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# Human THP-1 Monocytic Leukemic Cells Induced to Undergo Monocytic Differentiation by Bryostatin 1 Are Refractory to Proteasome Inhibitor-induced Apoptosis[1]

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## ABSTRACT

**The ubiquitin-proteasome pathway is the principal mechanism for the degradation of short-lived proteins in eukaryotic cells. We demonstrated that treatment of THP-1 human monocytic leukemia cells with Z-LLL-CHO, a reversible proteasome inhibitor, induced cell death through an apoptotic pathway. Apoptosis in THP-1 cells induced by Z-LLL-CHO involved a cytochrome *c*-dependent pathway, which included the release of mitochondrial cytochrome *c*, activation of caspase-9 and -3, and cleavage of Bcl-2 into a shortened 22-kDa fragment. Induction of apoptosis by protease inhibitor also was detected in U937 and TF-1 leukemia cell lines and cells obtained from acute myelogenous leukemia patients but not in normal human blood monocytes. Treatment of human blood monocytes with Z-LLL-CHO did not induce apoptosis or Bcl-2 cleavage in these cells that rarely proliferate. Interestingly, when THP-1 cells were induced to undergo monocytic differentiation by bryostatin 1, a naturally occurring protein kinase C activator, they were no longer susceptible to apoptosis induced by Z-LLL-CHO. Bryostatin 1-induced differentiation of THP-1 cells was associated with growth arrest, acquisition of adherent capacity, and expression of membrane markers characteristic of blood monocytes. Likewise, differentiated THP-1 cells were refractory to Z-LLL-CHO-induced cytochrome *c* release, caspase activation, and Bcl-2 cleavage. Resistance to Z-LLL-CHO-induced apoptosis in differentiated THP-1 cells was not due to cell cycle arrest. These findings show that the action of proteasome inhibitors is mediated primarily through a cytochrome *c*-dependent pathway and induces apoptosis in leukemic cells that are not differentiated.**

## INTRODUCTION

The ubiquitin-proteasome pathway is the major nonlysosomal tool in eukaryotic cells for the degradation of short-lived intracellular proteins for disposal via an ATP- and ubiquitin-dependent mechanism (1). In this pathway, specific proteins are marked for degradation by conjugation to multiple molecules of ubiquitin, which targets proteins for rapid hydrolysis by the 26S proteasome. The ubiquitin-proteasome pathway was initially regarded as a mechanism of destruction for old and damaged proteins. In recent years, however, it has become clear that proteolysis by the proteasome pathway is a crucial mechanism of regulation of many cellular processes, including cell cycle progression, gene expression, and cell differentiation. Known substrates of this pathway include mitotic and S-phase cyclins (2), $p21^{waf1}$ (3), cyclin-dependent kinase inhibitor p27 (4), I$\kappa$B$\alpha$ (5), Bax, Mdm2 (6), and transcriptional factors such as p53 (7, 8), Jun (9), and Fos (10). Alterations of proteasome function have been linked to cellular transformation by oncogenic viruses and immune escape, and correlated to poor prognosis in colon and breast cancer (11).

Furthermore, results from recent studies have suggested that the ubiquitin-proteasome pathway may be involved in the regulation of apoptosis (12). Shinohara *et al.* (13) showed that inhibition of the proteasome pathway can induce apoptosis in MOLT-4 cells by a p53-dependent mechanism. In contrast, Herrmann *et al.* (14) found that proteasome inhibitor-induced prostate carcinoma cell death is independent of functional Bcl-2 and p53. Drexler (15) reported that inhibition of proteasome function is associated with apoptosis in HL60 cells, primarily in the $G_1$ phase of the cell cycle. Kitagawa *et al.* (16) established that apoptosis of human glioma cells induced by proteasome inhibitors involves a mitochondria-independent mechanism. More recently, we showed that proteasome inhibitor-induced apoptosis in human M-07e leukemia cells is mediated through a caspase-3-dependent and Bcl-2-sensitive pathway (17). Induction of apoptosis by inhibition of the proteasome pathway appears to be cell cycle independent. There are examples where exposure of quiescent cells to proteasome inhibitors induces apoptosis (18, 19). However, there is evidence that proteasomes may be required for, or are protective against apoptosis under other conditions, such as growth factor withdrawal and ionizing irradiation (19, 20). Thus, the exact role of the ubiquitin-proteasome pathway in regulating apoptosis is far from clear.

During apoptosis, several effector proteases such as caspase-3 mediate the deliberate disassembly of the cell into apoptotic bodies (21). These downstream caspases are activated through proteolytic cleavage by either caspase-8 or caspase-9, two upstream initiator caspases. In the Fas pathway, the activation of caspase-8 involves the formation of a complex with the cytoplasmic death domain of TNF[3] receptor and its analogous receptors (22). In contrast, the activation of caspase-9 requires the participation of cytochrome *c* release from the mitochondria. In this pathway, caspase-9 is activated when complexed with extramitochondrial cytochrome *c* and apoptotic protease activating factor 1 (23, 24). Both initiator caspases are responsible for the activation of caspase-3 and other downstream effectors during apoptosis. In addition to caspases, other controllers of apoptosis are the Bcl-2 family proteins, which function upstream of caspases by either promoting or suppressing their protease activities. Several lines of evidences show that Bcl-2 family proteins are involved in controlling the release of cytochrome *c* from the mitochondria to activate caspase-9 (25–27). Apoptosis, which is essential for normal cell differentiation and development, is under strict physiological regulations. Deregulation of this process can lead to various defects ranging from embryonic lethality to a high susceptibility of malignant diseases such as leukemia (28). Indeed, leukemia is believed to be caused by impaired apoptosis in hematopoietic cells, resulting in the accumulation of immature nonfunctional cells (29).

We have shown that treatment of THP-1 monocytic leukemia cells

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[3] The abbreviations used are: TNF, tumor necrosis factor; bryo1, bryostatin 1; PKC, protein kinase C; PI, propidium iodide; AML, acute myelocytic leukemia; GM-CSF, granulocyte macrophage colony-stimulating factor.



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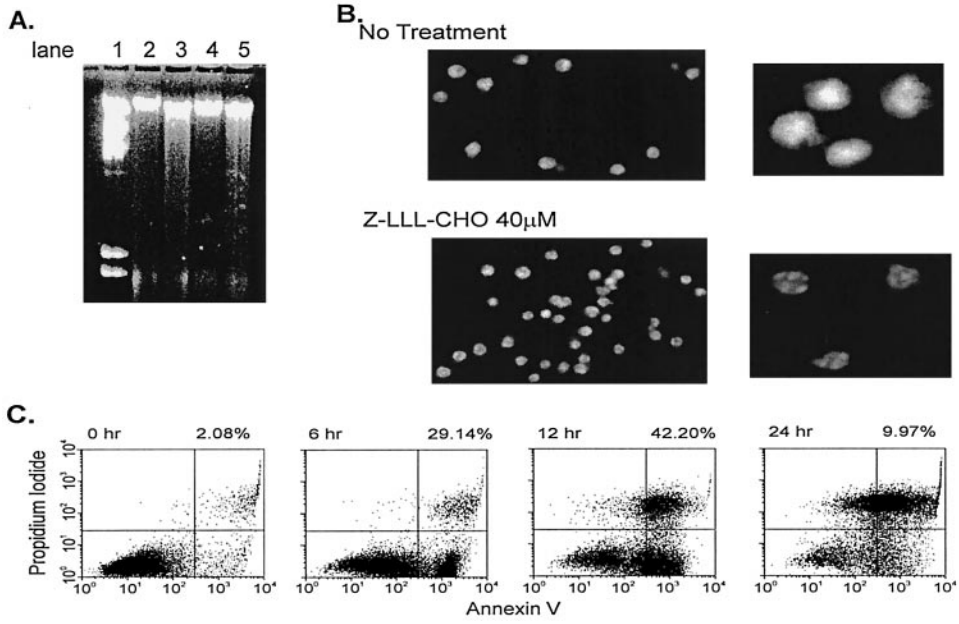


Fig. 1. Induction of apoptosis in THP-1 cells by Z-LLL-CHO treatment. *A*, DNA fragmentation occurred in Z-LLL-CHO-treated cells. Genomic DNA was isolated from THP-1 cells without treatment (*Lane 2*) or treatment with 5 $\mu$M Z-LLL-CHO (*Lane 3*), 10 nM bryo1 (*Lane 4*), and 40 $\mu$M Z-LLL-CHO (*Lane 5*). *Lane 1*, $\lambda$ DNA marker from *Hin*dIII digest. DNA (2.5 $\mu$g per sample/lane) was run on 1.2% agarose at 40 V. *B*, acridine orange staining of THP-1 cells with (*bottom panels*) or without (*top panels*) treatment with Z-LLL-CHO (40 $\mu$M) for 24 h. *C*, increased annexin V staining in Z-LLL-CHO-treated THP-1 cells. THP-1 cells were treated with Z-LLL-CHO (5 mM) for various times (h). Percentage of annexin V positive cells (*lower right quadrant*) is indicated at the *upper right corner* of each panel. Two hundred thousand cells per sample were analyzed on FACScan flow cytometer.

with Z-LLL-CHO, a reversible proteasome inhibitor, produced cell death through apoptotic pathways.[4] Biochemical analysis showed that apoptosis of THP-1 cells induced by Z-LLL-CHO was associated with the activation of a caspase-3-like protease that cleaved Bcl-2 into a shortened 22-kDa fragment. However, Z-LLL-CHO did not induce apoptosis in normal human blood monocytes, which rarely proliferate. This finding led us to hypothesize that proteasome inhibitors specifically target leukemic cells that are not differentiated. We now report that when THP-1 cells were induced to undergo monocytic differentiation by bryo1 (30), a naturally occurring PKC activator, they became refractory to the pro-apoptotic effect of proteasome inhibitors.

## MATERIALS AND METHODS

**Reagents.** Mouse anti-Bcl-2 monoclonal antibody (SC-509), anti-Bax (B9), and rabbit anti-c-fms were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal mouse anti-cytochrome *c* antibody and polyclonal rabbit anti-caspase-3 and caspase-9 antibodies were purchased from PharMingen Inc. (San Diego, CA). Polyclonal anti-Ron antibody was a gift from Dr. M. H. Wang (Department of Medicine, University of Colorado, Denver, CO). FCS and RPMI 1640 were products of Life Technologies (Grand Island, NY). Z-LLL-CHO, caspase inhibitors and protease inhibitors were obtained from Calbiochem (San Diego, CA). Other reagents were purchased from Sigma Co. (St. Louis, MO). bryo1 was prepared and purified from the marine bryozoan *Bugula neritina* in Dr. Pettit's laboratory as described previously (31).

**DNA Fragmentation Assay.** DNA was extracted according to the procedure of Miller *et al.* (32). THP-1 cell ($1 \times 10^7$) genomic DNA was extracted by adding 3 ml of nuclei lysis buffer [10 mM Tris-Cl, 400 mM NaCl, and 2 mM $Na_2EDTA$, (pH 8.2)], 0.2 ml of 10% SDS, and 0.5 ml proteinase K solution [1 mg proteinase K, 2 mM $Na_2EDTA$ (pH 8.2), and 1% SDS] and incubated at 37°C overnight. DNA was precipitated by adding 1 ml of 6 M NaCl and vortexed for 15 s. The supernatant was collected by spinning at 2500 rpm ($1300 \times g$) for 15 min. Two volumes of 95% ethanol were added to the supernatant and gently mixed. DNA precipitate was removed with a plastic spatula and placed in a tube containing 200 $\mu$l of Tris-EDTA buffer. DNA was allowed to dissolve at 37°C for 2 h; its absorbance was then determined by spectrophotometry. Fractionation of DNA by electrophoresis was performed on 1.2% agarose gel in 1× Tris-borate-EDTA buffer at a constant voltage of 40 V. The agarose gel was stained with ethidium bromide for visualization of DNA.

**Acridine Orange Staining.** Cells ($1 \times 10^6$/ml) were stained with acridine orange (10 $\mu$L of a 100 $\mu$g/ml solution into 100 $\mu$L of cell suspension) for 10 min at 37°C. Thereafter, cells were washed with cold 0.9% NaCl to remove excess stains. The cell pellet was left on ice until fluorescence microscopy was performed. A minimum of 100 cells per sample were counted under a fluorescence microscope. Cells with condensed chromatin and fragmented nuclei were counted as positive, whereas those with a normal chromatin pattern were counted as negative.

**Flow Cytometric Analysis.** Phosphatidylserine on the plasma membranes of cells was stained with Annexin V-FITC (Alexis Biochemicals) according to the protocol provided by the manufacturer. Briefly, 2–5 $\times 10^5$ cells/ml were washed in 1× PBS. The cells were resuspended in 198 $\mu$l of binding buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM $CaCl_2$; filtered in 0.2 $\mu$m pore filter] and 2 $\mu$l of FITC-labeled annexin V (annexin V-FITC). The mixtures were then incubated in the dark for 10 min, after which the cells were washed once with PBS and resuspended in 195 $\mu$l of binding buffer and 2 $\mu$l of 100 $\mu$g/ml PI. Apoptotic cells were defined as FITC positive and PI negative. Flow cytometry was analyzed on FACScan (Becton Dickinson), and data analysis was done on PC-LYSYS v1.1.

**Western Blots.** Whole cell lysates in 2× SDS loading buffer were fractionated by 12% SDS-PAGE at 100 V until the dye front reached the bottom of the gel. The proteins were transferred onto 0.2 $\mu$m pore nylon membrane (NYTRAN) at 40 V for 45–60 min. The membranes were blocked with 5% nonfat milk and probed with anti-Bcl-2 monoclonal antibody at a 1:200 dilution, anti-Bax monoclonal antibody at a 1:500 dilution, anti-c-fms polyclonal antibody at a 1:100 dilution, anti-Ron polyclonal antibody at a 1:5000 dilution, anti-caspase-3 polyclonal antibody at a 1:2000 dilution, anti-caspase-9 polyclonal antibody at a 1:1000 dilution, or with anti-cytochrome *c* monoclonal antibody at 1:500 for 1 h at room temperature. After extensive washes with 1× Tris-borate-EDTA buffer, the blots were incubated with appropriate secondary antibodies conjugated with horseradish peroxidase (1:5000 dilutions) for 1 h at room temperature. The blots were washed three times in 1× Tris-buffered saline, and the protein complexes were detected using enhanced chemiluminescence detection reagents according to the manufacturer's protocol (Amersham Life Science).

**Cytosolic Fraction Isolation.** The procedure for the isolation of the cytosolic fraction was described previously (33). Briefly, cells ($1 \times 10^8$) were washed in ice-cold PBS and spun at $300 \times g$ for 5 min at 4°C. The cell pellet was resuspended in 1 ml of ice-cold buffer A [250 mM sucrose, 20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1

[4] C. Chen, unpublished observation.



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mM EGTA, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride] with 1× protease inhibitor cocktail (Boehringer Mannheim). Cells were lysed by 20 strokes with a Dounce glass homogenizer (No. 7726) on ice. The lysate was centrifuged at 750 × *g* for 10 min at 4°C to remove nuclei and unbroken cells. The supernatant was removed and centrifuged at 15,000 × *g* for 15 min at 4°C to eliminate mitochondria. The resulting supernatant, the cytosolic fraction, was assayed for the protein concentration by the Bradford protein assay (Bio-Rad), and then boiled in 1× SDS sample loading buffer.

**Caspase-9 Assay.** The caspase-9 assay kit was purchased from Medical and Biological Laboratories Co., LTD. (Nagoya, Japan), and the assay was performed exactly according to the manufacturer's protocol. Briefly, THP-1 cells ($5 \times 10^6$) were resuspended in 50 $\mu$L of chilled cell lysis buffer and incubated on ice for 10 min. Cell debris was separated from the supernatant by centrifuging at 10,000 × *g* for 2 min. Equal volume of 2× reaction buffer and 5 $\mu$L of 2 mM LEHD-pNA substrate (100 $\mu$M final concentration) were added to the supernatant, and the mixtures were incubated at 37°C for another 2 h. Dilution buffer (500 $\mu$L) was added to the sample, and the absorbance at 405 nm was read. Enzyme activity was expressed as pmol/mg protein/min.

**Isolation of Human Monocytes and Leukemia Cells.** WBCs from healthy volunteers and AML patients with FAB M3 histological classification were isolated by layering whole blood over Histopaque 1077 (Sigma) at a volume of 1:1, and then centrifuging at 2000 rpm (1200 × *g*) for 30 min. The white mononuclear layer was carefully removed with a Pasteur pipette, washed once with PBS, and resuspended in cold medium. Human monocytes were isolated by incubating mononuclear cells in RPMI 1640 supplemented with 10% FCS in tissue culture dishes for an additional 3 h at 37°C and 5% $CO_2$, after which the nonadherent cells were removed. Over 90% of the adherent cells were identified as monocytes by morphology criteria. Each immunoblot lane contained $1 \times 10^6$ cells. Samples were obtained from the patients after informed consent for this study.

**Cell Cycle Arrest.** THP-1 cells were cultured in 0.2% FCS for 48 h, then in serum-free medium for 48 h to arrest the cell cycle at the $G_1$ phase. For $G_2$-M arrest, cells were starved in 0.2% serum for 48 h and then treated with colchicine ($2 \times 10^{-5}$ M), a microtubule inhibitor, for an additional 48 h. Growth arrest was confirmed using a [$^3$H]thymidine uptake technique. Briefly, THP-1 cells ($1 \times 10^5$/100 $\mu$l) cultured in 96-well plates in triplicate were labeled with 0.5 $\mu$Ci of tritiated [$^3$H]thymidine (6.7 Ci/mmol; NEN Life Science Products, Boston, MA) for 16 h. The cells were harvested onto fiberglass filters using an automated PHD cell harvester. The filters were dried and counted in 3 ml of scintillation fluid with a Beckman LS3801 scintillation counter.

## RESULTS

**Induction of Apoptosis in THP-1 Cells by Z-LLL-CHO.** THP-1 cell line was established from a patient with AML. The cells grew in culture as a suspension with a cell doubling time of ~16 h. THP-1 cells can be induced to differentiate into monocytes/macrophages but not cells of other hematopoietic lineages and are considered as the leukemic "counterpart" of blood monocytes. Treatment of THP-1 cells with Z-LLL-CHO resulted in growth arrest and cell death through an apoptotic pathway. Apoptosis was confirmed by a DNA fragmentation assay in cells that had been treated with Z-LLL-CHO (Fig. 1*A*). Under the light microscope, apoptotic cells, characterized by cytoplasmic vacuolation, membrane blebbing, and apoptotic bodies (34), could be seen at 12 h after Z-LLL-CHO treatment. Acridine orange staining showed nuclear fragmentation and condensed chromatin structures in >25% of the cells (Fig. 1*B*). In contrast, <2% of apoptotic cells were detected in control cultures of THP-1 cells. Flow cytometry study with FITC-labeled annexin V also showed a dramatic increase of annexin V-binding activity in Z-LLL-CHO-treated THP-1 cells (Fig. 1*C*). At 6 h, 29.14% of the treated cells were apoptotic, staining positive for annexin V and negative for PI, compared with 2.08% in control cultures without treatment. At 24 h, only 9.97% of the cells were identified as apoptotic because of procession of apoptotic cells to death, resulting in positive staining for both PI and annexin V-FITC.

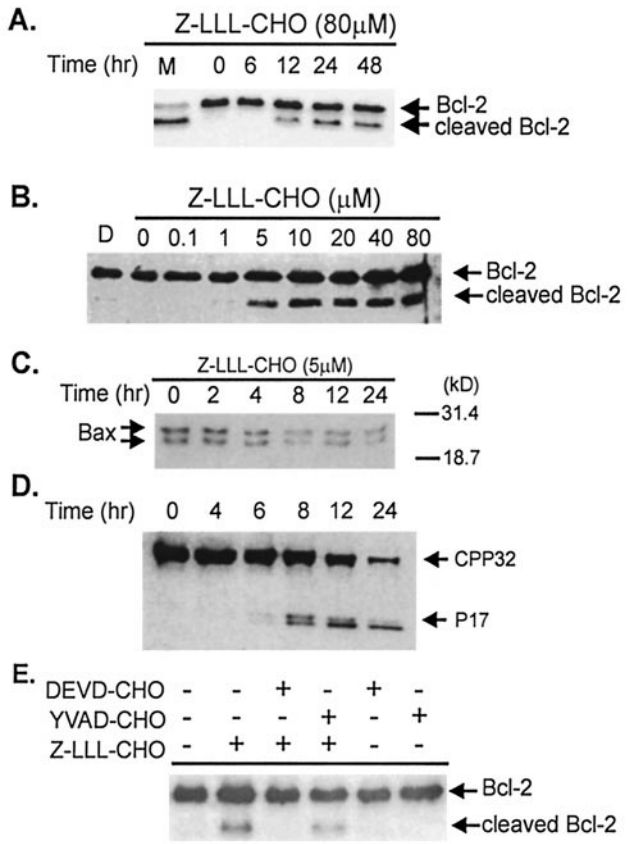


Fig. 2. Western blots of Bcl-2, Bax, and caspase-3 in apoptotic THP-1 cells. *A*, time dependence of Bcl-2 cleavage. THP-1 cells were treated with Z-LLL-CHO (80 $\mu$M) for various times (h). Total cell lysates were subjected to immunoblot analysis (10 $\mu$g protein/lane) with anti-Bcl-2 antibody as the probe. *Lane M*, marker for Bcl-2 cleavage prepared from M-07e cell lysates after starvation in cytokine-free medium for 48 h. *B*, cells were treated with increasing doses of Z-LLL-CHO as indicated for 12 h. Thereafter, total cell lysates were subjected to an immunoblot analysis (10 $\mu$g protein/lane) with anti-Bcl-2 antibody. DMSO (0.2%) was used as a control for drug solvent (*Lane D*). *C*, expression of Bax in Z-LLL-CHO-treated THP-1 cells. Cells were treated with Z-LLL-CHO (5 $\mu$M) for various times (h). *D*, THP-1 cells were treated with Z-LLL-CHO (5 $\mu$M) for various time periods as indicated. Total cell lysates were subjected to immunoblot analysis with anti-caspase-3 antibody. Note the appearance of the 17-kDa active caspase-3 fragment (*P17*) on the blot. *E*, inhibition of Bcl-2 cleavage by caspase-3 inhibitor. THP-1 cells were pretreated with DEVD-CHO (100 $\mu$M) or YVAD (100 $\mu$M) for 2 h, followed with Z-LLL-CHO (5 $\mu$M) for an additional 12 h. Thereafter, cells were lysed, and total cell lysates were subjected to immunoblot analysis with anti-Bcl-2 antibody.

**Z-LLL-CHO-induced Bcl-2 Cleavage and Caspase Activation.** To better understand the role of Bcl-2 in the process, we investigated the status of Bcl-2 during Z-LLL-CHO-induced apoptosis in THP-1 cells. Apoptosis of THP-1 cells induced by Z-LLL-CHO was clearly associated with the cleavage of Bcl-2 into a shortened 22-kDa fragment in a time- and dose-dependent manner (Fig. 2, *A* and *B*). The cleavage of Bcl-2 was detected at 12 h after the addition of the proteasome inhibitor. At the highest dose (80 $\mu$M) used in this study, Z-LLL-CHO treatment induced >25% cleavage of total cellular Bcl-2 as estimated from the intensity of the Bcl-2 bands in immunoblots. In contrast, no Bax cleavage was noticed in Z-LLL-CHO-treated cells although the levels of Bax appeared to be significantly reduced during apoptosis (Fig. 2*C*). Because of alternative splicing, Bax was detected as a doublet by the anti-Bax antibody obtained from commercial sources (Santa Cruz).

We next asked whether Z-LLL-CHO-induced apoptosis and Bcl-2 cleavage were mediated through a caspase-3-dependent pathway in THP-1 cells. Caspase-3 activation was monitored by cleavage from a 32-kDa precursor to a 17-kDa active fragment, using immunoblot analyses. As shown in Fig. 2*D*, the activation of caspase-3 was



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detected as early as 6 h after Z-LLL-CHO treatment. Caspase-3 activation also was confirmed by a colorimetric assay using Ac-DEVD-pNA as the substrate for caspase-3 (data not shown). To establish that Bcl-2 was cleaved by activated caspase-3, we treated the cells with a highly specific caspase-3 inhibitor, DEVD-CHO, prior to the addition of Z-LLL-CHO. In the presence of DEVD-CHO, the cleavage of Bcl-2 induced by Z-LLL-CHO treatment was inhibited (Fig. 2*E*). In contrast, cleavage of Bcl-2 was not inhibited by the caspase-1 inhibitor YVAD-CHO.

**Z-LLL-CHO Induced the Release of Mitochondrial Cytochrome *c* and Caspase-9 Activation in THP-1 Cells.** The preceding experiments showed that Z-LLL-CHO-induced apoptosis in THP-1 cells was associated with Bcl-2 cleavage by activated caspase-3. Because Bcl-2 has been implicated in the regulation of cytochrome *c* release from the mitochondria to the cytoplasm, we asked whether Z-LLL-CHO-induced apoptosis in THP-1 cells involved a cytochrome *c*-dependent pathway. THP-1 cells were treated with Z-LLL-CHO for various time periods. The levels of extramitochondrial cytochrome *c* were determined using an immunoblot analysis with anti-cytochrome *c* antibody. Treatment with Z-LLL-CHO readily induced the release of cytochrome *c* into the cytosolic fraction as early as 4 h (Fig. 3*A*). The release of cytochrome *c* was correlated with a transient activation of caspase-9 as indicated by cleavage of procaspase-9 (p48) into a shortened active p37 fragment in Z-LLL-CHO-treated cells (Fig. 3*B*). In parallel experiments, the activation of caspase-9 protease activity was detected using a colorimetric assay with a specific substrate, LEHD-pNA (Fig. 3*C*). To show that cytochrome *c* release is related to the activation of caspases, we pretreated THP-1 cells with a general caspase inhibitor, Boc-D-fmk, prior to the addition of Z-LLL-CHO. As shown in Fig. 3, *D* and *E*, pretreatment of THP-1 cells with the general caspase inhibitor reduced the amount of cell death and cytochrome *c* release induced by Z-LLL-CHO. Additional support of the activation of caspase-3 and -9 and Bcl-2 cleavage induced by Z-LLL-CHO treatment was shown in two other cell lines, TF-1, a GM-CSF-dependent eurythrocytic leukemia cell line, and U937, a human promonocytic cell line. Fig. 4 shows that when treated with Z-LLL-CHO, Bcl-2 was cleaved in both leukemia cell lines undergoing apoptosis. Likewise, the cleavage of Bcl-2 was associated with the activation of both caspase-3 and caspase-9.

**Human Peripheral Blood Monocytes Were Refractory to Z-LLL-CHO-induced Apoptosis.** To further analyze the mechanism whereby Z-LLL-CHO induces apoptosis, we examined the effect of Z-LLL-CHO on normal human blood monocytes and leukemia cells obtained from four AML patients. Compared with THP-1 cells and human leukemia cells, normal monocytes were highly resistant to Z-LLL-CHO-induced apoptosis (Fig. 5) from three normal volunteers. Immunoblot analysis showed that Z-LLL-CHO induced distinct Bcl-2 cleavage in both THP-1 cells and leukemic cells obtained from patients but not normal blood monocytes (Fig. 6). A very slight amount of cleaved Bcl-2 fragment was noticed in blood monocyte samples that had been treated with the highest dose of Z-LLL-CHO (100 $\mu$M) for an extended period (3 days).

**Z-LLL-CHO Induced Apoptosis in Leukemic Cells but not Differentiated Cells.** The preceding study shows that Z-LLL-CHO did not induce apoptosis in normal blood monocytes, raising the possibility that proteasome inhibitors may target specifically on leukemic cells that are not differentiated. To test this hypothesis, we induced THP-1 cells to undergo monocytic differentiation by bryo1, a naturally occurring PKC activator. Treatment with bryo1 for 12 h resulted in growth arrest of THP-1 cells and induced a major fraction (60%) of them to become adherent with distinct monocyte/macrophage differentiation markers (35), which included the expression of Ron and c-fms. Ron is a receptor for the human macrophage-stimu-

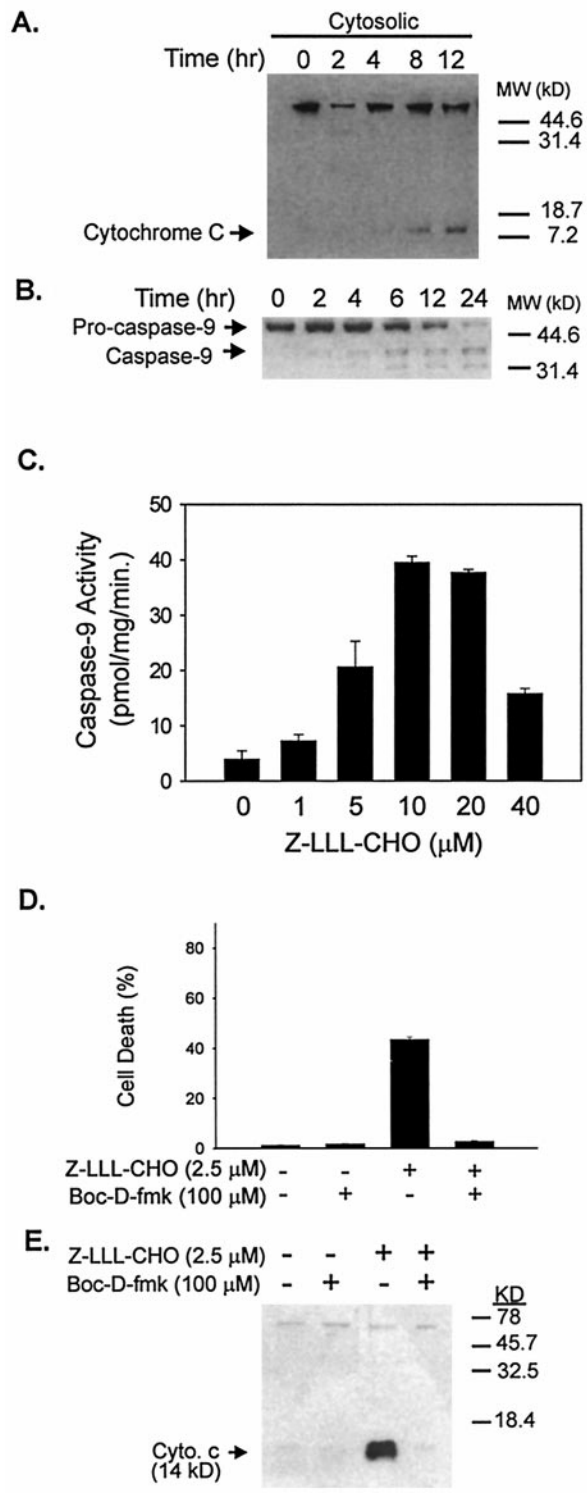


Fig. 3. Cytochrome *c* release and caspase-9 activation in Z-LLL-CHO-treated THP-1 cells. *A*, time-dependent release of cytochrome *c* (14 kDa) into the cytosol of THP-1 cells after treatment with Z-LLL-CHO (5 $\mu$M) for various times (h). *B*, time-dependent activation of caspase-9 by Z-LLL-CHO treatment. Cells were treated with Z-LLL-CHO (5 $\mu$M) for various times (h). After lysis, total cell lysates were subjected to immunoblot analysis with anti-caspase-9 antibody. Note the cleavage of procaspase-9 (48 kDa) to a 37-kDa active fragment. *C*, dose induction of caspase-9 activation in Z-LLL-CHO-treated THP-1 cells. Cells were treated with increasing doses of Z-LLL-CHO for 12 h. Protease activities in the cell lysates were determined using a colorimetric assay with LEHD-pNA as the substrate. *D*, general caspase inhibitor, Boc-D-fmk, reduced cell death in Z-LLL-CHO treated cells. Cells were pretreated with of Boc-D-fmk (100 $\mu$M) for 2 h, then with Z-LLL-CHO (2.5 $\mu$M) for 9 h. The percentage of cell death was determined by trypan blue exclusion. *E*, the cytosolic fraction of cells from *panel D* was immunoblotted with monoclonal antibody against cytochrome *c* (4 $\mu$g of protein/lane). Data are means from three experiments; *bars*, SD.



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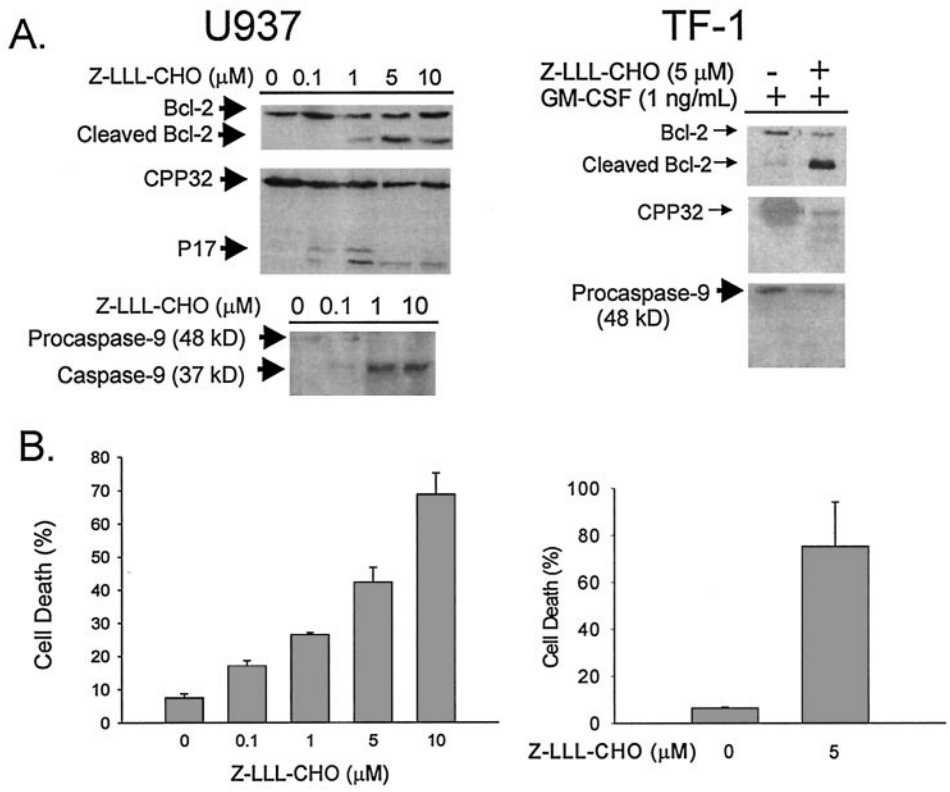


Fig. 4. Responses of U937 and TF-1 cells to Z-LLL-CHO. *A*, immunoblots of U937 cells treated with Z-LLL-CHO (0, 0.1, 1, 5, and 10 $\mu$M) for 24 h and probed for Bcl-2, caspase-3 (*CPP32*), and caspase-9. TF-1 cells were treated with 5 $\mu$M Z-LLL-CHO for 12 h, and the cell lysates were immunoblotted for Bcl-2, caspase-3, and caspase-9. In TF-1 cells, activation of caspase-3 and caspase-9 was detected as degradation or decreased expression of the proteins. *P17*, 17-kDa active caspase-3 fragment. *B*, Z-LLL-CHO-induced cell death (%) of U937 cells (*left*) and TF-1 cells (*right*). Data are means from three separate experiments; *bars*, SD.

lating protein, MSP, which regulates the motility and shape change of mature macrophages. The product of the c-fms proto-oncogene is the receptor for macrophage colony-stimulating factor (Fig. 7, *A* and *B*). The expression of these two receptors increases markedly during macrophage differentiation. Bryo1 treatment, however, neither significantly affected the levels of Bcl-2, Bax, and caspase-3 proteins from the same samples (Fig. 7, *C–E*), nor did it produce DNA fragmentation in THP-1 cells (Fig. 1*A*, *Lane 4*).

Unlike control THP-1 cells, treatment of differentiated THP-1 cells with Z-LLL-CHO did not induce Bcl-2 cleavage (Fig. 8*A*). The lack of Bcl-2 cleavage was associated with the failure of Z-LLL-CHO to activate caspase-3 in differentiated THP-1 cells (Fig. 8*B*). Because we have shown previously that the action of Z-LLL-CHO was mediated through a cytochrome *c*-dependent pathway, we asked whether bryo1 treatment affects the release of cytochrome *c* in Z-LLL-CHO-treated cells. As shown in Fig. 8, *C* and *D*, the release of cytochrome *c* and caspase-9 activation triggered by Z-LLL-CHO treatment were inhibited when THP-1 cells were induced to undergo monocytic differentiation by bryo1. Differentiated THP-1 cells became resistant to Z-LLL-CHO-induced apoptosis and also excluded trypan blue. Prolonged treatment (>48 h) of THP-1 cells with bryo1 eventually resulted in death but not apoptosis. Moreover, the induction of apoptosis by Z-LLL-CHO was also dramatically inhibited with prior bryo1 treatment (Fig. 8*E*).

To rule out the possibility that cell cycle arrest following bryo1 treatment was responsible for resistance to Z-LLL-CHO-induced apoptosis, we treated THP-1 cells with colchicine, which arrests the cells in $G_2$-M phase. Growth arrest in THP-1 cells was confirmed by reduced thymidine uptake in both cases. As shown in Fig. 9, quiescent cells arrested in $G_2$-M by colchicine were still responsive to Z-LLL-CHO-induced Bcl-2 cleavage and apoptotic cell death. In another experiment, THP-1 cells were cultured in serum-free medium for 48 h, which arrests cells in the $G_1$ phase. Likewise, nonproliferating cells arrested in the $G_1$ phase remained responsive to Z-LLL-CHO-

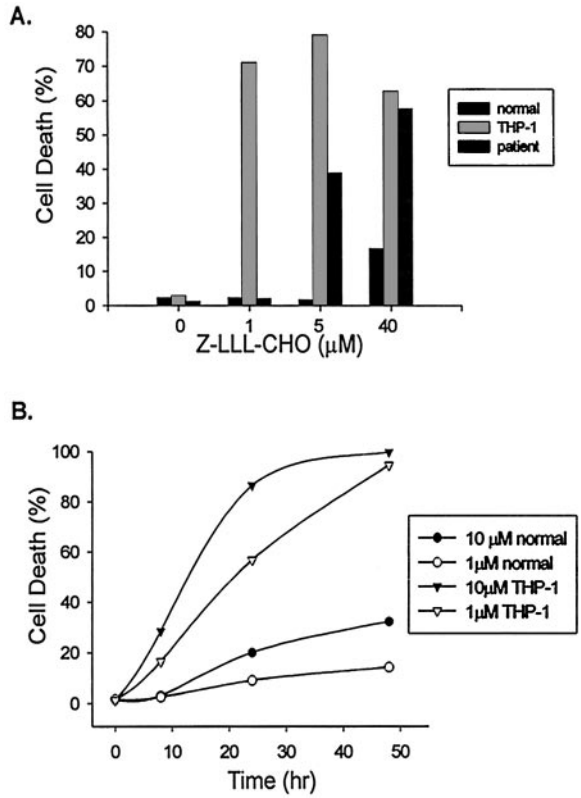


Fig. 5. Percentage of cell death after treatment with Z-LLL-CHO. *A*, normal blood monocytes, THP-1 cells, and leukemia cells obtained from an AML patient were treated with increasing doses of Z-LLL-CHO for 24 h. *B*, time course of cell death induced by Z-LLL-CHO. THP-1 cells and blood monocytes were treated with Z-LLL-CHO (1 and 10 $\mu$M) for various time periods. Cell death was determined by trypan blue exclusion method.



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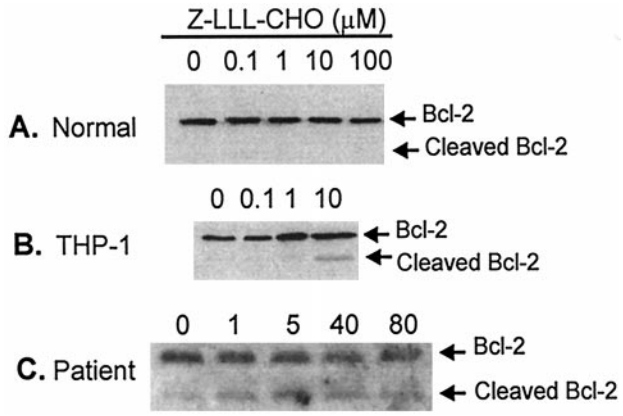


Fig. 6. Bcl-2 cleavage in THP-1 cells and leukemia patient samples but not normal blood monocytes. *A*, peripheral blood monocytes ($1 \times 10^6$/culture) from healthy normal volunteer were treated with varying doses of Z-LLL-CHO for 3 days. Cells were lysed in sample buffer and immunoblotted for Bcl-2. Representative blot from four healthy donors. *B*, THP-1 cells treated with increasing doses of Z-LLL-CHO as indicated for 24 h. Thereafter, cells were lysed in sample buffer and immunoblotted for Bcl-2. *C*, Bcl-2 cleavage in leukemia cells from AML patient treated with Z-LLL-CHO for 24 h. Cells ($1 \times 10^6$ cells/sample) were lysed in sample buffer, and total cell lysates were subjected to immunoblot analysis with anti-Bcl-2 antibody. Representative blot of four leukemia patient samples.

induced apoptosis. These findings excluded the possibility that cells in cell cycle arrest were not responsive for resistance to Z-LLL-CHO-induced apoptosis in differentiated THP-1 cells.

## DISCUSSION

A number of recent reports showed that inhibition of proteasome pathway induced apoptosis in various leukemic and nonhematological tumor cell lines (13, 14, 16, 17, 36). Here, we show that proteasome inhibitor-induced apoptosis in human leukemia THP-1 cells involves a cytochrome *c*-dependent pathway. The release of cytochrome *c* was accompanied by a transient activation of both caspase-9 and caspase-3 and apoptosis in Z-LLL-CHO-treated THP-1 cells. The release of cytochrome *c* and induction of cell death could be inhibited by the addition of a general caspase inhibitor. These results indicated that cytochrome *c* release is correlated with the activation of caspases and proteasome inhibitor-induced death of THP-1 cells proceeds in a caspase-dependent manner. In contrast to our finding, however, a recent study reported that proteasome inhibitor induces mitochondria-independent apoptosis in human glioma cells (16), raising the possibility that the execution of apoptosis induced by proteasome inhibitors is likely to be mediated, depending on the cell types and cellular factors, through several mechanisms, including both cytochrome *c*-dependent and -independent pathways.

In addition to THP-1 cells, Z-LLL-CHO induced apoptosis in leukemia cells obtained from AML patients. Two samples of AML were taken from patients who had undergone chemotherapy. Another two samples were taken from newly diagnosed leukemic patients before treatments. Despite the differences in samples, upon Z-LLL-CHO treatment, they invariably underwent apoptosis with distinct Bcl-2 cleavage. Serendipitously, we found that normal monocytes were highly resistant to Z-LLL-CHO-induced apoptosis compared with either THP-1 cells or leukemic cells from the patients. These observations led us to ask whether differentiated cells were less sensitive to proteasome inhibitor-induced apoptosis. To test this hypothesis, we induced THP-1 cells to undergo monocytic differentiation by bryo1, a naturally occurring PKC activator. Induction of monocytic differentiation in THP-1 cells was associated with growth arrest, acquisition of adherent capacity, and expression of some membrane markers characteristic of blood monocytes. As expected, we found that differentiated THP-1 cells were no longer susceptible to Z-LLL-CHO-induced cytochrome *c* release, caspase activation, and Bcl-2 cleavage, and thus were refractory to apoptosis. These results established that proteasome inhibitors specifically target leukemia cells that are not differentiated. Similar resistance to apoptotic agents after differentiation of U937 and THP-1 cells was reported recently (37, 38).

The mechanisms by which proteasome inhibitors induce apoptosis in leukemic cells are not known. Accumulation of short-lived proteins that are critical for cell proliferation and cell cycle regulation appears to be linked to Z-LLL-CHO-mediated apoptosis in THP-1 cells. For example, a recent study by Kitagawa *et al.* (16) reported that proteasome inhibitor-induced apoptosis in human glioma cells was associated with the up-regulation of short-lived proteins, including $p21^{Waf1}$, Mdm2, and $p27^{Kip1}$. Furthermore, Manna and Aggarwal (39) reported

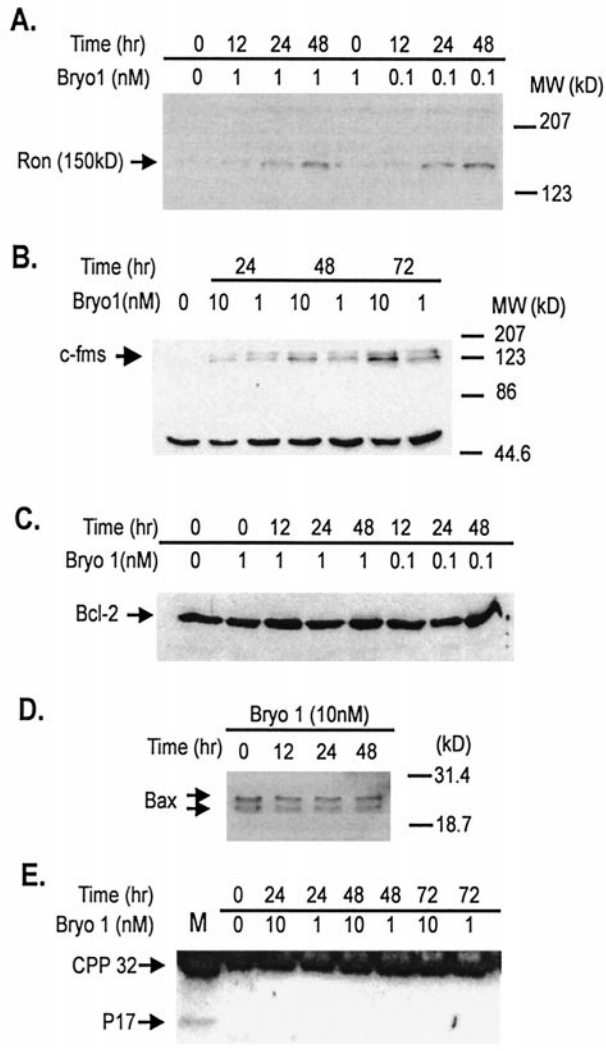


Fig. 7. Effect of bryo1 treatment on the levels of Bcl-2, Bax, caspase-3, Ron, and c-fms products. *A* and *B*, bryo1-induced expression of Ron (150 kDa) and macrophage colony-stimulating factor receptors (165-kDa doublet) in THP-1 cells. Cells were treated with bryo1 for various time periods. Cells were lysed in sample buffer and subjected to immunoblot analysis (20 $\mu$g/lane) with anti-Ron antibody (*A*) or anti-c-fms antibody (*B*). *C* and *D*, bryo1 treatment did not change the protein levels of Bcl-2 and Bax. Cells were treated with bryo1 (10 nM and 1 nM) for various times (h). Cells were lysed in sample buffer and subjected to immunoblot analysis with anti-Bcl-2 and anti-Bax antibodies. *E*, bryo1 treatment did not diminish caspase-3 protein level. Cells were treated with bryo1 (10 nM and 1 nM) for various times (h). Thereafter, cells were lysed in sample buffer and immunoblotted for caspase-3. *Lane M*, cell lysates obtained from M-07e cells that had been starved in GM-CSF-free medium for 48 h.



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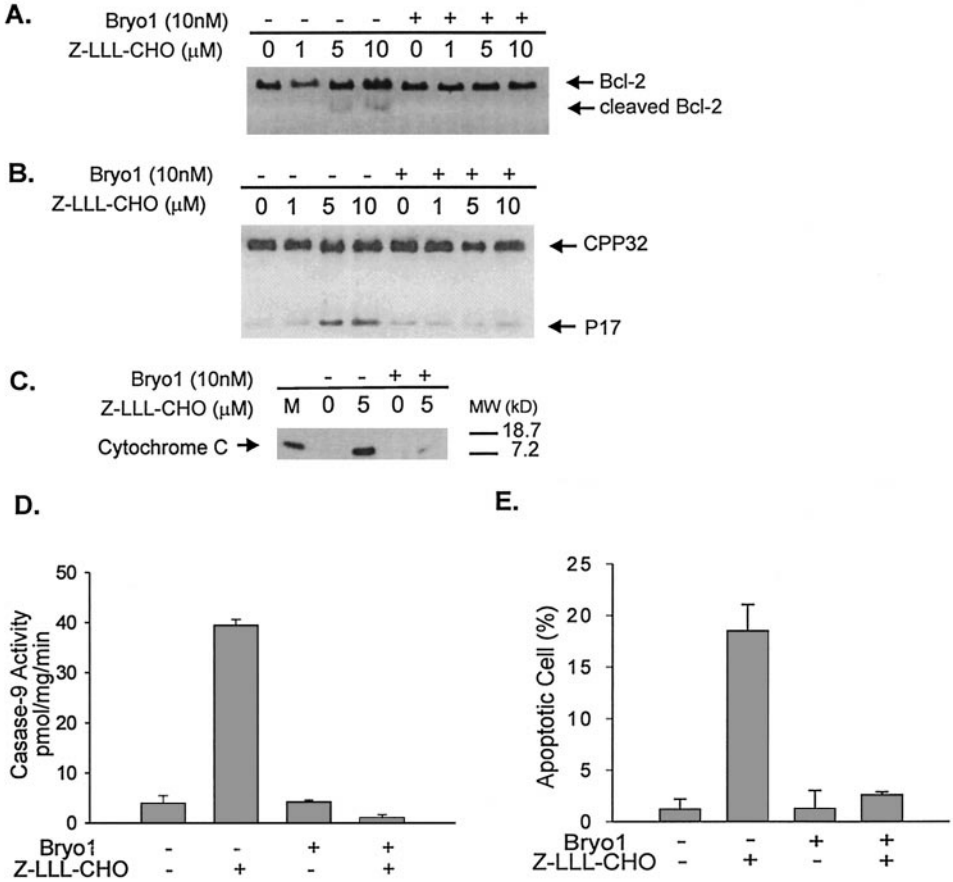


Fig. 8. Response of differentiated cell to Z-LLL-CHO. *A* and *B*, Bcl-2 cleavage and caspase-3 activation were inhibited in differentiated cells. THP-1 cells treated with bryo1 (10 nM) for 12 h. Differentiated THP-1 cells were further treated with Z-LLL-CHO for an additional 12 h. Thereafter, cells were lysed in sample buffer, and total cell lysates were subjected to immunoblot analyses with either anti-Bcl-2 (*A*) or anti-caspase-3 (*CPP32*) antibodies (*B*) as probes. *P17*, 17-kDa active caspase-3 fragment. *C*, diminished cytochrome *c* release in differentiated THP-1 cells. THP-1 cells were treated with bryo1 (10 nM) for 12 h. Differentiated cells were then treated with Z-LLL-CHO (5 $\mu$M) for an additional 12 h. Total cell lysates were immunoblotted for cytochrome *c* with anti-cytochrome *c* antibody. *Lane M*, standard marker for cytochrome *c*, 2.5 ng. *D*, decreased caspase-9 activation in differentiated THP-1 cells. THP-1 cells were treated with bryo1 (10 nM) for 12 h, and then with Z-LLL-CHO (5 $\mu$M) for an additional 12 h. Caspase-9 activity was determined using a colorimetric assay with LEHD-pNA as the protease substrate. Data are means from duplicate experiments; *bars*, SD. *E*, resistance of differentiated THP-1 cells to Z-LLL-CHO-induced apoptosis. THP-1 cells were treated with bryo1 (10 nM) for 12 h, and then with 5 $\mu$M Z-LLL-CHO for an additional 12 h. Apoptotic cells were counted after acridine orange staining; *bars*, SD.

that degradation of IkB, also a substrate of the proteasome degradation pathway, was accompanied by suppression of TNF-mediated apoptosis in human U937 cells. The relevance of these regulators in mediating apoptosis also is illustrated in our finding that THP-1 cells differentiated by bryo1 are highly resistant to Z-LLL-CHO-induced apoptosis, as described in this study. It has been shown that bryo1 treatment activates PKC and induced nuclear factor-$\kappa$B activation in a number of leukemic and tumor cell lines (40, 41). In addition, tumor cells that constitutively express nuclear factor-$\kappa$B were "resistant" to the apoptotic effects of TNF and a number of other apoptotic agents (42, 43). However, it should be pointed out that proteasome inhibitors, in addition to inducing apoptosis, have been reported to prevent apoptosis in sympathetic neurons upon deprivation of nerve growth factor (19) and in thymocytes treated with ionizing radiation, glucocorticoids, or phorbol ester (20), illustrating the complex nature of the proteasome systems in regulating apoptosis.

The biological significance of Bcl-2 cleavage is a matter of speculation. In addition to THP-1, we detected Bcl-2 cleavage and apoptosis in TF-1 and U937 human leukemia cells after treatment with Z-LLL-CHO. Bcl-2 cleavage also has been reported in HL-60 and other leukemic cells induced to undergo apoptosis, as reported previously (44). The observation that Bcl-2 was cleaved by activated caspase-3 was demonstrated using caspase-3 inhibitor. Cleavage of Bcl-2 may represent a means to effectively destroy and remove the antiapoptotic effect of Bcl-2. With Z-LLL-CHO treatment, the amount of Bcl-2 did not seem to decrease, although Bcl-2 was being cleaved, which indicated that Bcl-2 might be a proteasome substrate. However, the 22-kDa Bcl-2 fragment appears to be stable inside cells and becomes even more hydrophobic because of the loss of its hydrophilic $NH_2$ terminus, suggesting that it may be functional. Relevant to this study, a recent work showed that the cleaved Bcl-2 fragment adapted a Bax-like activity (45). Furthermore, Bax has been implicated in the promotion of cytochrome *c* release by forming membrane pores on the mitochondria (46). The widespread occurrences of Bcl-2 cleavage seem to suggest that the cleaved fragment may have a feedback role in further promoting the release of cytochrome and apoptosis in THP-1 cells. In support of this view, we showed in this study that the acquisition of resistance to apoptosis in differentiated THP-1 cells was correlated with inhibition of Bcl-2 cleavage and cytochrome *c* release.

In a previous study, Lopes *et al.* (18) described a wild-type p53-dependent induction of apoptosis by proteasome inhibitors. In our hands, Z-LLL-CHO-induced apoptosis in THP-1 cells appeared to involve a p53-independent mechanism because THP-1 cells possess mutated inactive p53 (47). Furthermore, cell cycle analysis of Z-LLL-CHO-treated THP-1 cells did not show changes in the percentage of cells in various phases (data not shown), and THP-1 cells arrested in $G_1$ and $G_2$-M by serum starvation and colchicine were still responsive to Z-LLL-CHO-induced apoptosis (Fig. 9). Therefore, we believe that the action of proteasome inhibitors is not dependent on $G_1$ and $G_2$ cell cycle arrest, which is under the control of p53 (48). The relation of proteasome inhibitor-induced apoptosis to cell cycle progression status remains to be clarified. The notion that differentiation, not growth arrest, in bryo1-treated THP-1 and blood monocytes is the determinant of their refractoriness to apoptosis was also corroborated by a recent study by Drexler (15), who showed that differentiated HL-60 cells had reduced sensitivity toward proteasome inhibition-induced cell death.

Lactacystin, an irreversible proteasome inhibitor isolated from a *Streptomyces* metabolite, has been used to induce apoptosis in B-



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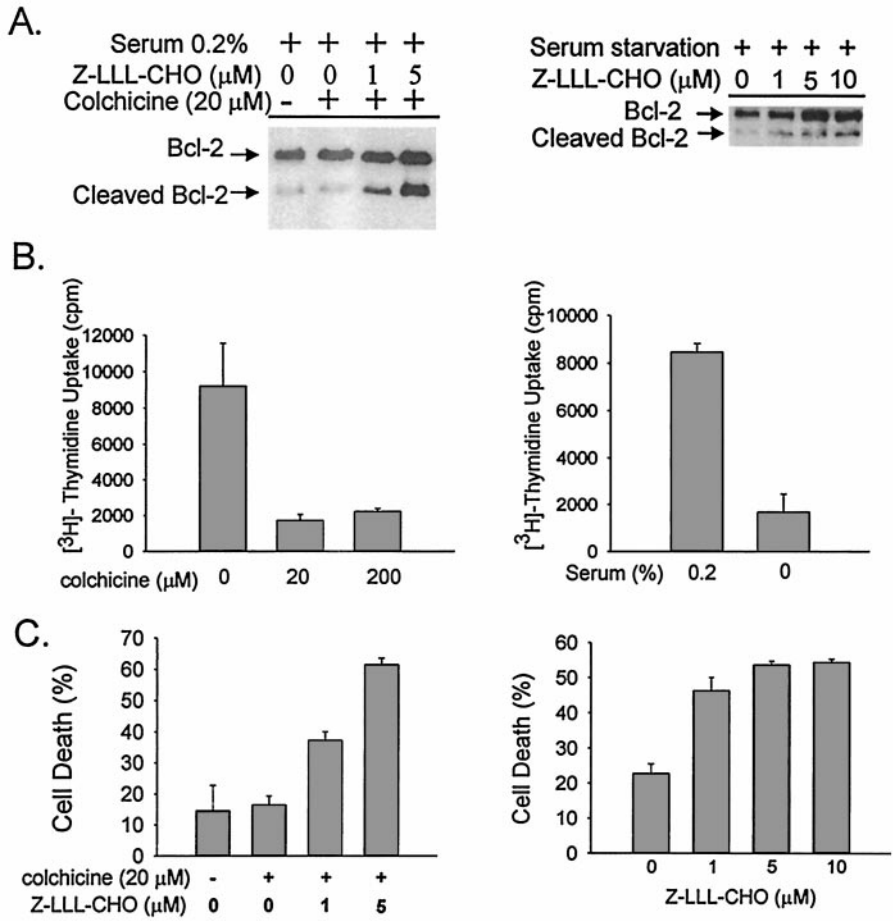


Fig. 9. Cell cycle inhibition response to Z-LLL-CHO. *A*, after incubation in 0.2% FCS for 48 h, THP-1 cells were arrested at $G_2$-M phase by treating with 20 $\mu$M colchicine for 48 h. Thereafter, cells were treated with Z-LLL-CHO (0, 1, and 5 $\mu$M) for an additional 24 h. After incubation in serum-free medium for 48 h, THP-1 cells were treated with Z-LLL-CHO (0, 1, 5, and 10 $\mu$M) for an additional 12 h. Immunoblots were performed using anti-Bcl-2 antibodies. *B*, [$^3$H]thymidine uptake for the colchicine-treated and serum-starved cells were done in triplicate wells, and the radioactivity was counted using a Beckman scintillation counter. *C*, the percentages of cell death were counted for the colchicine-treated and serum-starved cells. Data are means from three separate experiments; *bars*, SD.

CLL-3 cells obtained from leukemia patients (49). Therefore, modulation of the function of proteasomes may be therapeutically advantageous in the treatment of cancers. The role of proteasomes in normal and tumor cells could provide a rational basis for the use of proteasome-targeting drugs. Our finding that proteasome inhibitors specifically target leukemic cells but not differentiated normal cells is significant because a major complication associated with chemotherapy is marrow cytotoxicity. The selective killing of nondifferentiating leukemic cells may provide a means for the purging of leukemic cells from the peripheral bloodstream in patients undergoing autologous bone marrow transplantation. Given that the differentiated cells are highly resistant to proteasome inhibitors, our data also suggest possible adverse effects of using these agents, and perhaps other inducers of apoptosis, in combination with differentiation therapy for leukemia. Clearly, the effects of proteasome inhibitors on the induction of apoptosis in leukemic cells deserve further study.

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