

## Research Article

# Tetraarsenic Hexoxide Induces Beclin-1-Induced Autophagic Cell Death as well as Caspase-Dependent Apoptosis in U937 Human Leukemic Cells

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Tetraarsenic hexaoxide ( $\text{As}_4\text{O}_6$ ) has been used in Korean folk remedy for the treatment of cancer since the late 1980s, and arsenic trioxide ( $\text{As}_2\text{O}_3$ ) is currently used as a chemotherapeutic agent. However, evidence suggests that  $\text{As}_4\text{O}_6$ -induced cell death pathway was different from that of  $\text{As}_2\text{O}_3$ . Besides, the anticancer effects and mechanisms of  $\text{As}_4\text{O}_6$  are not fully understood. Therefore, we investigated the anticancer activities of  $\text{As}_4\text{O}_6$  on apoptosis and autophagy in U937 human leukemic cells. The growth of U937 cells was inhibited by  $\text{As}_4\text{O}_6$  treatment in a dose- and a time-dependent manner, and  $\text{IC}_{50}$  for  $\text{As}_4\text{O}_6$  was less than  $2 \mu\text{M}$ .  $\text{As}_4\text{O}_6$  induced caspase-dependent apoptosis and Beclin-1-induced autophagy, both of which were significantly attenuated by Bcl-2 augmentation and N-acetylcysteine (NAC) treatment. This study suggests that  $\text{As}_4\text{O}_6$  should induce Beclin-1-induced autophagic cell death as well as caspase-dependent apoptosis and that it might be a promising agent for the treatment of leukemia.

## 1. Introduction

Arsenic trioxide ( $\text{As}_2\text{O}_3$ ), a component of Chinese medicine, has been successfully employed for the treatment of acute promyelocytic leukemia (APL) [1, 2] and it has recently been shown to have some efficacy against a certain type of solid cancers [3, 4]. It is taken parenterally via an IV drip. With regard to anticancer effects of  $\text{As}_2\text{O}_3$ , many studies have shown that  $\text{As}_2\text{O}_3$  is capable of inducing programmed cell death. There are two types of programmed cell death reported. One is apoptosis, type I programmed

cell death which is characterized by a highly stereotypical series of morphological and biological changes, such as cytoplasmic shrinkage, blebbing of the plasma membrane, chromatin condensation, and DNA degradation [5]. Another is autophagy, type II programmed cell death [6]. Autophagy is originally named as a process of protein recycling. It begins with sequestering cytoplasmic organelles in a membrane vacuole called autophagosome, which are double-membrane cytoplasmic vesicles to engulf various cellular constituents, and to fuse with lysosomes, where the sequestered cellular constituents are degraded and recycled.

Tetraarsenic hexoxide ( $\text{As}_4\text{O}_6$ ) has been used as a Korean folk remedy for the management of cancer since the late 1980s because its toxicities were minimal compared to conventional cytotoxic chemotherapy. However, the anticancer effects of  $\text{As}_4\text{O}_6$  have not been investigated much although the anticancer effects of arsenic trioxide ( $\text{As}_2\text{O}_3$ ) have been investigated in many leukemic cells [7–9]. A comparison study of the anticancer effects between  $\text{As}_2\text{O}_3$  and  $\text{As}_4\text{O}_6$  demonstrated that  $\text{As}_4\text{O}_6$  was more effective in suppressing human cancer cells *in vitro* and *in vivo*, and that  $\text{As}_4\text{O}_6$ -induced cell death pathway was different from that of  $\text{As}_2\text{O}_3$  [10]. Upregulation of p53 and v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (ERBB2) was noted in  $\text{As}_4\text{O}_6$ -induced cell, but not in  $\text{As}_4\text{O}_6$ -induced cell death. In addition,  $\text{As}_4\text{O}_6$  has been used orally, whereas  $\text{As}_2\text{O}_3$  has been used as a parenteral drug. Oral agents are more convenient to take than parenteral agents. Hence identifying the molecular mechanisms involved in its anticancer effects would allow us to contribute to developing a new oral agent. Here, we investigated the mechanisms of anticancer effects of  $\text{As}_4\text{O}_6$  in U937 human leukemic cells.

## 2. Materials and Methods

**2.1. Cells and Reagents.** U937 human leukemic cells from the American type culture collection (Rockville, MD, USA) were cultured in RPMI 1640 medium (Invitrogen Corp, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (GIBCO BRL, Grand Island, NY, USA), 1 mM L-glutamine, 100 U/mL penicillin, and 100  $\mu\text{g}/\text{mL}$  streptomycin at 37°C in a humidified atmosphere of 95% air and 5%  $\text{CO}_2$ . The Bcl-2 overexpressing U937 cells were a generous gift from Dr T.K. Kwon (Department of Immunology, Keimyung University School of Medicine, Taegu, Republic of Korea) and were maintained in a medium containing 0.7  $\mu\text{g}/\text{mL}$  geneticin (G418 sulfate).  $\text{As}_4\text{O}_6$  was obtained from Chonjisan institute (Seoul, Republic of Korea). Antibodies against Bcl-2, Bax, Bad, Bcl-xL, XIAP, procaspase 3, procaspase 8, and procaspase 9 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against poly (ADP-ribose) polymerase (PARP), PLC $\gamma$ -1, LC3, and Beclin-1 were purchased from PharMingen (San Diego, CA, U.S.A.). Antibody against  $\beta$ -actin was from Sigma (Beverly, MA). Peroxidase-labeled donkey anti-rabbit and sheep anti-mouse immunoglobulin and an enhanced chemiluminescence (ECL) kit were purchased from Amersham (Arlington Heights, IL). Caspase activity assay kits were purchased from R&D systems (Minneapolis, MN, USA). All other chemicals not specifically cited here were purchased from Sigma Chemical Co. (St. Louis, MO). All these solutions were stored at –20°C. Stock solutions of DAPI (100  $\mu\text{g}/\text{mL}$ ) and propidium iodide (PI, 1 mg/mL) were prepared in phosphate-buffered saline (PBS).

**2.2. Cell Viability Assays.** For the cell viability assay, the cells were seeded onto 24-well plates at a concentration of  $5 \times 10^5$  cells/mL and then treated with the indicated concentration of  $\text{As}_4\text{O}_6$  for 24 h. MTT (0.5 mg/mL) was

subsequently added to each well. After 3 h of additional incubation, 100  $\mu\text{L}$  of a solution containing 10% SDS (pH 4.8) plus 0.01 N HCl was added to dissolve the crystals. The absorption values at 570 nm were determined with an ELISA plate reader.

**2.3. Nuclear Staining.** After treatment with the indicated concentration of  $\text{As}_4\text{O}_6$ , the cells were harvested, washed with phosphate-buffered saline (PBS), and fixed with 3.7% paraformaldehyde in PBS for 10 minutes at room temperature. Fixed cells were washed with PBS and stained with 2.5  $\mu\text{g}/\text{mL}$  4,6-diamidino-2-phenylindole (DAPI) solution for 10 min at room temperature. The cells were washed two times with PBS and analyzed by a fluorescent microscope.

**2.4. Flow Cytometry Assay.** The cells were plated at a concentration of  $2 \times 10^5$  cells/well in six-well plates. Reduced (sub-G<sub>1</sub>) DNA content was measured by PI staining. The DNA content in each cell nucleus was determined with a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, U.S.A.). Two independent experiments were performed [11].

**2.5. Western Blotting.** The cells were harvested and lysed, and protein concentrations were quantified using the BioRad protein assay (BioRad Lab., Hercules, CA, U.S.A.). The proteins of the extracts were resolved by electrophoresis, electrotransferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA), and then the membrane was incubated with the primary antibodies followed by a conjugated secondary antibody to peroxidase. Blots were developed with an ECL detection system.

**2.6. Caspase Activity Assay.** Caspase activity was determined by a colorimetric assay according to the manufacturer's protocol in a kit for caspase activity. In brief, the cells were lysed in the supplied lysis buffer. The supernatants were collected and incubated with the supplied reaction buffer containing dithiothreitol and substrates at 37°C. The reaction was measured by determining the change in absorbance at 405 nm using the microplate reader [12].

**2.7. Quantification of Acidic Vesicular Organelles (AVOs) with Acridine Orange Staining.** In acridine orange-stained cells, the cytoplasm and nucleolus fluoresce bright green and dim red, whereas acidic compartments fluoresce bright red. Therefore, we stained the cells with acridine orange for 17 min. Green (510–530 nm) and red (650 nm) fluorescence emission from  $1 \times 10^4$  cells illuminated with blue (488 nm) excitation light was measured with a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, U.S.A.). Three independent experiments were performed.

**2.8. Statistics.** Each experiment was performed in triplicate. The results were expressed as means  $\pm$  SD. Significant differences were determined using the one-way analysis of variance (ANOVA) with post-test Neuman-Keuls in the cases at least three treatment groups and Student's *t*-test for two

group comparison. Statistical significance was defined as  $P < 0.05$ .

### 3. Results

**3.1. Responses of U937 Human Leukemic Cells to  $As_4O_6$ .** To investigate the antitumor activity of  $As_4O_6$ , U937 cells were treated with various concentrations of  $As_4O_6$  for 24 h. The cell growth was assessed by MTT assay. The MTT assay revealed that the growth of U937 cells was inhibited by  $As_4O_6$  treatment in a dose- and time-dependent manner, and the 50% inhibition of cell growth ( $IC_{50}$ ) was less than  $2 \mu M$  (Figures 1(a) and 1(b)). The efficacy of  $As_4O_6$  was superior to that of  $As_2O_3$  in terms of growth inhibition (Figure 1(a)).

**3.2. Effects of  $As_4O_6$  on Apoptosis.** To determine whether the decrease in viability of U937 cells was caused by the induction of apoptosis, we assessed the changes in nuclear morphology of  $As_4O_6$ -treated cells by DAPI staining. The DAPI staining revealed the condensed and fragmented nuclei at a concentration of  $2 \mu M$  or higher. This is usually witnessed in apoptosis (Figure 1(c)). To estimate the population of the cell death, we measured cells with sub-G1 DNA content by flow cytometry. A significant accumulation of cells with sub-G1 DNA content was noted in a dose-dependent (Figures 1(d) and 1(e)) and time-dependent manner (Figures 1(f) and 1(g)).

**3.3. Caspases Activation and Subsequent Cleavage of Their Substrates by  $As_4O_6$ .** We then assessed the effects of  $As_4O_6$  on caspases and their substrates (PARP and  $PLC\gamma-1$ ).  $As_4O_6$  decreased the expression levels of procaspase-3, procaspase-8, and procaspase-9 in a dose- and time-dependent manner. With the decrease of procaspases, the cleavages of PARP and  $PLC\gamma-1$ , the substrates of caspases, were found to be progressed in a dose- and time-dependent manner (Figures 2(a) and 2(b)). These findings suggest that  $As_4O_6$  may induce apoptosis through caspase activation. To confirm and quantify the proteolytic activation of caspases, we assessed their activities using colorimetric assay kits. The caspase activity assay also showed that  $As_4O_6$  increased proteolytic activities of caspases in a dose- and time-dependent manner (Figures 2(c) and 2(d)).

**3.4. Effects of  $As_4O_6$  on Bcl-2 Family Members and X-Linked Inhibitor of Apoptosis (XIAP).** To elucidate further underlying mechanisms of  $As_4O_6$ -induced apoptosis, we assessed the levels of Bax, Bcl-2, Bad, Bcl-xL, and XIAP, which play a crucial role in apoptosis. Western blotting revealed that  $As_4O_6$  induced an increase in the expressions of Bax (proapoptotic protein) in a dose- and time-dependent manner whereas the expression of Bcl-2, Bad, Bcl-xL, and XIAP (antiapoptotic proteins) remained unchanged or slightly reduced (Figure 3(a)). The induction of Bax expression began to clearly be observed at 12 hours after the treatment (Figure 3(b)). This finding suggested the

possibility that the mechanism of Bax induction was related to the transcriptional activity. These findings suggested that upregulation of Bax protein and increased Bax/Bcl-2 ratio should be an important mechanism of  $As_4O_6$ -induced apoptosis in U937 cells.

**3.5. Effects of  $As_4O_6$  on Autophagy.** Many studies have demonstrated that  $As_2O_3$  can induce cell death through autophagy [13]. During autophagy, LC3-1 is converted to membrane-bound LC3-II that correlates with the extent of autophagosome formation which characterizes autophagy. For the autophagosome formation, Beclin-1 is important in mammalian cells. Hence, we assessed the expression of LC3 (a marker for autophagy) and beclin-1 to check whether  $As_4O_6$ -induced cell death is involved in type II programmed cell death, autophagy. Western blotting revealed that  $As_4O_6$  induced LC3 conversion (increase in the ratio of LC3-II/LC3-I) and increased the expressions of beclin-1 in a dose- and time-dependent manner (Figures 4(a) and 4(b)). The level of autophagosome formation corresponds with the ratio of LC3-II/LC3-I. Moreover, we also obtained evidence for  $As_4O_6$ -induced autophagy by measuring AVO formation through acridine orange staining. As shown in Figures 4(c) and 4(d),  $As_4O_6$  induced the accumulation of AVO in a dose- and time-dependent manner.

**3.6. Effects of Bcl-2 on  $As_4O_6$ -Induced Autophagy and Apoptosis.** From the above, we found that  $As_4O_6$  induced not only apoptosis through Bax induction but also autophagy through Beclin-1 induction. It has been suggested that the autophagy can be induced by apoptotic insults through upregulation of Beclin-1. Bcl-2 is a well-known antiapoptotic molecule, and the interaction between Bcl-2 and Beclin-1 is important in the induction of autophagy. Therefore, we assessed Beclin-1 response to Bcl-2 overexpression and the effects of Bcl-2 overexpression on  $As_4O_6$ -induced autophagy and apoptosis by comparing those between U937/vector and U937/Bcl-2 cells that constitutively express high levels of Bcl-2. As shown in Figure 5(a), Bcl-2 overexpression led to significantly suppress the apoptosis induced by  $As_4O_6$ . We assessed the changes in nuclear morphology of  $As_4O_6$ -treated cells by DAPI staining. The DAPI staining showed that Bcl-2 overexpression reduced the frequency of condensed and fragmented nuclei in the  $As_4O_6$ -treated U937 cells which indicate apoptosis (Figure 5(b)). We also assessed the effects of Bcl-2 overexpression on  $As_4O_6$ -induced autophagosome formation. It reduced the  $As_4O_6$ - $As_4O_6$ -induced AVO formation (Figure 5(c)). To confirm this finding at the molecular level, we performed western blotting for the molecules involved in  $As_4O_6$ -induced apoptosis and autophagy. It was observed on Western blotting that the overexpression of Bcl-2 suppressed the induction of Beclin-1 and LC3 conversion in response to  $As_4O_6$ , with the suppression of  $As_4O_6$ -induced caspase-3 activation and PARP cleavages (Figures 5(d) and 5(e)). These findings suggested that the increased Bcl-2 should significantly influence the antitumor effects of  $As_4O_6$  through suppressing autophagy as well as apoptosis, and that Beclin-1 induction by  $As_4O_6$  might be related to apoptosis induction.

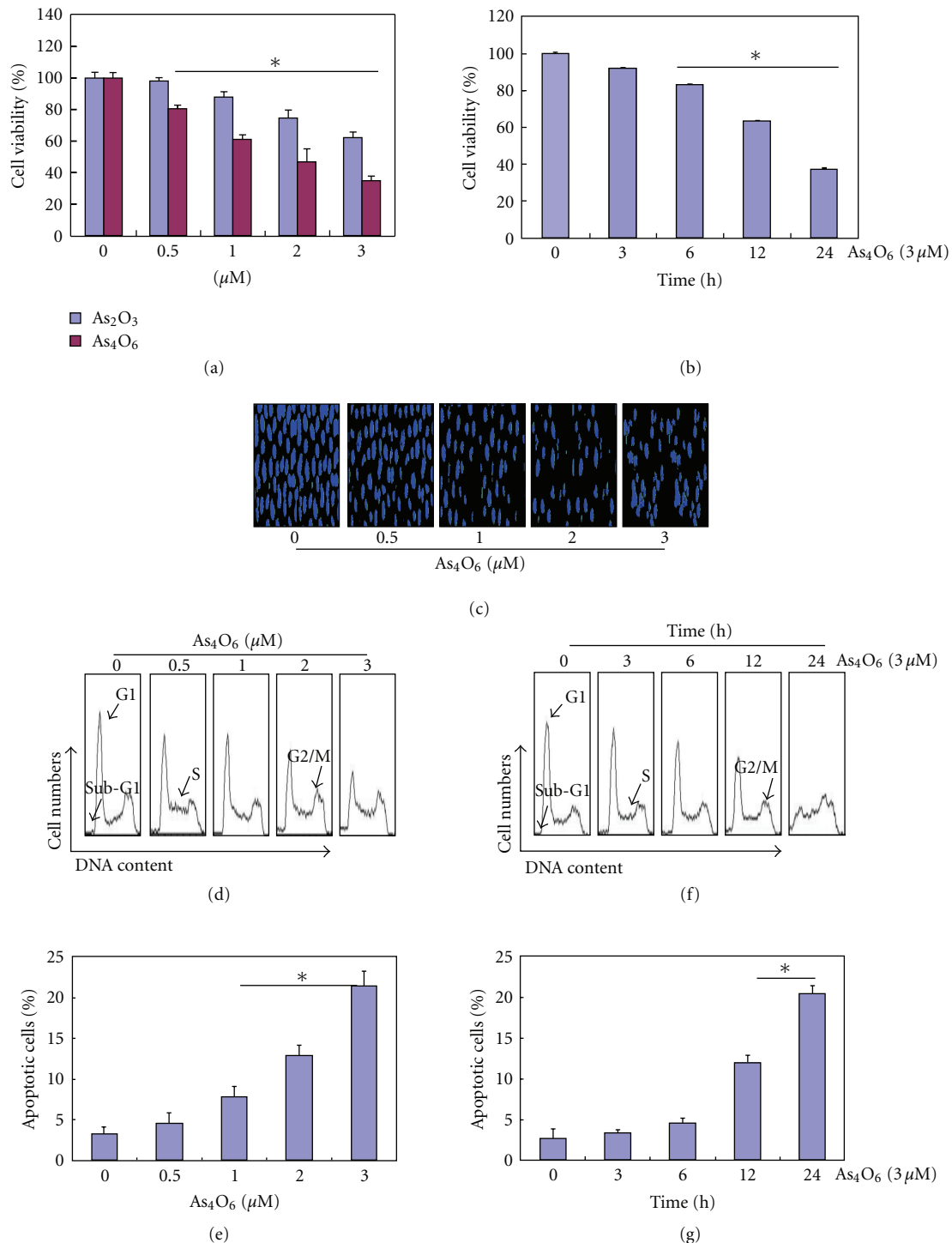


FIGURE 1: Inhibition of cell growth and induction of apoptosis by  $\text{As}_4\text{O}_6$  in U937 cells. The growth inhibition and cytotoxicity  $\text{As}_4\text{O}_6$  are a dose- and time-dependent manner. The efficacy of  $\text{As}_4\text{O}_6$  is superior to that of  $\text{As}_2\text{O}_3$ . The cells were seeded at the density of  $5 \times 10^4$  cells per mL. The inhibition of cell growth was measured by MTT assay. (a) and (c) The cells were treated with the indicated concentrations of  $\text{As}_4\text{O}_6$  and  $\text{As}_2\text{O}_3$  for 24 hours. (b) and (f) The cells were treated with  $3 \mu\text{M}$  of  $\text{As}_4\text{O}_6$  for the indicated times. The growth inhibition and cytotoxicity  $\text{As}_4\text{O}_6$  are exhibited in a time-dependent manner. (c) After fixation, the cells were stained with DAPI solution to observe apoptotic bodies, which were more frequently seen in higher doses. Stained nuclei were then observed under fluorescent microscope using a blue filter (Magnification, X 400). (d)–(g) To quantify the extent of  $\text{As}_4\text{O}_6$ -induced apoptosis, sub-G1 DNA content, which represents the fractions undergoing apoptotic DNA degradation, was analyzed by flow cytometry. The data are shown as means  $\pm$  SD of three independent experiments. \* $P < 0.05$  between the treated and the untreated control groups.

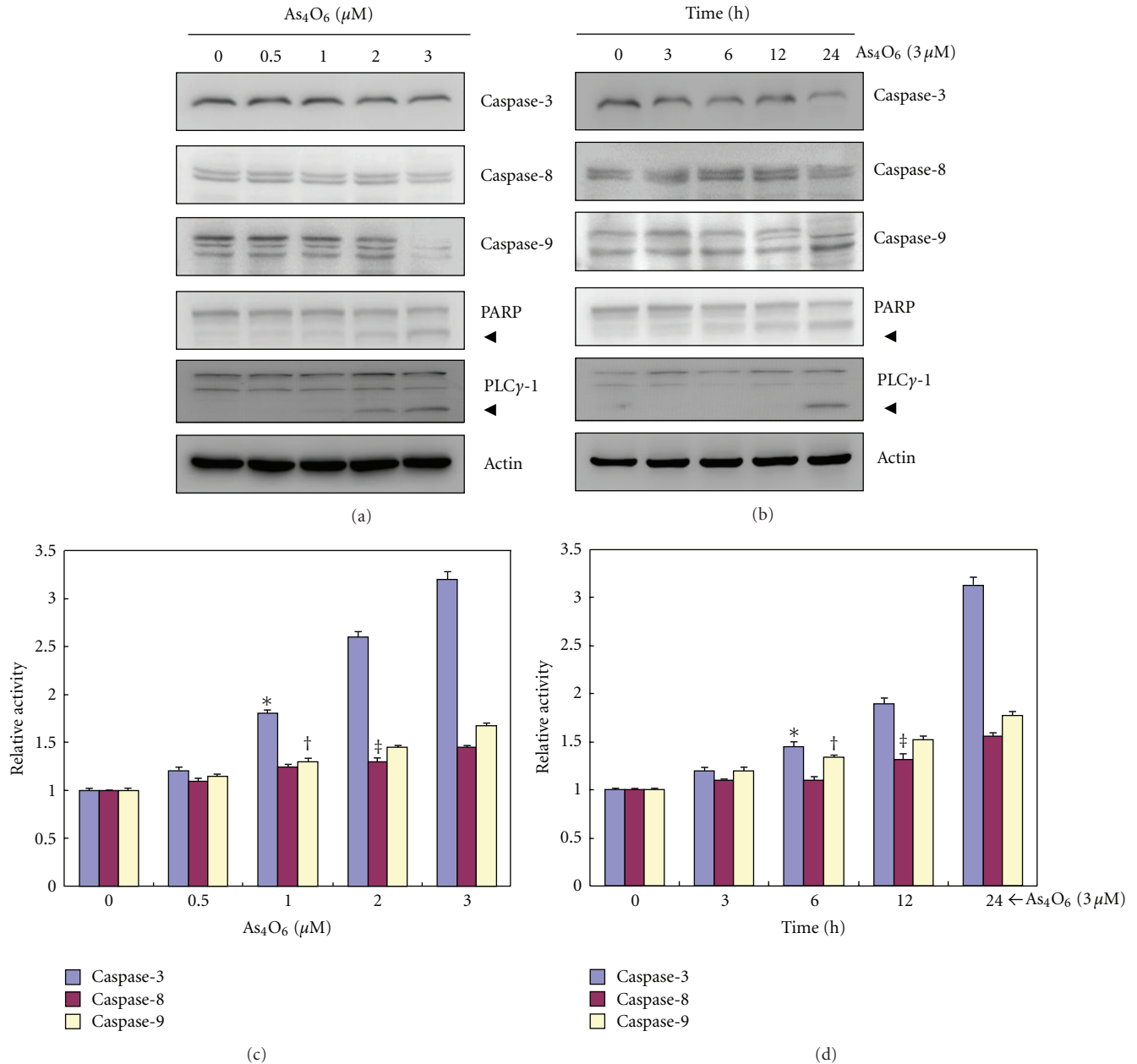


FIGURE 2: Activation of caspases and cleavage of PARP during the As<sub>4</sub>O<sub>6</sub>-induced apoptosis in U937 cells. The activation of caspases and cleavage of PARP by As<sub>4</sub>O<sub>6</sub> are a dose- and time-dependent. (a) and (c) The cells were incubated at the indicated concentrations of As<sub>4</sub>O<sub>6</sub> for 24 h. (b) and (d) The cells were treated with 3 μM of As<sub>4</sub>O<sub>6</sub> for the indicated times. (a) and (b) Total cell lysates were resolved by SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with the anticaspase-3, anticaspase-8, anticaspase-9, and anti-PARP antibodies. The proteins were visualized using an ECL detection system. β-Actin was used as an internal control. (c) and (d) The cell lysates from the cells treated with As<sub>4</sub>O<sub>6</sub> were assayed for *in vitro* caspase-3, caspase-8, and caspase-9 activity using DEVD-pNA, IETD-pNA, and LEHD-pNA, respectively, as substrates. The released fluorescent products were measured. Each bar graph represents mean ± SD of three independent experiments. \*P < 0.05 between the treated and the untreated control groups.

3.7. Inhibition of As<sub>4</sub>O<sub>6</sub>-Induced Apoptosis and Autophagy in U937 Cells by N-Acetylcysteine (NAC). A previous study showed that As<sub>4</sub>O<sub>6</sub> induced reactive oxygen species (ROS) leading to loss of mitochondrial potential (MMP, ΔΨ<sub>m</sub>) [14]. In addition, As<sub>2</sub>O<sub>3</sub> induced apoptosis in leukemic cell lines via modulation of the glutathione (GSH) redox system [15]. NAC is an antioxidant that functions by donating

a cysteine to the de novo synthesis of GSH. To assess the effects of NAC on As<sub>4</sub>O<sub>6</sub>-induced autophagy and apoptosis, we analyzed the cells with sub-G1 DNA content and AVOs using flow cytometry after As<sub>4</sub>O<sub>6</sub> treatment and observed changes in nuclear morphology of As<sub>4</sub>O<sub>6</sub>-treated cells by DAPI staining. We found that NAC reduced the As<sub>4</sub>O<sub>6</sub>-induced autophagosome formation as well as As<sub>4</sub>O<sub>6</sub>-induced



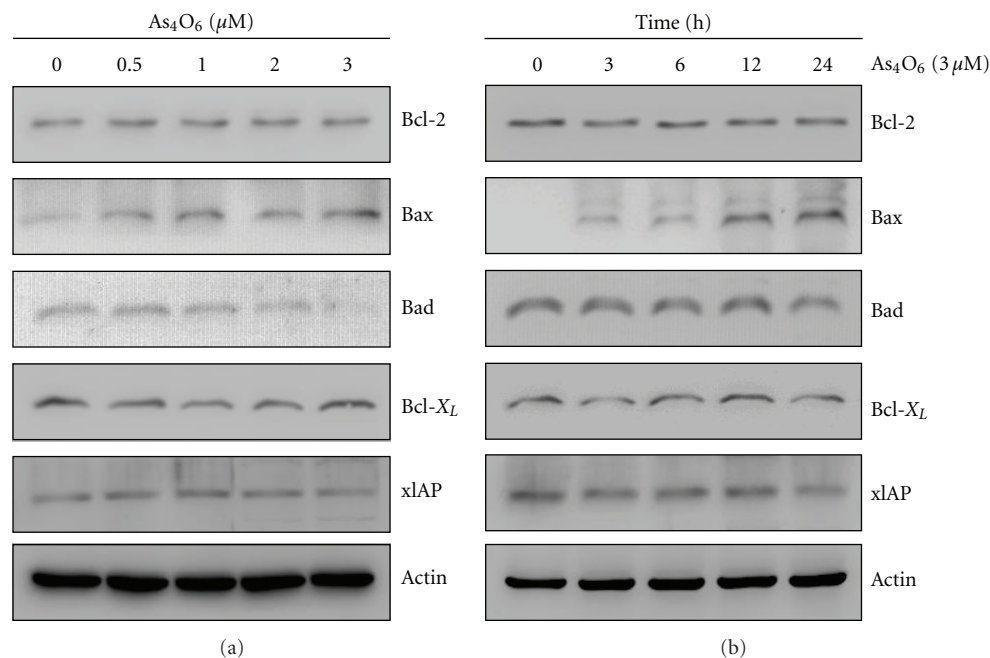


FIGURE 3: Regulation of Bcl-2 and IAP family proteins during As<sub>4</sub>O<sub>6</sub>-induced apoptosis. As<sub>4</sub>O<sub>6</sub> increases the expressions of Bax in a dose- and time-dependent manner whereas the expressions of Bcl-2, Bad, Bcl-xL, and XIAP remain unchanged or slightly reduced. (a) The cells were treated with the indicated concentrations of As<sub>4</sub>O<sub>6</sub> for 24 h. (b) The cells were treated with 3 μM of As<sub>4</sub>O<sub>6</sub> for the indicated times. The cells treated with As<sub>4</sub>O<sub>6</sub> were lysed and equal amounts of proteins were then separated by SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were probed with the indicated antibodies and detected by an ECL detection system. To confirm equal loading, the blot was stripped of the bound antibody and reprobed with the anti β-Actin antibody. The results are from one representative experiment of at least two independent experiments that showed similar patterns.

cell death (Figures 6(a) and 6(b)). The DAPI staining revealed that NAC reduced the frequency of condensed and fragmented nuclei in the As<sub>4</sub>O<sub>6</sub>-treated U937 cells (Figure 6(c)).

To confirm this finding at the molecular level and determine whether the Beclin-1-induction is associated with ROS production, we performed western blotting for the molecules involved in As<sub>4</sub>O<sub>6</sub>-induced apoptosis and autophagy. Western blotting revealed that NAC suppressed As<sub>4</sub>O<sub>6</sub>-induced Beclin-1 induction and LC3 conversion and As<sub>4</sub>O<sub>6</sub>-induced caspase-3 activation and PARP cleavages (Figures 6(d) and 6(e)). These findings suggested that the As<sub>4</sub>O<sub>6</sub>-induced autophagy as well as apoptosis should be related to ROS production. These findings suggested that ROS production by As<sub>4</sub>O<sub>6</sub> should be related to Beclin-1-induced autophagy as well as apoptosis.

#### 4. Discussion

This study was designed to determine whether As<sub>4</sub>O<sub>6</sub> has anticancer properties in human leukemic cells and further to investigate the underlying mechanisms as compared to that of the anticancer effects of As<sub>2</sub>O<sub>3</sub>. Regarding the As<sub>4</sub>O<sub>6</sub>-induced cell death, it has not been reported that autophagic cell death is a critical mechanism for the effects. To gain insights into the mechanisms for As<sub>4</sub>O<sub>6</sub>-induced cell death, we investigated the both apoptosis and autophagy. Here, we found that As<sub>4</sub>O<sub>6</sub> did not only induce caspase-dependent

apoptotic cell death but also induce autophagic cell death. Arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) is well known to have anticancer properties against leukemic cells as well as other cancer cells. The reported mechanisms of As<sub>2</sub>O<sub>3</sub>-induced cell death vary depending on the cell lines: caspase-dependent apoptosis [16, 17], caspase-independent [18], and autophagic cell death [13, 19]. Even in the studies on As<sub>2</sub>O<sub>3</sub>-induced cell death of U937 cells, some studies reported that caspase-dependent apoptosis is a major mechanism for the cell death [20] and other studies suggested that autophagic cell death is a critical mechanism for the antileukemic effects [13]. In other leukemic cell lines, arsenic trioxide did not only induce apoptosis but also induced autophagic cell death in leukemia cell lines via upregulation of Beclin-1 [21]. The mechanism for As<sub>2</sub>O<sub>3</sub>-induced cell death appears similar to that of As<sub>4</sub>O<sub>6</sub> although there is a report showing a significant difference between As<sub>2</sub>O<sub>3</sub>- and As<sub>4</sub>O<sub>6</sub>-induced cell death [10].

Apoptosis is the process of programmed cell death that can be executed through extrinsic pathway and intrinsic pathway. Either pathway is involved in mitochondrial outer membrane permeabilization which is a critical event in apoptosis [22]. The mitochondrial outer membrane permeabilization is controlled by several factors, such as the Bcl-2 and IAP protein family. The Bcl-2 family consists of proapoptotic factors (e.g., Bax, Bad, etc.) and antiapoptotic factors (e.g., Bcl-2, Bcl-xL, etc.). The Bax/Bcl-2 ratio is known as a key factor in triggering the apoptotic process. We found that caspase-dependent apoptosis was one of mechanisms for

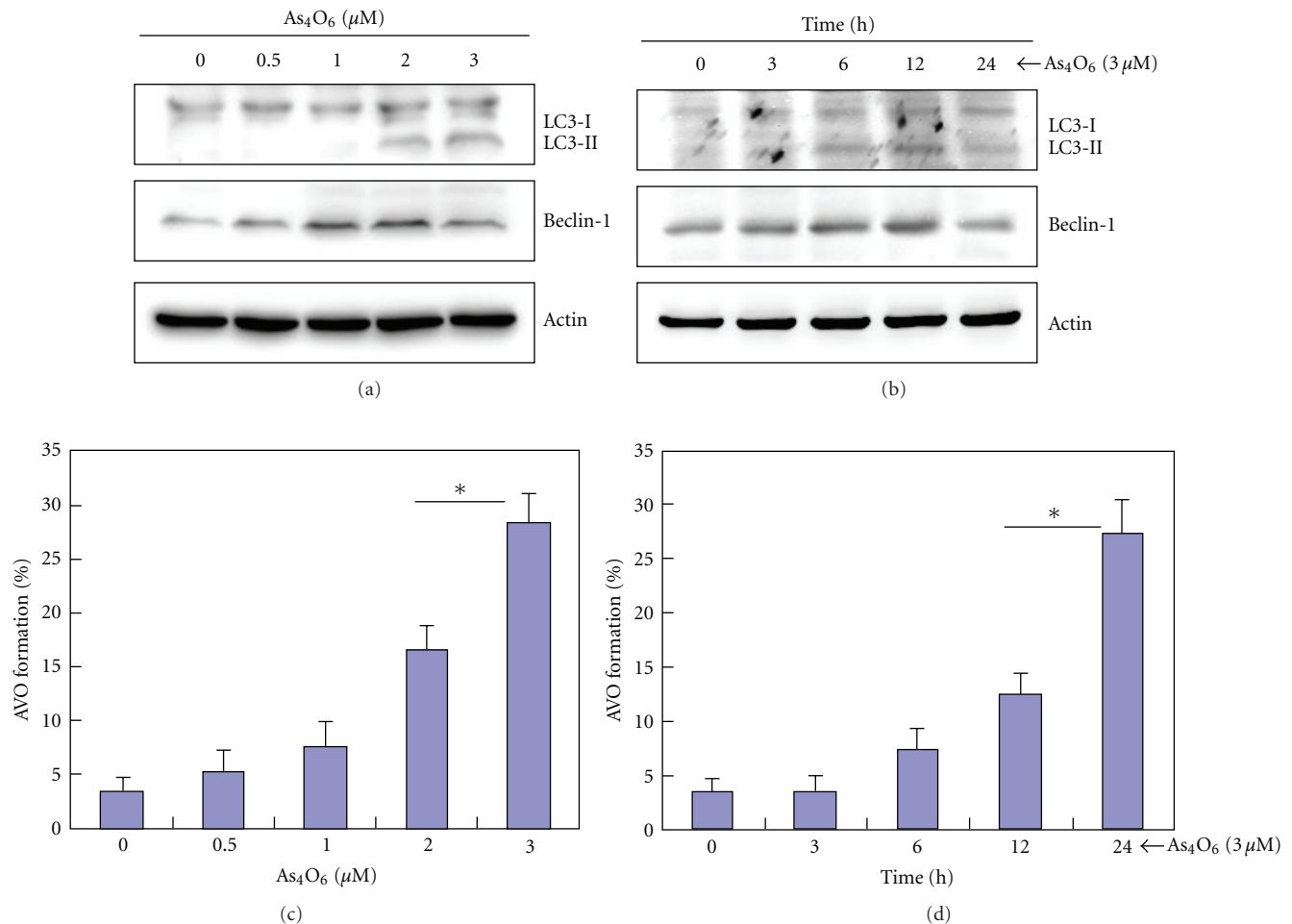


FIGURE 4: Effects of  $As_4O_6$  on the autophagy in U937 cells. AVO formation by  $As_4O_6$  is dose- and time-dependent. (a) and (c) The cells were treated with the indicated concentrations of  $As_4O_6$  for 24 h. (b) and (d) The cells were treated with 3  $\mu M$  of  $As_4O_6$  for the indicated times. (a) and (c) The cells treated with  $As_4O_6$  were lysed and equal amounts of proteins were then separated by SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were probed with the indicated antibodies and detected by an ECL detection system. To confirm equal loading, the blot was stripped of the bound antibody and reprobed with the anti  $\beta$ -Actin antibody. (b) and (d) The cells treated with  $As_4O_6$  were stained with 5  $\mu g/mL$  acridine orange for 17 min and collected in phenol red-free growth medium. Green (510–530 nm) and red (650 nm) fluorescence emission illuminated with blue (488 nm) excitation light was measured with a FACSCalibur (Becton Dickinson).

the antileukemic effects of  $As_4O_6$  through the induction of Bax protein. At first we were puzzled at this result (Bax induction by  $As_4O_6$ ) in p53-deficient U937 cells because tumor suppressor p53 plays the central role in regulating Bax protein, a proapoptotic protein. However, the previous report that Bax protein can be induced in U937 cells through the transaction of p73 gene can explain our results [23].

This study also suggested that the Beclin-1-induced autophagic cell death could be another mechanism for  $As_4O_6$ -induced cell death. This finding showing  $As_4O_6$ -induced autophagy in  $As_4O_6$ -induced cell death is also similar to that in  $As_2O_3$ -induced leukemic cell death [13, 21]. Recently it has been reported that arsenic trioxide induces a Beclin-1-independent autophagic cell death in ovarian cancer cells [24]. This finding suggested that mechanisms of  $As_2O_3$ -induced cell death should vary depending on the cell lines; so it is not unknown whether our results are applicable

to other cancer cells. Therefore, we are going to investigate the mechanism for  $As_4O_6$ -induced cell death in other solid cancer cells. Our results were derived from a single leukemic cell line; so it is difficult to generalize this finding to all leukemic cells. However, those indicated that  $As_4O_6$ -induced Beclin-1 induction which led to autophagy can be another mechanism for its antileukemic effects on U937 cells.

Another limitation is that we have not verified yet whether Beclin-1-induced autophagy is a critical mechanism for  $As_4O_6$ -induced cell death or a mechanism to rescue cancer cells from toxic damage. Now that the autophagic cell death is mainly a morphologic definition (i.e., cell death associated with autophagosomes/autolysosomes), there is still no definite evidence that a specific mechanism for autophagic death actually exists. Nonetheless, it is quite conceivable that the autophagy induced by  $As_4O_6$  could eventually destroy a cell because it has been reported

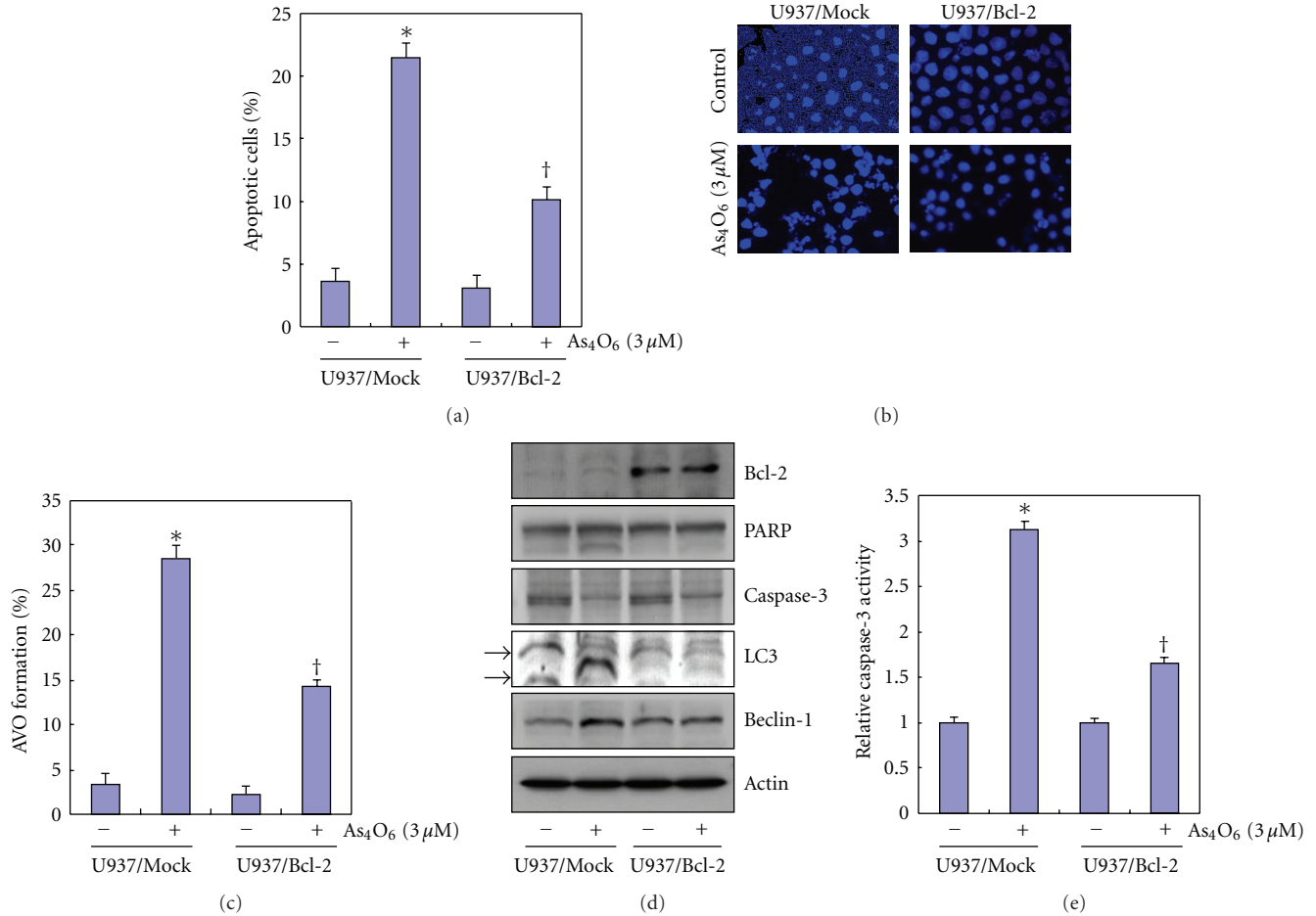


FIGURE 5: Effects of Bcl-2 overexpression in U937 cells on the apoptosis and autophagy induced by As<sub>4</sub>O<sub>6</sub>. Overexpression of Bcl-2 suppresses the induction of Beclin-1 and LC3 conversion in response to As<sub>4</sub>O<sub>6</sub> as well as As<sub>4</sub>O<sub>6</sub>-induced caspase-3 activation and PARP cleavages (a) U937/vector or U937/Bcl-2 cells were treated with 3 μM of As<sub>4</sub>O<sub>6</sub> for 24 h. Sub-G1 DNA content was analyzed by flow cytometry. (b) To confirm apoptosis, the cells were stained with DAPI solution after fixation. Stained nuclei were then observed under fluorescent microscope using a blue filter (Magnification, X 400). (c) The cells treated with As<sub>4</sub>O<sub>6</sub> were stained with 5 μg/mL acridine orange for 17 min, and collected in phenol red-free growth medium. Green (510–530 nm) and red (650 nm) fluorescence emission illuminated with blue (488 nm) excitation light was measured with a FACSCalibur (Becton Dickinson). (d) The cells were lysed and equal amounts of proteins were then separated by SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were probed with the indicated antibodies and detected by an ECL detection system. (e) The cell lysates from the cells treated with As<sub>4</sub>O<sub>6</sub> were assayed for *in vitro* caspase-3 activity using DEVD-pNA. The released fluorescent products were measured. The data are shown as means ± SD of three independent experiments. \**P* < 0.05 between the groups treated with and without As<sub>4</sub>O<sub>6</sub>, †*P* < 0.05 between the U937/vector and U937/Bcl-2 cells.

that autophagic cell death is a major mechanism for the anticancer activities of radiation [25] and temozolomide [26] as well as arsenic compounds [13, 21].

Unlike As<sub>2</sub>O<sub>3</sub>-induced cell death in U937 cells, As<sub>4</sub>O<sub>6</sub> did not suppress Bcl-2 expression in this study, but we tested the effects of augmented Bcl-2 on apoptosis and autophagy as well as apoptosis induced by As<sub>4</sub>O<sub>6</sub>. We observed that augmented Bcl-2 significantly suppressed the autophagic cell death as well as apoptotic cell death induced by As<sub>4</sub>O<sub>6</sub>. This finding is consistent with the previous study [27–29].

In aerobic organisms ROS is produced in the mitochondria via the electron transport chain during energy production. Under normal circumstances, reductive enzymes such as catalase and superoxide dismutase can defend cells from the ROS damage, but if ROS is produced

high enough to cause severe cellular damage, a cell may undergo programmed cell death [20, 30]. We observed that NAC suppressed As<sub>4</sub>O<sub>6</sub>-induced autophagy as well as As<sub>4</sub>O<sub>6</sub>-induced apoptosis. This finding suggested that ROS production should be greatly involved in As<sub>4</sub>O<sub>6</sub>-induced autophagy as well as As<sub>4</sub>O<sub>6</sub>-induced apoptosis. Although the possibility that Beclin-1-induced autophagy can be a process to rescue cancer cells from As<sub>4</sub>O<sub>6</sub>-induced apoptosis could not be excluded, our finding suggested that ROS induced by As<sub>4</sub>O<sub>6</sub> should lead to Beclin-1-induced autophagy.

In conclusion, we have demonstrated that As<sub>4</sub>O<sub>6</sub>-induced cell death is carried on through Beclin-1-induced autophagic cell death as well as caspase-dependent apoptosis, and that the ROS production by As<sub>4</sub>O<sub>6</sub> plays important roles in triggering both Beclin-1-induced autophagic cell



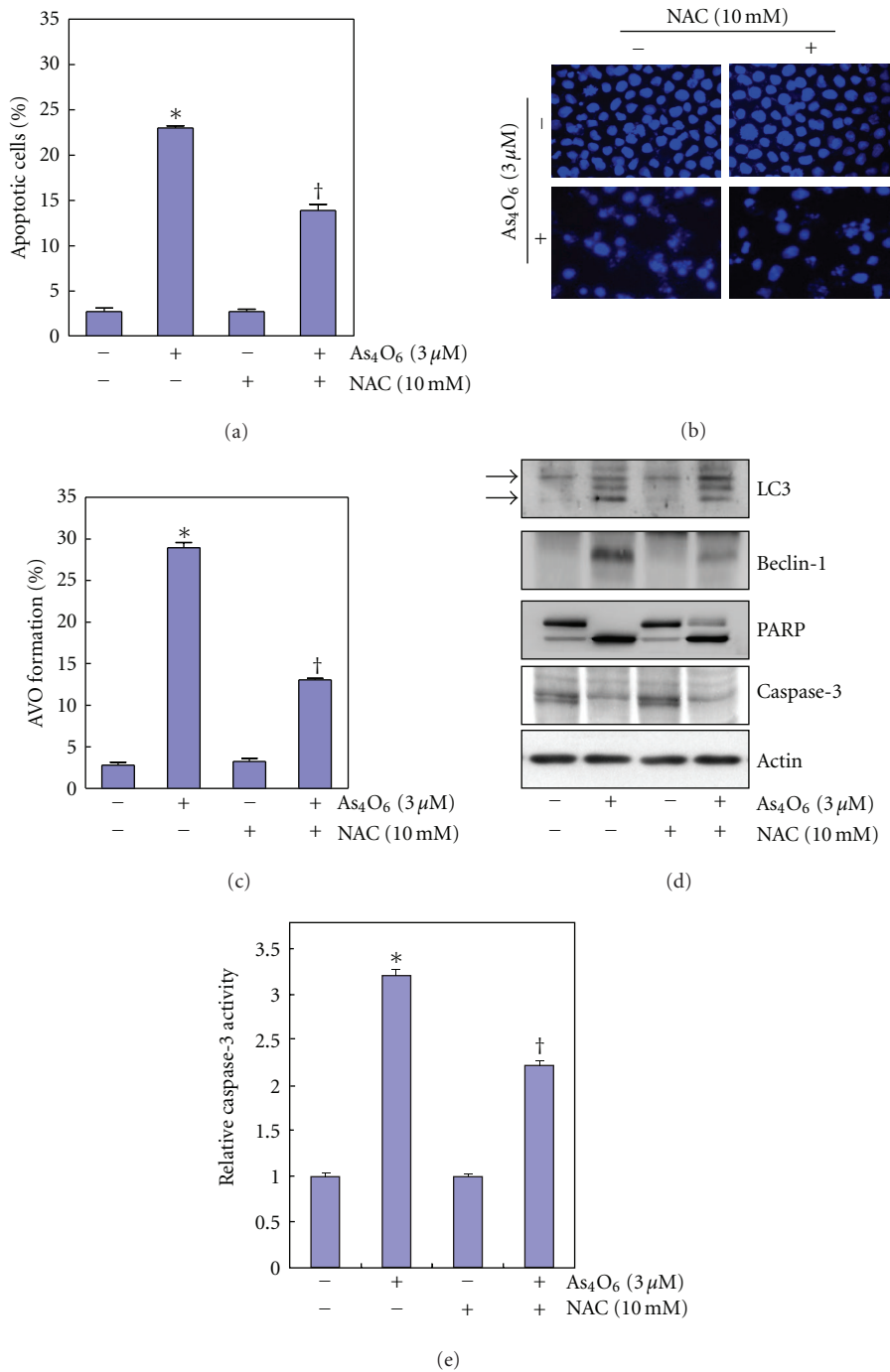


FIGURE 6: Inhibition of As<sub>4</sub>O<sub>6</sub>-induced apoptosis and autophagy in U937 cells by N-acetylcysteine (NAC). NAC reduces the As<sub>4</sub>O<sub>6</sub>-induced autophagosome formation as well as As<sub>4</sub>O<sub>6</sub>-induced cell death. (a) U937 cells were treated with NAC (10 mM) 30 min before As<sub>4</sub>O<sub>6</sub> (3 μM) for 24 h. The cells treated with As<sub>4</sub>O<sub>6</sub> were stained with 5 μg/mL acridine orange for 17 min and collected in phenol red-free growth medium. Green (510–530 nm) and red (650 nm) fluorescence emission illuminated with blue (488 nm) excitation light was measured with a FACSCalibur (Becton Dickinson). (b) Sub-G1 DNA content was analyzed by flow cytometry. (c) To confirm apoptosis, the cells were stained with DAPI solution after fixation. Stained nuclei were then observed under fluorescent microscope using a blue filter (Magnification, X 400). (d) The cells were lysed and equal amount of the lysate was separated by SDS-polyacrylamide gels and then transferred to nitrocellulose membranes. The membranes were probed with the indicated antibodies and detected by an ECL detection system. To confirm equal loading, the blot was stripped of the bound antibody and reprobed with the anti β-Actin antibody. (e) The cell lysates from the cells treated with As<sub>4</sub>O<sub>6</sub> were assayed for *in vitro* caspase-3 activity using DEVD-pNA. The released fluorescent products were measured. The data are shown as means ± SD of three independent experiments. \**P* < 0.05 between the groups treated with and without As<sub>4</sub>O<sub>6</sub>, †*P* < 0.05 between the groups treated with and without NAC.

death and caspase-dependent apoptosis. This study provides evidence that As<sub>4</sub>O<sub>6</sub>-induced cell death is related to Beclin-1-induced autophagy as well as caspase-dependent apoptosis and As<sub>4</sub>O<sub>6</sub> might be an effective agent for the treatment of leukemia similar to As<sub>2</sub>O<sub>3</sub>.

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