 25, 29]. Moreover, cells with increased GSH levels can be sensitized to As_2O_3 by agents that deplete intracellular GSH [7, 22]. The cytotoxic effects of As_2O_3 may be influenced by modulators of GSH [12, 13, 23, 32], especially in drug-resistant cell lines [10, 38].

A decrease in GSH levels in cells acts as a potent early activator of apoptosis signaling [1]. Depletion of GSH, especially mitochondrial GSH, is believed to induce the loss of MMP [31]. Mitochondrial permeability transition resulting from intracellular thiol depletion is known to be a critical event in apoptosis [26, 54]. Mitochondria undergo major changes in membrane integrity before classical signs of apoptosis become manifest, and these changes lead to disruption of the MMP [48]. These alterations in mitochondrial activity can be distinguished and monitored by the fluorescent dye Rh123 on flow cytometry [42, 50], which specifically stains mitochondria depending on the MMP [24, 35, 39]. Mitochondrial depolarization precedes caspase-3 activation and apoptosis [43], and apoptotic cells express high caspase-3 activity [16, 58]. Caspase-3 is considered to be a primary executioner of apoptosis [4]. 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 [27].

As_2O_3 is known to act at several points in apoptosis induced through mitochondrial pathways [8], which includes the forming of reversible bonds with thiol groups [49] and the depletion of GSH [7, 22, 34, 55], loss of MMP [17], and activation of caspase-3 [10, 17, 18, 30, 36]. From these points of view, we examined the apoptosis-inducing effect of As_2O_3 in the presence of GSH modulators in MOLT-4 and MOLT-4/DNR cells.

We conclude 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.

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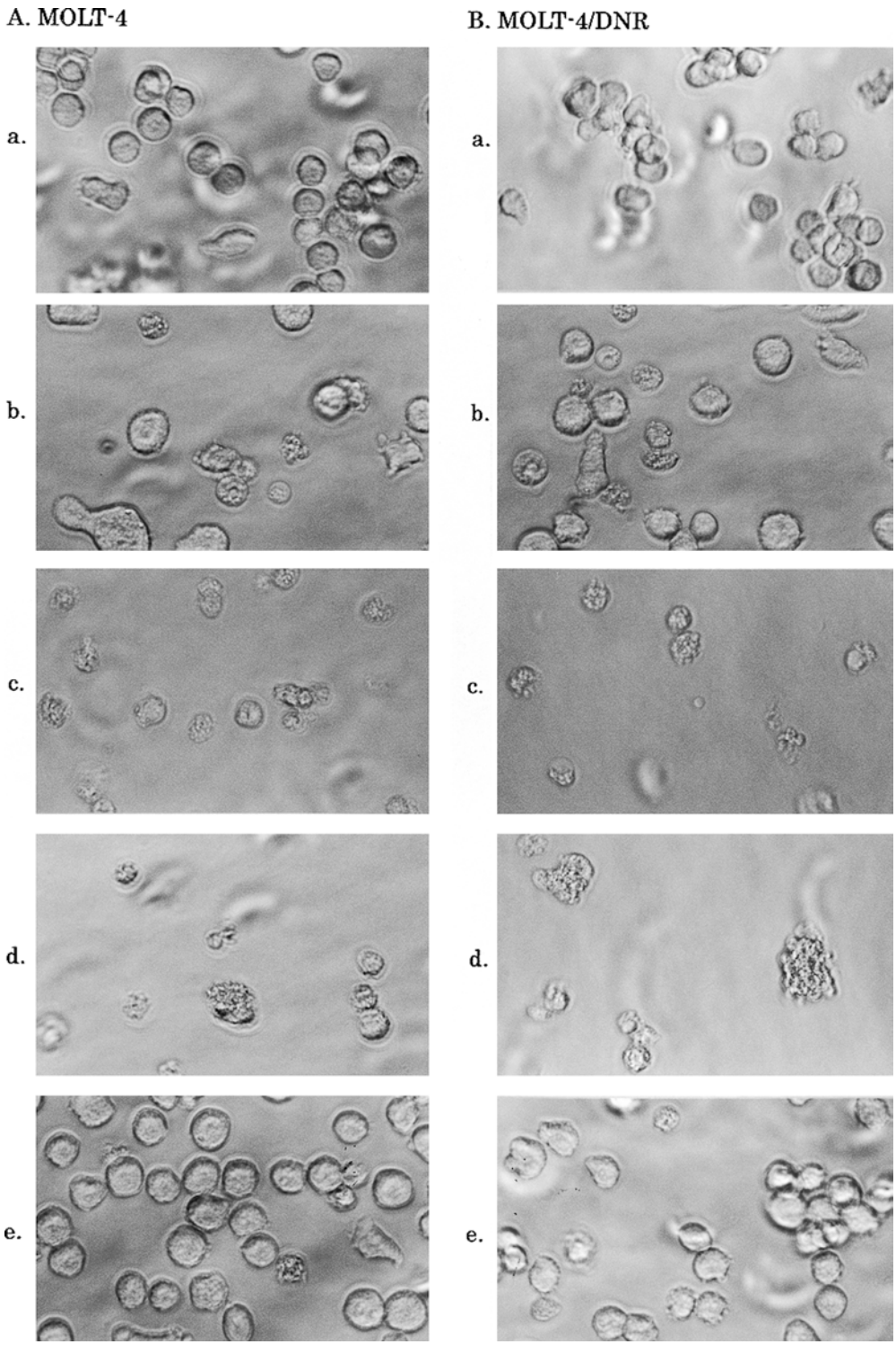

Fig. 10A, B Morphology of MOLT-4 cells (**A**) and MOLT-4/DNR cells (**B**) after treatment with 5 μmol/l As_2O_3 or combination of 5 μmol/l As_2O_3 with 100 μmol/l BSO, 125 μmol/l AA, or 10 mmol/l NAC for 72 h (*a* untreated cells, *b* cells treated with 5 μmol/l As_2O_3 alone, *c* cells treated with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

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