Research Article

Involvement of MAPK, Bcl-2 family, cytochrome *c*, and caspases in induction of apoptosis by 1,6-*O,O*-diacetylbritannilactone in human leukemia cells

Min-Hsiung Pan[1], Yi-Siou Chiou[1], An-Chin Cheng[2], Naisheng Bai[3], Chih-Yu Lo[3], Di Tan[3] and Chi-Tang Ho[3]

[1] Department of Seafood Science, National Kaohsiung Marine University, Kaohsiung, Taiwan
[2] Department of Nutrition and Health Science, Toko University, Chia-Yi County, Taiwan
[3] Department of Food Science, Cook College, Rutgers University, New Brunswick, NJ, USA

1,6-*O,O*-diacetylbritannilactone (OODBL) isolated from *Inula britannica*, exhibits potent antitumor activity against several human cancer cell lines. However, the molecular mechanism of OODBL in the induction of anticancer activity is still unclear. In the present study, we demonstrated that OODBL induced the occurrence of apoptosis in human leukemic (HL-60) cells and cell arrest at the S phase. On the other hand, activation of caspase-8, -9, and -3, phosphorylation of Bcl-2 and Bid, and increased release of cytochrome *c* from mitochondria into cytosolic fraction were detected in OODBL-treated HL-60 cells. We further demonstrated that production of reactive oxygen species (ROS), activation of mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) signaling pathways may play an important role in OODBL-induced apoptosis. The results from the present study highlight the molecular mechanisms underlying OODBL-induced anticancer activity.

Keywords: Apoptosis / Bax / Bcl-2 / Bid / Caspase-3

Received: April 25, 2006; revised: June 16, 2006; accepted: June 18, 2006

1 Introduction

During the past decade, a large number of natural products and dietary components have been evaluated as potential chemopreventive agents [1]. Dietary factors play important roles in human health and the development of certain chronic diseases, including cancer [2, 3].

Inula, from Compositae, exists as more than 100 species, and it is found mainly in Europe, Africa, and Asia. *Inula britannica* is a wild plant found in Eastern Asia, namely China, Korea, and Japan. Britannilactone (BL), 1-*O*-acetylbritannilactone (OABL), and 1,6-*O,O*-diacetylbritannilactone (OODBL) (Fig. 1), isolated from *I. britannica*, exhibit potent antitumor activities against several human cancer cell lines [4, 5]. The antitumor properties of OODBL has been studied with respect to apoptosis and cell cycle arrest [4, 6]. The molecular mechanisms of apoptosis by OODBL remain largely unclear, but appear to involve modulation of multiple apoptotic regulatory proteins.

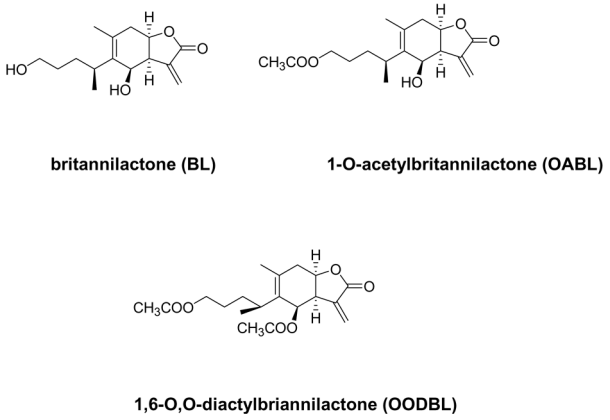

Figure 1. The chemical structures of BL, OABL, and OODBL.

Many recent studies have indicated that anticancer drugs or cancer chemopreventive agents act through the induction of apoptosis to prevent tumor promotion, progression, and

Correspondence: Dr. Min-Hsiung Pan, Department of Seafood Science, National Kaohsiung Marine University, No. 142, Hai-Chuan Rd, Nan-Tzu, Kaohsiung 811, Taiwan
E-mail: mhpan@mail.nkmu.edu.tw
Fax: +886-7-361-1261

Abbreviations: BL, britannilactone; **DCFH-DA,** dichlorodihydrofluorescein diacetate; **DFF,** DNA fragmentation factor; **JNK,** c-Jun N-terminal kinase; **MAPK,** mitogen-activated protein kinase; **OODBL,** 1,6-*O,O*-diacetylbritannilactone; **PARP,** poly(ADP-ribose)polymerase; **ROS,** reactive oxygen species; **SEM,** scanning electron microscopy

the occurrence of cellular inflammatory responses other than necrosis [7, 8]. Apoptosis is also a gene-directed form of cell death with well-characterized morphological and biochemical features [9]. Initiation of apoptosis appears to be a common mechanism of many cytotoxic agents used in chemotherapy. The Bcl-2 family of proteins is predominantly situated upstream of irreversible cellular damage in the intrinsic apoptotic pathway, including antiapoptotic proteins such as Bcl-2 and Bcl-X_L and proapoptotic proteins such as Bad, Bid, Bim, Bax, and Bak [10]. A recent study suggests that mitogen-activated protein kinases (MAPKs) include stress-activated protein kinases such as c-Jun NH2-terminal kinases (JNK) and p38 play important roles in triggering apoptosis in response to various cellular stressors including oxidative stress. Bcl-2 has been reported to be phosphorylated by JNK in response to different stimuli [11]. The phosphorylation of Bcl-2 has been described as an important step from microtubule damage to apoptosis [12]. Furthermore, Bid is believed to be relatively inactive in the cytosol until proteolytically cleaved by caspase-8. However, the apoptotic pathways in which Bid plays a role are not yet fully characterized. Recent studies suggest that Bid is phosphorylated by DNA-damage kinase ATM (Ataxia-telangiectasia mutated) and may play an important role for S phase arrest [13, 14].

In the current study, we examined the antiproliferative effects of OODBL and structurally related compounds on human leukemia cells. Our results demonstrate that OODBL can induce apoptosis in a dose-dependent manner in HL-60 cells. We further evaluated the molecular mechanisms of apoptotic effects induced by OODBL. It is suggested that OODBL modulates the production of reactive oxygen species (ROS), the phosphorylation of Bcl-2 and Bid through the activation of JNK/p38, the release of cytochrome *c*, and the activation of caspases in OODBL-induced apoptosis.

2 Materials and methods

2.1 Cell culture and chemicals

Human promyelocytic leukemia (HL-60) cells obtained from American Type Culture Collection (Rockville, MD, USA) were grown in 90% RPMI 1640 and 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA), supplemented with 2 mM glutamine (Gibco BRL), 1% penicillin/streptomycin (10 000 U of penicillin/mL and 10 mg streptomycin/mL). Medium was normally changed to phenol red-free RPMI 1640 before polyphenol treatment. Propidium iodide was obtained from Sigma Chemical (St. Louis, MO, USA).

2.2 Extraction and isolation

The air-dried, powered flower material was percolated with 95% EtOH at room temperature. A combination of column chromatography on silica gel, Sephadex LH-20, and preparative HPLC of the chloroform-soluble portion of the ethanol extract from the flowers of *I. britannica* var. *chinensis* gave three compounds, BL, OABL, and 1,6-*O,O*-diacetylbriannilactone (OODBL) [4].

2.3 Determination of cell viability

Cell viability was determined at indicated compounds based on the trypan blue exclusion method. Briefly, HL-60 cells were plated at a density of 1×10^5 cells/mL into 24-well plates. After overnight growth, cells were pretreated with a series of concentrations of OODBL for 24 h. The final concentrations of DMSO in the culture medium were <0.05%. The viability percentage was calculated based on the percentage of unstained cells as described previously [15].

2.4 Acridine orange staining assay

Cells (5×10^5) were seeded into 60-mm Petri dishes and incubated at 37°C for 24 h. The cells were harvested after treatment for 24 h, and 5 μL of cell suspension was mixed on a slide with an equal volume of acridine orange solution (10 μg/mL in PBS). Green fluorescence was detected between 500 and 525 nm by using an Olympus microscope (Olympus America, Lake Success, NY, USA). Bright-staining condensed chromatin was detected in apoptotic cells.

2.5 Scanning electron microscopy analysis

Scanning electron microscopy (SEM) was used to examine the surface topography of the OODBL-treated HL-60 cells (Hitachi S-3500N). Cells were seeded at a density of 2×10^5 cells/mL onto six-wells tissue culture plates. After overnight growth, cells were treated with 50 μM OODBL, and the final concentrations of DMSO in the culture medium were <0.1%. Following 24 h of incubation with 50 μM OODBL, the cell morphology was assessed by SEM. Briefly, cells were rinsed three times with PBS and then fixed with 3% paraformaldehyde for 10 min. After a final rinse with PBS, a contrast treatment in 1% osmium tetroxide (Alfa, Ward Hill, MA, USA) for 1 h was performed, followed by extensive rinsing in PBS and dehydration through a graded series of ethanol from 30, 50, 70, 90, and 100%. After air-drying, surfaces were thinly sputter coated with platinum (Ion Sputter, E-1010, Hitachi; with 15 mA for ~5 min). Cells on polished titanium disks and on glass cover slips were used as controls.

2.6 Flow cytometry

HL-60 cells (2×10^5) were cultured in 60-mm Petri dishes and incubated for a further 24 h. The cells treated with various concentrations (5, 10, 25, 50, and 100 μM) of com-

pounds for 24 h or 50 μM OODBL for the indicated time (3, 6, 9, 12, 18, and 24 h). The cells were then harvested, washed with PBS, resuspended in 200 μL of PBS, and fixed in 800 μL of iced 100% ethanol at −20°C. After being left to stand overnight, the cell pellets were collected by centrifugation, resuspended in 1 mL of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 μg/mL RNase), and incubated at 37°C for 30 min. Next, 1 mL of propidium iodide solution (50 μg/mL) was added, and the mixture was allowed to stand on ice for 30 min. Fluorescence emitted from the propidium iodide-DNA complex was quantitated after excitation of the fluorescent dye by FACScan cytometry (Becton Dickinson, San Jose, CA, USA).

2.7 ROS production determination

ROS production was monitored by flow cytometry using dichlorodihydrofluorescein diacetate (DCFH-DA). This dye is readily diffused into cells and is hydrolyzed by intracellular esterase to yield DCFH, which is trapped within the cells. Hydrogen peroxide or low M_r peroxides produced by the cells oxidize DCFH to the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF). Thus, the fluorescence intensity is proportional to the amount of peroxide produced by the cells. Cells were treated with OODBL (50 μM) for different time periods, and DCFH-DA (30 μM) was added into the medium for a further 30 min at 37°C.

2.8 Analysis of mitochondrial transmembrane potential

The change of the mitochondrial transmembrane potential was monitored by flow cytometry. Briefly, HL-60 cells were exposed to OODBL (50 μM) for different time periods, and the mitochondrial transmembrane potential was measured directly using 40 nM 3,3′-dihexyloxacarbocyanine [DiOC6(3)] (Molecular Probes, Eugene, OR, USA). Fluorescence was measured after staining of the cells for 30 min at 37°C. Histograms were analyzed using Cell Quest software and were compared with histograms of control untreated cells.

2.9 Western blotting

The nuclear and cytosolic proteins were isolated from HL-60 cells after treatment with 50 μM OODBL for 0, 3, 6, 9, 12, 18, and 24 h. The total proteins were extracted *via* the addition of 200 μL of gold lysis buffer (50 mM Tris-HCl, pH 7.4; 1 mM NaF; 150 mM NaCl; 1 mM EGTA; 1 mM phenylmethanesulfonyl fluoride; 1% NP-40; and 10 μg/mL leupeptin) to the cell pellets on ice for 30 min, followed by centrifugation at 10 000 × *g* for 30 min at 4°C. The cytosolic fraction (supernatant) proteins were measured by BioRad protein assay (BioRad Laboratories, Munich, Germany). The samples (50 μg of protein) were mixed with 5 × sample buffer containing 0.3 M Tris-HCl (pH 6.8), 25% 2-mercaptoethanol, 12% SDS, 25 mM EDTA, 20% glycerol, and 0.1% Bromophenol blue. The mixtures were boiled at 100°C for 5 min and were subjected to 12% SDS-polyacrylamide minigels at a constant current of 20 mA. Subsequently, electrophoresis was ordinarily carried out on SDS-polyacrylamide gels. After electrophoresis, proteins on the gel were electrotransferred onto an immobile membrane (PVDF; Millipore, Bedford, MA, USA) with transfer buffer composed of 25 mM Tris-HCl (pH 8.9), 192 mM glycine, and 20% methanol. The membranes were blocked with blocking solution containing 20 mM Tris-HCl and then immunoblotted with primary antibodies including anti-Bcl-2, anti-Bcl-X_L, anti-Bad, anti-Bax, anti-β-actin (Santa Cruz Biotech.), antipoly(ADP-ribose)polymerase (PARP; UBI, Lake Placid, NY, USA), anti-Bid, anticaspase-8, anticaspase-3, anticaspase-9, anti-Fas, and Fas-L (Transduction Laboratory, Lexington, KY), and anti-DNA fragmentation factor (DFF)-45/inhibitor of caspase-activated DNase (ICAD) antibody (MBL, Naka-Ku, Nagoya, Japan) at room temperature for 1 h. The antiphospho-JNK, antiphospho-p38 (Thr180/Tyr182), JNK, and p38 obtained from Cell Signaling Technology (Beverly, MA, USA) were used to determine the level of phosphorylated proteins. Detection was achieved by measuring the chemiluminescence of blotting agent (ECL, Amersham, Arlington Heights, IL, USA), after exposure of the filters to Kodak X-Omat films. The cytochrome *c* protein was detected by using anticytochrome *c* antibody (Research Diagnostic, Flanders, NJ, USA).

2.10 Activity of caspase

HL-60 cells (2×10^5) were cultured in 100-mm Petri dishes. After 24 h of incubation, cells were treated with OODBL (50 μM) for various times. Cells were collected and washed with PBS and suspended in 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothione, 2 mM phenylmethanesulfonyl fluoride, 10 μg/mL pepstatin A, and 10 μg/mL leupeptin after treatment. Cell lysates were clarified by centrifugation at 12 000 × *g* for 20 min at 4°C. Caspase activity in the supernatant was determined by a fluorogenic assay (Promeaga's CaspACE Assay System, Madison, WI, USA). Briefly, 50 μg of total protein, as determined by BioRad protein assay (BioRad Laboratories), was incubated with 50 μM substrate Ac-Try-Val-Ala-Asp-AMC (Ac-YVAD) (caspase-1-specific substrate), Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC) (caspase-3-specific substrate), Ac-Ile-Glu-Thr-Asp-AMC (Ac-IETD-AMC) (caspase-8-specific substrate), or Ac-Leu-Glu-His-Asp-AMC (Ac-LEHD-AMC) (caspase-9-specific substrate) at 30°C for 1 h. The release of methylcoumaryl-7-amine (AMC) was measured by excitation at 360 nm and emission at 460 nm using a fluorescence spectrophotometer (Hitachi, F2000).

3 Results

3.1 Treatment with BL, OABL, and OODBL cause dose-dependent reductions in cell survival

Previous studies have shown that sesquiterpene lactones from *I. britannica* are potent antiproliferation and anticancer agents [4]. Here we investigated the cytotoxicity of BL, OABL, and OODBL. The structure of compounds is illustrated in Fig. 1. We first tested the effect of BL, OABL, and OODBL on cell viability. Human leukemia HL-60 cells were treated with different concentrations of BL, OABL, and OODBL. After 24 h of treatment, the number of live cells was determined by means of trypan blue exclusion method. As shown in Fig. 2, OODBL is a more potent inhibitor of cell viability than BL and OABL. The inhibition of cell viability of OODBL was found to be dose-dependent with an IC_{50} of 18 μM.

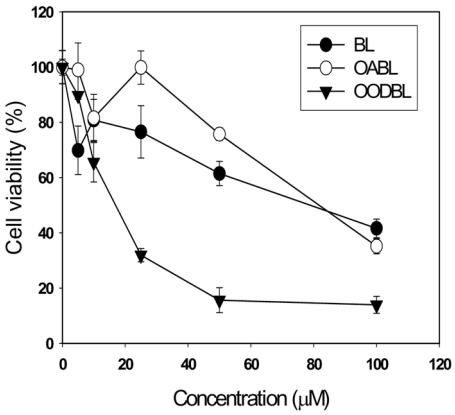

Figure 2. Effect of BL, OABL, and OODBL on the cell survival of HL-60 cells. HL-60 cells were treated with different concentrations of BL, OABL, and OODBL for 24 h. HL-60 cells were either treated with 0.1% DMSO as vehicle control. Cell viability was then determined by trypan blue assay as described in Section 2. Data were represented as means ± SE for three determinations.

3.2 OODBL-induced apoptosis in human leukemia cells

To characterize the cell death induced by OODBL, we examined the nuclear morphology of dying cells with a fluorescent DNA-binding agent, acridine orange, and SEM. As shown in Fig. 3, within 24 h of treatment with 50 μM OODBL, cells clearly exhibited significant chromosomal condensation (Fig. 3A) and morphological changes (Fig. 3B), which is indicative of apoptotic cell death. Such results imply that the cytotoxic action of OODBL was due to its ability to induce apoptosis. As described previously [16], the apoptotic cell is characterized by specific changes in the presence of a subdiploid peak. To investigate the induction of a sub-G1 cell population, the DNA content of HL-60

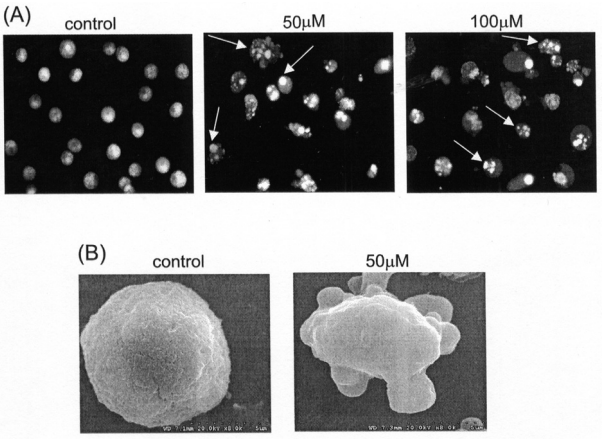

Figure 3. Chromatin condensation and morphological changes induced by OODBL in HL-60 cells. (A) HL-60 cells were treated with 0.05% DMSO as vehicle control or with 50 and 100 μM OODBL for 24 h, and cells were harvested and washed with PBS followed by staining with acridine orange. The nuclear staining was examined by fluorescence microscopy. Apoptotic cells are shown as white arrowheads. (B) HL-60 cells were treated with 50 DBL for 24 h, the morphological change was determined by SEM (8000 ×). The data presented are representative of three independent experiments.

cells treated with BL, OABL, and OODBL for various concentrations was analyzed by flow cytometry (Fig. 4). As seen in Fig. 4A, the data indicated that BL had less ability of apoptosis in HL-60 cells. OABL obviously induced apoptosis after incubation with 50 and 100 μM, the percentages of apoptotic cells were 20.13 and 40.22% (Fig. 4B). Among them, OODBL was the most potent inducer of apoptosis in HL-60 cells. The percentages of apoptotic cells were 20.04, 20.51, 49.86, 58.76, and 64.23% after incubation with 5, 10, 25, 50, and 100 μM OODBL, respectively (Fig. 4C). As shown in Fig. 4D, OODBL-induced apoptosis in HL-60 cells in a time dependent manner.

3.3 Activation of caspases-9, -3, and -8 but not caspase-1, are involved in OODBL-induced apoptosis

We then asked whether caspases were involved in the cell death response induced by OODBL. Caspases are activated in a sequential cascade of cleavages from their inactive forms [17]. As shown in Fig. 5A, caspases-8, -9, and -3 were time-dependently activated by OODBL. Once activated, caspases can subsequently cleave their substrates at specific sites. For example, caspase-3 cleaves preferentially after a DXXD↓X, whereas caspase-1 cleaves at YXXD↓X. To monitor the enzyme activity of caspases during OODBL-induced apoptosis, we used four fluorogenic peptide substrates. Ac-IETD-AMC, Ac-LEHD-AMC, Ac-DEVD-AMC, and Ac-YVAD-AMC are specific substrate for caspases-8, -9, -3, and -1 activity, respectively. As illu-

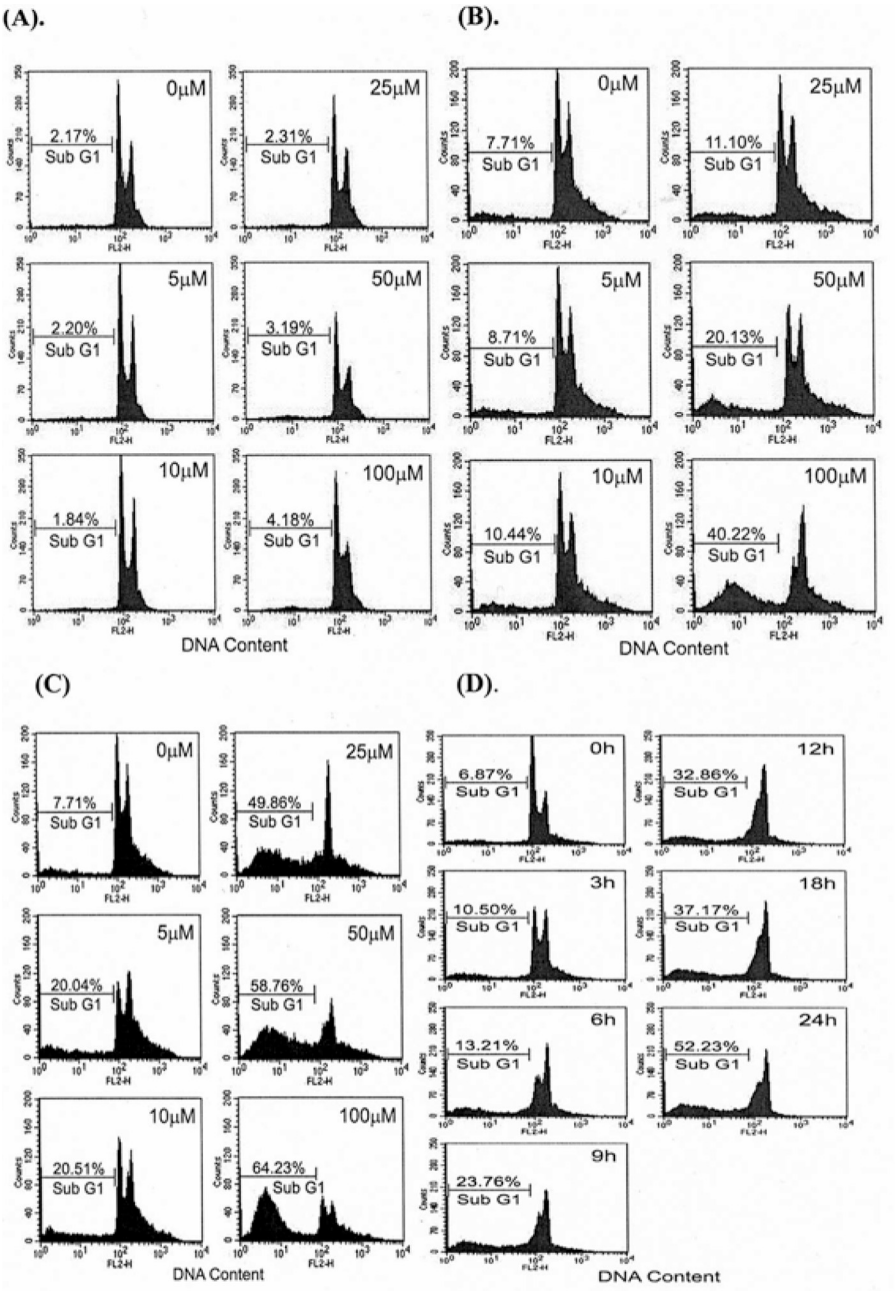

Figure 4. Induction of apoptosis in HL-60 cells by different concentrations of BL, OABL, and OODBL. HL-60 cells were treated with 0, 5, 10, 25, 50, and 100 μM of BL (A), OABL (B), and OODBL (C), respectively, and treated with 50 μM OODBL for indicated time and Sub-G1 cells in compounds-treated HL-60 cells were determined by flow cytometry. The method of flow cytometry used is described in Section 2. Sub-G1 represents apoptotic cells with a lower DNA content. The data presented are representative of three independent experiments.

strated in Fig. 5B, OODBL (50 μM) induced a dramatic increase in caspases-3, -8, and -9 activity in treated HL-60 cells. In contrast to the increase in caspase-3 activity, negligible caspase-1 activity was observed. Activation of caspase-3 leads to the cleavage of a number of proteins, one of which is PARP. The cleavage of PARP is the hallmark of apoptosis. PARP (116-kDa) is cleaved to produce an 85-kDa fragmentation during apoptosis [18]. As was already described, ICAD is a mouse homolog of human DFF-45. Caspase-3 cleaves DFF-45, and once caspase-activated deoxyribonuclease (CAD) is released, it can enter the nucleus where it degrades chromosomal DNA to produce interchromosomal DNA fragmentation [19, 20]. Figure 5C shows that exposure of HL-60 cells to OODBL causes

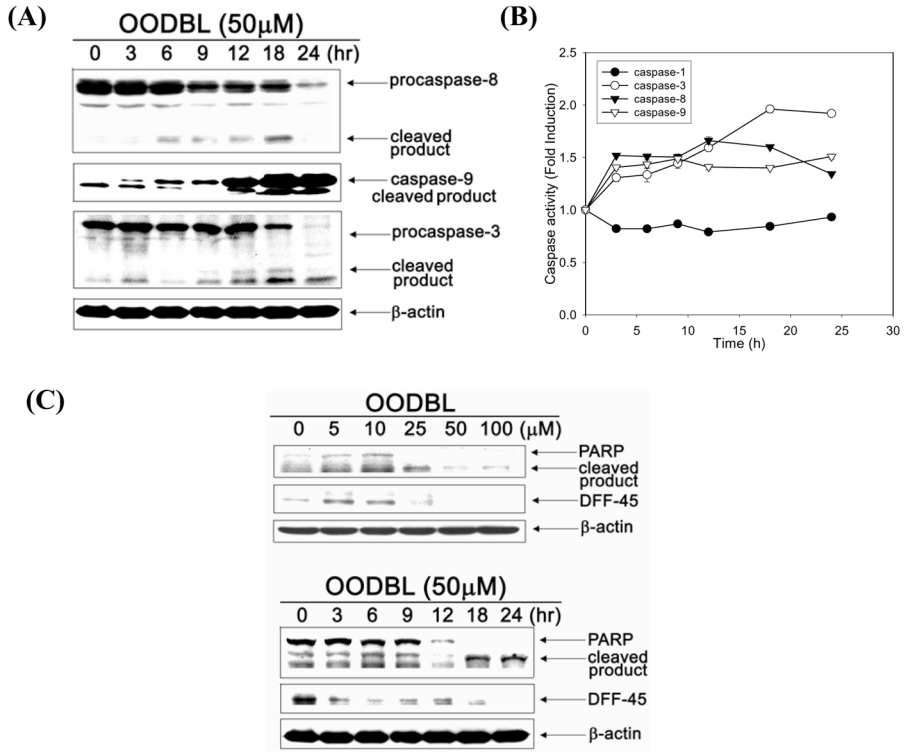

Figure 5. Intracellular response of caspases activation in OODBL-treated HL-60 cells. (A) HL-60 cells were treated with OODBL (50 μM) for various times. Proteins (50 μg/lane) were separated by SDS-PAGE, immunoblotted with anticaspases-8, -9, -3 antibody. (B) Kinetics of caspase activation. To determine the kinetics of caspases-9, -8, -3, and -1 activation, cells were treated with 50 μM OODBL for different time periods or treated with 0.05% DMSO as vehicle control. Cells were harvested and lysed in lysis buffer. Enzymatic activity of caspases-9, -8, -3, and -1 proteases was determined by incubation of 50 μg of total protein with fluorogenic substrates, Ac-LEHD-AMC; Ac-IETD-AMC; Ac-DEVD-AMC; Ac-YVAD-AMC, respectively, for 1 h for 30°C. The release of AMC was monitored (excitation = 360 nm; emission = 460 nm). Data represent means ± SE for three determinations. (C) Cleavage of PARP and DFF-45 during OODBL-induced apoptosis. Cells were treated as indicated and examined by western blotting as described in Section 2. This experiment was repeated three times with similar results.

degradation of 116-kDa PARP to 85-kDa fragments and the cleavage of DFF-45.

3.4 Involvement of ROS production, mitochondrial dysfunction, and release of cytochrome *c* from mitochondria to cytosol in OODBL-induced apoptosis

Growing evidence has indicated that ROS played an important role in the induction of apoptosis. We studied the generation of ROS using the fluorescent probe DCFH-DA and monitoring by flow cytometry. HL-60 cells were treated with 50 μM OODBL for 0.5 h. As shown in Fig. 6A, the fluorescence intensity shifted to the right from 103.54 to 205.15 in OODBL-induced apoptotic HL-60 cells. These data indicated that the increment of ROS might play a role as an early mediator in OODBL-induced apoptosis. We next evaluated the effects of OODBL on the mitochondrial transmembrane potential ($\Delta\Psi_m$) and the release of mitochondrial cytochrome *c* into cytosol. We measured $\Delta\Psi_m$ using the fluorescent probe DiOC6(3) fluorescence and monitored it *via* flow cytometry. As shown in Fig. 6A, which compares HL-60 cells exposed to OODBL and control cells, the DiOC6(3) fluorescence intensity shifted to the left from 106.9 to 72.77 in OODBL-induced apoptotic HL-60 cells at 2 h. These results demonstrate that OODBL caused a decrease in mitochondrial transmembrane potential in HL-60 cells. As shown in Fig. 6B, the release of mitochondrial cytochrome *c* into the cytosol was detected at 3 h in OODBL-treated HL-60 cells.

3.5 Effect of OODBL on the expression of Bcl-2 family and S phase-arrest in HL-60 cells

The Bcl-2 family of proteins is predominantly situated upstream of the irreversible cellular damage in the intrinsic apoptosis pathway [21]. Previous study has demonstrated that OODBL induces phosphorylation of Bcl-2 [6]. As shown in Fig. 7A (upper panel), the phosphorylation of Bcl-2 was detected at 3 h and increased when the time increased

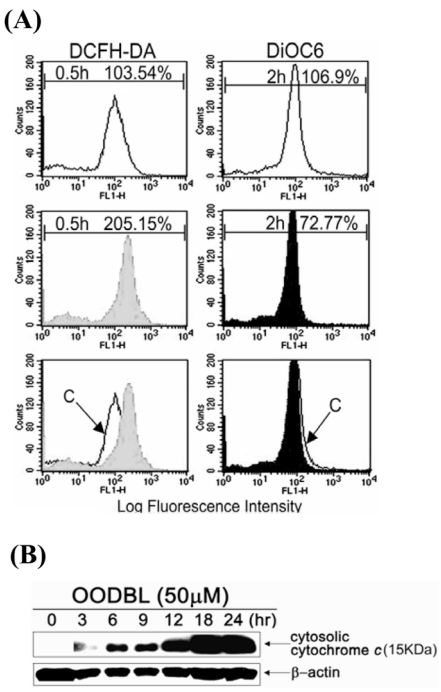

Figure 6. Induction of ROS generation, mitochondrial dysfunction, and cytochrome *c* release in OODBL-induced apoptosis. (A) HL-60 cells were treated with 50 μM OODBL for indicated time and were then incubation with 20 μM DCFH-DA and 40 nM 3,3′-dihexyloxacarbocyanine (DiOC6), respectively, and analyzed by flow cytometry. Data are presented as log fluorescence intensity. C, control. (B) HL-60 cells were treated with 50 μM OODBL at indicated periods. Subcellular fractions were prepared as described in Section 2, and cytosolic cytochrome *c* was detected by cytochrome *c* antibody.

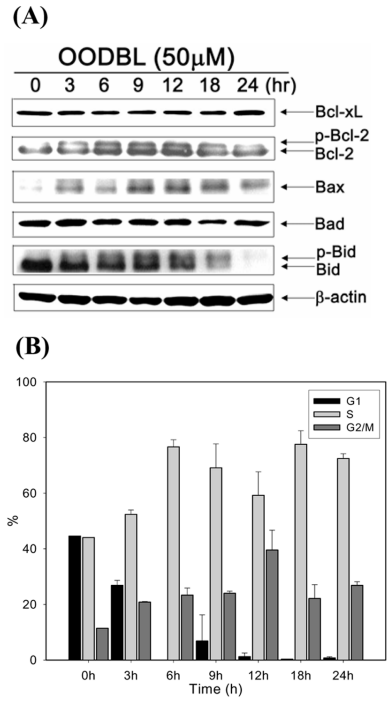

Figure 7. Effect of OODBL on Bcl-2 protein family and S phase arrest in OODBL-treated HL-60 cells. HL-60 cells were treated with 50 μM OODBL for the indicated time point. (A) Eexpression of Bcl-xL, Bcl-2, Bax, Bad, and Bid was detected by Western blotting analysis using specific antibodies and then detected using the ECL system. Each blot is representative results of three similar experiments. (B) The population of cells at the S phase arrest was measured by flow cytometry at the indicated time points after exposure to OODBL. In the case, plots are as means ± SE.

in OODBL-treated cells, but no change was observed for Bcl-X_L protein. We examined the expression of proapoptotic protein, Bax, which inserts into the outer member of mitochondria and forms a large channel, allowing the release of cytochrome *c*; this process can be prevented by Bcl-2 or Bcl-X_L [22]. Figure 7A (middle panel) shows a marked time-dependent increase of Bax protein. On the other hand, a negligible change of Bad protein was detected in OODBL-treated HL-60 cells. Furthermore, the marked phosphorylation of Bid was also observed in OODBL-treated cells (Fig. 7A, lower panel). Phosphorylation seems to regulate Bid activity, since it was recently demonstrated that its phosphorylation by casein kinase 1 and/or 2 inhibits its cleavage by caspase-8 [23]. Phosphorylation may also play a prosurvival role important for S phase arrest [13]. To examine this possibility, cells were treated with OODBL (50 μM) for 3–24 h and assessed for effects on cell cycle by flow cytometry. Interestingly, we first found that OODBL-induced accumulation of HL-60 cells in S and G2 phases (Fig. 7B).

3.6 Effects of OODBL on activation of p38 (MAPK) and JNK in HL-60 cells

To assess whether OODBL promoted apoptosis *via* receptor-mediated pathway, the Fas and Fas ligand (FasL) protein levels were determined by western blotting. The result showed that OODBL could not stimulate the expression of Fas and FasL after treatment with OODBL (Fig. 8A). Other studies suggest that apoptosis signal-regulating kinase 1 (ASK1) mediated the sustained activation of JNK/p38 and apoptosis [24, 25]. To identify the kinase responsible in response to OODBL, we examined the activation state of two classes of MAPK, namely p38 and JNK. Using immunoblot analysis with antiphospho-specific antibody, we found activation of p38 at 2.5 h (Fig. 8B) and activation of JNK at 2 h (Fig. 8C) in OODBL-treated cell, suggesting that p38 and JNK might be involved in OODBL-induced phosphorylation of Bcl-2 and apoptosis.

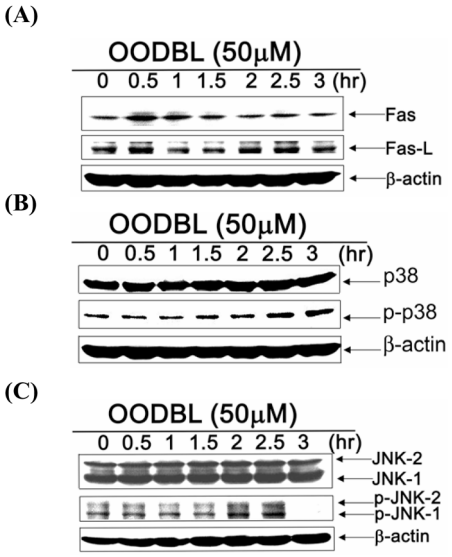

Figure 8. Effect of the expression of Fas, Fas-L, p38, p-p38, JNK1/2, and p-JNK1/2 protein in OODBL-treated HL-60 cells. HL-60 cells were treated with 50 μM OODBL for 0, 0.5, 1, 1.5, 2, 2.5, and 3 h, and the expression of Fas and Fas-L (A), p38 and p-p38 (B), JNK1/2 and p-JNK1/2 (C) protein was analyzed by immunoblotted with anti-Fas, Fas-L, antiphospho-p38, and phosphor-JNK1/2 antibodies, and then detected using the ECL system. The levels of β-actin, total-p38, and total-JNK1/2 protein were used as controls for equal loading of protein in different lanes.

4 Discussion

Our previous studies have demonstrated that the sequiterpenelactone, OODBL, isolated from *I. Britannica* was cytotoxic, induced the phosphorylation of Bcl-2, G2/M cell cycle arrest, and apoptosis in human cancer cells [4, 6]. The present study demonstrated that OODBL, acetyl group in positions 1 and 6, is more cytotoxic in HL-60 cells than BL and OABL (Fig. 1). The result suggests that acylation of the hydroxyl group is important to its bioactivity. OODBL is one α,β unsaturated and has two acetyl moiety sesquiterpene lactones present. Previous studies reported that the bioactivity of the structure element was caused by the reaction of α-methylene-γ-lactone with nucleophiles by a Michael addition reaction [26, 27]. The difference in bioactivity between OODBL and OABL is the number of acetyl groups. The reason OODBL displays potent cytotoxicity in human HL-60 cells might be due to lipophilic properties of OODBL and how it easily penetrates the cell membrane into the cytosol. Carcinogens usually cause genomic damage in exposed cells. As a consequence, the damaged cells may be triggered either to undergo apoptosis or to proliferate with genomic damage, leading to the formation of cancerous cells that usually exhibit cell cycle abnormalities which are more susceptible to various apoptosis-inducing agents [28, 29]. Therefore, identifying active compounds from herbs with apoptosis-inducing activity against cell lines is considered to be a primary mechanism for the chemoprevention of cancer.

OODBL has been reported to exhibit many biological effects including anticancer activity [4–6], but their anticancer mechanism is still elusive. In this study, we clarified the molecular mechanism by which OODBL triggered human leukemia HL-60 cells undergoing apoptosis. As shown in Fig. 2, OODBL was the potent inhibitor of cell viability and caused the potent and rapid induction of apoptosis, concurrent with sub-G1 peak appearance, chromatin condensation, and apoptotic appearance in HL-60 cells. This induction of apoptosis occurred within hours, consistent with the view that OODBL-induced apoptosis by activating preexisting apoptosis machinery. Indeed, treatment with OODBL caused an induction of caspases-3, -8, and -9 but not caspase-1, associated with the degradation of DFF-45 and PARP, which preceded the onset of apoptosis. Recent studies have shown that caspase-3 can activate caspase-8 [30, 31]. Thus, we propose that caspase-8 is involved in cytochrome *c*-mediated apoptosis and participates in a feedback amplification loop involving caspase-3 in OODBL-treated HL-60 cells. Mitochondrial transmembrane potential ($\Delta\Psi_m$) is often employed as an indicator of cellular viability, and its disruption has been implicated in a variety of apoptosis phenomena [32]. Mitochondria have also been implicated as a source of ROS during apoptosis. Reduced mitochondria membrane potential has recently been shown to lead to increased generation of ROS and apoptosis [33]. Herein, we demonstrated that OODBL could induce ROS generation and disrupt the functions of mitochondria at the early stages of apoptosis while subsequently coordinating caspase-9 activation, but not caspase-1, through the release of cytochrome *c* (Fig. 6). The increase in ROS was probably due to OODBL which penetrates the cell membrane into the cytosol and affects mitochondria cycling dioxygen through the electron transport assembly, and generating ROS by one-electron-transfer mitochondria could be a main target of a nonspecific damage through oxidative stress at the level of the outer and inner membranes. The Bcl-2 family of proteins, whose members may be antiapoptotic or proapoptotic, regulates cell death by controlling the mitochondria membrane permeability during apoptosis [34, 35]. We, therefore, inferred that Bcl-2 family proteins might participate in the event that controlled the change in mitochondrial membrane potential and trigger cytochrome *c* release during apoptosis induced by OODBL. Previous reports have demonstrated that Paclitaxel, an anticancer drug, phosphorylates Bcl-2 and inactivates its antiapoptotic effects, thereby inducing apoptosis [36]. Moreover, when DNA-damaged Bid was phosphorylated by ATM kinase, it in turn leads to cell cycle arrest at the S phase [13]. In our present study, Bcl-2 and Bid phosphorylation were observed at 3 h after OODBL treatment and caused cell cycle arrest at the S phase in HL-60 cell (Fig. 7).

Recent study indicated Bid phosphorylation is linked to maintenance of the S phase checkpoint. Following death-receptor-induced apoptotic signals, Bid is post-tranlsationally modified by caspase cleavage and myristoylation and translocates to the mitochondrion to activate the downstream apoptotic program. However, Bid has a distinct role downstream of DNA damage. Following treatment with agents inducing DNA damage such as mitomycin c, Bid accumulates in the nucleus and is phosphorylated by the DNA-damage kinase ATM. Bid, with its dual function in both apoptosis and the intra-S phase checkpoint, is well positioned to serve as a mediator of two pathways [14]. Interestingly, our findings differ somewhat in that OODBL induced the phosphorylation of Bcl-2, and induced G2/M arrest in T47D cells [6]. The ability to cause ROS production and penetrate the cell membrane of OODBL, might induce DNA damage. A recent study suggests that stress-activated protein kinases such as JNK1/2 and p38 play important roles in triggering apoptosis in response to various cellular stressors including oxidative stress [37]. Several kinases including JNK, Raf-1, and PKA have been identified to be involved in Bcl-2 phosphorylation [11, 38, 39]. Our finding suggested that JNK1/2 and p38 activation were observed at 2 and 2.5 h, respectively, in OODBL-treated HL-60 cells (Fig. 8). All these results implied that the OODBL-induced apoptosis in HL-60 cells resulted from JNK1/2 activation and phosphorylation of Bcl-2. In the present study, our data suggest that activation of Bax and Bcl-2 phosphorylation might promote the release of cytochrome *c* from mitochondria and cause activation of the caspase cascade.

This study was supported by the National Science Council under the grant numbers NSC 94-2321-B-022-001 and NSC 95-2313-B-022-003-MY3.

5 References

[1] Sharma, S., Stutzman, J. D., Kelloff, G. J., Steele, V. E., Screening of potential chemopreventive agents using biochemical markers of carcinogenesis, *Cancer Res.* 1994, *54*, 5848 – 5855.

[2] Doll, R., The lessons of life: Keynote address to the nutrition and cancer conference, *Cancer Res.* 1992, *52*, 2024s – 2029s.

[3] Rogers, A. E., Zeisel, S. H., Groopman, J., Diet and carcinogenesis, *Carcinogenesis* 1993, *14*, 2205 – 2217.

[4] Bai, N., Lai, C. S., He, K., Zhou, Z. *et al.*, Sesquiterpene lactones from *Inula britannica* and their cytotoxic and apoptotic effects on human cancer cell lines, *J. Nat. Prod.* 2006, *69*, 531 – 535.

[5] Liu, S., Liu, H., Yan, W., Zhang, L. *et al.*, Studies on 1-*O*-acetylbritannilactone and its derivative, (2-*O*-butyloxime-3-phenyl)-propionyl-1-O-acetylbritannilactone ester, *Bioorg. Med. Chem. Lett.* 2004, *14*, 1101 – 1104.

[6] Rafi, M. M., Bai, N. S., Ho, C. T., Rosen, R. T. *et al.*, A sesquiterpenelactone from *Inula britannica* induces anti-tumor effects dependent on Bcl-2 phosphorylation, *Anticancer Res.* 2005, *25*, 313 – 318.

[7] Nicholson, D. W., From bench to clinic with apoptosis-based therapeutic agents, *Nature* 2000, *407*, 810 – 816.

[8] Jacks, T., Weinberg, R. A., Taking the study of cancer cell survival to a new dimension, *Cell* 2002, *111*, 923 – 925.

[9] Boise, L. H., Gonzalez-Garcia, M., Postema, C. E., Ding, L. *et al.*, Bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death, *Cell* 1993, *74*, 597 – 608.

[10] Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M. *et al.*, Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade, *Cell* 1997, *91*, 479 – 489.

[11] Yamamoto, K., Ichijo, H., Korsmeyer, S. J., BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/M, *Mol. Cell Biol.* 1999, *19*, 8469 – 8478.

[12] Blagosklonny, M. V., Giannakakou, P., el-Deiry, W. S., Kingston, D. G. *et al.*, Raf-1/bcl-2 phosphorylation: A step from microtubule damage to cell death, *Cancer Res.* 1997, *57*, 130 – 135.

[13] Kamer, I., Sarig, R., Zaltsman, Y., Niv, H. *et al.*, Proapoptotic BID is an ATM effector in the DNA-damage response, *Cell* 2005, *122*, 593 – 603.

[14] Zinkel, S. S., Hurov, K. E., Ong, C., Abtahi, F. M. *et al.*, A role for proapoptotic BID in the DNA-damage response, *Cell* 2005, *122*, 579 – 591.

[15] Pan, M. H., Chen, W. J., Lin-Shiau, S. Y., Ho, C. T., Lin, J. K., Tangeretin induces cell-cycle G1 arrest through inhibiting cyclin-dependent kinases 2 and 4 activities as well as elevating Cdk inhibitors p21 and p27 in human colorectal carcinoma cells, *Carcinogenesis* 2002, *23*, 1677 – 1684.

[16] Enari, M., Sakahira, H., Yokoyama, H., Okawa, K. *et al.*, A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD, *Nature* 1998, *391*, 43 – 50.

[17] Enari, M., Talanian, R. V., Wong, W. W., Nagata, S., Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis, *Nature* 1996, *380*, 723 – 726.

[18] Tewari, M., Quan, L. T., O'Rourke, K., Desnoyers, S. *et al.*, Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase, *Cell* 1995, *81*, 801 – 809.

[19] Liu, X., Zou, H., Slaughter, C., Wang, X., DFF a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis, *Cell* 1997, *89*, 175 – 184.

[20] Sakahira, H., Enari, M., Nagata, S., Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis, *Nature* 1998, *391*, 96 – 99.

[21] Danial, N. N., Korsmeyer, S. J., Cell death: Critical control points, *Cell* 2004, *116*, 205 – 219.

[22] Antonsson, B., Conti, F., Ciavatta, A., Montessuit, S. *et al.*, Inhibition of Bax channel-forming activity by Bcl-2, *Science* 1997, *277*, 370 – 372.

[23] Desagher, S., Osen-Sand, A., Montessuit, S., Magnenat, E. *et al.*, Phosphorylation of bid by casein kinases I and II regulates its cleavage by caspase 8, *Mol. Cell* 2001, *8*, 601 – 611.

[24] Ichijo, H., Nishida, E., Irie, K., Ten, D. P. *et al.*, Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways, *Science* 1997, *275*, 90 – 94.

[25] Hatai, T., Matsuzawa, A., Inoshita, S., Mochida, Y. *et al.*, Execution of apoptosis signal-regulating kinase 1 (ASK1)-induced apoptosis by the mitochondria-dependent caspase activation, *J. Biol. Chem.* 2000, *275*, 26576 – 26581.

[26] Kupchan, S. M., Fessler, D. C., Eakin, M. A., Giacobbe, T. J., Reactions of alpha methylene lactone tumor inhibitors with model biological nucelophiles, *Science* 1970, *168*, 376 – 378.

[27] Schmidt, T. J., Helenanolide-type sesquiterpene lactones – III. Rates and stereochemistry in the reaction of helenalin and related helenanolides with sulfhydryl containing biomolecules, *Bioorg. Med. Chem.* 1997, *5*, 645 – 653.

[28] Steller, H., Mechanisms and genes of cellular suicide, *Science* 1995, *267*, 1445 – 1449.

[29] Thompson, C. B., Apoptosis in the pathogenesis and treatment of disease, *Science* 1995, *267*, 1456 – 1462.

[30] Slee, E. A., Harte, M. T., Kluck, R. M., Wolf, B. B. *et al.*, Ordering the cytochrome c-initiated caspase cascade: Hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner, *J. Cell Biol.* 1999, *144*, 281 – 292.

[31] Tang, D., Lahti, J. M., Kidd, V. J., Caspase-8 activation and bid cleavage contribute to MCF7 cellular execution in a caspase-3-dependent manner during staurosporine-mediated apoptosis, *J. Biol. Chem.* 2000, *275*, 9303 – 9307.

[32] Marchetti, P., Castedo, M., Susin, S. A., Zamzami, N. *et al.*, Mitochondrial permeability transition is a central coordinating event of apoptosis, *J. Exp. Med.* 1996, *184*, 1155 – 1160.

[33] Zamzami, N., Marchetti, P., Castedo, M., Decaudin, D. *et al.*, Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death, *J. Exp. Med.* 1995, *182*, 367 – 377.

[34] Adams, J. M., Cory, S., The Bcl-2 protein family: Arbiters of cell survival, *Science* 1998, *281*, 1322 – 1326.

[35] Tsujimoto, Y., Role of Bcl-2 family proteins in apoptosis: Apoptosomes or mitochondria? *Genes Cells* 1998, *3*, 697 – 707.

[36] Blagosklonny, M. V., Giannakakou, P., el-Deiry, W. S., Kingston, D. G. *et al.*, Raf-1/bcl-2 phosphorylation: A step from microtubule damage to cell death, *Cancer Res.* 1997, *57*, 130 – 135.

[37] Inoshita, S., Takeda, K., Hatai, T., Terada, Y. *et al.*, Phosphorylation and inactivation of myeloid cell leukemia 1 by JNK in response to oxidative stress, *J. Biol. Chem.* 2002, *277*, 43730 – 43734.

[38] Blagosklonny, M. V., Giannakakou, P., el-Deiry, W. S., Kingston, D. G. *et al.*, Raf-1/bcl-2 phosphorylation: A step from microtubule damage to cell death, *Cancer Res.* 1997, *57*, 130 – 135.

[39] Srivastava, R. K., Srivastava, A. R., Korsmeyer, S. J., Nesterova, M. *et al.*, Involvement of microtubules in the regulation of Bcl2 phosphorylation and apoptosis through cyclic AMP-dependent protein kinase, *Mol. Cell Biol.* 1998, *18*, 3509 – 3517.

