Apoptosis and Growth Inhibition in Malignant Lymphocytes After Treatment With Arsenic Trioxide at Clinically Achievable Concentrations

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Background: **Arsenic trioxide (As_2O_3) can induce clinical remission in patients with acute promyelocytic leukemia via induction of differentiation and programmed cell death (apoptosis). We investigated the effects of As_2O_3 on a panel of malignant lymphocytes to determine whether growth-inhibitory and apoptotic effects of As_2O_3 can be observed in these cells at clinically achievable concentrations.** ***Methods:*** **Eight malignant lymphocytic cell lines and primary cultures of lymphocytic leukemia and lymphoma cells were treated with As_2O_3, with or without dithiothreitol (DTT) or buthionine sulfoximine (BSO) (an inhibitor of glutathione synthesis). Apoptosis was assessed by cell morphology, flow cytometry, annexin V protein level, and terminal deoxynucleotidyl transferase labeling of DNA fragments. Cellular proliferation was determined by 5-bromo-2′-deoxyuridine incorporation into DNA and flow cytometry and by use of a mitotic arrest assay. Mitochondrial transmembrane potential ($\Delta\Psi_m$) was measured by means of rhodamine 123 staining and flow cytometry. Protein expression was assessed by western blot analysis or immunofluorescence.** ***Results:*** **Therapeutic concentrations of As_2O_3 (1–2 μ*M*) had dual effects on malignant lymphocytes: 1) inhibition of growth through adenosine triphosphate (ATP) depletion and prolongation of cell cycle time and 2) induction of apoptosis. As_2O_3-induced apoptosis was preceded by $\Delta\Psi_m$ collapse. DTT antagonized and BSO enhanced As_2O_3-induced ATP depletion, $\Delta\Psi_m$ collapse, and apoptosis. Caspase-3 activation, usually resulting from $\Delta\Psi_m$ collapse, was not always associated with As_2O_3-induced apoptosis. As_2O_3 induced PML (promyelocytic leukemia) protein degradation but did not modulate expression of cell cycle-related proteins, including c-myc, retinoblastoma protein, cyclin-dependent kinase 4, cyclin D1, and p53, or expression of differentiation-related antigens.** ***Conclusions:*** **Substantial growth inhibition and apoptosis without evidence of differentiation were induced in most malignant lymphocytic cells treated with 1–2 μ*M* As_2O_3. As_2O_3 may prove useful in the treatment of malignant lymphoproliferative disorders. [J Natl Cancer Inst 1999;91: 772–8]**

Apoptosis (i.e., programmed cell death) has emerged as an important biologic mechanism that contributes to the maintenance of the integrity of multicellular organisms. Impairment of apoptosis has been implicated in many human diseases including cancers *(1,2)*. For instance, deregulation of apoptosis is regarded as a major factor contributing to the development of chronic lymphocytic leukemia (CLL) and some types of lymphoma, such as follicular B-cell lymphoma with the chromosomal translocation t(14;18), which results in overexpression of the death antagonist Bcl-2 *(3–5)*. Therefore, understanding the basic mechanisms that underlie apoptosis will help identify new potential targets for treatment of these diseases *(6,7)*.

Recently, arsenic trioxide (As_2O_3), an ancient drug used in Traditional Chinese Medicine and, in the last century, in western medicine, attracted wide interest for its ability to induce complete remission in most patients with acute promyelocytic leukemia (APL), mainly through the induction of apoptosis and differentiation *(8–14)*. Moreover, preliminary *in vitro* studies (Chen GQ, Chen Z, Jing YK, Waxman S: unpublished data) revealed that clinically achievable concentrations of As_2O_3 can also trigger apoptosis in chronic myelogenous leukemia cells, some multiple myeloma cells, and some solid tumor cells, such as esophageal cancer and neuroblastoma cells, suggesting that the ability of As_2O_3 to induce apoptosis is not limited to APL. On the other hand, Konig et al. *(15)* reported that melarsoprol, an organic arsenic compound synthesized by complexing melarsen oxide with the metal-chelating drug dimercaprol, can induce cell apoptosis with concentration-dependent (0.1–1 μ*M*) inhibition of Bcl-2 messenger RNA (mRNA) expression in chronic B-cell leukemia cell lines WSU-CLL, 183CLL, and JVM-2, whereas 0.1 μ*M* As_2O_3 had no effect on the growth, survival, or Bcl-2 mRNA expression in the same cells. However, the toxic effects of melarsoprol restrict its clinical use; in contrast, the intravenous infusion of 10 mg of As_2O_3 results in plasma concentrations higher than 1–2 μ*M* without causing substantial hematopoietic toxicity *(16)*. Hence, 0.1 μ*M* may not be the ideal concentration of As_2O_3 for *in vitro* pharmacologic studies.

In this study, we investigated the response of eight malignant lymphocytic cell lines and primary acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), and lymphoma cells to a wide range of As_2O_3 concentrations (0.1–2 μ*M*). We examined the effect of As_2O_3 on apoptosis and/or growth inhibition as well as on mitochondrial transmembrane potential ($\Delta\Psi_m$) collapse in malignant lymphocyte populations. Since studies in the early 1900s already proposed that the interaction with active sulf-

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See "Notes" following "References."

hydryl (SH) groups of biologic molecules is the most important mechanism by which trivalent arsenicals exert their toxic effects [reviewed in *(17)*], we addressed the possible mechanisms of As_2O_3 treatment in malignant lymphocytes by using buthionine sulfoximine (BSO), a selective inhibitor of γ-glutamylcysteine synthetase and thus of glutathione synthesis, and dithiothreitol (DTT), a widely used disulfide-bond-reducing agent.

Materials and Methods

Reagents. A solution of As_2O_3 for intravenous administration (0.1% or 5 m*M*) was prepared by the Department of Pharmacy in the First Affiliated Hospital of Harbin Medical University (Harbin, People's Republic of China). A stock solution was prepared from the above formulation by dilution to 1 m*M* in phosphate-buffered saline (PBS) and stored at 4 °C; further dilutions to working concentrations were made before use. DTT and BSO were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell culture, cell viability, and cell morphology. The following eight human cell lines were used: B-lymphocytic lineages from pre-B-cell ALL (Nalm-6), Burkitt's lymphoma (Namalwa and Raji), B-cell lymphoma (BJAB), follicular B-cell lymphoma with t(14;18) chromosomal translocation (su-DHL-4), T-lymphocytic lineages from T-cell ALL (Molt-4 and Jurkat), and CLL (SKW-3). Primary malignant lymphocytes were prepared from bone marrow or lymph node biopsy specimens obtained from patients who gave written informed consent. The study was approved by the Institutional Review Board of Shanghai Institute of Hematology. Briefly, bone marrow or lymph nodes were minced with scissors, and a fraction enriched (>85%) in malignant lymphocytes was obtained by centrifugation (1000*g* at 4 °C for 10 minutes) through Ficoll–Paque (Pharmacia Biotech AB, Uppsala, Sweden). The cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Cell viability was estimated by trypan blue dye exclusion. Cell morphology was evaluated by Wright's staining of cells prepared by cytospin centrifugation (250*g* for 4 minutes at room temperature).

DNA proliferation assays. DNA flow cytometry was conducted by use of propidium iodide (PI) staining. To perform flow cytometry of 5-bromo-2′-deoxyuridine (BUdR)-labeled cells, we utilized anti-BUdR antibody and a protocol from Boehringer Mannheim GmbH (Mannheim, Germany). A mitotic arrest assay was performed as described previously *(18)*. Briefly, after pretreatment with 1 μ*M* As_2O_3 for 72 hours at 4 °C, 10^5 cells/mL were incubated with 0.2 mg/mL Colcemid (Life Technologies, Inc.) for 4, 8, 12, and 16 hours. Cells were then centrifuged (300*g* at 4 °C for 10 minutes), washed, and fixed overnight in 75% ethanol, and the number of cells in the G_2 + M phase (sub-G_1 cells) was determined by flow cytometry. The percentage of cells in the G_2 + M phase (*Y*) and the length of Colcemid treatment, in hours (*X*), were linearly related ($Y = kX + b$). The cell cycle time was estimated by adding the absolute *X* values for $Y = 0$ and $Y = 100\%$.

Cell differentiation assays. The expression of lymphocyte surface differentiation antigens, including CD19, CD20, CD22, SmIg, CD2, CD7, CD4, CD8, and CD3, was determined by flow cytometry. All monoclonal antibodies, including Simultest™ control immunoglobulin (Ig) G1/IgG2a (negative control), were purchased from Becton Dickinson (San Jose, CA).

Determination of cellular adenosine triphosphate (ATP) levels. Cellular ATP levels were measured by the bioluminescence assay. Briefly, after treatment with As_2O_3 (1–2 μ*M*) for 2, 6, 12, 24, or 48 hours, 2×10^5 cells were resuspended in 3 mL of boiled distilled water containing 1 m*M* magnesium sulfate, maintained at 100 °C for 10 minutes, and stored at −20 °C for further analysis. The diluted cell extract (0.2 mL) was added to 0.8 mL of luciferin–luciferase reaction buffer (Institute of Plant Physiology, Academia Sinica, Shanghai, People's Republic of China) in a polystyrene cuvette, and the ATP-dependent luciferase activity was measured by use of a luminometer. The light emission (300–900 nm) was determined in a counter, and ATP standard curves were constructed each time.

Apoptosis assays. Cell morphology was evaluated, and the percentage of hypodiploid cells was quantitated as described previously *(11,12)*. Annexin V was assayed by flow cytometry (antibody and protocol from Boehringer Mannheim GmbH), and poly-adenosine diphosphate (ADP) ribose polymerase (PARP) degradation was assayed by western blot analysis. *In situ* terminal deoxynucleotidyl transferase labeling was performed on cytospin slides according to the protocols recommended by Clontech Laboratories, Inc. (Palo Alto, CA). Mitochondrial transmembrane potential ($\Delta\Psi_m$) was determined by flow cytometry. Briefly, As_2O_3-treated and -untreated cells (about 10^6 cells) were washed twice with PBS and incubated with 10 mg/mL rhodamine 123 (Rh123) at 37 °C for 30 minutes. Subsequently, PI was added, and Rh123 and PI staining intensity was determined by flow cytometry. All data were collected, stored, and analyzed with the use of LYSIS II software (Becton Dickinson).

Western blot analysis. Protein extracts (20 μg) were prepared from 2×10^7 cells. They were loaded onto an 8%–12% polyacrylamide gel containing sodium dodecyl sulfate, subjected to electrophoresis, and transferred to nitrocellulose membranes. The blots were stained with 0.2% Ponceau S red to ensure equal protein loading, blocked with 10% defatted milk powder, and incubated for 90 minutes at 37 °C with human polyclonal (retinoblastoma protein [Rb], c-myc, cyclin D1, cyclin-dependent kinase [CDK] 4, CDK inhibitor p16, and PARP) or anti-human Cpp32 (anti-caspase-3) and p53 monoclonal antibodies. All antibodies were purchased from Santa-Cruz Biotechnology (Santa Cruz, CA). Immunocomplexes were visualized by chemiluminescence (ECL kit RPN2108; Amersham Life Science, Buckinghamshire, U.K.).

Immunofluorescence analysis. Cells were centrifuged (250*g* for 4 minutes at room temperature) onto slides as described above and rapidly air-dried. Promyelocytic leukemia (PML) protein immunofluorescence was performed with the use of an anti-PML antiserum raised against the N-terminal region of PML, provided by Dr. T. Naoe (Branch Hospital, School of Medicine, Nagoya University, Nagoya, Japan).

Results

Growth Inhibition and Apoptosis in Malignant Lymphocytic Cell Lines and Primary Cells

As_2O_3 exerted substantial dose- and time-dependent growth inhibition in all malignant lymphocytic cell lines examined, but different cells exhibited distinct sensitivities to As_2O_3. As_2O_3 at 0.1–0.25 μ*M* did not substantially inhibit the growth of most cells during 3–5 days of treatment (data not shown). At 1–2 μ*M* As_2O_3, however, growth inhibition was evident after 1–3 days and became substantial after 3–5 days in almost all cell lines, but the response in Jurkat cells was weaker than in the other cell lines (Table 1). Furthermore, As_2O_3 reduced cell viability, an effect that depended on drug concentration and cell type; e.g., 1 μ*M* As_2O_3 decreased the viability of Molt-4, BJAB, and SKW-3 cells but not the viability of the other cells tested, whereas 2 μ*M* As_2O_3 decreased the viability of all cell lines except Jurkat cells. The decrease in cell viability was accompanied by the appearance of morphologic characteristics of apoptosis, such as shrinking cytoplasm, condensed chromatin, and nuclear fragmentation with intact cell membrane, as observed in SKW-3 and Molt-4 cells (data not shown). Cultures of these cells demonstrated a dose- and time-dependent increase in the number of cells with a sub-G_1 DNA content. (A representative result with SKW-3 cells is shown in Fig. 1, A.) Terminal deoxynucleotidyl transferase labeling demonstrated DNA fragmentation in 1 μ*M* As_2O_3-treated SKW-3 cells and 2 μ*M* As_2O_3-treated Namalwa cells (data not shown). In addition, as shown in Fig. 1, B, the fraction of annexin V-positive SKW-3 cells increased to 73% after treatment with 1 μ*M* As_2O_3 for 48 hours. These results suggested that 2 μ*M* As_2O_3 or less induced apoptosis in most malignant lymphocytic cell lines studied.

Because some morphologic features, such as chromatin condensation, could also be associated with cell differentiation, we analyzed B- and T-lymphocyte differentiation-related antigens. However, As_2O_3 did not affect differentiation of these cell lines, since there were no substantial alterations in the expression of CD19, CD20, CD22, SmIg, CD7, CD2, CD4, CD8, and CD3 in all cell lines treated with 1 μ*M* As_2O_3 for 9 days (data not shown).

To further understand the effect of

Table 1. Effects of arsenic trioxide (As_2O_3) treatment on growth and survival of malignant lymphocytes

	Cell line*							
	SKW-3	Molt-4	BJAB	su-DHL-4	Namalwa	Raji	Nalm-6	Jurkat
				1 μM As_2O_3				
Inhibition, %								
Day 1	7.5 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	14.0 ± 2.1	13.5 ± 2.1	23.2 ± 4.2	27.6 ± 13.9	0.0 ± 0.0
Day 3	63.9 ± 1.2	44.7 ± 3.6	32.7 ± 3.9	30.3 ± 1.1	57.3 ± 10.3	42.7 ± 4.6	42.6 ± 6.5	15.8 ± 0.5
Day 5	77.5 ± 14.7	82.1 ± 7.6	81.0 ± 5.6	64.3 ± 2.9	82.0 ± 11.1	77.5 ± 2.3	72.3 ± 13.1	25.8 ± 1.6
Viability, %								
Day 1	87.0 ± 2.9	96.4 ± 5.1	94.0 ± 2.6	92.0 ± 2.7	97.4 ± 3.2	93.2 ± 2.0	100 ± 0.0	95.8 ± 0.6
Day 3	75.3 ± 1.3	60.6 ± 3.4	85.4 ± 1.1	82.0 ± 3.1	90.6 ± 4.5	88.5 ± 3.3	97.7 ± 2.5	97.0 ± 1.8
Day 5	63.8 ± 2.2	37.9 ± 1.4	38.9 ± 0.9	87.0 ± 4.3	93.0 ± 5.5	88.2 ± 5.2	80.4 ± 3.1	97.7 ± 1.6
				2 μM As_2O_3				
Inhibition, %								
Day 1	55.0 ± 9.2	12.0 ± 1.2	0.0 ± 0.0	36.8 ± 1.7	8.1 ± 1.1	36.0 ± 4.7	17.2 ± 1.4	0.0 ± 0.0
Day 3	93.2 ± 0.0	69.8 ± 20.0	76.9 ± 25.6	84.0 ± 2.7	73.5 ± 11.8	57.3 ± 1.4	72.3 ± 18.0	31.0 ± 3.1
Day 5	98.5 ± 11.6	99.6 ± 1.1	99.1 ± 0.0	98.0 ± 1.1	86.9 ± 19.3	90.4 ± 23.4	100 ± 0.0	41.7 ± 11.4
Viability, %								
Day 1	74.7 ± 2.3	87.7 ± 1.1	83.2 ± 1.4	82.0 ± 4.2	92.4 ± 0.1	88.6 ± 0.3	84.7 ± 8.1	92.0 ± 2.4
Day 3	25.0 ± 1.3	30.1 ± 2.5	25.9 ± 2.7	76.0 ± 3.9	71.5 ± 5.6	50.1 ± 2.5	60.6 ± 4.7	87.6 ± 3.2
Day 5	17.5 ± 1.1	4.2 ± 0.8	5.5 ± 1.7	10.0 ± 1.0	61.0 ± 3.7	34.8 ± 5.2	0.0 ± 0.0	80.3 ± 3.6

*Data are means ± standard deviations of triplicate experiments. SKW-3 = human T-cell chronic lymphocytic leukemia; Molt-4 and Jurkat = human T-cell acute lymphocytic leukemia; BJAB = B-cell lymphoma; su-DHL-4 = follicular B-cell lymphoma; Namalwa and Raji = Burkitt's lymphoma; Nalm-6 = pre-B-cell acute lymphocytic leukemia.

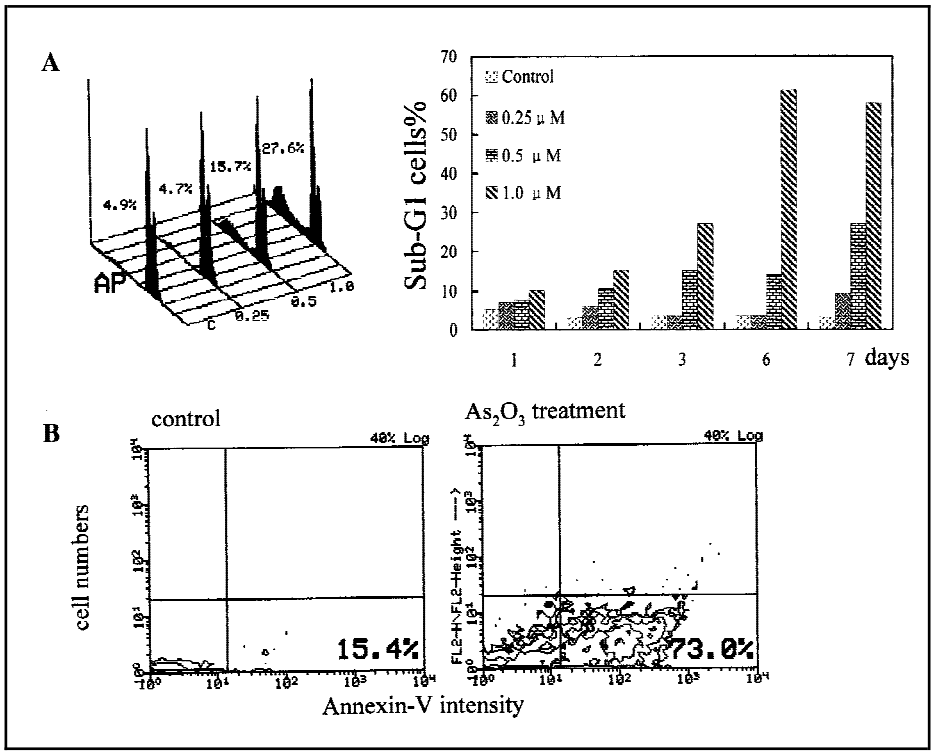

Fig. 1. Distribution of nuclear DNA contents and detection of annexin V protein, indicating apoptosis induced by arsenic trioxide (As_2O_3) in SKW-3 (human T-cell chronic lymphocytic leukemia) cells. **A) Left panel:** Distribution of nuclear DNA contents, measured by flow cytometry, in SKW-3 cells treated with As_2O_3 for 3 days; AP indicates the position of sub-G_1 cells, and c indicates findings for control cells (no As_2O_3); 0.25, 0.5, and 1.0 represent micromolar concentrations of As_2O_3. The percentage of AP cells at each As_2O_3 concentration is indicated. **Right panel:** Percentages of sub-G_1 cells induced by different concentrations of As_2O_3 in SKW-3 cells. **B)** SKW-3 cells were treated with or without (control) 1.0 μM As_2O_3 for 48 hours. Annexin V-positive cells were measured by flow cytometry, and the percentage of positive cells in each case is indicated.

As_2O_3 on malignant lymphocytes, we used primary cultures to study lymphocytes obtained from six patients with malignant lymphoproliferative disorders. As_2O_3 reduced the viability of bone marrow cells isolated from four CLL patients and from one patient with primary B-lineage ALL and of lymph node cells isolated from one patient with B-cell lymphoma. This reduction was time (2–5 days) and dose (0.1–1 μ*M* As_2O_3) dependent. After 5 days of treatment with 1 μ*M* As_2O_3, cell viability ranged from 5% to 35% of untreated cells in the above studies.

Lengthened Cell Cycle Time and Decreased Expression of PML Protein

As mentioned above, 1 μ*M* As_2O_3 inhibited the growth of nearly all cell lines examined, but it did not induce apoptosis in Nalm-6, Namalwa, Raji, su-DHL-4, and Jurkat cells. These results indicated that 1 μ*M* As_2O_3 exerted a cytostatic effect in these cells. DNA flow cytometry and BUdR-incorporation assays showed that 1 μ*M* As_2O_3 did not substantially alter the distribution of these cells throughout the phases of the cell cycle (data not shown). However, the mitotic arrest assay using Colcemid revealed that the cell cycle time was prolonged in 1 μ*M* As_2O_3-treated cells. The cell cycle time was 35.4 hours versus 63.4 hours and 49.9 hours versus 75.3 hours for untreated and 1 μ*M*

As_2O_3-treated Namalwa and Raji cells, respectively. No substantial alteration in cell cycle time was found in Jurkat cells upon treatment with 1 μM As_2O_3 (28.1 hours), as compared with untreated cells (32.2 hours). These results suggest that 1 μM As_2O_3 can inhibit proliferation of some malignant lymphocytic cell lines by prolonging the cell cycle instead of arresting cells in a specific phase. Moreover, western blotting revealed that expression of cell cycle-related proteins, including Rb, cyclin D1, CDK4, p16, p53, and c-myc, was not substantially altered in a number of cell lines after treatment with 1 μM As_2O_3 for 12, 24, and 48 hours (Fig. 2, A; data not shown). However, immunofluorescence analysis with an anti-PML antibody showed that the number of PML speckles, which in untreated cells ranged from 15 to 25 speckles per nucleus, decreased to fewer than five speckles per nucleus after a 24-hour treatment with 1 μM As_2O_3 (data not shown). This finding is consistent with the observation that the PML protein is degraded in NB4 cells treated with 1 μM As_2O_3 *(11)*.

ATP Levels, Mitochondrial Transmembrane Potential, and Activation of Caspase-3

The above data suggest that As_2O_3 treatment had dual effects on malignant lymphocytic cells, i.e., growth arrest and apoptosis. To find an association with growth inhibition, we measured cellular ATP contents in Namalwa, SKW-3, and Jurkat cells after treatment with As_2O_3. Jurkat cells, consistent with their insensitivity to As_2O_3, had basal ATP levels that were twofold higher than those of Namalwa and SKW-3 cells. Cellular ATP levels decreased approximately 25% in these cell lines after treatment with As_2O_3 (1–2 μM) for 24 hours.

Data *(19)* suggest that $\Delta\Psi_m$ collapse is a critical step that occurs in all cell types undergoing apoptosis, regardless of the inductive signal. To assess the effect of As_2O_3 on the $\Delta\Psi_m$ and to determine whether cells with a low $\Delta\Psi_m$ also lose plasma membrane integrity, we double-stained As_2O_3-treated and -untreated SKW-3, Namalwa, and Jurkat cells with PI and Rh123, a lipophilic cation that is taken up by mitochondria in proportion to the $\Delta\Psi_m$ *(20)*. As shown in Fig. 3 (left panel), untreated living cells were PI negative and strongly stained by Rh123 (PI−, $\Delta\Psi_m$ high). With 1 or 2 μM As_2O_3 treatment, a fraction of PI-negative and low-Rh123-staining (PI−, $\Delta\Psi_m$ low) SKW-3 and Namalwa cells, but not Jurkat cells, appeared in a dose- and time-dependent manner (Fig. 3, right upper panel). These results suggest that As_2O_3 decreased the $\Delta\Psi_m$ without altering plasma membrane permeability in SKW-3 and Namalwa cells, in which apoptosis was induced by 1 μM and 2 μM As_2O_3, respectively.

It is known that $\Delta\Psi_m$ collapse results in the activation of caspases, which play a central role in the apoptosis-signaling pathway *(21,22)*. We found that caspase-3 precursor was cleaved into its active form in 2 μM As_2O_3-treated Namalwa and Raji cells. At the same time, its substrate PARP was also cleaved (Fig. 2, B). However, activation of caspase-3 and degradation of PARP were not observed in As_2O_3-treated BJAB, Molt-4, and SKW-3 cells, all of which underwent substantial apoptosis in response to 1 μM As_2O_3 (data not shown).

DTT-, BSO-, and As_2O_3-Induced Apoptosis

The interaction with active SH groups has long been suspected to be the most important mechanism by which trivalent arsenicals exert their toxic effects *(17)*. To understand whether such a mechanism is involved in As_2O_3-induced $\Delta\Psi_m$ collapse, ATP depletion, and apoptosis, we treated SKW-3, Namalwa, and Jurkat cells simultaneously with 1–2 μM As_2O_3 and 0.2 mM DTT or 1 mM BSO. DTT treatment alone had no substantial effect on $\Delta\Psi_m$, cell viability, and the percentage of sub-G_1 cells in SKW-3 and Namalwa cells. However, in these two cell lines, 0.2 mM DTT blocked the $\Delta\Psi_m$ collapse induced by 2 μM As_2O_3. DTT also blocked the As_2O_3-induced decrease in cell viability, ATP depletion, and the increase in the sub-G_1 cell population (Fig. 3, right lower panel). On the other hand, treatment with 1 mM BSO alone for 24 hours induced slight $\Delta\Psi_m$ collapse and apoptosis in Namalwa cells. Although 1 μM As_2O_3 had no effect on cell viability and $\Delta\Psi_m$ in Namalwa cells, simultaneous treatment with 1 μM As_2O_3 and 1 mM BSO substantially enhanced the magnitude of $\Delta\Psi_m$ collapse and apoptosis. This cooperative effect was also evident in Jurkat cells: 2 μM As_2O_3 or 1 mM BSO treatment alone for 1–3 days did not cause $\Delta\Psi_m$ collapse, cell viability reduction, and sub-G_1 cell appearance; however, after simultaneous treatment for 1 and 2 days, the percentage of PI-negative, $\Delta\Psi_m$-low cells was 19.71% and 95.96%, respectively. Correspondingly, cell viability was decreased and sub-G_1 cells increased (data not shown).

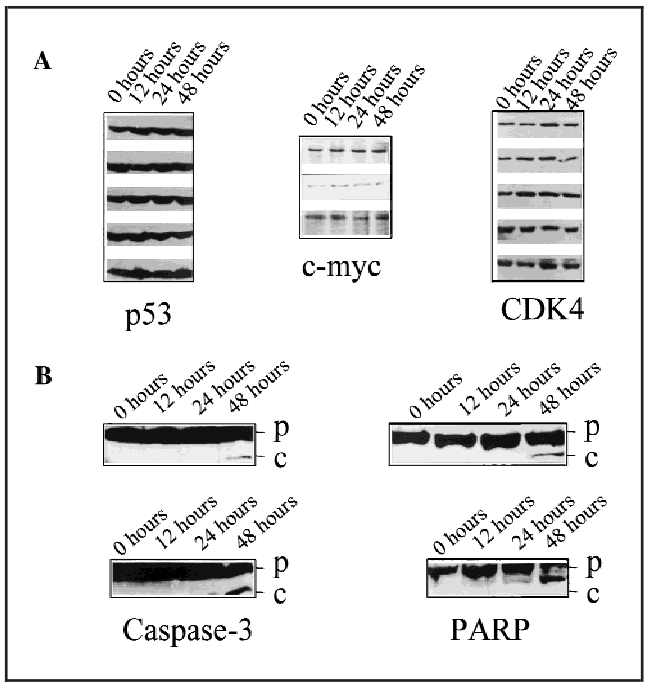

Fig. 2. Expression of cell cycle-related and apoptosis-related proteins in cells treated with arsenic trioxide (As_2O_3). **A)** Western blot analysis of cell cycle-related proteins p53, c-myc, and cyclin-dependent kinase (CDK)-4 in cells treated with 1 μM As_2O_3. **From top to bottom,** the cell lines studied for p53 and CDK4 were Namalwa, Raji, Nalm-6 (pre-B-cell acute lymphocytic leukemia), Molt-4 (T-cell acute lymphocytic leukemia), and BJAB (B-cell lymphoma); the cell lines studied for c-myc were Namalwa, Raji, and Nalm-6. **B)** Western blot analysis of $\Delta\Psi_m$-related proteins caspase-3 and poly-adenosine diphosphate (ADP) ribose polymerase (PARP) in 2 μM As_2O_3-treated Namalwa (**upper panels**) and Raji (**lower panels**) cells. Both cell lines are derived from Burkitt's lymphoma. p and c indicate progenitor and cleaved forms of caspase-3 or PARP, respectively. In both **A** and **B,** time indicates hours of exposure to As_2O_3.

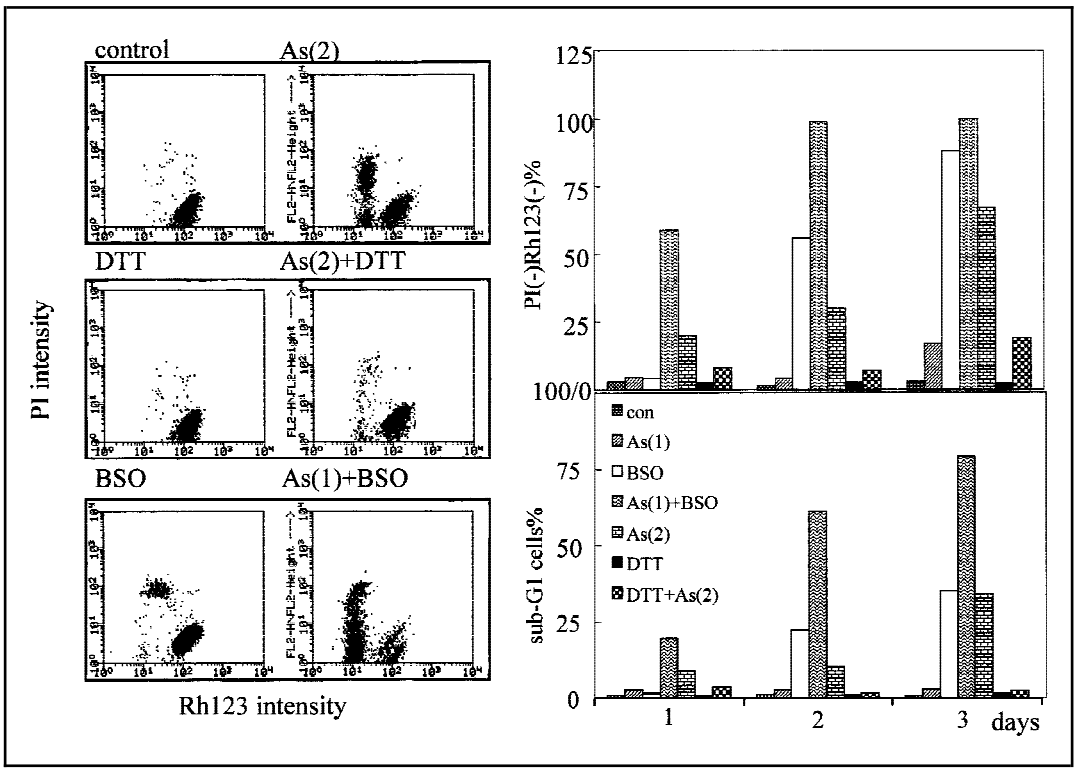

Fig. 3. Effects of arsenic trioxide (As_2O_3), dithiothreitol (DTT), or buthionine sulfoximine (BSO) on the mitochondrial transmembrane potential ($\Delta\Psi_m$) (assessed by uptake of rhodamine 123 [Rh123]) and sub-G_1 cell distribution in SKW-3 (human T-cell chronic lymphocytic leukemia) and Namalwa (Burkitt's lymphoma) cells. After treatment without or with 1 μM [As(1)] or 2 μM [As(2)] As_2O_3 alone or with 0.2 m*M* DTT or 1 m*M* BSO, cells were double stained with propidium iodide (PI) and Rh123, with fluorescence intensities measured by flow cytometry. **Left panel:** scatter plots of SKW-3 cells. **Right panel:** percentages of PI-negative and Rh123-low [PI(−)Rh123(−)] Namalwa cells (**upper axes**) and sub-G_1 Namalwa cells (**lower axes**). The key is the same for both sets of axes. con = untreated control.

DISCUSSION

In this study, 0.1 μM As_2O_3 did not show substantial effects on the growth and survival of the malignant lymphocyte lines tested, a finding that is consistent with the report of Konig et al. *(15)*, although they used different cell lines. However, at 1–2 μM, i.e., at concentrations that are clinically achievable and do not cause severe side effects *(16)*, As_2O_3 inhibited cell viability and/or cell proliferation in most of the malignant lymphocyte lines tested, depending on cell type and drug concentration. This result is consistent with that reported recently by Zhang et al. *(23)*. Thus, As_2O_3 at 1 μM decreased the viability of Molt-4, BJAB, and SKW-3 cells and inhibited the proliferation of Namalwa, Nalm-6, and Raji cells. As_2O_3 at 2 μM decreased the viability of all cell lines except Jurkat cells, which showed only a slight growth inhibition. The As_2O_3-induced decrease in cell viability was due mainly to the induction of apoptosis, as confirmed by morphologic changes, appearance of sub-G_1 cells, and terminal deoxynucleotidyl transferase labeling, indicating DNA fragmentation typical of apoptosis. It was recently shown *(24)* that loss of plasma membrane asymmetry resulting in the externalization of phosphatidyl serine, a membrane phospholipid normally restricted to the inner leaflet of the lipid bilayer, is an early event in apoptosis, independent of cell type. Annexin V was shown to interact strongly and specifically with phosphatidyl serine and could be used *in vitro* to detect apoptosis before other well-described morphologic or nuclear changes associated with apoptosis *(24)*. Therefore, the increase in annexin V-positive cells after As_2O_3 treatment further points to As_2O_3-induced apoptosis.

$\Delta\Psi_m$ collapse with evidence of intact plasma membrane was seen in 1 μM As_2O_3-treated SKW-3 and in 2 μM As_2O_3-treated Namalwa cells, but not in Jurkat cells. $\Delta\Psi_m$ collapse after As_2O_3 treatment was also observed in the APL cell line NB4 and in some multiple myeloma cell lines (Chen GQ, Chen Z, Jing YK, Waxman S: unpublished data). These data suggest that, as was found for other apoptosis-inducing agents, the $\Delta\Psi_m$ collapse is an important event in As_2O_3-induced apoptosis. Pharmacologic and functional studies have indicated that the $\Delta\Psi_m$ collapse can be attributed to the opening of mitochondrial permeability transition (MPT) pores or mitochondrial megachannels, which are formed by multiprotein complexes at the contact sites between the mitochondrial inner and outer membranes *(25)*. In addition to ATP levels, which we found to be reduced in some As_2O_3-treated malignant lymphocyte cell lines, other factors including the state of thiol oxidation and reactive oxygen species may influence the opening and/or closing characteristics of MPT pores *(26–28)*. It is known that arsenite at low concentrations selectively binds to closely spaced (vicinal) thiol groups in proteins. We found that 0.2 m*M* DTT did not modify the $\Delta\Psi_m$, but it could inhibit the decrease of the $\Delta\Psi_m$ as well as apoptosis as a result of As_2O_3 treatment. In contrast, BSO accelerated As_2O_3-induced $\Delta\Psi_m$ collapse and cell apoptosis. These results suggest that the binding of arsenic to protein thiols, perhaps within protein constituents of MPT pores, underlies the As_2O_3 induction of $\Delta\Psi_m$ collapse and apoptosis. For instance, the function of the adenine nucleotide translator, a fundamental component of the MPT multiprotein complex, is regulated by the redox state of vicinal SH groups. Thus, when these SH groups form a disulfide bond, MPT pores are opened and the $\Delta\Psi_m$ is disrupted; disulfide bond reduction returns MPT pores to a closed conformation *(25,29)*.

Recently, $\Delta\Psi_m$ collapse has been shown *(30)* to play an essential role in mediating apoptosis in that it allows the release into cytoplasm of apoptotic mediators, such as cytochrome c and apoptosis-inducing factor. In turn, cytochrome c and the apoptosis-inducing factor directly or indirectly activate members of the caspase family, which are regarded as death effector molecules *(21,22,30)*.

However, we found that caspase-3, an important member of the caspase family, was activated in 2 μM As_2O_3-treated Namalwa and Raji cells but not in 1 μM As_2O_3-treated BJAB, Molt-4, and SKW-3 cells, despite substantial apoptosis. This observation implies that different downstream cell-specific death effector molecules of the $\Delta\Psi_m$ collapse may contribute to As_2O_3-induced apoptosis. Recent reports have revealed that different cells may have distinct, even opposite, responses to arsenic. For example, PARP is one of the important substrates of the caspase family. It has been reported that sodium arsenite decreases PARP activity in a dose-dependent manner (from 2.5 μM up to 25 μM) in Molt-3 cells *(31)*. In contrast, arsenite may generate nitric oxide that can damage DNA and stimulate poly-ADP-ribosylation in CHO-K1 cells *(32)*.

Several years ago, Meng et al. *(33,34)* reported that the effects of inorganic arsenicals on DNA synthesis in unsensitized human blood lymphocytes were biphasic: Low concentrations of trivalent (As_2O_3 and sodium arsenite, 0.8–10 μM) or pentavalent (sodium arsenate, 2–100 μM) arsenic compounds enhanced DNA synthesis in human lymphocytes, whereas higher concentrations inhibited DNA synthesis. In contrast to malignant lymphocytes, mitogen-stimulated normal human lymphocytes in primary cultures did not exhibit growth inhibition after treatment with 1 μM As_2O_3 *(35)*. Moreover, we have not observed an increase in malignant lymphocyte proliferation after treatment of the cells with 1–2 μM As_2O_3. On the contrary, we found that, at 1 μM, As_2O_3 inhibited proliferation of BJAB, Namalwa, and Nalm-6 cells by extending the duration of the cell cycle, without affecting any specific checkpoints or expression of the cell cycle-related proteins Rb, cyclin D1, CDK4, p16, p53, and c-myc.

We and others *(11,12,36–39)* previously revealed that, in APL, wild-type PML protein and the PML-retinoic acid receptor α (RARα) chimeric protein were rapidly degraded upon As_2O_3 treatment. PML-RARα is believed to suppress the apoptosis and differentiation of APL cells. PML is a nuclear phosphoprotein with growth suppressor activity and a component of the nuclear organelle PML oncogenic domain that fluctuates in concentration during the cell cycle *(40–42)*. We found PML to be expressed as typical PML oncogenic domain speckles in all malignant lymphocytes investigated. Upon As_2O_3 treatment, PML was degraded to the same extent in all cells regardless of their sensitivity to As_2O_3 in terms of growth regulation. This finding suggests that PML is only an affected bystander rather than a key player in the control of lymphocyte proliferation. The mechanisms through which As_2O_3 modulates cell proliferation remain to be clarified.

In summary, As_2O_3 at concentrations of 1–2 μM, shown to be clinically well tolerated in APL patients, inhibited proliferation and/or induced apoptosis in several cell lines derived from patients with lymphoproliferative disorders characterized by defective apoptosis. The ability of As_2O_3 to exert the same effects on primary cultures of malignant lymphocytes further suggests the feasibility of its use in the treatment of lymphoma.

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NOTES

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