



## L-Ascorbic acid induces apoptosis in acute myeloid leukemia cells via hydrogen peroxide-mediated mechanisms 625

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### Abstract

L-Ascorbic acid (LAA) is being investigated clinically for the treatment of patients with acute myeloid leukemia (AML) based on the observed effects of LAA on AML progenitor cells in vitro. However, the mechanism for LAA-induced cytoreduction remains to be elucidated. LAA at concentrations of 0.25–1.0 mM induced a dose- and time-dependent inhibition of proliferation in three AML cell lines and also in leukemic cells from peripheral blood specimens obtained from three patients with AML. In contrast, ovarian cancer cell lines were only minimally affected. Flow cytometric analysis showed that LAA at concentrations of 0.25–1.0 mM could significantly induce apoptosis in the AML cell lines. LAA induced oxidation of glutathione to oxidized form (GSSG) and subsequent H<sub>2</sub>O<sub>2</sub> accumulation in a concentration-dependent manner, in parallel to induction of apoptosis. The direct role of H<sub>2</sub>O<sub>2</sub> in the induction of apoptosis in AML cells was clearly demonstrated by the finding that catalase could completely abrogate LAA-induced apoptosis. Induction of apoptosis in LAA-treated AML cells involved a dose-dependent increase of Bax protein, release of cytochrome C from mitochondria to cytosol, activation of caspase 9 and caspase 3, and cleavage of poly[ADP-ribose]polymerase. In conclusion, LAA can induce apoptosis in AML cells, and this is clearly due to H<sub>2</sub>O<sub>2</sub> which accumulates intracellularly as a result of oxidation of reduced glutathione by LAA. © 2004 Elsevier Ltd. All rights reserved.

**Keywords:** L-ascorbic acid; Apoptosis; Hydrogen peroxide; Glutathione; Acute myeloid leukemia

**Abbreviations:** AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; BSO, buthionine sulfoximine; DCF, dichlorodihydrofluorescein; DHA, dehydroascorbic acid; FITC, fluorescein-isothiocyanate; GSH, reduced glutathione; H<sub>2</sub>DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; HPLC, high performance liquid chromatography; LAA, L-ascorbic acid (vitamin C); MDS, myelodysplastic syndromes; MPA, meta-phosphoric acid; O<sub>2</sub><sup>-</sup>, superoxide; PARP, poly[ADP-ribose]polymerase; PI, propidium iodide; RA, retinoic acid; SBA, sodium 5,6-benzylidene-L-ascorbate; SOD, superoxide dismutase

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## 1. Introduction

There are a number of studies demonstrating that L-ascorbic acid (LAA), one of the major water-soluble anti-oxidant present in cells and plasma, can under certain conditions function as a pro-oxidant and increase DNA damage (Speit, Wolf, & Vogel, 1980; Stich, Karim, Koropatnick, & Lo, 1976). There is also increasing evidence that LAA is selectively toxic to some types of tumor cells, functioning as a pro-oxidant, rather than anti-oxidant (Bram, Froussard, & Guichard, 1980; Bruchelt et al., 1993; Fujinaga et al., 1994). LAA at concentrations of 10 nM–1 mM induced apoptosis in neuroblastoma and melanoma cells (De Laurenzi et al., 1995). LAA was also shown to be an important modulator for the growth of mouse myeloma cells in an *in vitro* colony assay (Park, Bergsagel, & McCulloch, 1971). Our series of studies have established that the growth of leukemic progenitor cells from patients with acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS) can be profoundly modulated by LAA (Park, 1985; Park, Kimler, Bodensteiner, Lynch, & Hassanein, 1992). Intravenous administration of sodium 5,6-benzylidene-L-ascorbate (SBA) to inoperable cancer patients induced a significant reduction in the tumor volume, without any adverse side effects (Sakagami et al., 1991). Further, our recent clinical studies indicate that manipulation of LAA levels *in vivo* can result in clinical benefit for patients with AML and solid tumors (Kim et al., 2001; Park et al., 1999).

Apoptosis is an active process of cell death characterized by cellular shrinkage, membrane blebbing, chromatin condensation, and DNA fragmentation. Impairment of apoptosis has been implicated in many human diseases including malignancies (Chinnaiyan & Dixit, 1996). Several mitochondrial specific events have been known to precede apoptosis, including alteration of the ratio of Bcl-2/Bax and cytosolic translocation of cytochrome C which in turn can activate a downstream apoptotic cascade of activation of caspases and cleavage of PARP (poly[ADP-ribose]polymerase) (Jayshree et al., 2000; Kluck et al., 1997). Recently, LAA in combination with arsenic trioxide has shown clinical responses in chemorefractory multiple myeloma (Dai, Weinberg, Waxman, & Jing, 1999). There are data suggesting

that oxidative stress triggers signal transduction cascade leading to apoptosis by arsenic trioxide (Bahlis et al., 2001; Dai et al., 1999; Grad et al., 2001; Jing, Dai, Chalmers-Redman, Tatton, & Waxman, 1999). Nevertheless, the mechanism by which LAA initiates signaling toward cell death is unclear. To understand this mechanism, we investigated the effect of LAA on proliferation, redox system modulation and induction of apoptosis, using human acute myeloid leukemia cells.

We show here that LAA alone induces apoptosis in HL-60, NB4, NB4-derived all-*trans*-retinoic acid-resistant R1 cells; and that the induction of apoptosis in these cells is explained, at least in part, by the accumulation of higher H<sub>2</sub>O<sub>2</sub> levels. It has been known that when ascorbic acid is administered into blood vessels, it is oxidized to the dehydroascorbic acid (DHA), then is transported into cells through the type 1 glucose transporter and reduced into LAA nonenzymatically using reduced glutathione (Vera, Rivas, Fischbarg, & Golde, 1993; Welch et al., 1995). To further investigate this mechanism, we determined the intracellular reduced glutathione (GSH) level. Next, we measured the intracellular accumulation of H<sub>2</sub>O<sub>2</sub> using H<sub>2</sub>DCFDA and flow cytometric analysis. We have found that LAA resulted in decreased GSH/GSSG ratio accompanied by intracellular H<sub>2</sub>O<sub>2</sub> accumulation, with H<sub>2</sub>O<sub>2</sub> playing a major role in the induction of apoptosis. We also observed here that LAA induced apoptosis via a decrease in Bcl-2/Bax ratio, cytochrome C release from mitochondria, activation of caspase-9 and caspase-3, and cleavage of PARP.

## 2. Materials and methods

### 2.1. Cell culture

Human leukemia cell lines, HL-60, NB4, NB4-R1 and K562 cells were maintained in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (100 U penicillin and 100 µg streptomycin per ml) at 37 °C in a humidified 5% CO<sub>2</sub> incubator. Human ovarian cancer cell lines, SK-OV-3, OVCAR-3 and 2774 cells were cultured in DMEM media supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. HL-60, K562, SK-OV-3,

OVCAR-3 and 2774 cells were purchased from American Type Culture Collection, Rockville, MD. Retinoic acid (RA)-sensitive NB4 and RA-resistant NB4-R1 promyelocytic cell lines were a gift from Dr. M. Lanotte (Hospital St. Louis, Paris, France). Cells from patients with AML were used in the [<sup>3</sup>H]-thymidine uptake assay and for measurement of GSH/GSSG ratio and H<sub>2</sub>O<sub>2</sub> accumulation. Peripheral blood specimens containing over 90% blasts were obtained from patients with AML, after informed consent. Blast cells were separated by Ficoll sedimentation and cells were cultured in RPMI 1640 media in the presence or absence of LAA.

### 2.2. [<sup>3</sup>H]-Thymidine uptake cell proliferation assay

Leukemic cells ( $3 \times 10^4$  cells/well) were cultured in 96-well flat bottom microtiter plates (Costar, Cambridge, MA, USA) in 0.2 ml of RPMI 1640 containing antibiotics and 10% FBS. Ovarian cancer cell lines were cultured in 96-well flat bottom microtiter plates in 0.2 ml of DMEM containing antibiotics (100 U penicillin and 100  $\mu$ g streptomycin) and 10% FBS. Cultures were incubated at 37 °C with 5% CO<sub>2</sub> for 24 h and were pulsed with 1  $\mu$ Ci of [<sup>3</sup>H] thymidine (2 Ci/mmol; New England Nuclear, Boston, MA, USA) during the last 4 h of the culture period. Cultures were harvested and [<sup>3</sup>H]-thymidine incorporation was determined by liquid scintillation counter. The statistical significance of the differences between treated and untreated samples were evaluated using Student's test. The difference was judged to be statistically significant if  $P < 0.05$ .

### 2.3. Measurement of cell apoptosis and cell cycle

For early apoptosis detection, cells were harvested and stained with fluorescein–isothiocyanate (FITC)-labeled annexin V and propidium iodide (PI) (Roche, Mannheim, Germany) according to the manufacturer's manual, and analyzed with a four-color fluorescence flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). For cell cycle analysis, the DNA content was measured by staining with PI (0.05 mg/ml). The percentage of cells in each phase of the cell cycle was calculated using the Mod-Fit LT (Verity Software House, Inc., Topsham, ME, USA) program.

### 2.4. Measurement of intracellular total glutathione (GSH<sub>t</sub>) and oxidized form (GSSG)

Intracellular GSH contents were measured using a GSH:GSSG ratio assay kit (Calbiochem, San Diego, CA, USA). In brief, for the accurate determination of the low amount of GSSG in cells,  $5 \times 10^6$  cells were harvested and treated with a thiol-scavenging reagent to prevent oxidation of GSH to GSSG during sample preparation. Cells were freeze-thawed and the specimen centrifuged. Supernatant was used for GSSG measurement according to the manufacturer's instruction, while the pellet was dissolved in 1 M NaOH and analyzed for protein by Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA, USA). To measure the total GSH level, cells were assayed using the same methods without treatment with thiol-scavenging reagent.

### 2.5. Determination of intracellular H<sub>2</sub>O<sub>2</sub> accumulation

Intracellular H<sub>2</sub>O<sub>2</sub> production was measured using H<sub>2</sub>DCFDA (Sigma, St. Louis, MO, USA), an H<sub>2</sub>O<sub>2</sub> sensitive fluorescent dye. Briefly, cells ( $5 \times 10^5$ ) were incubated in 0.5  $\mu$ M H<sub>2</sub>DCFDA for 30 min and then, incubated in the presence or absence of LAA for 1 h. Cells were washed and resuspended in PBS, and then analyzed by flow cytometry. H<sub>2</sub>DCFDA diffuses into cells and, after conversion by non-specific esterases, reacts with H<sub>2</sub>O<sub>2</sub> so as to form a fluorescent molecule (Grad et al., 2001). The channel number of the peak of the fluorescence intensity distribution is used as a measure of the intracellular H<sub>2</sub>O<sub>2</sub> content. To compare levels of H<sub>2</sub>O<sub>2</sub>, the peak for treated cells was expressed as relative to the peak for control cells.

### 2.6. Western blot analysis

HL-60 or NB4 cells ( $5 \times 10^6$ ) were harvested and washed, and the pellet was suspended with 1.0 ml of ice-cold RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 ng/ml PMSF, 0.03% aprotinin, 1  $\mu$ M sodium orthovanadate) and incubated on ice for 30 min. After incubation on ice, the lysates were centrifuged and the supernatants were boiled in a SDS sample buffer. The proteins were separated

on SDS–polyacrylamide gel electrophoresis and electroblotted to the nitrocellulose membrane. The proteins were detected by the ECL Western blotting analysis system (Amersham Pharmacia, Piscataway, NJ, USA) using anti Bcl-2, Bax, cytochrome C, caspase 9, caspase 3 and PARP antibody (Santa Cruz, Santa Cruz, CA, USA), respectively.

### 2.7. Preparation of mitochondrial and cytosolic extracts from HL-60 cells

$5 \times 10^6$  cells were harvested, washed once with ice-cold PBS and resuspended with 300  $\mu$ l of buffer A (20 mM HEPES–KOH (pH 7.5), 10 mM KCl, 1.5 mM  $MgCl_2$ , 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM DTT, and 0.1 mM PMSF) containing 250 mM sucrose. The cells were homogenized for 20 s and the homogenates were centrifuged at  $23\,100 \times g$  for 30 min at 4 °C, and the resulting mitochondria pellets were resuspended in 150  $\mu$ l lysis buffer (150 mM NaCl, 0.5% Triton X 100, 50 mM Tris–HCl (pH 7.4), 20 mM EGTA, 1 mM DTT, 1 mM sodium orthovanadate, protease inhibitor cocktail tablet (Boehringer Mannheim, Mannheim, Germany)) and frozen at –70 °C until use. The supernatant was further centrifuged at  $23\,100 \times g$  for 1 h at 4 °C, aliquoted, and stored at –70 °C for subsequent analysis. The protein content of the final extracts was estimated using the BCA kit according to the manufacturer's protocol from Bio-Rad Laboratories.

### 2.8. Measurement of intracellular ascorbate

After incubation with LAA, cells were washed three times with PBS and harvested. Cell pellets were resuspended in 5% meta-phosphoric acid (MPA) and centrifuged at 14 000 rpm for 10 min. The supernatant was applied to high performance liquid chromatography (HPLC) system (Shimadzu Corporation, Tokyo, Japan) equipped with Shim-pack CLC-ODS column (6 mm  $\times$  15 cm) connected to a Shim-pack G-ODS guard column (4 mm  $\times$  1 cm) (Shimadzu) and L-ECD electrochemical detector. The mobile phase was 50 mM potassium phosphate with 100  $\mu$ M ethylenediaminetetraacetic acid and was adjusted at pH 2.5 with phosphoric acid (Iwase & Ono, 1994). Column was equilibrated with the mobile phase and analyzed

at a flow rate of 1.0 ml/min. Ascorbate concentration was calculated from a standard curve from each experiment.

### 2.9. Measurement of glutathione peroxidase activity

Intracellular glutathione peroxidase (GPx) activity was measured using a Cellular glutathione peroxidase assay kit (Calbiochem). In brief,  $6 \times 10^6$  cells were harvested in cold buffer (50 mM Tris–HCl, pH 7.5, 5 mM EDTA, 1 mM DTT), freeze-thawed and separated by centrifugation. Supernatant was used for GPx assay according to the manufacturer's instruction, while the pellet was dissolved in 1 M NaOH and analyzed for protein by Bio-Rad protein assay (Bio-Rad Laboratories).

## 3. Results

### 3.1. Effect of LAA on cell proliferation

To investigate the effects of LAA on cell proliferation, human myeloid leukemia cell line HL-60, retinoic acid (RA)-sensitive acute promyelocytic leukemia (APL) cell line NB4 and RA-resistant APL cell line NB4-R1 cells were used as targets. Treatment of each of the cell line with LAA (0.25–1 mM) for 24 h led to a marked dose-dependent decrease of cell proliferation as determined using [<sup>3</sup>H]-thymidine uptake assay (Fig. 1A). A similar result was obtained with cells containing over 90% blasts from patients with AML (Fig. 1B). In contrast, exposure of three ovarian cancer cell lines including SK-OV-3, OVCAR-3 and 2774 to the same concentrations of LAA had little effect on the proliferation of these cells, as shown in Fig. 1A. To investigate the response on different type of leukemia cells, K562 chronic myelogenous leukemia cell line was also used.

DNA histograms were obtained for HL-60 and NB4 cell following LAA treatment for 16 h (Fig. 1C and D). As compared with control cells, LAA treatment resulted in increases in the G1 fraction ranging from 7% (0.25 mM) to 25% (1 mM), with concomitant reduction in the S phase fraction of 3 and 30%, respectively. This indicates the induction of a G1/S arrest by LAA.

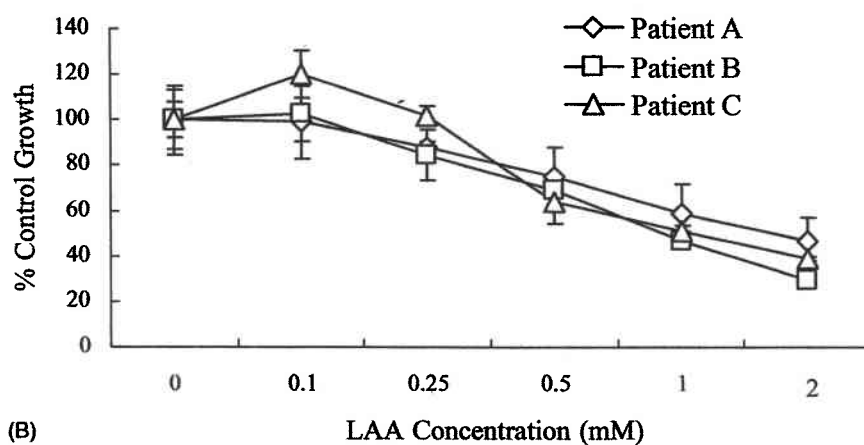
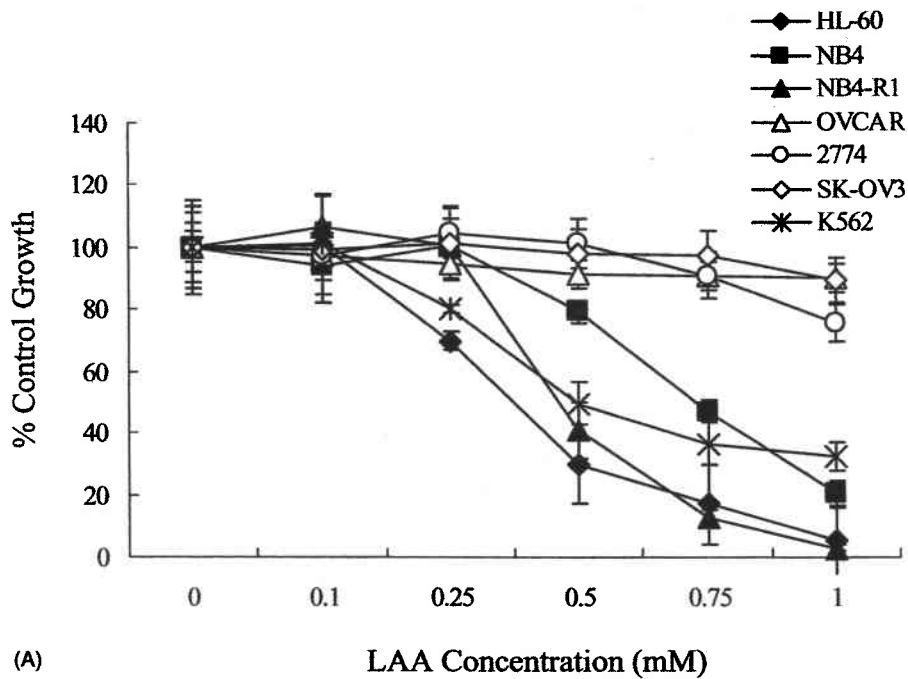


Fig. 1. Effect of LAA on the proliferation of cells. (A) HL-60, NB4, NB4-R1, K562, OVCAR-3, 2774, SK-OV-3 cells or (B) cells from three patients with AML were incubated in culture media with the indicated concentrations of LAA for 24 h. Cell proliferation was determined by measuring  $[H^3]$ -thymidine uptake. Data represent the mean  $\pm$  S.E. of triplicate determinations and are representative of three experiments. Results are presented relative to cell growth under control conditions (absence of LAA). (C) Cell cycle distributions of HL-60 cells after treatment for 24 h with various concentrations of LAA. The percentage of HL-60 and NB4 cells within the G1, S, and G2-M phases of the cell cycle was determined using the ModFit program and plotted in (D).

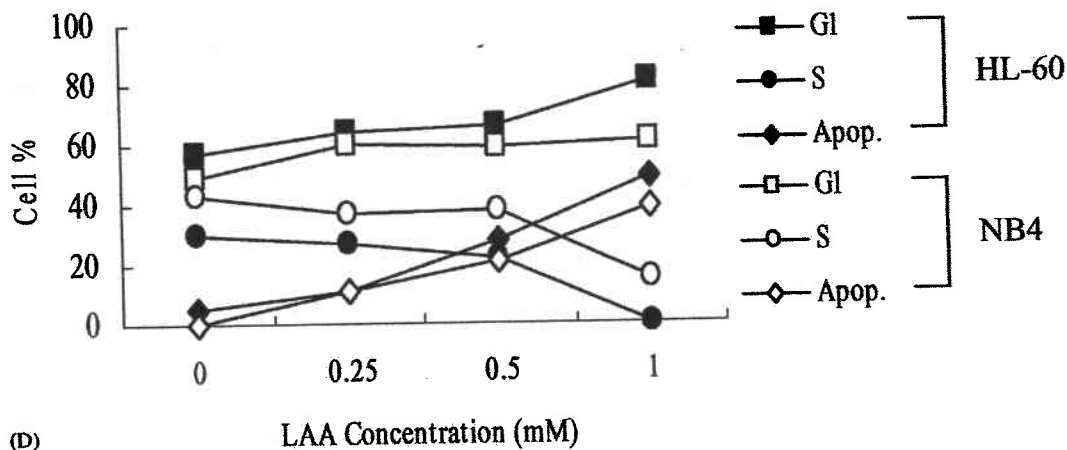
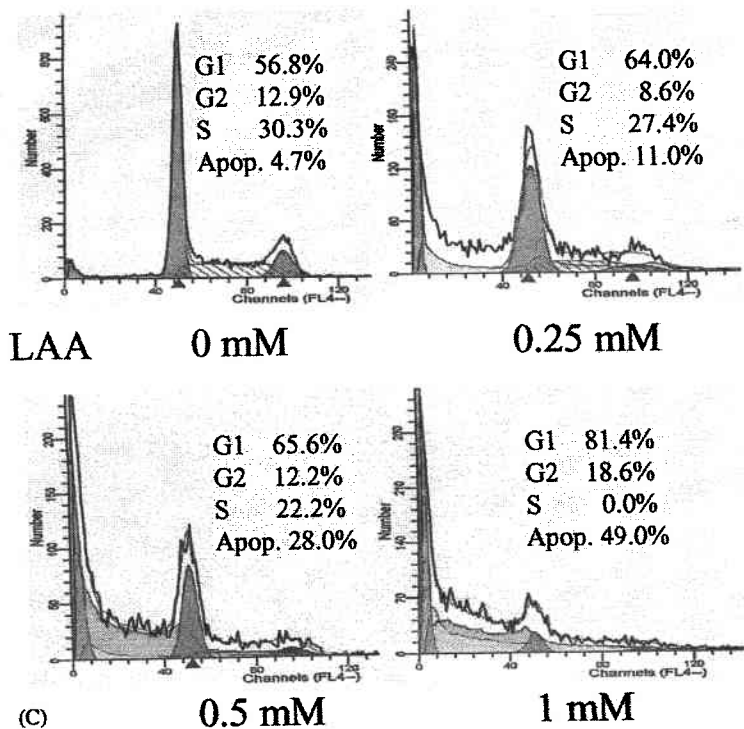


Fig. 1. (Continued).

### 3.2. Correlation of LAA effect with redox status modulation

It is well known that an important function of GSH is the elimination of ROS and the reduction of DHA

to LAA by GSH-dependent mechanisms in the cell (Meister, 1994). Therefore, to determine whether LAA affects the redox status of AML cells, we investigated the correlation of the GSH level with the inhibitory effect of LAA on the proliferation of HL-60, NB4 cells

and cells from a patient with AML. These AML cells were cultured with various concentrations of LAA in the presence or absence of 1 mM BSO or 0.2 mM DTT for 24 h, respectively. As shown in Fig. 2A, the addition of 1 mM BSO, which inhibited GSH synthesis resulting in lower GSH levels, increased the growth inhibition of HL-60 cells induced by LAA. However, the treatment of 0.2 mM DTT, which reduced GSSG back to GSH, decreased the LAA-induced growth inhibition of HL-60 cells. We also investigated whether

the inhibitory effect of LAA is correlated with  $H_2O_2$  level. Various concentrations of LAA in the absence or presence of 500 U/ml catalase or 100  $\mu$ M  $NaN_3$  were added to HL-60 culture for 24 h. A striking finding is that the inhibitory effect of LAA was completely abrogated by the addition of 500 U/ml catalase, regardless of LAA concentration; whereas the addition of 100  $\mu$ M  $NaN_3$  synergistically enhanced the effect of LAA. As shown in Fig. 2B, these results were also observed in NB4 cells and in cells from a patient

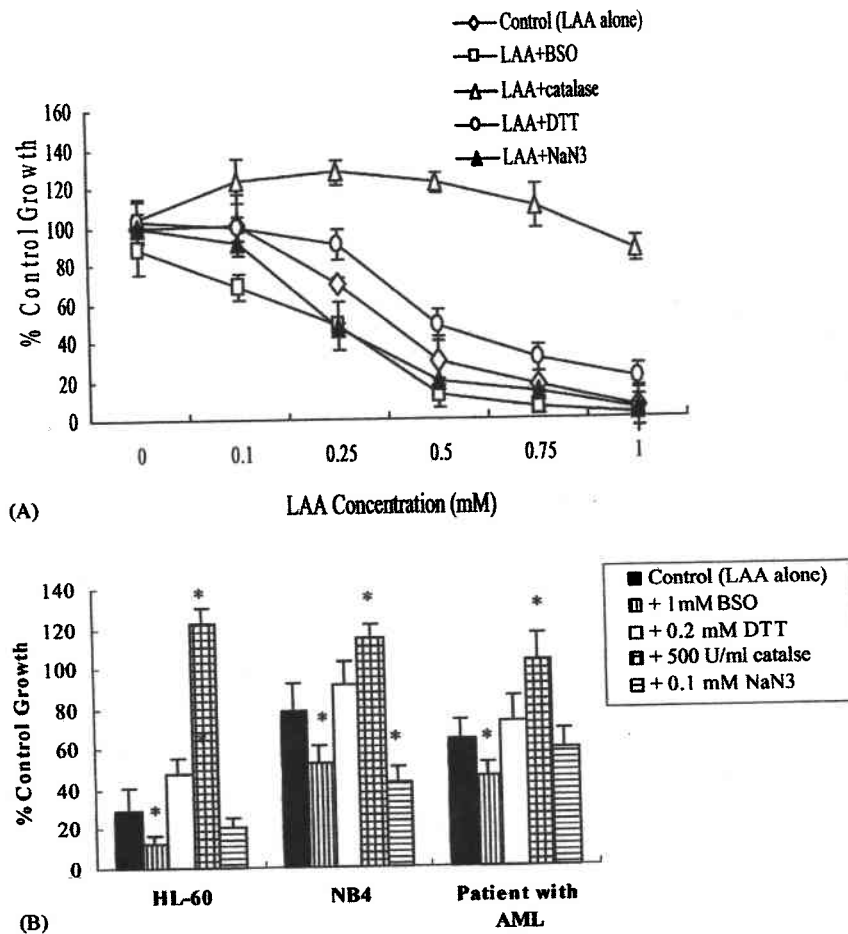


Fig. 2. Effect of BSO, catalase, DTT, and  $NaN_3$  on LAA-induced inhibition of cell proliferation. Data represent the mean  $\pm$  S.E. of triplicate determinations and are representative of three experiments. Results are presented relative to cell growth under control conditions (absence of LAA or other chemicals). (A) HL-60 cells ( $3 \times 10^4$  per well) were cultured in various concentrations of LAA in the presence or absence of 1 mM BSO or 0.2 mM DTT or 500 U/ml catalase or 100  $\mu$ M  $NaN_3$  for 24 h. Cell proliferation was determined by measuring [ $H^3$ ]-thymidine uptake. (B) Effects of BSO, catalase, DTT or  $NaN_3$  on proliferation inhibition induced by 0.5 mM LAA were represented in HL-60 cells, NB4 cells, and cells from a patient with AML. Cells ( $3 \times 10^4$  per well) were cultured in 0.5 mM LAA with or without 1 mM BSO, 0.2 mM DTT, 500 U/ml catalase or 100  $\mu$ M  $NaN_3$  for 24 h. Cell proliferation was determined by measuring [ $H^3$ ]-thymidine uptake. Asterisks (\*) indicate  $P < 0.05$  compared to LAA only.

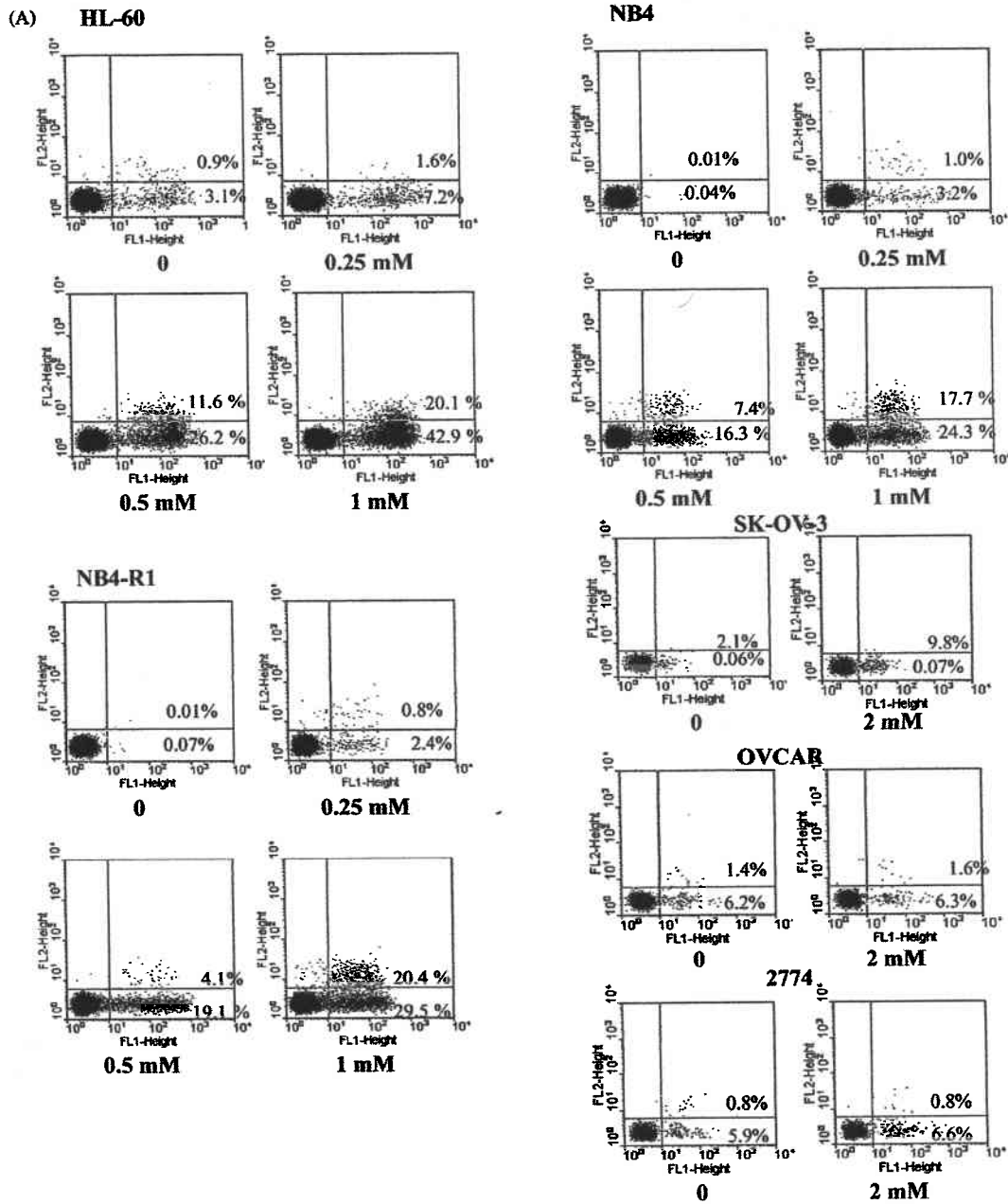


Fig. 3. Effect of LAA on apoptotic cell induction. After cells were incubated with LAA for 24 h, cells were harvested, stained with Annexin V-FITC (FL-1) and propidium iodide (FL-2), and analyzed by flow cytometry. (A) Viable cells (lower left), apoptotic cells (lower right), and necrotic cells (upper right) are shown. An increase in apoptotic cells is evident after incubation with LAA in a concentration dependent manner in HL-60, NB4 and NB4-R1 cell lines, but not in three ovarian cancer cell lines. The results are representative of at least four experiments. (B) Proportion of apoptotic cells. LAA or H<sub>2</sub>O<sub>2</sub> rarely induced apoptosis in ovarian cancer cell lines SK-OV-3, OVCAR-3 and 2774. Data represent the mean  $\pm$  S.E. of four determinants. Asterisk (\*) indicates statistically significant differences between 0.5-mM-treated and 1-mM-treated samples ( $P < 0.05$ ). (C) HL-60 cells were cultured with the indicated concentrations of LAA in the presence or absence of 1 mM BSO, 0.2 mM DTT or 500 U/ml catalase. Proportion of apoptotic cells was determined by flow cytometry. Data represent the mean  $\pm$  S.E. of triplicate determinations. DTT and catalase antagonizes but BSO promotes the LAA-induced apoptosis in these cells.

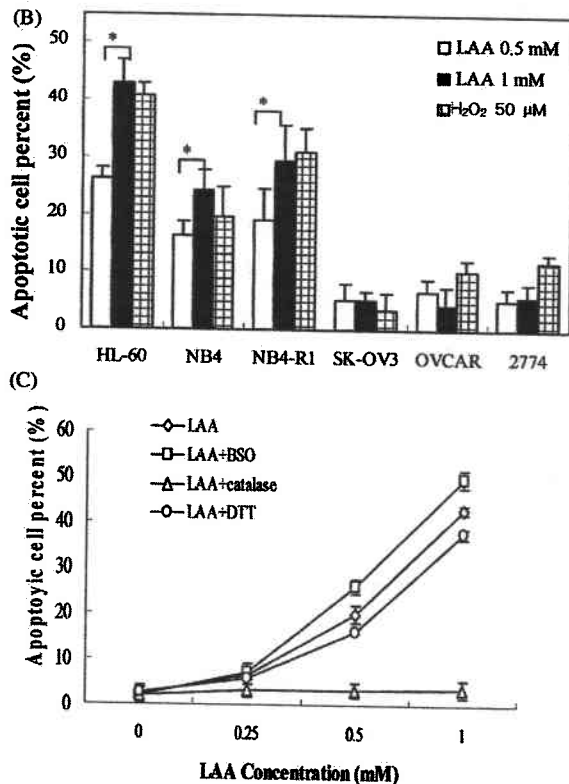


Fig. 3. (Continued).

with AML. These results indicated that the removal of H<sub>2</sub>O<sub>2</sub> by catalase completely abolished the inhibitory effect of LAA; but the inhibition of endogenous catalase by NaN<sub>3</sub> accelerated it. The results indicated that at least the major portion, if not all, of the growth inhibitory effect of LAA is due to the generation of H<sub>2</sub>O<sub>2</sub>.

The above results urged us to investigate whether superoxide anion (O<sub>2</sub><sup>-</sup>) is also involved in the inhibitory effect of LAA. Various concentrations of LAA in the presence or absence of 1000 U/ml SOD were added to HL-60 culture for 24 h. The inhibitory effect of LAA was not significantly affected by the addition of 1000 U/ml SOD (data not shown). This result indicated that superoxide anion had no appreciable role in the inhibitory effect of LAA.

### 3.3. Effect of LAA on cell apoptosis

To quantify the frequency of apoptotic cells induced by LAA, Annexin-V/PI double staining was

performed. Cells were treated with LAA (0.25–1 mM) for 24 h. The results indicated that for three acute myeloid leukemia cell lines (HL-60, NB4 and NB4-R1), 1 mM LAA for 24 h induced apoptosis in approximately 24–43% of the treated cells (Fig. 3A). Induction of apoptosis by LAA in these cell lines demonstrated a dose-dependent pattern. LAA failed to promote apoptosis in ovarian cell lines including SK-OV-3, OVCAR-3 and 2774 as measured by the annexin-V/PI staining (Fig. 3B). We also investigated apoptosis induction by addition of H<sub>2</sub>O<sub>2</sub> in both the ovarian cancer and leukemia cell lines. The addition of H<sub>2</sub>O<sub>2</sub> up to 50 μM for 24 h had no significant change in apoptotic cells in ovarian cancer cells, while the addition of H<sub>2</sub>O<sub>2</sub> induced apoptosis in three leukemia cell lines, as shown in Fig. 3B. In accordance with cell proliferation assay, LAA-induced apoptosis was affected by the modulation of cellular redox status (Fig. 3C). The addition of 1 mM BSO increased the apoptosis in HL-60 cells induced by LAA. However, treatment of 0.2 mM DTT decreased the LAA-induced apoptosis of HL-60 cells. We also investigated whether the inhibitory effect of LAA is correlated with H<sub>2</sub>O<sub>2</sub> level. Various concentrations of LAA in the absence or presence of 500 U/ml catalase were treated to HL-60 culture for 24 h. Here again, apoptosis induction by LAA was completely abrogated by the addition of 500 U/ml catalase, regardless of LAA concentrations. These data were particularly striking in that the apoptotic cell proportion was reduced to essentially zero. These results indicated that the removal of H<sub>2</sub>O<sub>2</sub> by catalase completely abolished the induction of apoptosis by LAA.

### 3.4. Effect of LAA on intracellular GSH/GSSG ratio

As shown in Fig. 4, incubation with LAA at each concentration for 1 h resulted in the decrease of GSH/GSSG ratio in not only three myeloid leukemia cell lines but also cells from patients with AML, in concentration dependent manners. Interestingly, however, incubation with LAA did not show such decrease of GSH/GSSG ratio in three ovarian cancer cell lines.

### 3.5. H<sub>2</sub>O<sub>2</sub> accumulation induced by LAA

It follows that if LAA-mediated augmentation in intracellular GSH oxidation increased intracellular

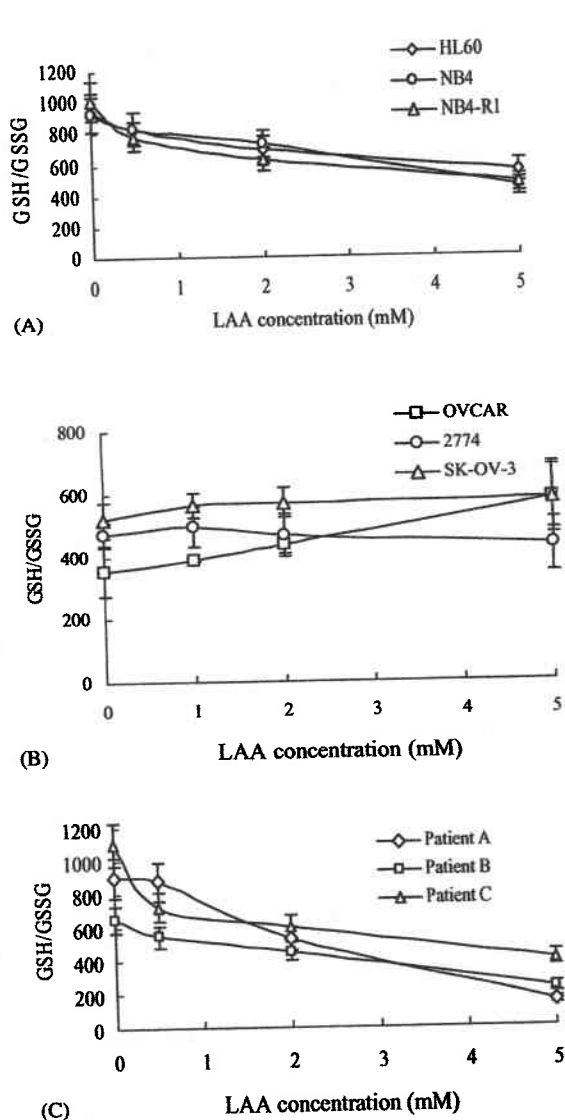


Fig. 4. Effect of LAA on the GSH/GSSG ratio. (A) AML cell lines (HL-60, NB4 and NB4-R1), (B) ovarian cancer cell lines (OVCAR-3, 2774 and SK-OV-3) or (C) cells from three patients with AML were incubated with the indicated concentrations of LAA for 1 h, and the intracellular GSH and GSSG levels were estimated as described in Section 2. Values are means  $\pm$  S.D. of four experiments. LAA induced GSH/GSSG ratio decreases in HL-60, NB4, NB4-R1 and cells from patients with AML; but not in OVCAR-3, 2774 and SK-OV-3 cells.

$H_2O_2$  level, the LAA is likely to induce intracellular oxidative stress via GSH/GSSG ratio changes. Thus, we tested the intracellular  $H_2O_2$  accumulation using  $H_2DCFDA$  and flow cytometric analysis. The oxida-

tion of  $H_2DCFDA$  in HL-60 cells treated with  $50 \mu M$   $H_2O_2$  for 1 h served as a positive control (Fig. 5A). The oxidized DCF mean peak dropped under level 5 within 1 h of incubation after the co-treatment with 1 mM LAA and 500 U/ml catalase in HL-60 cells. In contrast, co-treatment of LAA with BSO caused the cell percentage representing oxidized DCF to increase compared with the only LAA-treated cells (Fig. 5B). Consistent with its proposed pro-oxidant activity, the LAA-mediated decrease in GSH/GSSG ratio correlated functionally with an intracellular increase in  $H_2O_2$  level for each of the three AML cell lines (Fig. 5C). After treatment with 1 mM LAA, the marker area percentage representing oxidized DCF increased compared with the untreated controls from 3.7 to 19.7% in HL-60 cells, and increased from 0.1 to 11.9% in NB4 cells, from 1.0 to 10.4% in NB4-R1 cells within 1 h. LAA-mediated increase in  $H_2O_2$  level was also observed in cells from patients with AML consistent with its GSH oxidation activity in these cells (Fig. 5D). HL-60, NB4 or NB4-R1 cells had higher oxidized DCF contents than OVCAR-3, 2774 or SK-OV-3 cells 1 h after adding LAA (Fig. 5E). This demonstrates that the level of  $H_2O_2$  was correlated with the apoptotic activity of LAA.

### 3.6. LAA-induced apoptosis through an apoptotic cascade

We investigated whether LAA-induced apoptosis is mediated through an apoptotic cascade. Many previous reports demonstrated that free radical-induced apoptosis may involve the mitochondrial permeability transition pore and is associated with the suppression of Bcl-2 and activation of Bax (Kroemer, 1997). First, in order to determine whether LAA affects the Bcl-2/Bax level in HL-60 and NB4 cells, we examined whether the ratio of Bcl-2/Bax is regulated by LAA. HL-60 or NB4 cells were cultured with various concentrations of LAA and Western blot assay was performed. Treatment of LAA for 24 h in HL-60 cells and for 36 h in NB4 cells repressed the constitutive expression of Bcl-2 while strongly increased the Bax expression via a dose-dependent manner (Fig. 6A). This result indicated that LAA decreased the ratio of Bcl-2/Bax. It is well known that the decrease of Bcl-2/Bax ratio alters mitochondrial membrane permeability resulting in the release of cy-

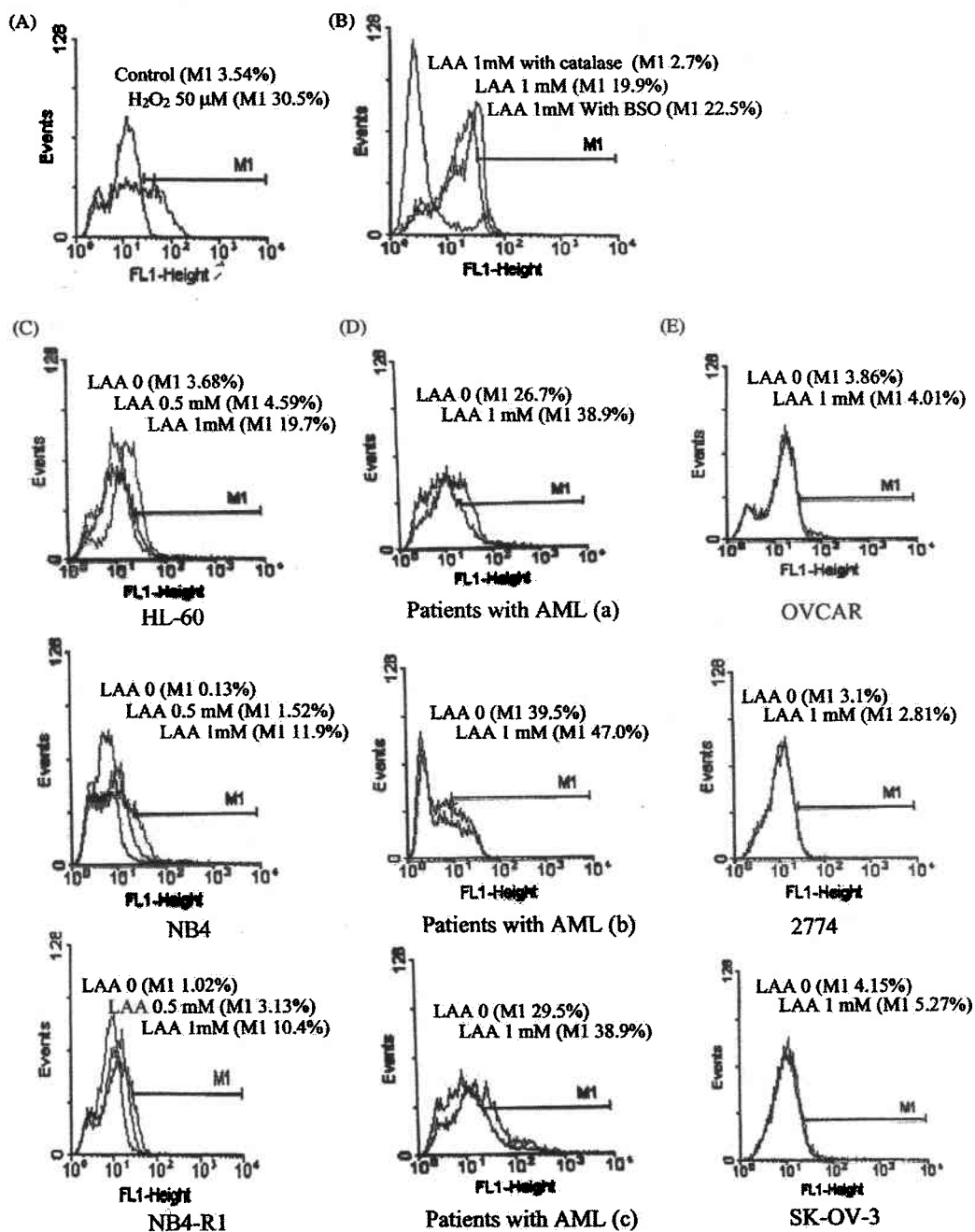


Fig. 5. H<sub>2</sub>O<sub>2</sub> accumulation induced by LAA. (A) HL-60 cells were labeled with H<sub>2</sub>DCFDA probe for 30 min. and then treated with or without 50 μM H<sub>2</sub>O<sub>2</sub> for 1 h as a positive control. (B) HL-60 cells were pre-treated with H<sub>2</sub>DCFDA probe for 30 min. and then treated with 1 mM LAA in the presence of or absence of 500 U/ml catalase or 1 mM BSO for 1 h. The oxidized DCF was analyzed by flow cytometry. (C) AML cell lines (HL-60, NB4 and NB4-R1), (D) Cells from three patients with AML and (E) Ovarian cancer cell lines (OVCAR-3, 2774 and SK-OV-3) were treated with the indicated concentrations of LAA for 1 h, with H<sub>2</sub>DCFDA probe being added 30 min before the addition of LAA. M1 gate represents cells undergoing H<sub>2</sub>O<sub>2</sub> accumulation and is presented as a percentage of the total events collected. Results are from one representative experiment of three. Standard deviations (not shown) were less than ±10%.

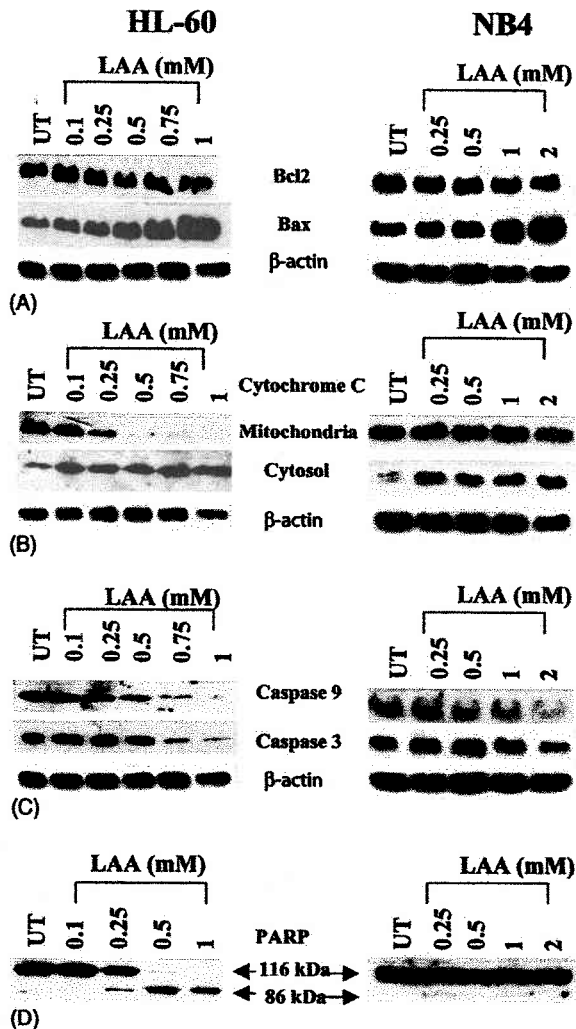


Fig. 6. Western blot analysis for apoptotic signal proteins. (A) Bcl-2, Bax; (B) cytochrome C; (C) pro-caspase 9, pro-caspase 3 and (D) PARP protein in HL-60 and NB4 cells after treatment with 0.1–1 mM (for HL-60 cells) or 0.25–2 mM (for NB4 cells) LAA. Protein total extracts were prepared as described in Section 2. For the detection of cytochrome C, mitochondrial and cytosolic fractions were extracted separately. Equal amounts of protein were loaded onto each lane.

cytochrome C from mitochondria into the cytosol (Kluck et al., 1997). We investigated whether LAA causes the release of cytochrome C from mitochondria to cytosol. HL-60 cells were cultured with various concentrations of LAA for 24 h and Western blot was performed (Fig. 6B). Cytochrome C in mitochondria fraction had

gradually decreased to a barely detectable level at 0.5 mM and then completely disappeared at 1 mM of LAA. On the contrary, cytochrome C in cytosolic fraction was increased. Although the band of cytochrome C in the mitochondrial fraction did not completely disappear, that in the cytosolic fraction was observed to be intensified in NB4 cells, cultured with LAA for 36 h.

Next, we investigated whether LAA-induced release of cytochrome C activates caspases. Caspase-9 is known to be activated by proteolytic cleavage when complexed with dATP, Apaf-1, and extramitochondrial cytochrome C (Li et al., 1997). Caspase-9 can activate caspase-3 by proteolytic cleavage and the activated caspase-3 may then cleave vital cellular proteins such as PARP or activate additional caspases by proteolytic cleavage. Caspase-9 and caspase-3 were both activated through the cleavage of the inactive form of procaspase-9 and procaspase-3 via a dose-dependent manner, after being treated for 24 h in HL-60 cells and for 36 h in NB4 cells (Fig. 6C).

As shown in Fig. 6D, we found that constitutive PARP was cleaved by the treatment of LAA. PARP is an enzyme involved in DNA repair and genomic integrity. 116 kDa of PARP is proteolytically cleaved into 86 kDa and 23 kDa fragments during apoptosis by caspase-3 which reduces PARP's enzymatic activity, thereby inhibiting DNA repair. These results indicated that LAA caused apoptosis by way of inducing a decrease in Bcl-2/Bax ratio, release of cytochrome C from mitochondria to cytosol, activation of caspase-9 and caspase-3, and cleavage of PARP.

### 3.7. Influence of inhibition of caspases in HL-60 cells

Since activation of caspase-9 and caspase-3 was observed in LAA-treated HL-60 cells, we examined whether caspases were functionally involved in apoptosis induced by LAA. HL-60 cells were treated with 1 mM LAA in the presence or absence of 100  $\mu$ M ZVAD-fmk, which is a caspase inhibitor, for 24 h. [ $^3$ H]-thymidine incorporation assay was performed as well as demonstration of apoptotic cells by flow cytometry assessment of Annexin-V and PI staining. We also cultured HL-60 cells in 1 mM LAA in the presence or absence of 20  $\mu$ M DEVD-CHO, a caspase-3 specific inhibitor, for 24 h. Treatment of

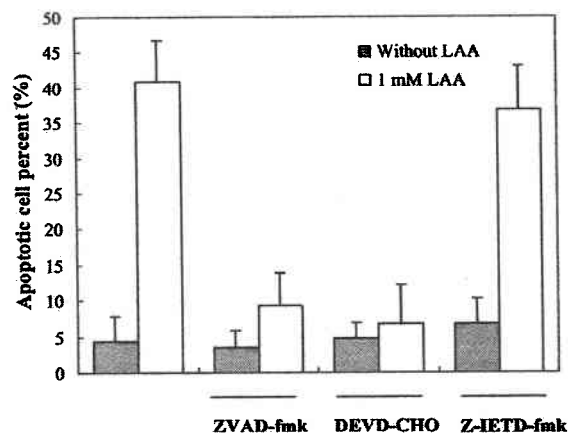


Fig. 7. Functional implication of caspases in LAA-induced apoptosis. Cells were incubated with or without 1 mM LAA in the presence or absence of the caspase inhibitors ZVAD-fmk (100  $\mu$ M), DEVD-CHO (20  $\mu$ M), and Z-IETD-fmk (40  $\mu$ M). After 24 h, cells were harvested, stained with Annexin V-FITC (FL-1) and propidium iodide (FL-2), and analyzed by flow cytometry for the proportion of apoptotic cells. Data represent the means  $\pm$  S.D. of triplicate determinations.

100  $\mu$ M ZVAD-fmk or 20  $\mu$ M DEVD-CHO did not lessen proliferation inhibition by LAA in HL-60 cells (data not shown), but completely inhibited apoptosis induced by 1 mM LAA (Fig. 7). These results confirm that caspase 9 are functionally implicated in the induction of apoptosis downstream of cell-cycle events and that caspase-3 is indeed involved in the LAA-induced apoptosis of HL-60 cells. To investigate the involvement of other initiator caspase such

as caspase 8, 40  $\mu$ M of Z-IETD-fmk, a caspase 8 inhibitor was co-treated with 1 mM LAA. Z-IETD-fmk did not lessen apoptosis induced by LAA suggesting caspase 8 was not involved in this system.

### 3.8. Intracellular uptake of LAA

We measured the uptake of LAA by each cell line. A 1 h incubation of each cell line with LAA resulted in its uptake in a concentration-dependent manner (Fig. 8). As can be seen, three AML cell lines can effectively accumulate LAA, compared to three ovarian cancer cell lines. This result provides one possible explanation for the cell line-specific differences in LAA sensitivity.

### 3.9. Intracellular GSH level and glutathione peroxidase activity

To determine whether the ability to catabolize  $H_2O_2$  in cells, the innate antioxidant, GSH and the major cellular scavenging enzymes GPx were measured in leukemia cell lines and ovarian cancer cell lines with different sensitivities to LAA-induced apoptosis. Table 1 shows that the level of GSH and the activity of GPx were rather higher in three ovarian cancer cells, which were less sensitive to LAA-induced apoptosis, than in AML cells. Thus, the difference in innate oxidant-metabolizing activity also provides another basis for the cell line-specific differences in LAA sensitivity.

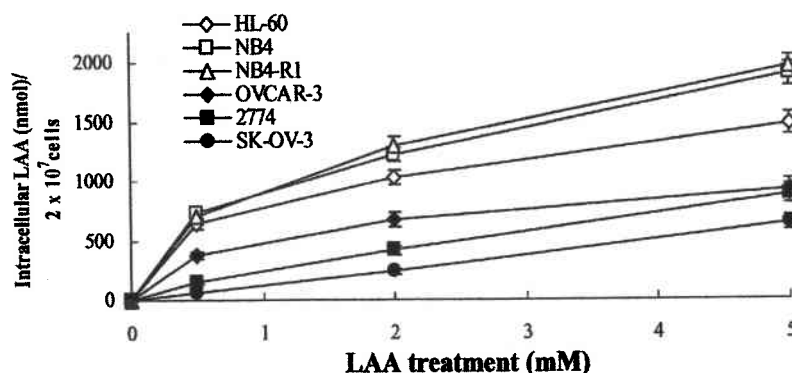


Fig. 8. Intracellular uptake of LAA. Cells were supplemented with the indicated concentration of LAA for 1 h, and intracellular LAA concentration was measured by HPLC. Means  $\pm$  S.D. of three determinations are shown.

Table 1  
The basal activity of glutathione peroxidase (GPx) and GSH levels in different cells

Cell	GPx (mU/mg protein)	GSH ( $\mu$ g/mg protein)
HL-60	11 $\pm$ 7.2	4.7 $\pm$ 1.2
NB4	18 $\pm$ 2.3	2.8 $\pm$ 0.8
NB4-R1	17 $\pm$ 4.6	2.1 $\pm$ 0.6
OVCAR	29 $\pm$ 2.1	8.9 $\pm$ 1.7
2774	39 $\pm$ 5.4	7.6 $\pm$ 1.6
SK-OV-3	92 $\pm$ 7.6	12.3 $\pm$ 2.1

#### 4. Discussion

There is evidence that apoptosis plays a role in the response of leukemia patients to chemotherapy, and there is probably an association between therapy-induced apoptosis and therapeutic efficiency (Kerr, Winterford, & Harmon, 1994; Sachs & Lotem, 1993). Several groups have shown that LAA augments arsenic trioxide ( $As_2O_3$ )-mediated apoptosis (Grad et al., 2001). We, however, present data that LAA alone can modulate malignant cell growth in vitro. Also, LAA alone can effectively induce apoptosis of the AML cell lines HL-60, NB4 and NB4-R1. In contrast, the same concentrations of LAA had no significant effect on three ovarian cancer cell lines (SK-OV-3, OVCAR-3, 2774), demonstrating that the effect of LAA was restricted. This differential sensitivity could be partly associated with differences in LAA uptake when compared to AML cell lines, showing lower uptake efficiency in these cell lines. As shown in Fig. 8, 1 mM of LAA in the incubation media, results in an uptake from approximately 750 to 900 nmol per  $2 \times 10^7$  cells in each of the three AML cell lines, while from 120 to 495 nmol per  $2 \times 10^7$  cells in three ovarian cancer cell lines.

As a putative apoptosis initiator, LAA treatment induced immediate intracellular GSH oxidation and  $H_2O_2$  accumulation, not only in three human acute myeloid leukemia cell lines but also in cells from patients with AML. However, treatment of three human ovarian cancer cells with even 2 mM LAA did not induce GSH/GSSG ratio decrease or  $H_2O_2$  accumulation. These findings are consistent with our observation that LAA induces apoptosis with the generation of GSH oxidation and  $H_2O_2$  accumulation. This differential sensitivity to LAA-induced apoptosis in cells was associated with differences in cellular  $H_2O_2$  levels

that were determined by flow cytometry. Although the differential rate of LAA uptake is likely to be related to differences in cellular  $H_2O_2$  accumulation, we need to investigate intracellular ability to metabolize the GSH oxidation during LAA treatment. Based on the data that ovarian cancer cells have relatively high basal activities of glutathione peroxidase and high basal level of GSH compared to leukemic cells (Table 1), it can be hypothesized that the sensitivity of cells towards LAA-induced apoptosis partly depends on their ability to restore the GSH level as reduction power for the elimination of  $H_2O_2$  by metabolizing GSH oxidation/reduction.

The family of Bcl-2 related proteins is known to regulate apoptosis, consisting of apoptosis-inducing and apoptosis-inhibitory members (Kroemer, 1997). It has been established that free radical-induced cell death may involve the mitochondrial permeability transition pore while Bcl-2/Bax ratio determines the survival or death of cells following an apoptotic stimulus (Bossy-Wetzel, Newmeyer, & Green, 1998; Korsmeyer, Yin, Oltvai, Veis-Novack, & Linette, 1995; Kroemer, 1997). Our result also showed that treatment with LAA repressed the constitutive expression of Bcl-2 whereas it strongly increased Bax expression in a dose-dependent manner (Fig. 6). This implied that the increase of the Bcl-2/Bax ratio by LAA might lead to alteration of the mitochondrial membrane permeability resulting in the release of cytochrome C into the cytosol (Kluck et al., 1997). Our results indicated that cytochrome C in the mitochondrial fraction gradually decreased to a barely detectable level and then completely disappeared; whereas in the cytosolic fraction it was increased by LAA in a dose-dependent manner (Fig. 6). This finding is well matched with a previous finding that demonstrated that suppression of the anti-apoptotic members or activation of the pro-apoptotic members of the Bcl-2 family alters mitochondrial membrane permeability, which results in the release of cytochrome C into the cytosol.

From our clinical studies, LAA administered at doses up to 100 g per day showed beneficial effects in five of eight patients, without significant toxicities (Kim et al., 2001). Under these conditions of high dose treatment, the plasma LAA level rose to 20 mM, 200 times the renal threshold of 0.1 mM. Another development recently is that  $As_2O_3$  could induce in

vitro growth inhibition and/or apoptosis of malignant lymphocytes and myeloma cells (Akao et al., 1998; Akao, Nakagawa, & Akiyama, 1999). The findings that arsenites can induce apoptosis in CHO cells (Wang, Kuo, Jan, & Huang, 1996), that hydrogen peroxide-resistant CHO cells are less responsive (Cantoni, Hussain, Guidarelli, & Cattabeni, 1994), and that catalase-deficient CHO cells are hypersensitive suggests an important role for hydrogen peroxide as a mediator of arsenic-induced apoptosis (Cantoni et al., 1994). The  $H_2O_2$  levels increased up to ten times the control level in AML cells when cells in vitro were treated with 20 mM LAA. This accumulation of  $H_2O_2$  in AML cells was not increased further by the addition of  $As_2O_3$ , suggesting that high dose LAA alone is sufficient to induce  $H_2O_2$ -mediated apoptosis.

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