Zoledronic acid overcomes adriamycin resistance in acute myeloid leukemia cells by promoting apoptosis

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Abstract. Zoledronic acid (ZOL), a nitrogen-containing bisphosphonate, is widely used in metastatic bone disease. Previous studies indicate that ZOL has marked anti-leukemia activity, however, the underlying mechanism of action remains to be elucidated. The present study aimed to explore the mechanism of the anti-leukemia effect of ZOL in leukemia cells. It was observed that ZOL inhibited the proliferation of HL-60 and adriamycin-resistant HL-60 (HL-60/A) cells using a WST-8 assay. An Annexin V-propidium iodide indicated that ZOL induced apoptosis of the two cell types in a dose- and time-dependent manner. Hoechst 33342 staining was also used to verify the levels of apoptosis. The colony formation assay demonstrated that ZOL significantly inhibited colony formation capacity in acute myeloid leukemia (AML) cells. This was achieved by the induction of S-phase cell cycle arrest, downregulation of B-cell lymphoma 2 (Bcl-2) and upregulation of Bcl-2 associated X protein and cleaved poly (ADP-ribose) polymerase. The results indicate that ZOL inhibited cell proliferation by inducing apoptosis via the mitochondrial apoptotic pathway and this anti-leukemic activity appeared notably enhanced in HL-60/A cells. As ZOL is already available for clinical use, these results indicate that it may be an effective addition to the chemotherapeutic strategies for AML.

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Abbreviations: AML, acute myeloid leukemia; ZOL, zoledronic acid; HL-60/A, adriamycin-resistant HL-60; CML, chronic myeloid leukemia; MDR-1, multidrug resistance

Key words: zoledronic acid, acute myeloid leukemia, apoptosis, adriamycin resistance

Introduction

Acute leukemia is a clonal malignant hematopoietic disorder that results from acquired genetic alterations and epigenetic changes in normal hematopoietic stem/progenitor cells (1). Since the use of all trans-retinoic acid in acute promyelocytic leukemia, an increasing number of therapeutic agents have been developed. Tyrosine-kinases and proteasome inhibitors (2,3) were used in leukemia and multiple myeloma respectively, and these exhibited marked anti-tumor activities.

ZOL is used in metastatic bone disease, and functions by accumulating in the bone and inhibiting osteoclastic bone resorption (4). Preclinical studies have suggested that ZOL had marked anti-tumor activities in numerous types of solid and hematological malignancy by reducing proliferation and inducing apoptosis, in addition to inhibiting angiogenesis and tumor cell invasion (5-7). ZOL also inhibited the prenylation of rat sarcoma (RAS) proteins by inhibiting key enzymes, including farnesyl transferase and geranylgeranyl transferase enzymes within the mevalonate pathway. Blocking the prenylation of RAS proteins resulted in reduced cellular proliferation and induced apoptosis of tumor cells (8-10). ZOL alone exhibited marked inhibitory effects on acute and chronic leukemic cell growth *in vitro* and *in vivo* (11-13). In chronic myeloid leukemia (CML) cells, previous studies indicated that ZOL had anti-leukemic activity via suppression of the proliferation and clonogenicity of imatinib-sensitive and imatinib-resistant cells (11,14). However, the underlying mechanism of the anti-leukemic activity of ZOL in acute leukemia cells remains to be elucidated.

The present study revealed that ZOL inhibited cell proliferation and induced cell apoptosis, with S phase cell cycle arrest on HL-60 and HL-60/A cells. In addition, the present study investigated the potential mechanism of apoptosis induced by ZOL, which was accompanied by downregulation of B-cell lymphoma 2 (Bcl-2), upregulation of Bcl-2 associated X protein (Bax) and cleaved poly (ADP-ribose) polymerase (PARP). These results suggested that ZOL exerted an anti-leukemic effect via the mitochondrial

pathway, suggesting that ZOL may be useful as a novel therapeutic agent in the treatment of leukemia.

Materials and methods

Cell culture and chemicals. HL-60 and HL-60/A cells were cultured in RPMI 1640 medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) containing 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences) at 37˚C in 5% CO_2. ZOL was obtained from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany).

Cell proliferation and viability. Cell proliferation was evaluated by WST-8 assay using a Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Rockville, MD, USA) according to the manufacturer's protocols. Briefly, $1x10^4$ cells were seeded into 96-well plates and were then treated with increasing concentrations of ZOL (0, 0.025, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mM) and incubated at 37˚C in 5% CO_2 for 24, 48 and 72 h. Subsequently, 10 μl WST-8 solution was added to each well and incubated at 37˚C for 4 h, and the plates were read at a wavelength of 450 nm using a microplate reader.

Measurement of apoptosis by flow cytometry analysis and microscopic analysis. Apoptosis was evaluated using an Annexin V-propidium iodide (Annexin V-PI) binding assay (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) according to the manufacturer's protocols. HL-60 and HL-60/A cells were treated with 0, 0.2, 0.4 mM ZOL for 24, 48 and 72 h were collected and stained with Annexin V-PI for 15 min at 37˚C in the dark. Apoptosis analysis was conducted using flow cytometry and the data was analyzed using FlowJo software version 7.6 (FlowJo, LLC., Ashland, OR, USA).

Hoechst 33342 (Sigma-Aldrich; Merck Millipore) was used to examine nuclear fragmentation of apoptotic cells. Cells were harvested and stained with Hoechst 33342 (10 μg/ml) for 15 min and then slides were viewed using a fluorescence microscope. The nuclei of normal cells, and those that had undergone apoptosis, were counted in ten random areas per coverslip, with at least 100 cells counted. The data collected were from three independent experiments.

Cell cycle analysis. Cells were collected, fixed and resuspended in phosphate-buffered saline containing 100 μg/ml RNaseA, 0.2% Triton X-100, and 50 μg/ml PI. The cell cycle was analyzed by flow cytometry and the data was analyzed using Modfit LT for Mac Version 2.0 software (BD Biosciences, Franklin Lakes, NJ, USA).

Western blot analysis. HL-60 and HL-60/A cellular proteins were isolated using a lysis buffer. Protein concentrations were measured using Bradford's method. Proteins (40 μg) were separated on 8 and 12% SDS-PAGE gels and transferred to nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked with 5% bovine serum albumin (MP Biomedicals, Santa Ana, CA, USA) for 1 h at room temperature and then incubated with rabbit primary antibodies against Bcl-2 (1:1,000; catalog no. 4223), Bax (1:1,000; catalog no. 5023), PARP (1:1,000; catalog no. 9532) and β-actin (1:2,000; catalog no. 4970), obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Subsequently, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000; catalog no. SA00001-2; ProteinTech Group, Inc., Chicago, IL, USA) and detected using enhanced chemiluminescence reagents (Sigma-Aldrich; Merck Millipore) according to the manufacturer's protocols.

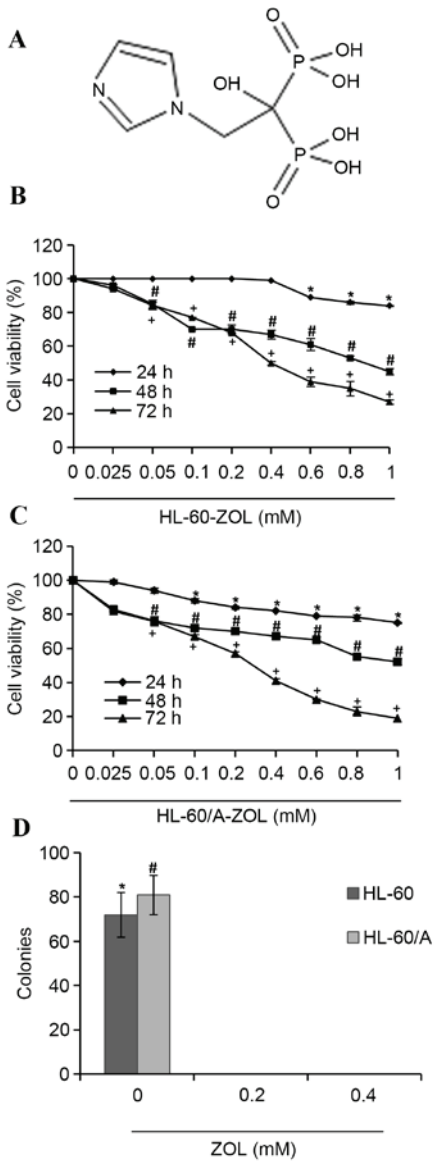

Figure 1. ZOL inhibits cell proliferation and decreases colony formation capacity of acute myeloid leukemia cells. (A) Chemical structure of ZOL. (B) HL-60 and (C) HL-60/A cells were treated with various concentrations of ZOL for 24, 48 and 72 h, and the cell viability was determined by WST-8 assay. *P<0.05 vs. 0 mM group 24 h; #P<0.05 vs. 0 mM group 48 h; +P<0.05 vs. 0 mM group 72 h. (D) HL-60 and HL-60/A cells treated with various concentrations of ZOL were incubated in methylcellulose culture for two weeks, and observed under a microscope (magnification, x200). ZOL blocked colony formation in the two cell lines. *P<0.05 vs. 0.2 and 0.4 mM groups, HL-60; #P<0.05 vs. 0.2 and 0.4 mM groups, HL-60/A. Data are presented as the mean ± standard error. ZOL, zoledronic acid.

Colony formation assay. HL-60 and HL-60/A cells were incubated in methylcellulose culture in triplicate as described previously (15). Briefly, 1 ml culture mixture containing $2x10^3$ cells, 0.9% methylcellulose (R&D Systems, Inc.,

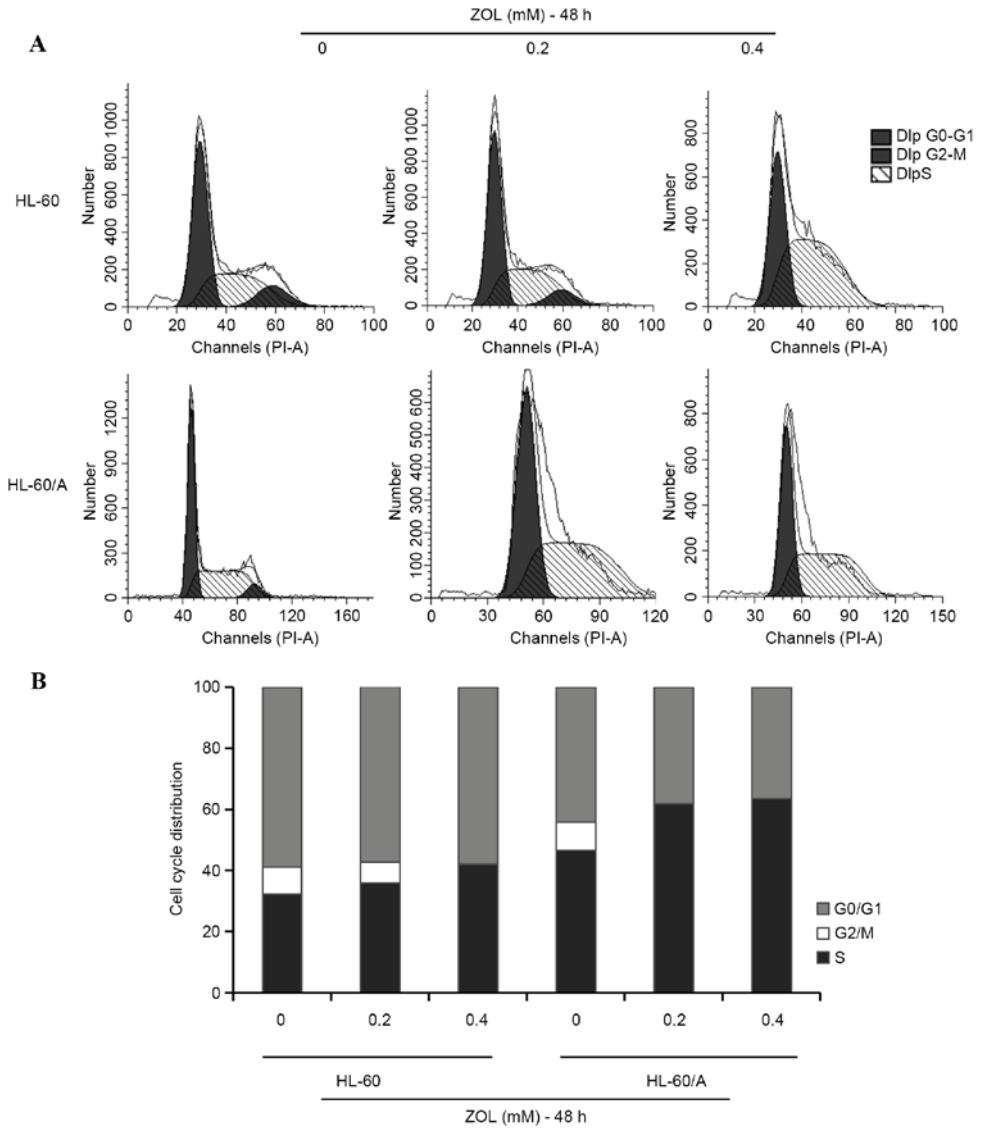

Figure 2. ZOL induces S phase arrest in cell cycle of acute myeloid leukemia cells. (A) HL-60 and HL-60/A cells were stained with propidium iodide and subjected to flow cytometric analysis. (B) Analysis revealed the proportion of cells in each phase of the cell cycle. ZOL, zoledronic acid.

Minnneapolis, MN, USA), and various concentrations of ZOL (0, 0.2 and 0.4 mM) was plated and incubated at 37˚C in 5% CO_2 for two weeks. The colonies (>40 cells) were evaluated by direct counting under an inverted microscope.

Statistical analysis. SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA) was used to analyze data, which are presented as the mean ± standard deviation. One-way analysis of variance was performed to compare groups, followed by Fisher's *post hoc* test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

ZOL inhibits cell proliferation in AML cells in a dose- and time-dependent manner. The structure of ZOL is presented in Fig. 1A. Initially, the anti-proliferative effect of ZOL in AML cells was detected by the CCK-8 assay. Compared with HL-60 cells, HL-60/A cells were resistant to adriamycin (data not shown). Subsequently, HL-60 and HL-60/A cells were exposed to a series of concentrations (0, 0.025, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 mM) of ZOL for 24, 48 and 72 h. As presented in Fig. 1B and C, the cell viability inhibition was greater at 72 h compared with 24 and 48 h. ZOL inhibited the proliferation of HL-60 and HL-60/A cells in a dose- and time-dependent manner. The half maximal inhibitory concentration value at 48 and 72 h was 1.10±0.08 and 0.41±0.03 mM for HL-60 cells, and 1.56±0.20 and 0.25±0.02 mM for HL-60/A cells, respectively.

ZOL reduces colony formation capacity in AML cells. In order to identify the effect of ZOL on colony formation in AML cells, HL-60 and HL-60/A cells treated with various concentrations of ZOL (0, 0.2 and 0.4 mM) were incubated in methylcellulose culture for two weeks. Results demonstrated that ZOL blocked colony formation in the two types of cells, suggesting that ZOL significantly inhibited the colony formation capacity of AML cells (Fig. 1D).

ZOL induces cell cycle arrest in S phase. In order to determine whether ZOL induces cell cycle arrest, the effect of ZOL on the

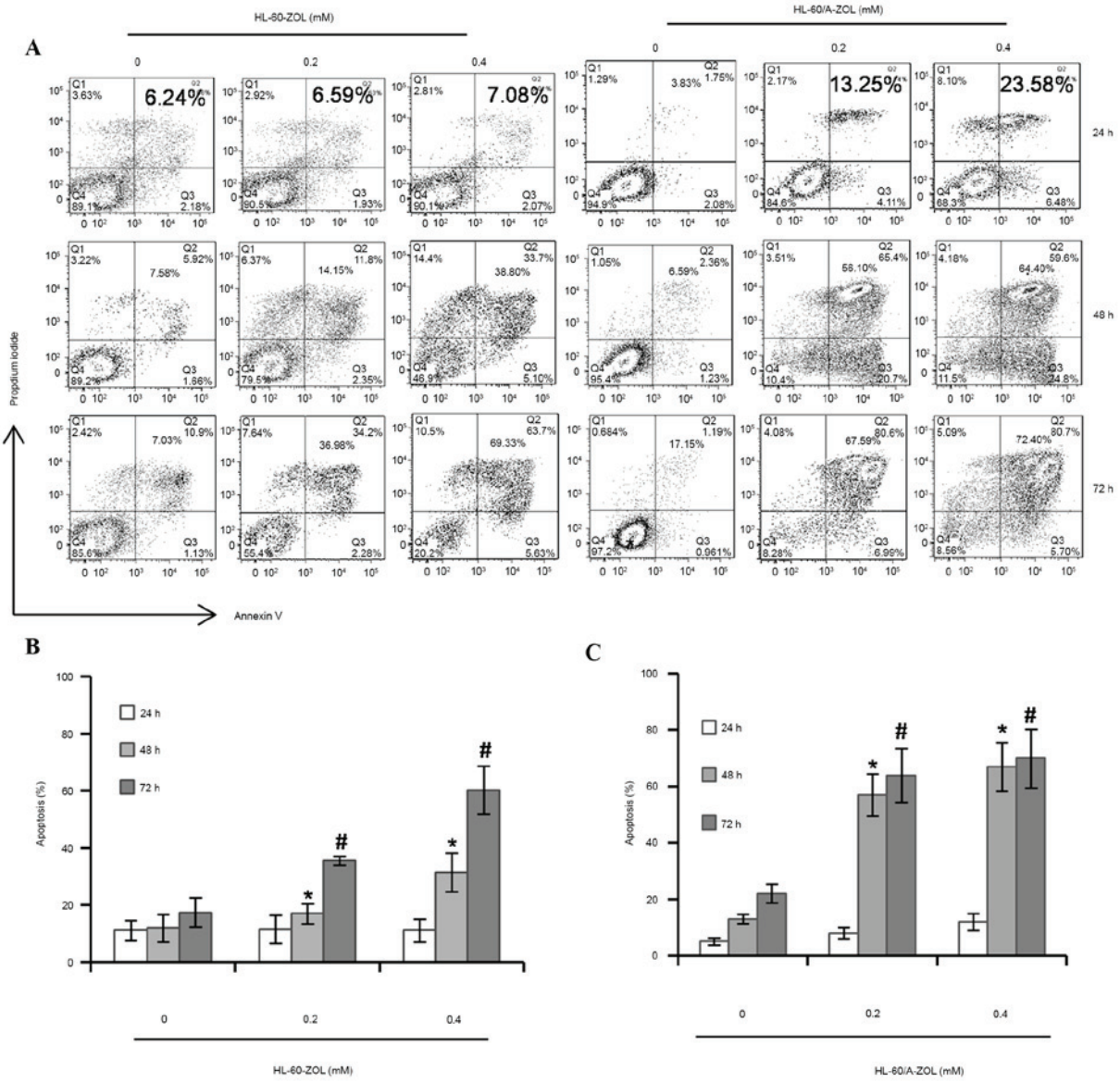

Figure 3. ZOL induces apoptosis of acute myeloid leukemia cells, as demonstrated by Annexin V/propidium iodide staining. HL-60 and HL-60/A cells were treated with ZOL at different concentrations for 24, 48 and 72 h. (A) Apoptotic cells were measured by Annexin V/propidium iodide staining. Analysis of (B) HL-60 and (C) HL-60A cells in three independent experiments. Data is presented as the mean ± standard error. *P<0.05 vs. 0 mM group 48 h; #P<0.05 vs. 0 mM group 72 h. ZOL, zoledronic acid.

AML cell cycle distribution was analyzed using PI staining. Following treatment of cells with various concentrations of ZOL (0, 0.2 and 0.4 mM) for 48 h, the proportion of cells in S phase increased (Fig. 2A). Treatment of HL-60 and HL-60/A cells with 0.2 mM ZOL for 48 h resulted in an increase in the proportion of S phase cells from 32.14±1.78 to 41.23±2.31% in HL-60 cells and from 46.56±1.34 to 61.87±14.18% in HL-60/A cells (Fig. 2B).

ZOL induces apoptosis in AML cells. The Annexin V-PI double staining assay demonstrated that ZOL induced apoptosis of AML cells in a dose- and time-dependent manner (Fig. 3A). Flow cytometry analysis indicated that the total apoptosis rates were 11.57±4.94 and 16.18±4.00% in HL-60 cells treated with ZOL at the concentrations of 0.2 and 0.4 mM for 24 h and increased to 35.58±1.49 and 60.24±8.50% at 72 h, respectively (Fig. 3B). However, in HL-60/A cells treated with ZOL, the total apoptosis rates were 13.05±4.22 and 22.49±5.12% at the concentrations of 0.2 and 0.4 mM for 24 h and significantly increased to 65.14±6.11 and 71.24±7.98% for 72 h respectively (P<0.05; Fig. 3B). Subsequently, AML cells were stained with Hoechst 33342 dye following exposure to ZOL for 48 h and observed under a fluorescence microscope. The results demonstrated that the nuclei of untreated cells were round in shape, whilst the nuclei of cells treated with ZOL were condensed or ruptured (Fig. 4A). The total apoptosis rates were 15.01±4.22 (0.2 mM) and 27.35±5.78% (0.4 mM) in HL-60 cells and 43.38±7.35 (0.2 mM) and 61.68±8.1% (0.4 mM) in HL-60/A cells (Fig. 4B and C). In order to determine the apoptotic mechanism of ZOL, the expression levels of apoptosis-associated proteins were measured using the western blotting method. Treatment of cells with 0.4 mM ZOL for 48 h induced the expression of cleaved PARP and Bax, as well

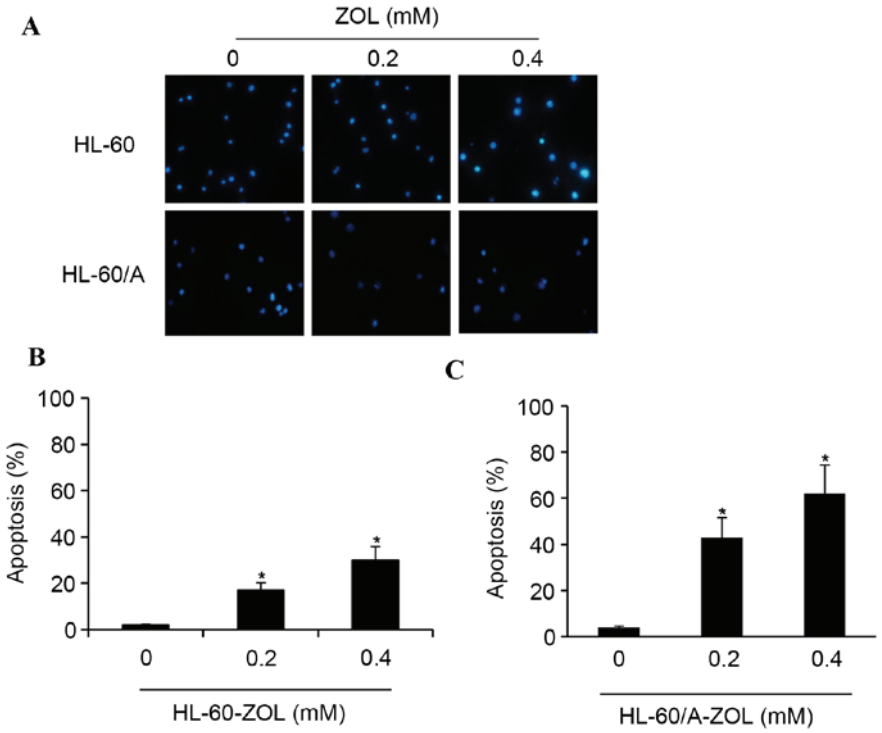

Figure 4. ZOL induces apoptosis of acute myeloid leukemia cells by Hoechst 333342 staining. (A) HL-60 and HL-60/A cells were treated with different concentrations of ZOL for 48 h, stained with Hoechst 333342 and examined under a fluorescence microscope (magnification, x200). Analysis of (B) HL-60 and (C) HL-60A cells in three independent experiments. Data is presented as the mean ± standard error. *P<0.05 vs. 0 mM group. ZOL, zoledronic acid.

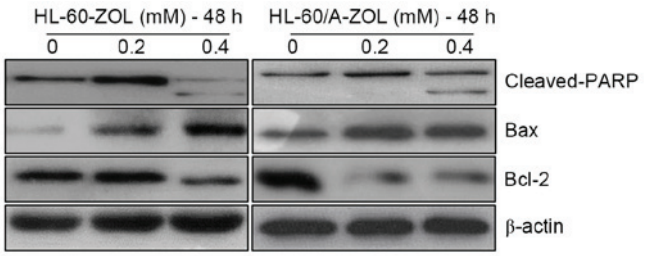

Figure 5. ZOL upregulates the expression levels of Bax and cleaved PARP and downregulates the protein expression level of Bcl-2 in HL-60 and HL-60/A cells. HL-60 and HL-60/A cells were treated with ZOL at the concentrations of 0.2 and 0.4 mM for 48 h, and expression levels of proteins associated with apoptosis, including Bax and cleaved PARP were increased, whilst Bcl-2 levels were decreased. ZOL, zoledronic acid; Bcl-2, B cell lymphoma 2; Bax, Bcl-2 associated X protein; PARP, poly (ADP ribose) polymerase.

as decreased the expression of Bcl-2. The upregulation of cleaved PARP and Bax, and the downregulation of Bcl-2 are presented in Fig. 5.

Discussion

Over the past half century, progress has been made regarding the treatment of AML, due to the development of molecular targeted therapy and the development of chemotherapy and hematopoietic stem cell transplantation. Long-term disease-free survival can be achieved, however certain patients will relapse following remission. Relapse is the predominant reason for treatment failure. The cause of relapse remains controversial, with minimal residual disease and multiple drug resistance as key factors. Recently, molecular targeted therapy has become an area of interest, with regards to overcoming the problems presented by relapse (16).

ZOL, a third-generation nitrogen-containing bisphosphonate, is used for the treatment of cancer-induced bone disease in solid tumors and multiple myeloma. Accumulating evidence has demonstrated that ZOL has marked anti-tumor activities in a variety of cancer cells. The first study to assess the anti-leukemic effect of ZOL investigated primary Philadelphia chromosome-positive CML cells (11). Chuah *et al* (14) later demonstrated that ZOL was effective in inhibiting the proliferation and clonogenicity of imatinib-sensitive and -resistant CML cells, regardless of the mechanism of resistance. The present study indicated that ZOL inhibited the proliferation of AML cells in a dose- and time-dependent manner, and this was consistent with results obtained in previous studies (12,17). To investigate the anti-proliferative efficacy on cell lines refractory to other anti-cancer therapeutic agents, the current study evaluated the ability of ZOL to suppress the growth of HL-60/A cells. ZOL was demonstrated to have a marked effect in HL-60/A cells. The present study also investigated the effect of ZOL on colony formation activity in HL-60 and HL-60/A cells, and it was demonstrated that 0.2 mM ZOL inhibited the colony formation activities of HL-60 and HL-60/A cells. Previous studies demonstrated that adriamycin-resistant tumor cells were associated with the overexpression of multidrug resistance (MDR-1) and activation of Akt/mammalian target of rapamycin signaling. Knockdown of MDR-1, however, reversed drug resistance (18). Adriamycin-resistant gastric cancer cells (SGC-7901/ADR) also exhibited activation

of the Wnt/β-catenin signaling pathway. The proton pump inhibitor pantoprazole could block SGC7901/ADR cell invasiveness (19). The present study demonstrated that ZOL overcomes adriamycin-induced drug resistance, indicating a novel application in leukemia therapy.

The manner by which ZOL affects the cell cycle remains to be elucidated. Forsea *et al* (20) indicated melanoma cells treated with ZOL were arrested in S phase, however, Chuah *et al* (14) demonstrated that various CML cells treated with ZOL were resistant to imatinib-induced S phase arrest. In addition, ZOL altered the cell cycle distribution of the BV173 leukemia cell line from S phase to the boundary of G_2/M phase (11), and this effect was tumor protein 53-independent. The present study demonstrated that with an increase of therapeutic agent concentration, and length of exposure time, the cell cycle could be arrested in the S phase, indicating that the anti-proliferative effect of ZOL was achieved by the induction of S-phase cell cycle arrest.

Apoptosis is defined as programmed cell death and associated signaling pathways include the mitochondria-mediated pathway and the death receptor-mediated pathway. The apoptosis-associated Bcl-2 protein family regulates the mitochondria-mediated pathway. The increase in the Bax/Bcl-2 ratio results in an increase in the permeability of the mitochondrial outer membrane and induces the release of high molecular weight pro-apoptotic effectors from the mitochondrial inner membrane to the cytoplasm (21). The present study investigated the effect of ZOL on cell apoptosis using the Annexin V-PI assay. The results indicated that ZOL significantly induced apoptosis of HL-60 and HL-60/A cells compared with the control group. As presented in Fig 3B, following exposure to 0.2 mM ZOL for 48 and 72 h, the apoptotic rate was determined to be 17.04 and 35.58% for HL-60 cells, and 76.2 and 75.14% for HL-60/A cells. Similar results were observed in Hoechst 333342 staining. In order to investigate the mechanism of the pro-apoptotic effect of ZOL, the effect of ZOL on apoptosis-associated proteins, such as Bax, Bcl-2 and cleaved PARP were investigated, and the levels of these proteins were demonstrated to be consistent. Thus, the proapoptotic effect of ZOL may be associated with the mitochondrial-mediated pathway of cell apoptosis. Ottewell *et al* (22) indicated that ZOL and doxorubicin induced cleavage of caspase 8 resulting in activation of the mitochondria-independent pathway, which was consistent with results observed in the present study.

In conclusion, the present study demosntrated that ZOL inhibited proliferation and induced apoptosis in HL-60 and HL-60/A cells, and its anti-leukemic activity was more predominantly observed in HL-60/A cells, suggesting that ZOL could overcome adriamycin resistance, providing a novel approach and possible therapeutic strategy for the treatment of leukemia.

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