

# Caspase-dependent and caspase-independent apoptosis induced by evodiamine in human leukemic U937 cells

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## Abstract

Evodiamine is one of the major bioactive compounds that have been isolated and purified from the fruit of *Evodia fructus*. Evodiamine exhibits antitumor activities against the human tumor cells, including multidrug-resistant tumor cells. However, the molecular mechanism involved in cell death induced by evodiamine treatment remains poorly understood. In the present study, we showed that evodiamine activated the caspase-dependent apoptotic pathway. This apoptosis was only partially inhibited by a pancaspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone, which suggested that evodiamine-induced apoptosis in leukemic U937 cells is partially caspase independent. We observed the nuclear translocation of apoptosis-inducing factor in evodiamine-induced apoptosis of U937 cells, which may be responsible for the caspase-independent apoptotic execution. We next showed that evodiamine induced the substantial amount of apoptosis both in Bcl-2- and Akt-overexpressing U937 cells but not in human peripheral blood mononuclear cells. Although benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone inhibited caspase activity in Bcl-2-overexpressing U937 cells, it completely prevented neither the induction of apoptosis or the nuclear translocation of apoptosis-

inducing factor, which suggests that evodiamine is, at least in part, able to bypass the resistance of leukemia cells via caspase-independent apoptotic pathways. Thus, therapeutic strategy using evodiamine may warrant further evaluation. [Mol Cancer Ther 2006;5(9):2398–407]

## Introduction

Most anticancer agents eradicate tumor cells by the induction of apoptosis. Recent studies have suggested that the various anticancer agents used against cancer mediate their effects by induction of apoptosis of the cancer cells (1–3). It is suggested that there are several apoptotic pathways in cells responsive to apoptotic stimuli, such as the death receptor-mediated pathway, the mitochondrial apoptotic pathway, and the endoplasmic reticulum pathway (4–6). Although each pathway is initially mediated by different mechanisms, they share a common final phase of apoptosis, consisting of the activation of the executioner caspases and dismantling of substrates critical for cell survival (7).

Induction of apoptosis by chemotherapeutic agents, such as DNA-damaging agents, topoisomerase inhibitors, and antimetabolic agents, is largely mediated by the mitochondrial apoptotic pathways (2), which in turn increase the permeability of outer mitochondrial membrane (8–10). This involves release of mitochondrial apoptotic components, such as cytochrome *c*, apoptosis-inducing factor (AIF), second mitochondrial-derived activator of caspase, endonuclease G, and Omi1/HtrA2 (11–14). The released apoptotic proteins initiate caspase activation and trigger caspase-mediated apoptotic DNA fragmentation and eventually cell death. On the other hand, mammalian cells in a certain circumstance can undergo caspase-independent apoptosis that is mediated by the disruption of the mitochondrial membrane potential and the translocation of AIF and endonuclease G to nucleus where they induce chromatin condensation and/or large-scale DNA fragmentation (15).

Leukemia is grouped into two. The one is lymphocytic leukemia originated from lymphocytes in the bone marrow and the other is myelogenous leukemia mainly originated from granulocytes or monocytes (16). Both acute myelogenous leukemia and adult-onset acute lymphocytic leukemia are aggressive diseases that respond poorly to conventional chemotherapy. The primary cause of treatment failures in patients with these diseases is the emergence of multidrug resistance, including decreased drug uptake, increased drug export, modification of targets to drug action, decreased apoptosis, and increased drug metabolism (17). Leukemia cells, in turn, have adopted various mechanisms to resist apoptosis. Natural inhibitors of apoptosis, such as Bcl-2 and inhibitor of apoptosis protein (IAP) family members, protect the tumor cells from the apoptotic effects of various antineoplastic agents. These

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proteins may impair the ability to achieve remission and cure with chemotherapy. In trying to overcome drug resistance and improve clinical outcomes, attention is turning to developing therapeutic agents that overcome defects in the apoptotic pathways. Current chemotherapy protocols for leukemia commonly use drugs that interfere with DNA replication and induce apoptosis primarily in replicating cells (18, 19). However, these regimens may not effectively discriminate between normal and malignant cells, thus causing substantial damage to normal tissues. For this reason, it is important to develop treatments that can more specifically target the leukemic cell population without showing the cytotoxicity to normal cells.

A variety of plant antioxidants, such as dietary and herbal polyphenols and carotenoids, with anticancer activity have been identified in both epidemiologic studies and experimental model systems (20–23). Natural products have provided a large number of currently used chemotherapeutics and will continue to be an important component of drug discovery (24, 25). Evodiamine, an alkaloidal component extracted from the fruit of *Evodiae fructus*, has been reported to inhibit various tumor cell proliferations, including monocytic leukemia (26), melanoma (27), prostate cancer (28), and breast cancer cell (29). However, the mechanism underlying its antiproliferative, antimetastatic, and apoptotic activities is poorly defined yet.

In the present study, we examined the apoptosis-inducing potential of evodiamine in U937 cell lines and dissected the evodiamine-induced apoptotic signaling pathway. Our results showed that evodiamine induced relatively high levels of apoptosis in the majority of U937 cells through both caspase-dependent and caspase-independent pathways. Furthermore, evodiamine induced cell death in both Bcl-2-overexpressed and active Akt-overexpressed U937 cells without effect on the viability of human normal peripheral blood mononuclear cells (PBMC).

## Materials and Methods

### Cell Lines

The human leukemic U937 cells were obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in RPMI 1640 supplemented with 2 mmol/L L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum. Evodiamine was directly added to cell cultures at the indicated concentrations, whereas untreated cells contained the solvent alone. Anti-poly(ADP-ribose) polymerase (PARP), anti-phospholipase C-γ1 (PLC-γ1), anti-procaspase-3, anti-Bcl-2, anti-AIF, and anti-cytochrome *c* antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody against X-linked IAPs (XIAP) was obtained from R&D Systems (Minneapolis, MN), and benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk) was obtained from Biomol (Plymouth Meeting, PA). Evodiamine was purchased from Calbiochem (San Diego, CA).

### Annexin V and 7-Aminoactinomycin D Staining

Enumeration of apoptotic cells was done using FITC-conjugated Annexin V (BD Pharmingen, San Jose, CA) and

7-aminoactinomycin D (7-AAD). Cells were washed twice in cold PBS and resuspended in Annexin V-binding buffer (BD Pharmingen) at a concentration of  $3 \times 10^6$  per mL. This suspension (100 µL) was stained with 5 µL of Annexin V-FITC and 5 µL 7-AAD. 7-AAD (BD Pharmingen) is a nucleic acid dye that was used for the exclusion of nonviable cells. The cells were gently vortexed and incubated for 15 minutes at room temperature in the dark. After addition of 400 µL of binding buffer to each tube, cells were analyzed by flow cytometry.

### Western Blotting

Cellular lysates were prepared by suspending  $1 \times 10^6$  cells in 100 µL of lysis buffer (137 mmol/L NaCl, 15 mmol/L EGTA, 0.1 mmol/L sodium orthovanadate, 15 mmol/L MgCl<sub>2</sub>, 0.1% Triton X-100, 25 mmol/L MOPS, 100 µmol/L phenylmethylsulfonyl fluoride, 20 µmol/L leupeptin, adjusted to pH 7.2). The cells were disrupted by sonication and extracted at 4°C for 30 minutes. The proteins were electrotransferred to Immobilon-P membranes (Millipore Corp., Bedford, MA). Detection of specific proteins was carried out with an enhanced chemiluminescence Western blotting kit according to the manufacturer's instructions (Amersham, Arlington Heights, IL).

### Fluorescence-Activated Cell Sorting Analysis for Apoptosis

Approximately  $1 \times 10^6$  U937 cells were suspended in 100 µL PBS, and 200 µL of 95% ethanol were added while vortexing. The cells were incubated at 4°C for 1 hour, washed twice with PBS, and resuspended in 250 µL of 1.12% sodium citrate buffer (pH 8.4) together with 12.5 µg RNase (Sigma, St. Louis, MO). Incubation was continued at 37°C for 30 minutes. The cellular DNA was then stained by applying 250 µL of propidium iodide (50 µg/mL) for 30 minutes at room temperature. The stained cells were analyzed by fluorescence-activated cell sorting (FACS) on a FACScan flow cytometer for relative DNA content based on red fluorescence.

### DNA Fragmentation Assay

After treatment with drugs, U937 cells were lysed in buffer containing 10 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, and 0.5% Triton X-100 for 30 minutes on ice. Lysates were vortexed and cleared by centrifugation at  $10,000 \times g$  for 20 minutes. Fragmented DNA in the supernatant was extracted with an equal volume of neutral phenol-chloroform-isoamyl alcohol mixture (25:24:1) and analyzed electrophoretically on 2% agarose gels containing 0.1 µg/mL ethidium bromide.

### Asp-Glu-Val-Asp-ase Activity Assay for Evaluation of Caspase Activation

To evaluate Asp-Glu-Val-Asp-ase (DEVDase) activity, cell lysates were prepared after their respective treatment with evodiamine. Assays were done in 96-well microtiter plates by incubating 20 µg of cell lysates in 100 µL reaction buffer [1% NP40, 20 mmol/L Tris-HCl (pH 7.5), 137 mmol/L NaCl, 10% glycerol] containing the caspases substrate (DEVD-chromophore *p*-nitroanilide) at 5 µmol/L. Lysates were incubated at 37°C for 2 hours. Thereafter, the absorbance at 405 nm was measured with a spectrophotometer.

#### **4',6-Diamidino-2-Phenylindole Staining for Nuclei Condensation and Fragmentation**

U937 cells were treated with 0.6  $\mu\text{mol/L}$  evodiamine or evodiamine plus 50  $\mu\text{mol/L}$  z-VAD for 18 hours. The cells were fixed with 1% paraformaldehyde on slide glass for 30 minutes at room temperature. After washing with PBS, 300 nmol/L 4',6-diamidino-2-phenylindole (Roche, Indianapolis, IN) was added to the fixed cells for 5 minutes, after which they were examined by fluorescence microscopy. Apoptotic cells were identified by condensation and fragmentation of nuclei. The 4',6-diamidino-2-phenylindole staining experiments were done in duplicate.

#### **Confocal Immunofluorescence Microscopy for AIF Translocation**

Cells ( $5 \times 10^4$ ) were cytospun onto noncharged slides (Becton Dickinson), fixed for 20 minutes in 4% paraformaldehyde, washed again with PBS, and permeabilized with 1% Triton X-100 for 30 minutes at room temperature and washed with TBS containing 0.1% sodium azide. To reduce nonspecific antibody binding, slides were incubated in 1% bovine serum albumin in TBS for 1 hour at room temperature before incubation with rabbit polyclonal antibody to human AIF (Santa Cruz Biotechnology) overnight at 4°C. Slides were then washed for 30 minutes in TBS containing 0.1% sodium azide and incubated for 1 hour with FITC-conjugated secondary antibody (Vector, Burlingame, CA). Nuclei were stained with propidium iodide (Sigma) for 15 minutes at room temperature. Slides were washed and air dried before they were mounted on coverslips with ProLong Antifade mounting medium (Molecular Probes, Eugene, OR), and then they were examined under a Zeiss LSM 510 multiphoton confocal microscope (Zeiss, Göttingen, Germany).

#### **Separation of Human PBMCs**

Blood was obtained from three healthy adult volunteers (10 mL plus 0.1 mL of heparin, 1,000 units/mL). The blood was centrifuged at 2,000 rpm with a vasculant rotor for 10 minutes at room temperature. The layer of white cells plus some RBCs was taken and transferred to tubes with 0.1 mol/L sodium phosphate buffer (PBS; pH 7.4) and centrifuged at 1,000 rpm for 10 minutes. The white layer was taken, completed to 10 mL PBS, and placed on 5 mL Ficoll-Hypaque, and after centrifugation at 2,000 rpm for 30 minutes, the monocyte layer was taken and placed and cultured in RPMI 1640 containing 10% fetal bovine serum.

#### **Cell Counting Kit-8 Assay**

Cell counting kit-8 assay is nearly proportional to the cell number and thus can be used to determine cell proliferation and cytotoxicity. PBMCs ( $2 \times 10^3$ ) per well were added to the 96-well culture plates. The cells were exposed to various concentrations of evodiamine. The cells then were grown for an additional 18 hours, and the cell counting kit-8 assay was carried out using the manufacturer's instructions (Dojindo, Kumamoto, Japan).

#### **Measurement of Reactive Oxygen Species Generation**

The generation of reactive oxygen species (ROS) was measured by a flow cytometry analysis using 2',7'-dichloro-

odihydrofluorescein diacetate as a substrate. Briefly, U937 cells were incubated with 0.6  $\mu\text{mol/L}$  evodiamine in the presence or absence of 10 mmol/L *N*-acetyl-L-cysteine (NAC) for 18 hours and loaded with 10  $\mu\text{mol/L}$  2',7'-dichlorodihydrofluorescein diacetate 1 hour before harvesting. The fluorescence was measured at the desired time intervals by flow cytometry. The ROS generation was assessed by the dichlorofluorescein fluorescence intensity (FL-1, 530 nm) from 10,000 cells with a FACSCalibur flow cytometer (Becton Dickinson). Control cells were subjected to the same manipulation, except for treatment with the evodiamine.

## **Results**

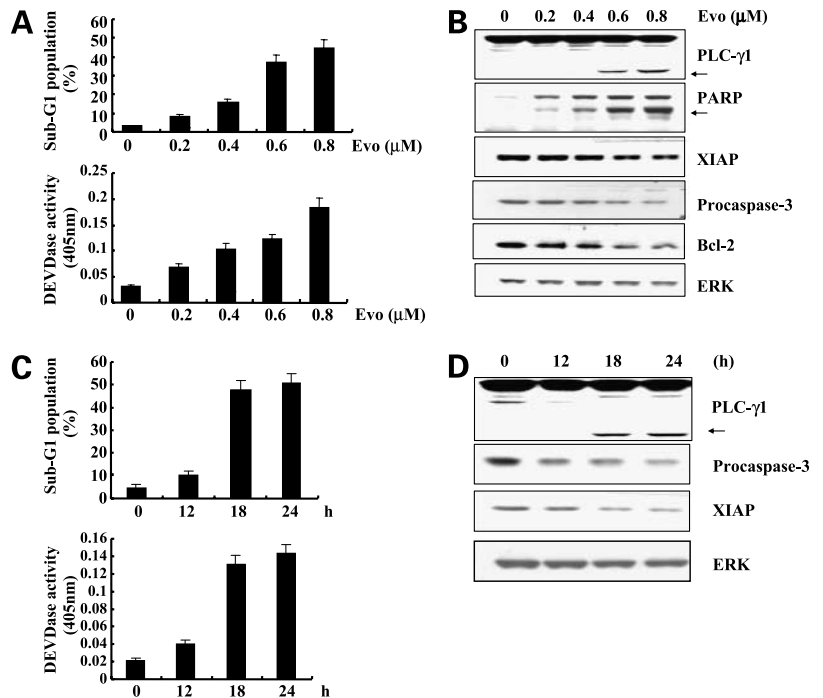
### **Evodiamine Induces Apoptosis in U937 Cells**

Human leukemic U937 cells were treated with evodiamine (0.2–0.8  $\mu\text{mol/L}$ ). We first determined apoptosis in U937 cells using flow cytometric analysis to detect hypodiploid cell populations. As shown in Fig. 1A, treatment of U937 cells with evodiamine resulted in a markedly increased accumulation of sub-G<sub>1</sub> phase cells in a dose-dependent manner. We next analyzed whether treatment with evodiamine caused the activation of caspases, a key executioner of apoptosis. Exposure of U937 cells to evodiamine strongly stimulated DEVDase activity and led to a reduction of the protein levels of XIAP and 32-kDa caspase-3 precursor together with a concomitant cleavage of PLC- $\gamma$ 1 and PARP, a substrate protein of caspases (Fig. 1B). Next, we carried out time kinetics studies of the apoptotic effects of evodiamine on U937 cells. Because the dose required for half-maximal inhibition of viability was  $\sim 0.6 \mu\text{mol/L}$ , this single concentration was used for further assessment of apoptosis. Evodiamine treatment induced markedly increase of sub-G<sub>1</sub> population after 18 hours of treatment (Fig. 1C). The levels of procaspase-3 were progressively degraded from 12 to 24 hours after evodiamine treatment in U937 cells. We further assessed the DEVDase activity and proteolytic cleavage of PLC- $\gamma$ 1. In parallel with the degradation of caspase-3, the activity of DEVDase and the 60-kDa cleavage product of PLC- $\gamma$ 1 were progressively increased from 18 to 24 hours in U937 cells following treatment with 0.6  $\mu\text{mol/L}$  evodiamine (Fig. 1C and D).

### **Evodiamine-Induced Apoptosis Was Partly Mediated by Caspase-Dependent Pathway**

We examined the involvement of caspase in evodiamine-induced apoptosis. Not only evodiamine induced the degradation of procaspase-3 and produced 60-kDa cleavage product of PLC- $\gamma$ 1, a well-known substrate of caspase (Fig. 2A), but it increased DEVDase activity (Fig. 2B). Cells treated with evodiamine showed DNA fragmentation, which is a hallmark of apoptosis (Fig. 2C). We also found that pretreatment with z-VAD-fmk, a pancaspase inhibitor, prevented DNA fragmentation in cells treated with evodiamine (Fig. 2C). A general and potent inhibitor of caspases z-VAD-fmk prevented all these caspase-related events (Fig. 2A–C). However, z-VAD-fmk did not completely block the nuclear condensation and formation of apoptotic

**Figure 1.** Evodiamine-induced apoptosis in U937 cells. **A**, evodiamine (Evo)-induced apoptosis in a dose-dependent manner. U937 cells were treated with various concentrations of evodiamine for 18 h. Apoptosis was analyzed as a sub-G<sub>1</sub> fraction by FACS. Enzymatic activities of DEVDase were determined by incubation of 20 μg of total protein with 200 μmol/L chromogenic substrate (DEVD-*p*-nitroanilide) in a 100 μL assay buffer for 2 h at 37°C. The release of chromophore *p*-nitroanilide was monitored spectrophotometrically (405 nm). **Columns**, mean of three independent experiments; **bars**, SD. **B**, equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western blot for PLC-γ1, PARP, XIAP, procaspase-3, Bcl-2, and extracellular signal-regulated kinase (ERK). ERK was used as control for the loading of protein level. **Arrow**, proteolytic cleavage of PARP and PLC-γ1. **C**, evodiamine-induced apoptosis in a time-dependent manner. U937 cells were treated with 0.6 μmol/L evodiamine for the indicated times. Apoptosis was analyzed as a sub-G<sub>1</sub> fraction by FACS. DEVDase activity was determined. **D**, equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western blot for PLC-γ1, procaspase-3, XIAP, and ERK.



bodies caused by evodiamine treatment (Fig. 2D). In addition, z-VAD-fmk treatment partly prevented evodiamine-induced cell death as determined by FACS analysis (Fig. 2E). These results suggest that evodiamine-induced cell death was partly mediated by caspase-dependent pathway and caspase-independent cell death in the presence of z-VAD-fmk.

#### Caspase-Independent Cell Death Was Not Caused by Necrotic Cell Death

To reveal whether caspase-independent cell death induced by evodiamine treatment in U937 cells is apoptosis or necrosis, we examined the translocation of membrane phosphatidylserine from the inner to outer leaflet of the plasma membrane. Because translocation of phosphatidylserine to external cell surface occurs during early apoptosis and necrosis, Annexin V-FITC and vital dye, such as 7-AAD, were used to distinguish apoptotic cells from necrotic cells (30). As shown in Fig. 3, the incidence of early apoptotic 7-AAD<sup>-</sup>/Annexin V<sup>+</sup> cells was increased in evodiamine-treated cells but the incidence of necrotic 7-AAD<sup>+</sup>/Annexin V<sup>-</sup> or 7-AAD<sup>+</sup>/Annexin V<sup>+</sup> cells was not. In addition, pretreatment with 50 μmol/L z-VAD-fmk decreased the population of early apoptotic 7-AAD<sup>-</sup>/Annexin V<sup>+</sup> cells increased by evodiamine treatment. These results suggest that evodiamine-induced cell death was not mediated by necrotic cell death but apoptosis.

#### Evodiamine-Induced Apoptosis Seems to be Independent on the Formation of ROS

Numerous investigations have documented that oxidative stress-mediated cellular changes are frequently induced in cells exposed to cytotoxic drugs, UV, or γ-irradiation (31, 32), and ROS is a mediator of caspase-independent cell death (33). We here examined whether

evodiamine affects the cellular levels of peroxide by measuring the changes in the fluorescence using 2',7'-dichlorodihydrofluorescein diacetate. As shown in Fig. 4A, treatment with evodiamine markedly increased the 2',7'-dichlorodihydrofluorescein diacetate-derived fluorescence. This evodiamine-mediated increase in fluorescence was markedly inhibited by pretreatment with antioxidant NAC. Then, we next asked whether ROS generation induced by evodiamine is directly associated with the induction of apoptosis. However, as shown in Fig. 4B, pretreatment with NAC did not prevent evodiamine-induced increase of sub-G<sub>1</sub> population. In addition, pretreatment with NAC prevented neither the production of 60-kDa cleavage of PLC-γ1 nor the degradation of procaspase-3 (Fig. 4C). These data indicate that ROS generation is not critical for the induction of apoptosis by evodiamine.

#### Evodiamine-Induced Caspase-Independent Pathway Was Mediated by AIF Translocation

Because AIF is involved in induction of apoptotic cell death through caspase-independent pathway, we examined whether AIF plays a role in evodiamine-induced apoptotic cell death. Translocation of AIF was analyzed by the observation of its release from mitochondria and translocation to the nucleus by confocal microscopy. As shown in Fig. 5, analysis by confocal microscopy showed that AIF was translocated into the nucleus and caused nuclear condensation after treatment with evodiamine. In addition, caspase inhibitor z-VAD-fmk did not affect AIF translocation into the nucleus after the evodiamine treatment. These results suggest that evodiamine-induced apoptotic cell death in U937 cells is mediated by the AIF translocation from mitochondria into nucleus via a caspase-independent pathway. It was reported that

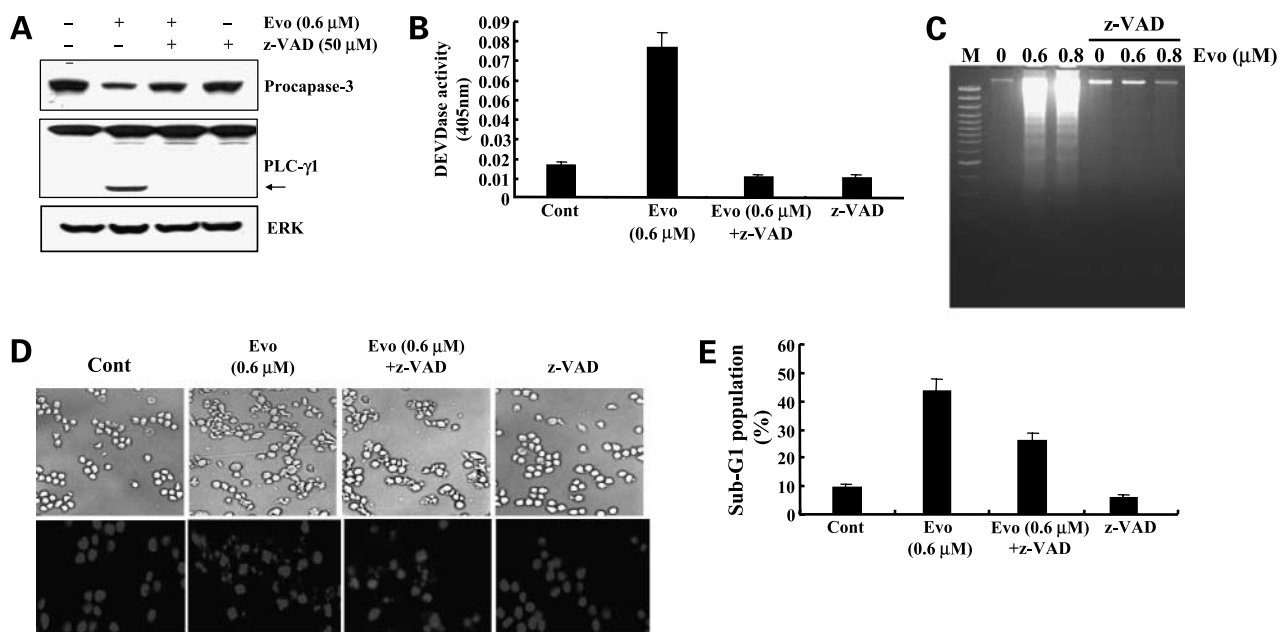
internucleosomal DNA fragmentation of nuclei induces chromatin condensation in apoptotic cells (34). As shown in Fig. 5, we found that the chromatin was less condensed in evodiamine plus z-VAD-fmk-treated cells compared with evodiamine-treated cells.

#### Evodiamine Could Induce Apoptosis Even in Bcl-2-Overexpressing Cells

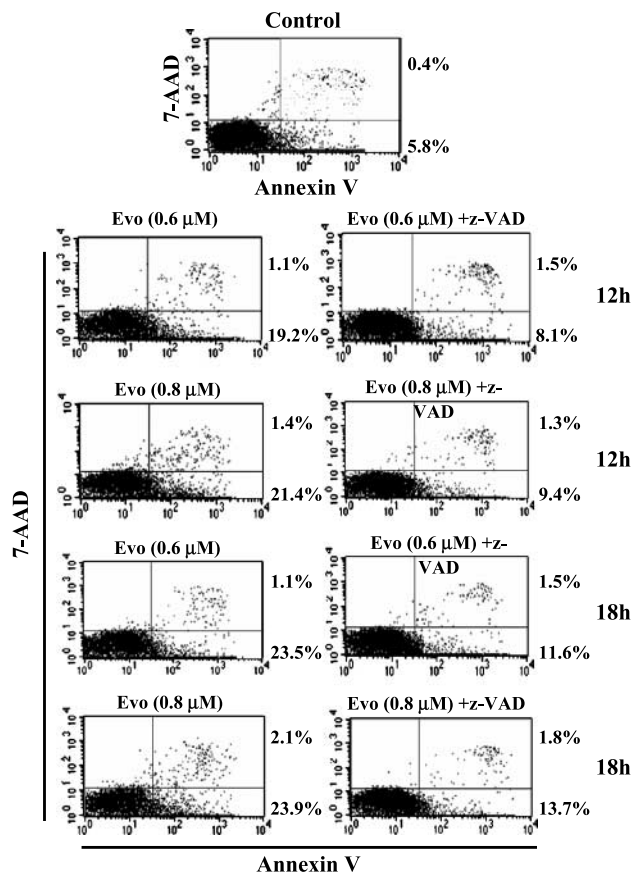
Increased expression level of Bcl-2 in leukemia cells is correlated with elevated resistance to cytotoxic drugs (35). Thus, bypassing this chemoresistance by Bcl-2 is an important therapeutic strategy. We first established Bcl-2-overexpressing cells. U937 cells were transfected with an expression vector containing Bcl-2 cDNA. After 4 weeks, geneticin-resistant (G418) cells were isolated and the relative expression level of Bcl-2 was determined by Western blot. In our previous study, we showed that U937/Bcl-2 cells exhibited approximately 3- to 4-fold increase in Bcl-2 expression compared with cells containing empty vector only (36). We here evaluated whether evodiamine could induce cell death even in Bcl-2-overexpressing leukemia cells. As shown in Fig. 6A, overexpression of Bcl-2 did not completely prevent the production of cleavage products of PLC- $\gamma$ 1 and PARP and the degradation of XIAP and procaspase-3. In addition, overexpression of Bcl-2 did not completely prevent

evodiamine-induced cell death as determined by accumulation of sub-G<sub>1</sub> phase (Fig. 6B), which suggests that evodiamine can bypass the inhibition of apoptosis originated from increased expression of Bcl-2 in leukemia cells.

Because evodiamine was revealed to induce apoptosis in U937 cells both by caspase-dependent and caspase-independent pathways, we next examined whether induction of cell death by evodiamine in Bcl-2-overexpressing U937 cells is caspase dependent or caspase independent. z-VAD-fmk pretreatment completely prevented the production of cleavage products of PLC- $\gamma$ 1 and the degradation of procaspase-3 and XIAP in Bcl-2-overexpressing U937 cells (Fig. 7A). z-VAD-fmk pretreatment abolished DEVDase activity, which was maintained with a lower level in Bcl-2-overexpressing U937 cells compared with the U937/vector cells (Fig. 7B). However, z-VAD-fmk pretreatment prevented only in part the accumulation of sub-G<sub>1</sub> phase in Bcl-2-overexpressing U937 cells as it did in U937/vector cells (Fig. 7C). Accordingly, we deliberately assumed that evodiamine at least in part could bypass the Bcl-2-mediated chemoresistance in leukemia cells via caspase-independent apoptosis, which is supported by confocal microscopy data showing that Bcl-2 overexpression could not prevent the release from mitochondria and translocation onto nucleus of AIF (Fig. 7D).



**Figure 2.** Evodiamine-induced apoptosis was partly mediated by caspase-dependent pathway. **A**, pancaspase inhibitor z-VAD-fmk prevented evodiamine-induced cleavage of PLC- $\gamma$ 1 and procaspase-3 proteins. U937 cells were incubated with 50  $\mu$ mol/L z-VAD-fmk or solvent for 1 h before challenge with evodiamine (0.6  $\mu$ mol/L) for 18 h. Equal amounts of cell lysates (40  $\mu$ g) were subjected to electrophoresis and analyzed by Western blot for PLC- $\gamma$ 1 and procaspase-3 antibodies. Western blotting of ERK levels was included to show that equivalent amounts of protein were loaded in each lane. **B**, DEVDase activity was determined as described in Fig. 1A. *Columns*, mean ( $n = 3$ ); *bars*, SD. **C**, fragmentations of genomic DNA in U937 cells treated for 18 h with the indicated concentrations of evodiamine or evodiamine plus z-VAD-fmk (50  $\mu$ mol/L). Fragmented DNA was extracted and analyzed on 2% agarose gel. **D**, detection of evodiamine-induced apoptosis in U937 cells by 4',6'-diamidino-2-phenylindole staining. U937 cells were incubated with 50  $\mu$ mol/L z-VAD-fmk or solvent for 1 h before challenge with evodiamine (0.6  $\mu$ mol/L) for 18 h. The morphologies of cells were determined by light microscopy. For 4',6'-diamidino-2-phenylindole staining, cells were harvested and fixed in 1% paraformaldehyde for 30 min. The cells were stained with 300 nmol/L 4',6'-diamidino-2-phenylindole for 5 min, and changes in nuclear morphology were observed by fluorescence microscopy. **E**, apoptosis was analyzed as sub-G<sub>1</sub> fraction by FACS.

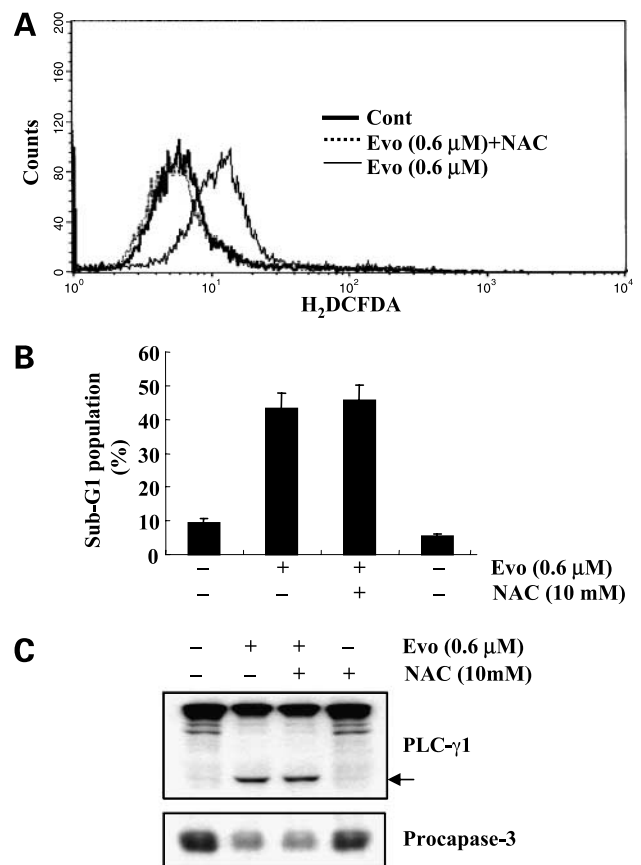


**Figure 3.** Caspase-independent cell death was not caused by necrotic cell death. U937 cells were treated with indicated concentrations of evodiamine alone or evodiamine plus z-VAD-fmk for 12 or 18 h, harvested, and stained with 7-AAD and Annexin V. Cell death was determined by flow cytometry. *Bottom right quadrant*, Annexin V<sup>+</sup>, early apoptotic cells; *top right quadrant*, Annexin V<sup>+</sup>/7-AAD<sup>+</sup>, late apoptotic cells. Values correspond to the percentage of cells in those quadrants. Two additional studies yielded equivalent results.

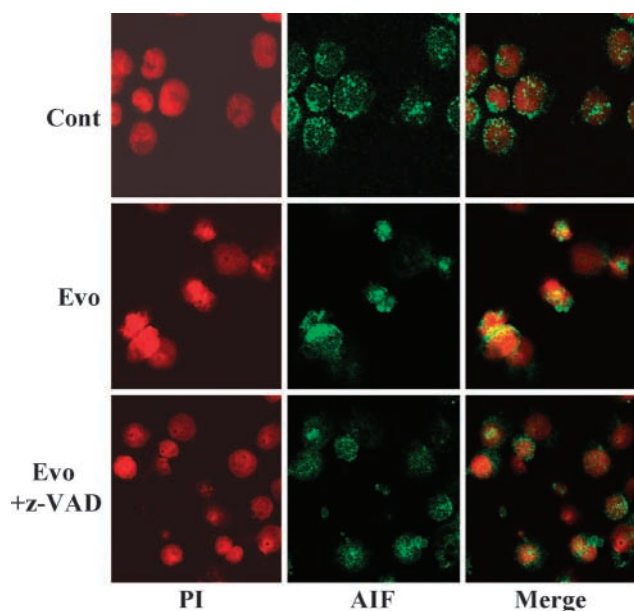
### Evodiamine Treatment Enhanced Cell Death in U937/ Akt and Renal Cancer Cells Overexpressing cFLIPs but Not in Human PBMCs

Previous studies have shown that constitutively activated Akt significantly protected cells from apoptosis (37). To determine whether overexpression of Akt prevented evodiamine-induced apoptosis in leukemic cells, we used U937/vector and U937/dominant-active Akt cells generated by transfection of the constitutively active Myc-tagged form of Akt. As shown in Fig. 8A and B, overexpression of constitutively active Akt did weakly inhibit evodiamine-induced apoptosis in leukemia cells. We examined next whether evodiamine could induce cell death in different type of cancer cells overexpressing antiapoptotic genes, such as cFLIPs. We established Caki/cFLIP cell lines transfected with the expression vectors encoding cFLIPs. Stable cFLIP-overexpressing cell lines were treated with various concentrations of evodiamine and examined cytotoxicity using FACS analysis. As shown in Fig. 8C,

overexpression of cFLIPs was not enough to prevent evodiamine-induced apoptosis even if overexpression of cFLIPs prevented cisplatin-mediated apoptosis in human renal Caki cells. In addition, we examined whether evodiamine shows cytotoxicity in normal PBMCs. Primary PBMCs isolated from three volunteers were treated for 18 hours with 0.6 to 1 μmol/L evodiamine, and cell viabilities were assessed using cell counting kit-8 assay kit. As shown in Fig. 8D, we did not observe increased cell death in PBMCs treated with evodiamine compared with untreated PBMCs. These results suggest that treatment of evodiamine may have a therapeutic effect on leukemia cells overexpressing Bcl-2 and Akt and renal cancer cells overexpressing cFLIPs, which are resistant to many other chemotherapeutic drugs.



**Figure 4.** Evodiamine-induced cell death seems to be independent on the formation of ROS. **A**, evodiamine-induced ROS generation was prevented by pretreatment of NAC. U937 cells were loaded with 2',7'-dichlorodihydrofluorescein diacetate, and fluorescence was measured by flow cytometry. Cells were incubated with 10 mmol/L NAC for 30 min before challenge with evodiamine for 1 h, and fluorescence was measured by flow cytometry. **B**, U937 cells were stimulated with evodiamine in the presence or absence of NAC (5 mmol/L). After 18 h, cells were harvested and sub-G<sub>1</sub> populations were measured by flow cytometry. **C**, equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western blot for PLC-γ1 and procaspase-3 antibodies. Western blotting of ERK levels was included to show that equivalent amounts of protein were loaded in each lane.



**Figure 5.** Evodiamine-induced caspase-independent pathway was mediated by AIF translocation. U937 cells were treated with evodiamine (0.8  $\mu\text{mol/L}$ ) in the presence or absence of z-VAD-fmk (50  $\mu\text{mol/L}$ ) and incubated with antibody against AIF followed by labeling with the FITC-conjugated secondary antibody. Nuclei were stained with propidium iodide (PI). Yellow, nuclear translocation of AIF is shown by overlap of AIF (green fluorescence) and nuclear staining (red fluorescence).

## Discussion

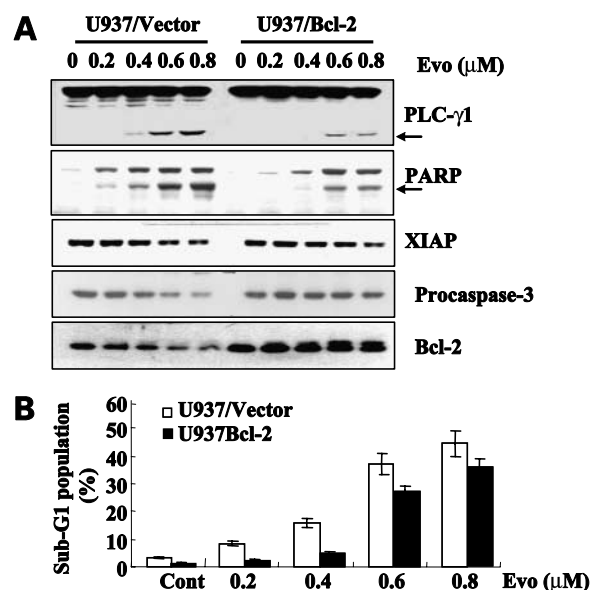
In the present study, evodiamine was found to exhibit significant antitumor activity against human leukemic cancer cells. Evodiamine-induced apoptosis was mediated by two different types of pathways (e.g., caspase-dependent and caspase-independent pathways) in human U937 leukemia cells. We also found that evodiamine significantly induced apoptosis in both human leukemia and renal cancer cells that are overexpressed antiapoptotic proteins, including Bcl-2, Akt, and cFLIPs, whereas evodiamine did not cause significant normal PBMC death.

Multiple myeloma and acute myelogenous leukemia are cancers with high mortality rates, where novel strategies are required to improve on current treatment standards (38). To overcome drug resistance and improve clinical outcomes, identification and evaluation of novel therapeutic agents that have less toxicity in normal cells for treatment of multiple myeloma and acute myelogenous leukemia are important and challenging tasks.

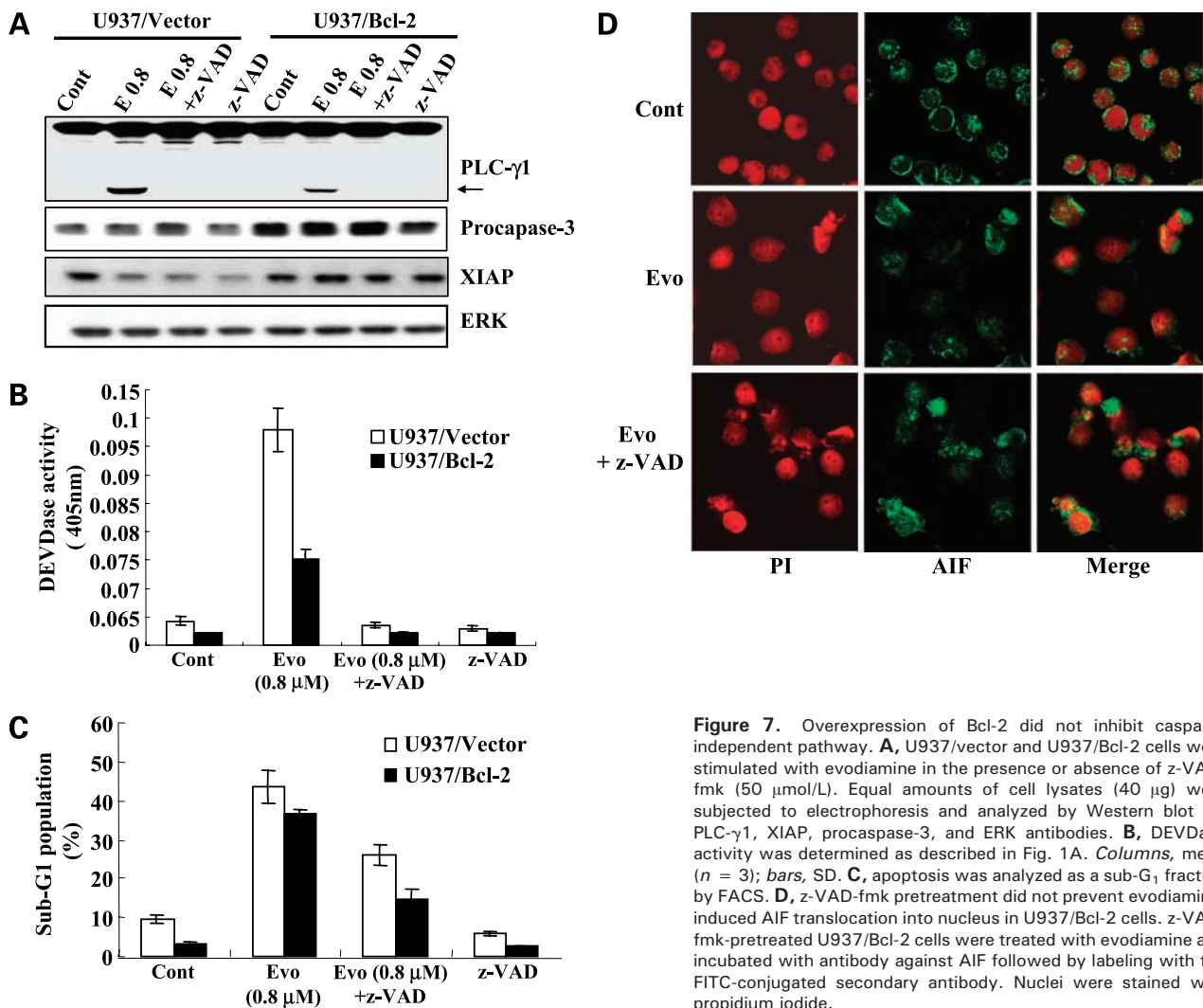
Evodiamine is an alkaloidal component isolated from *Evodiae fructus* and has anticarcinogenic, antiproliferative, and antimetastatic effects (26–29, 39). However, the molecular mechanism involved in evodiamine-induced apoptosis was poorly understood. In this study, we tested whether evodiamine treatment could be a new possibility for the treatment of human leukemia and examined the mechanism of evodiamine-induced apoptosis in human leukemia cells. We observed that evodiamine activated the mitochondrial caspase-dependent apoptotic pathway in

dose- and time-dependent manners in U937 cells, which were partly prevented by pretreatment of a pancaspase inhibitor z-VAD-fmk. This result suggested that evodiamine-induced apoptosis was mediated by caspase-independent as well as caspase-dependent apoptotic pathways. Previous reports have shown that caspase-dependent pathways play essential roles in evodiamine-induced apoptosis (26). They have shown that z-VAD-fmk and z-DEVD effectively prevented evodiamine-induced DNA fragmentation and apoptosis in HeLa cells. However, in another study, evodiamine-induced cell death in A375-S2 cells was partially prevented by z-VAD-fmk treatment (40). Furthermore, evodiamine was shown to induce necrosis and caspase inhibitors failed to block A375-S2 cell death induced by evodiamine (27, 40). Our data are first consistent with these previous findings (27, 40).

We first define the caspase-independent cell death machinery involved in the alternate pathway activated by evodiamine. To examine which type of cell death is induced by evodiamine, evodiamine-treated cells were stained with FITC-conjugated Annexin V and 7-AAD. As shown in Fig. 3, caspase-independent cell death was not caused by necrotic cell death. Mitochondrial ROS are used as active mediators in the regulation of cell death, including caspase-dependent and caspase-independent pathways and necrosis (41–43). Evodiamine exposure leads to increase of ROS accumulation, but inhibition of ROS by NAC did not prevent evodiamine-induced apoptosis,



**Figure 6.** Overexpression of Bcl-2 was not enough to inhibit evodiamine-induced cell death. **A**, overexpression of Bcl-2 attenuated evodiamine-induced PLC- $\gamma$ 1 cleavage, degradation of XIAP, and procaspase-3 proteins. U937/vector and U937/Bcl-2 cells were treated with various concentrations of evodiamine for 18 h. Equal amounts of cell lysates (40  $\mu\text{g}$ ) were subjected to electrophoresis and analyzed by Western blot for PLC- $\gamma$ 1, PARP, XIAP, procaspase-3, and Bcl-2 antibodies. **B**, U937/vector and U937/Bcl-2 cells were treated with various concentrations of evodiamine for 18 h, and apoptosis was analyzed as a sub-G<sub>1</sub> fraction by FACS.



**Figure 7.** Overexpression of Bcl-2 did not inhibit caspase-independent pathway. **A**, U937/vector and U937/Bcl-2 cells were stimulated with evodiamine in the presence or absence of z-VAD-fmk (50  $\mu$ mol/L). Equal amounts of cell lysates (40  $\mu$ g) were subjected to electrophoresis and analyzed by Western blot for PLC- $\gamma$ 1, XIAP, procaspase-3, and ERK antibodies. **B**, DEVDase activity was determined as described in Fig. 1A. *Columns*, mean ( $n = 3$ ); *bars*, SD. **C**, apoptosis was analyzed as a sub-G<sub>1</sub> fraction by FACS. **D**, z-VAD-fmk pretreatment did not prevent evodiamine-induced AIF translocation into nucleus in U937/Bcl-2 cells. z-VAD-fmk-pretreated U937/Bcl-2 cells were treated with evodiamine and incubated with antibody against AIF followed by labeling with the FITC-conjugated secondary antibody. Nuclei were stained with propidium iodide.

indicating that ROS generation was not involved in evodiamine-mediated cell death.

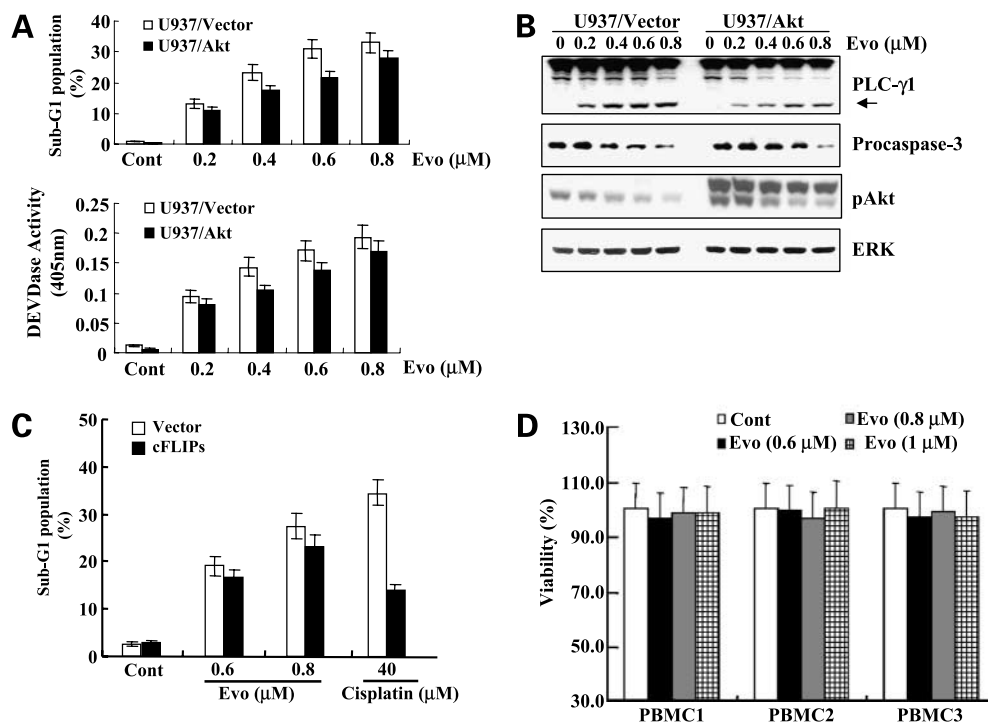
It has been reported that AIF mediates cell death through a caspase-independent pathway. Mitochondrial AIF translocates to the nucleus on death stimuli and initiates nuclear condensation that leads to large-scale chromatin fragmentation followed by the cell death (44, 45). Confocal microscopy showed that translocation of AIF into nucleus was found in U937 cells, which were not inhibited by z-VAD-fmk. This result suggested that the activation of caspase-independent apoptotic route was mediated through the translocation of AIF into nucleus.

Bcl-2 protein can act at multiple levels to inhibit apoptosis (46), and increased expression level of Bcl-2 is correlated with elevated resistance to cytotoxic drugs in leukemia cells (35). In this study, we showed that Bcl-2 overexpression partly attenuated caspase-dependent PLC- $\gamma$ 1 and PARP cleavage, decreased DEVDase activity, and degradation of XIAP proteins but did not completely

prevent evodiamine-induced cell death, suggesting that Bcl-2 overexpression could partly block the caspase-dependent pathway but not caspase-independent apoptotic pathway. In addition, we showed that translocation of AIF into nucleus was found in U937/Bcl-2 cells, which were not inhibited by z-VAD-fmk. This result suggested that evodiamine treatment can activate an AIF-mediated apoptotic cell death that is independent of caspase activation and not inhibited by overexpression of Bcl-2. Our data are consistent with the data from previous studies. Zhang et al. (47) reported that overexpression of Bcl-2 inhibited the early onset of caspase-dependent pathway but not the later caspase-independent pathway induced by addition of staurosporine through the AIF translocation to nucleus.

Tumor cells expressed several proteins that render them resistant to apoptosis. Increased activity of antiapoptotic proteins, such as Bcl-2, Akt, and cFLIPs, may result in aggressively growing and therapy-resistant tumors. In our





**Figure 8.** Treatment with evodiamine enhanced cell death in U937/Akt and renal cancer cells overexpressing cFLIPs but not in human normal PBMCs. **A**, effect of constitutive active Akt overexpression on cell death induced by the evodiamine treatment. U937/vector and U937/Akt cells were treated with the indicated concentrations of evodiamine for 18 h and harvested, and sub-G<sub>1</sub> populations were measured by FACS. DEVDase activity was determined as described in Fig. 1A. Columns, mean ( $n = 3$ ); bars, SD. **B**, cell lysates were prepared and immunoblotted with anti-PLC- $\gamma$ 1, procaspase-3, phosphorylated Akt (pAkt), and ERK antibodies. **C**, effects of cFLIP overexpression on cell death induced by the evodiamine treatment. Caki/vector and Caki/cFLIPs cells were treated with the indicated concentrations of evodiamine and cisplatin for 18 h and harvested, and sub-G<sub>1</sub> populations were measured by FACS. **D**, effect of evodiamine on the viability of human normal PBMCs. Human normal PBMCs were treated with or without the indicated concentrations of evodiamine for 18 h, and cell viability was analyzed using cell counting kit-8 assay kit.

present study, we showed that exogenous Bcl-2, constitutive active Akt, and cFLIP overexpression cannot prevent completely evodiamine-induced apoptosis in human leukemia cells and human renal Caki cells and that evodiamine did not cause significant normal PBMC death. These results raise the possibility that a strategy treating evodiamine may be effective against drug-resistant leukemia cells overexpressing Bcl-2 and Akt without any cytotoxicity to normal cells. These findings may have implications for the rational development of novel regimens in hematologic malignancies.

## References

- Ferreria CG, Epping M, Krzyt FAE, Giaccone G. Apoptosis: target of cancer therapy. *Clin Cancer Res* 2002;8:2024–34.
- Debatin KM, Poncet D, Kroemer G. Chemotherapy: targeting the mitochondrial cell death pathway. *Oncogene* 2002;21:8786–803.
- Norbury CJ, Zhivotovsky B. DNA damage-induced apoptosis. *Oncogene* 2004;23:2797–808.
- Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. *Science* 1998;281:1305–8.
- Budihardjo I, Oliver H, Lutter M, Luo X, Wang X. Biochemical pathways of caspase activation during apoptosis. *Annu Rev Cell Dev Biol* 1999;15:269–90.
- Xu C, Bailly-Maitre B, Reed JC. Endoplasmic reticulum stress: cell life and death decisions. *J Clin Invest* 2005;115:2656–64.
- Cryns V, Yuan J. Proteases to die for. *Genes Dev* 1998;12:1551–70.
- Henry-Mowatt J, Dive C, Martinou JC, James D. Role of mitochondrial membrane permeabilization in apoptosis and cancer. *Oncogene* 2004;23:2850–60.
- van Gurp M, Festjens N, van Loo G, Saelens X, Vandenabeele P. Mitochondrial intermembrane proteins in cell death. *Biochem Biophys Res Commun* 2003;304:487–97.
- Cande C, Vahsen N, Garrido C, Kroemer G. Apoptosis-inducing factor (AIF): caspase-independent after all. *Cell Death Differ* 2004;11:591–5.
- Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 1996;86:145–57.
- Susin SA, Lorenzo HK, Zamzami N, et al. Mitochondrial release of caspase-2 and -9 during the apoptotic process. *J Exp Med* 1999;189:381–93.
- Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* 2000;102:33–42.
- Hegde R, Srinivasula SM, Zhang Z, et al. Identification of Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts inhibitor of apoptosis protein-caspase interaction. *J Biol Chem* 2002;277:432–8.
- Cregan SP, Dawson VL, Slack RS. Role of AIF in caspase-dependent and caspase-independent cell death. *Oncogene* 2004;23:2785–96.
- Abramson N, Melton B. Leukocytosis: basics of clinical assessment. *Am Fam Physician* 2000;62:2053–60.
- Schimmer AD, Hedley DW, Penn LZ, Minden MD. Receptor- and mitochondrial-mediated apoptosis in acute leukemia: a translational view. *Blood* 2001;98:3541–53.
- Lowenberg B, Downing JR, Burnett A. Acute myeloid leukemia. *N Engl J Med* 1999;341:1051–62.
- Smith M, Barnett M, Bassan R, Gatta G, Tondini C, Kern W.

- Adult acute myeloid leukaemia. *Crit Rev Oncol Hematol* 2004;50:197–222.
20. Sharoni Y, Stahl W, Danilenko M, Levy J. The anticancer activity of carotenoids: from human studies to cellular processes and gene regulation. In: Krinsky NI, Mayne ST, Sies H, editors. *Carotenoids in health and disease*. New York: Marcel Dekker; 2003. p. 165–96.
  21. Chung FL, Schwartz J, Herzog CR, Yang YM. Tea and cancer prevention: studies in animals and humans. *J Nutr* 2003;133:3268–74S.
  22. Arts IC, Hollman PC. Polyphenols and disease risk in epidemiologic studies. *Am J Clin Nutr* 2005;81:317–25S.
  23. Lee TJ, Kim OH, Kim YH, et al. Quercetin arrests G<sub>2</sub>/M phase and induces caspase-dependent cell death in U937 cells. *Cancer Lett* 2006;240:234–42.
  24. Mann J. Natural products in cancer chemotherapy: past, present, and future. *Nat Rev Cancer* 2002;2:143–8.
  25. Newman DJ, Cragg GM, Holbeck S, Sausville EA. Natural products and derivatives as leads to cell cycle pathway targets in cancer chemotherapy. *Curr Cancer Drug Targets* 2002;2:279–308.
  26. Fei XF, Wang BX, Li TJ, et al. Evodiamine, a constituent of *Evodia fructus*, induces anti-proliferating effects in tumor cells. *Cancer Sci* 2003;94:92–8.
  27. Zhang Y, Wu LJ, Tashiro S, Onodera S, Ikejima T. Intracellular regulation of evodiamine-induced A375-2 cell death. *Biol Pharm Bull* 2003;26:1543–7.
  28. Kan SF, Huang WJ, Lin LC, Wang PS. Inhibitory effects of evodiamine on the growth of human prostate cancer cell line LNCaP. *Int J Cancer* 2004;110:641–51.
  29. Liao CH, Pan SL, Guh JH, et al. Antitumor mechanism of evodiamine, a constituent from Chinese herb *Evodia fructus*, in human multiple-drug resistant breast cancer NCI/ADR-RES cells *in vitro* and *in vivo*. *Carcinogenesis* 2005;26:968–75.
  30. Darzynkiewicz Z, Juan G, Li X, Gorczyca W, Murakami T, Traganos F. Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis). *Cytometry* 1997;27:1–20.
  31. Wen J, You KR, Lee SY, Song CH, Kim DG. Oxidative stress-mediated apoptosis. The anticancer effect of the sesquiterpene lactone parthenolide. *J Biol Chem* 2002;277:38954–64.
  32. Sheng-Tanner X, Bump EA, Hedley DW. An oxidative stress-mediated death pathway in irradiated human leukemia cells mapped using multilaser flow cytometry. *Radiat Res* 1998;150:636–47.
  33. Fleury C, Mignotte B, Vayssiere JL. Mitochondrial reactive oxygen species in cell death signaling. *Biochimie* 2002;84:131–41.
  34. Widlak P, Palyvoda O, Kumala S, Garrard WT. Modeling apoptotic chromatin condensation in normal cell nuclei. Requirement for intranuclear mobility and actin involvement. *J Biol Chem* 2002;277:21683–90.
  35. Reed JC, Kitada S, Takayama S, Miyashita T. Regulation of chemoresistance by the bcl-2 oncoprotein in non-Hodgkin's lymphoma and lymphocytic leukemia cell lines. *Ann Oncol* 1994;5:61–5.
  36. Woo KJ, Yoo YH, Park JW, Kwon TK. Bcl-2 attenuates anticancer agents-induced apoptosis by sustained activation of Akt/protein kinase B in U937 cells. *Apoptosis* 2005;10:1333–43.
  37. Zhou H, Li XM, Meinkoth J, Pittman RN. Akt regulates cell survival and apoptosis at a postmitochondrial level. *J Cell Biol* 2000;151:483–94.
  38. Carroll WL, Bhojwani D, Min DJ, et al. Pediatric acute lymphoblastic leukemia. *Hematology Am Soc Hematol Educ Program* 2003;102–31.
  39. Takada Y, Kobayashi Y, Aggarwal BB. Evodiamine abolishes constitutive and inducible NF- $\kappa$ B activation by inhibiting I $\kappa$ B $\alpha$  kinase activation, thereby suppressing NF- $\kappa$ B-regulated antiapoptotic and metastatic gene expression, up-regulating apoptosis, and inhibiting invasion. *J Biol Chem* 2005;280:17203–12.
  40. Zhang Y, Wu LJ, Tashiro S, Onodera S, Ikejima T. Evodiamine induces tumor cell death through different pathways: apoptosis and necrosis. *Acta Pharmacol Sin* 2004;25:83–9.
  41. Jacobson MD. Reactive oxygen species and programmed cell death. *Trends Biochem Sci* 1996;21:83–6.
  42. Guenal I, Sidoti-de Fraise C, Gaumer S, Mignotte B. Bcl-2 and Hsp27 act at different levels to suppress programmed cell death. *Oncogene* 1997;15:347–60.
  43. Kang YH, Yi MJ, Kim MJ, et al. Caspase-independent cell death by arsenic trioxide in human cervical cancer cells: reactive oxygen species-mediated poly(ADP-ribose) polymerase-1 activation signals apoptosis-inducing factor release from mitochondria. *Cancer Res* 2004;64:8960–7.
  44. Susin SA, Lorenzo HK, Zamzami N, et al. Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* 1999;397:441–6.
  45. Joza N, Susin SA, Daugas E, et al. Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. *Nature* 2001;410:549–54.
  46. Yang J, Liu X, Bhalla K, et al. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science (Washington DC)* 1997;275:1129–32.
  47. Zhang XD, Gillespie SK, Hersey P. Staurosporine induces apoptosis of melanoma by both caspase-dependent and -independent apoptotic pathways. *Mol Cancer Ther* 2004;3:187–97.

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