RESEARCH ARTICLE

Eupafolin, a flavonoid isolated from *Artemisia princeps*, induced apoptosis in human cervical adenocarcinoma HeLa cells

Kyung-Sook Chung[1,2], Jung-Hye Choi[3], Nam-In Back[4], Myung-Sook Choi[5], Eun-Kyung Kang[6], Hae-Gon Chung[6], Tae-Sook Jeong[7] and Kyung-Tae Lee[1,2]

[1] Department of Biomedical Science, College of Medical Science, Kyung-Hee University, Seoul, South Korea
[2] Department of Pharmaceutical Biochemistry, College of Pharmacy, Kyung-Hee University, Seoul, South Korea
[3] Department of Oriental Pharmaceutical Science, College of Pharmacy, Kyung-Hee University, Seoul, South Korea
[4] Graduate School of Biotechnology and Plant Metabolism Research Center, Kyung-Hee University, Suwon, South Korea
[5] Department of Food Science and Nutrition, Kyungpook National University, Daegu, South Korea
[6] GangHwa Agricultural R&D Center, Incheon, South Korea
[7] National Research Laboratory of Lipid Metabolism, Atherosclerosis, Korea Research Institute of Bioscience and Biotechnology, Daejeon, South Korea

Although eupafolin, a flavone found in *Artemisia princeps* Pampanini, has been shown to inhibit the growth of several human cancer cells, its mode of action is poorly understood. In this study, we investigated the pro-apoptotic activities of eupafolin in human cervical carcinoma HeLa cells. It was found that eupafolin induced apoptosis in a dose-dependent manner, as evidenced by DNA fragmentation and the accumulation of positive cells for annexin V. In addition, eupafolin triggered the activations of caspases-3, -6, -7, -8, and -9 and the cleavages of their substrates, such as, poly (ADP-ribose) polymerase and lamin A/C. Furthermore, treatment with eupafolin resulted in a loss of mitochondrial membrane potential ($\Delta\Psi_m$), increased the release of cytochrome *c* to the cytosol, and altered the expression levels of B-cell lymphoma 2 (Bcl-2) family proteins. Interestingly, caspase-8, an initiator caspase, was activated after the loss of $\Delta\Psi_m$ and the activations of caspases-3 and -9. Moreover, treatment with *z*-DEVD-fmk (a specific caspase-3 inhibitor) and the overexpression of Bcl-2 prevented eupafolin-stimulated caspase-8 activation. Altogether, these results suggest that the eupafolin-induced apoptosis in HeLa cells is mediated by caspase-dependent pathways, involving caspases-3, -9, and -8, which are initiated by the Bcl-2-dependent loss of $\Delta\Psi_m$.

Received: June 30, 2009
Revised: November 10, 2009
Accepted: November 24, 2009

Keywords:
Apoptosis / Bcl-2 / Caspase / Eupafolin / Mitochondria

1 Introduction

Apoptosis, a morphologically and biochemically defined form of cell death, plays a key role in the regulation of the growth of normal and neo-plastic tissues [1]. The mechanisms that trigger apoptosis are often referred to the extrinsic-

Correspondence: Professor Kyung-Tae Lee, Department of Pharmaceutical Biochemistry, College of Pharmacy Kyung-Hee University Dongdaemun-Gu, Hoegi-Dong, Seoul, 130-701, South Korea
E-mail: ktlee@khu.ac.kr
Fax: +82-2-966-3885

Abbreviation: **$\Delta\Psi_m$,** mitochondria membrane potential; **Apaf-1,** apoptotic protease activating factor-1; **Bcl-2,** B-cell lymphoma 2; **CCCP,** carbonyl cyanide *m*-chlorophenylhydrazone; **CsA,** cyclosporine A; **DAPI,** 4′,6-diamidino-2-phenylindole-dihydrochloride; **$DiOC_6$,** 3,3′-dihexyloxacarbocyanine iodide; **FBS,** fetal bovine serum; **PARP,** poly (ADP-ribose) polymerase; **PI,** propidium iodide; **PVDF,** polyvinylidene difluoride; **TBS,** Tris-buffered saline; **VDAC,** voltage-dependent anion-selective channel; **XIAP,** X-linked inhibitor of apoptosis protein

and intrinsic-mediated pathways. The extrinsic-mediated pathway is also called the receptor-mediated pathway, and is characterized by the activations of cell surface ligand-gated death receptors, such as tumor necrosis factor receptor 1, 2 (TNFR 1, 2), Fas, and death receptors 3–6 (DR 3−6) [2]. When specific ligands bind to the extracellular domains of death receptors, receptor trimerization is triggered. Tumor necrosis factor receptor 1-associated death domain protein (TRADD) and Fas-associated death domain protein (FADD) adapter molecules then bind to the cytoplasmic domains of death receptors. Initiator caspase-8, then initiates a sequence that leads to caspase activation, either directly or through Bid cleavage, and thus, induces irreversible cell death [3]. On the other hand, the intrinsic-mediated pathway is activated directly by a variety of extracellular and intracellular death stimuli by a death receptor. During this process, mitochondria undergoing permeability transition causes a loss of mitochondrial membrane potential ($\Delta\Psi_m$) and releases apoptogenic proteins, such as, cytochrome *c*, into cytosol. This released cytochrome *c* then binds to apoptotic protease activating factor-1 (Apaf-1), which causes its oligomerization [4]. Procaspase-9 then binds to Apaf-1 oligomers to form a high-molecular-mass complex called the apoptosome [5]. In addition, this interaction activates caspase-9, which activates caspase-3, and thus, triggers the irreversible apoptotic program [6].

Artemisia herbs are widely cultivated, and their leaves have long been used in foods and in traditional medicines in East Asia. *Artemisia* species belong to the genus *Compositae*, which is composed of more than 350 species. The compounds isolated from *Artemisia* species include terpenoids, flavonoids, coumarins, acetylenes, caffeoylquinic acids, and sterols, and their constituents have been shown to have anti-malarial, anti-viral, anti-tumor, anti-pyretic, anti-hemorrhagic, anti-coagulant, anti-anginal, anti-oxidant, anti-hepatitis, anti-ulcerogenic, and anti-spasmodic activities [7].

Eupafolin (6-methoxy 5,7,30,40-tetrahydroxyflavone) (its chemical structure is shown in Fig. 1A), the flavone used in this study, was isolated from *Artemisia princeps* Pampanini (family Asteraceae). Eupafolin is known to have several biological effects, for example, it promotes iron release from ferritin, and donates electrons to the stable free radical DPPH [8]. Furthermore, it protects cultured neurons against glutamate-induced oxidative stress and inhibits xanthine oxidase activity [9, 10]. In addition, eupafolin has anti-proliferative effects on MK-1 (human gastric adenocarcinoma), B16-F10 (murine

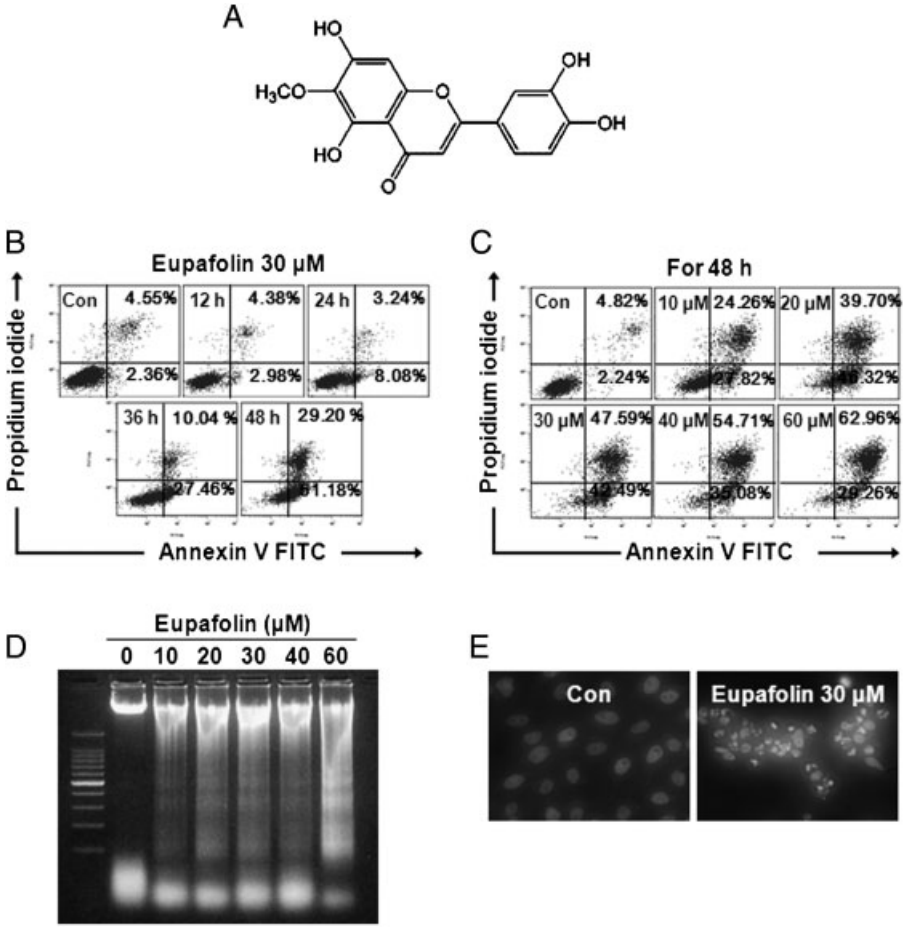

Figure 1. Effects of eupafolin on apoptotic induction in HeLa cells. (A) The chemical structure of eupafolin. (B and C) Cells were treated with eupafolin for the indicated times and concentrations and then were co-stained with PI and FITC-conjugated annexin V. The translocation of phosphatidylserine was detected by flow cytometry after eupafolin treatment. (D) Cells were treated with eupafolin (10–60 μM, 48 h) and genomic DNA was extracted and resolved on 2% agarose gels. Apoptotic DNA fragmentation was visualized by ethidium bromide staining. (E) Cells were treated with 30 μM eupafolin for 48 h, and DNA fragmentation was examined by DAPI staining by immunofluorescence microscopy.

melanoma), and HeLa (human cervical adenocarcinoma) cells [11]. However, no report has been issued on the molecular mechanism underlying its anti-proliferative effects.

Despite the widespread use of the Papanicolaou smear and HPV (human papollomavirus) vaccine, cervical cancer remains the most common cancer in women under 45 years of age in developing countries. Furthermore, almost half of all women with invasive cervical cancer are diagnosed at the later stage. Accordingly, there is an urgent need to identify an agent that can be used alone or in combination with a conventional anti-cancer drug such as cisplatin, to improve the prognosis of this disease. Cisplatin was first reported to have a therapeutic effect in cervical cancer in the early 1980s, and recently, several studies have shown that combination therapies including cisplatin (multiple drug regimens or chemo-radiation) are more active than cisplatin alone against metastatic disease and as neo-adjuvant treatment followed by surgery in patients with local disease [12]. In this study, we initially tested the anti-cancer effect of eupafolin in HeLa cells. We found that eupafolin-induced apoptosis involves the mitochondrial pathway *via* loss of $\Delta\Psi_m$, a hallmark of permeability transition, and this causes the release of cytochrome *c* to cytosol, and the sequential activations of caspases-3, -9, and -8 *via* the modulation of Bcl-2 in human cervical adenocarcinoma HeLa cells.

2 Materials and methods

2.1 Materials

Eupafolin used in this study was isolated from *Artemisia princeps* Pampanini. The physicochemical data of eupafolin were as follows: yellow amorphous powder ($CHCl_3$-MeOH); m.p. 272–274°C; IR (KBr, cm^{-1}): 3410, 1662, 1601; EI/MS *m/z* (70 eV): 316 $[M]^+$, 301, 298, 273, 167; ^{1}H-NMR (400 MHz, C_5D_5N,δ_H): 7.31 (1H, s, H-2′), 7.30 (1H, d, *J*=8.0 Hz, H-6′), 6.85 (1H, d, *J* = 8.0 Hz, H-5′), 6.46 (2H, s, H-3, 8), 3.86 (3H, s, OCH_3). ^{13}C-NMR (100 MHz, C_5D_5N,δ_C): 183.88 (C-4), 166.10 (C-2), 158.42 (C-9), 154.34 (C-5), 153.91 (C-7), 150.73 (C-4′), 146.75 (C-3′), 132.61 (C-6), 123.46 (C-1′), 120.16 (C-6′), 116.59 (C-5′), 113.98 (C-2′), 105.59 (C-10), 103.25 (C-3), 95.13 (C-8), 60.91 (OCH_3). The identity of isolated compound was confirmed by LC-MS and was found to be >98% pure. RPMI 1640 medium, FBS, penicillin, and streptomycin were obtained from Life Technologies (Grand Island, NY, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), DMSO, RNase A, leupeptin, aprotinin, phenylmethylsulfonylfluoride (PMSF), 4′,6-diamidino-2-phenylindole-dihydrochloride (DAPI), triton X-100, and propidium iodide (PI) were purchased from Sigma Chemical (St. Louis, MO, USA). Antibodies for anti-caspase-3, anti-caspase-7, anti-poly (ADP-ribose) polymerase (PARP), anti-lamin A/C, anti-Bax, anti-Bcl-2, anti-Apaf-1, anti-voltage-dependent anion-selective channel (VDAC), anti-α-tubulin, and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for anti-X-linked inhibitor of apoptosis protein (XIAP), anti-caspase 6, anti-caspase-8, and anti-cytochrome *c* antibodies were purchased from BD Biosciences, Pharmingen (San Diego, CA, USA). Antibody for anti-caspsae-9 and anti-Bid antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). *z*-VAD-fmk and *z*-DEVD-fmk, *z*-IETD-fmk, and *z*-LEHD-fmk were purchased from Calbiochem (Bad Soden, Germany).

2.2 Cell culture and MTT assay

HeLa (human cervical adenocarcinoma), A549 (human lung adenocarcinoma), A172 (human glioblastoma), SK-OV-3 (human ovarian adenocarcinoma), L-132 (human lung epithelial cell line), Chang (human epithermal conjunctival cell line) were obtained from the Korean cell line bank (KCLB, Seoul, South Korea) and IOSE-80PC (immortalized ovarian surface epithelial cell line) was kindly provided by Dr. Auersperg (University of British Columbia, Canada) and A. Godwin (Fox Chase Cancer Center, Philadelphia, PA, USA). Cells were cultured in RPMI 1640 or DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 U/mL) and streptomycin sulfate (100 μg/mL) (Life Technologies). Cells were cultured at 37°C in an atmosphere of 5% CO_2 in the presence or absence of the eupafolin. The cytotoxicity was assessed using a MTT assay. Briefly, the cells (5×10^4) were seeded in each well containing 100 μL of the RPMI medium supplemented with 3% FBS in a 96-well plate. After 24 h, various concentrations of eupafolin were added. After 48 h, 50 μL of MTT (5 mg/mL stock solution) was added and the plates were incubated for an additional 4 h. The medium was discarded and the formazan blue, which was formed in the cells, was dissolved with 100 μL DMSO. The OD was measured at 540 nm.

2.3 Annexin V and PI double staining by flow cytometry

During apoptosis, exposure of phosphatidylserine on the exterior surface of the plasma membrane can be detected by the binding of fluoresceinated annexin V (annexin V-FITC). This assay is combined with analysis of the exclusion of the plasma membrane integrity probe PI [13, 14]. For annexin V and PI double staining, cells were suspended with 100 μL of binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) and stained with 5 μL of FITC-conjugated annexin V and 5 μL of PI (50 μg/mL). The mixture was incubated for 15 min at room temperature in dark place and analyzed by the fluorescence-activated cell sorting (FACS) cater-plus flow cytometry (Becton Dickinson, Heidelberg, Germany).

2.4 DNA fragmentation assay

Analysis of DNA fragmentation by agarose gel electrophoresis was performed as described previously [15]. Briefly, cells were

washed in PBS and resuspended in ice-cold lysis buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.2% triton X-100). After incubating for 15 min at 48°C, cell lysates were centrifuged at 14 000 × *g* for 15 min to separate low molecular weight DNA from intact chromatin. The supernatant was treated with 0.2 mg/mL of proteinase K in a buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 40 mM EDTA and 1% SDS for 4 h at 37°C. The DNA preparations were phenol/chloroform extracted twice to remove proteins. DNA was precipitated with 140 mM NaCl and two volumes of ethanol at −20°C overnight. DNA precipitates were recovered by centrifugation at 14000 × g for 15 min at 4°C, washed twice in cold 70% ethanol and air-dried. DNA pellets were resuspended in 15 mL of TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) and treated with RNase-DNase free (Boehringer Mannheim, Mannheim, Germany) for 1 h at 37°C. Electrophoresis was performed in a 2.0% w/v agarose gel in 40 mM Tris-acetate buffer (pH 7.4) at 50 V for 1 h. The fragmented DNA was visualized by staining with ethidium bromide after electrophoresis.

2.5 DAPI staining

DAPI, a DNA-binding fluorescent dye, was used to determine whether the mechanism of growth inhibition after eupafolin treatment is through apoptosis. After treatment with eupafolin (30 μM) for 48 h, the cells were washed three times with PBS, fixed in a 3.7% formaldehyde solution for 10 min, then stained with DAPI for 10 min. Apoptotic cells were identified by features characteristic of apoptosis (*e.g.* nuclear condensation, formation of membrane blebs and apoptotic bodies) and then examined in a Olympus IX51 fluorescent microscopy (Olympus, Tokyo, Japan).

2.6 Cell fractionation and Western blot analysis

Eupafolin-treated cells were washed with ice-cold PBS and extracted by mitochondrial fractionation kit (Activemotif, CA, USA). Cells were collected by centrifugation (600 × g, 5 min, 4°C). The cells were then washed twice with ice-cold PBS, and centrifuged (600 × g, 5 min, 4°C). The cell pellet obtained was then resuspended in ice-cold cytosolic buffer for 15 min on ice. The cells were then homogenized with a glass dounce and a B-type pestle (80 strokes), homogenates were spun at 10 000 × *g* for 20 min at 4°C, and the supernatant (cytosolic fraction) was removed whilst taking care to avoid the pellet. The resulting pellet (mitochondrial fraction) was resuspended in completed mitochondria buffer. For total cell protein extracts, eupafolin-treated cells were washed with ice-cold PBS and extracted in protein lysis buffer (Intron, Seoul, South Korea). Protein concentration was determined by Bradford assay. Protein samples of cell lysate were mixed with an equal volume of 5 × SDS sample buffer, boiled for 4 min, and then separated by 10–12% SDS-PAGE gels. After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked in 5% non-fat dry milk for 1 h, rinsed, and incubated with specific antibodies against Apaf-1, Bid, Bcl-2, Bax, caspases-3, -6, -7, -8, and -9, PARP, lamin A/C, XIAP, cytochrome *c*, VDAC, α-tubulin, and β-actin in Tris-buffered saline (TBS) containing Tween-20 (0.1%) overnight at 4°C. Primary antibody was removed by washing the membranes three times in TBS-T, and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (1:1000–2000). Following three times of washing in TBS-T, immuno-positive bands were visualized by enhanced chemiluminescence and exposed to X-ray film (Amersham, Piscataway, NJ, USA).

2.7 Determination of caspase activity

The enzymatic activity of the caspases induced by eupafolin was assayed using a colorimetric assay kit (Calbiochem) according to the manufacturer's protocol. Briefly, the cells were harvested and lysed in a lysis buffer for 5 min on an ice bath. The lysed cells were centrifuged at 10 000 × *g* for 10 min, and 10 μg of the protein was incubated with 80 μL of an assay buffer and 10 μL of the substrate, respectively, at 37°C for 2 h. The OD of the reaction mixture was quantified spectrophotometrically at a wavelength of 405 nm.

2.8 Analysis of $\Delta\Psi_m$

Dissipation of $\Delta\Psi_m$ occurs early during apoptosis and is detected using the 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$), membrane-permeable lipophilic cationic fluorochromes. The $DiOC_6$ uptake by charged mitochondria driven by the transmembrane potential is detected by the shift in color of fluorescence. However, depolarization is evidenced by a decrease in $DiOC_6$ uptake when undergoing apoptosis [16]. Changes in $\Delta\Psi_m$ were monitored by flow cytometric analysis. Cells were incubated with 50 nM $DiOC_6$ for 30 min, washed twice with PBS, and analyzed by flow cytometric analysis (Becton Dickinson) with excitation and emission settings of 484 and 500 nm, respectively. To ensure that $DiOC_6$ uptake was specific for $\Delta\Psi_m$, we also treated cells with 100 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) or 5 μM cyclosporine A (CsA). CCCP was used as a reference depolarizing agent and CsA was used as an inhibitor of mitochondrial permeability transition.

2.9 Transfections

Wild-type HeLa cells were transfected with 16 μg of plasmid DNA (pMig-Bcl-2 and pc DNA 3.1) by Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and then incubated for 36–48 h prior to the treatment of eupafolin. The pMig-Bcl-2 is made

available at the non-profit plasmid repository Addgene (http://www.addgene.org).

2.10 Statistical analysis

Data presented are the means ± SD of results from three independent experiments with similar patterns. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ *versus* eupafolin-treated group, significance of difference between treated groups by Student's *t*-test.

3 Results

3.1 Eupafolin-induced apoptosis in HeLa cells

The cytotoxic effects of eupafolin were examined in various cancer cell lines and in normal cell lines using MTT assays. Its effects were assessed using IC_{50} values (Table 1). Interestingly, eupafolin was found to be less cytotoxic on normal cells than cisplatin (the positive control). In HeLa human cervical cancer cells, eupafolin showed a substantial cytotoxic effect with an IC_{50} of 26.75 μM at 48 h. Accordingly, further experiments were performed to evaluate the effects of eupafolin on apoptosis and to identify the molecular mechanisms involved in HeLa cells. To determine whether the cytotoxic effect of eupafolin is associated with the induction of apoptosis, HeLa cells were treated with eupafolin, and then analyzed by flow cytometry for the early apoptotic marker annexin V. Proportions of annexin V-positive cells (early apoptotic cells) were found to increase in a time- and dose-dependent manner after treatment with eupafolin (Fig. 1B and C). To further characterize the end stage of apoptosis induced by eupafolin, we examined whether treatment with eupafolin induces a typical ladder pattern of internucleosomal DNA fragmentation in HeLa cells. As shown in Fig. 1D, this laddering pattern of internucleosomal DNA fragmentation was observed after treating HeLa cells with eupafolin, and it was found to occur in a dose-dependent manner. In addition, eupafolin-induced apoptosis was also confirmed by DAPI staining (Fig. 1E), and eupafolin was found to significantly increase the formation of apoptotic bodies and chromatin condensation. Altogether, these findings indicate that eupafolin-induced cell death in HeLa cells is due to apoptotic rather than necrotic cell death.

Table 1. Cytotoxic activity of eupafolin on cancer cell growth *in vitro*

Cell lines	Origin	IC_{50} (μM)[a)]	
		Eupafolin	Cisplatin
HeLa	Human cervical adenocarcinoma	26.75	52.26
A549	Human lung adenocarcinoma	53.34	82.92
A172	Human glioblastoma	145.70	137.77
SKOV-3	Human ovarian adenocarcinoma	423.13	153.80
L-132	Human lung epithelial	46.10	8.32
Chang	Human epithelial conjunctival	49.77	5.94
IOSE-80PC	Immortalized ovarian surface epithelial	203.95	30.38

a) IC_{50} is defined as the concentration that results in a 50% decrease in the number of cells compared to that of the control cultures in the absence of eupafolin. The values represent the means of three independent experiments with similar patterns. Cisplatin is treated as the positive control.

3.2 The apoptosis of eupafolin-induced HeLa cells required caspase activities

The apoptosis triggered by various cytotoxic agents is highly dependent on the activations of caspases, which play pivotal roles in the proteolysis of specific targets [17]. To characterize eupafolin-triggered apoptosis, we investigated whether treatment with eupafolin leads to the activations of caspases in HeLa cells. Accordingly, cells were treated with serial concentrations of eupafolin for 48 h, and then Western blotting was performed. As shown in Fig. 2A, eupafolin was found to induce the dose-dependent activations of initiator caspases, including caspase-8 and -9. The activations of these caspases were demonstrated by decreases in the levels of procaspase forms and the appearances of their corresponding cleaved bands. In addition, we examined the activations of effector caspases, such as, caspase-3, -6, and -7, after treatment with eupafolin. The cleavage forms of caspases-3, -6, and -7 were found to increase in a dose-dependent manner after eupafolin treatment, and these inductions were accompanied by dose-dependent increases in the cleavages of their substrates (PARP and lamin A/C) (Fig. 2B). To monitor the enzymatic activities of caspases-3, -8, and -9, caspase activity assays were performed after treatment of cells with eupafolin. As shown in Fig. 2C, eupafolin induced significant dose-dependent increases in the activities of caspases-3, -8, and -9.

To further confirm the involvement of caspases in eupafolin-induced apoptosis, various caspase inhibitors, namely, *z*-VAD-fmk (a broad caspase inhibitor), *z*-DEVD-fmk (a specific caspase-3 inhibitor), *z*-IETD-fmk (a specific caspase-8 inhibitor), and *z*-LEHD-fmk (a specific caspase-9 inhibitor) were used at concentrations that completely blocked the activations of their corresponding caspases. As shown in Fig. 2D, *z*-VAD-fmk, *z*-DEVD-fmk, *z*-IETD-fmk, and *z*-LEHD-fmk all significantly suppressed eupafolin-induced apoptosis by 89.11, 80.16, 76.14, and 80.94%, respectively. Similarly, pretreatment with *z*-VAD-fmk completely attenuated eupafolin-induced apoptotic body formation (Fig. 2E). These observations indicate that eupafolin-induced apoptosis involves the caspase-dependent pathway in HeLa cells.

To elucidate the signaling events involved in eupafolin-induced apoptosis, we examined the effects of eupafolin on

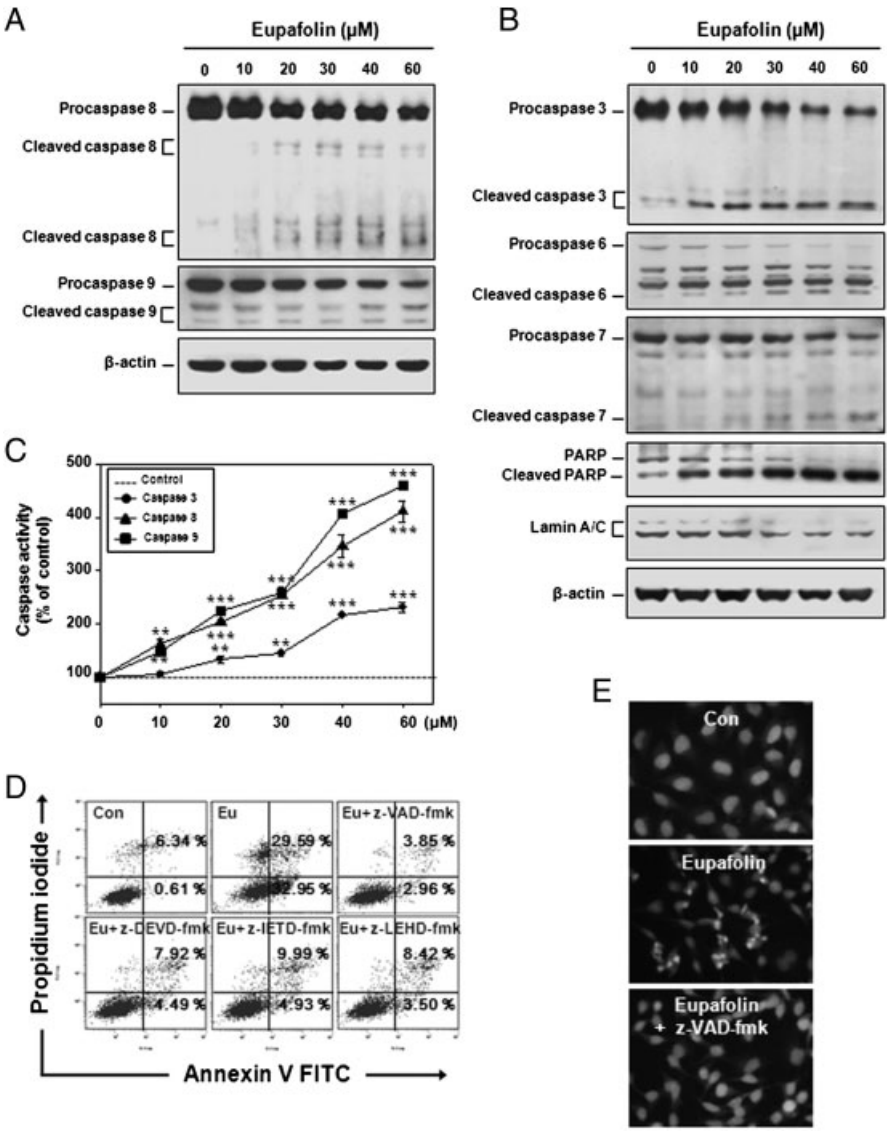

Figure 2. Activations of caspases during eupafolin-induced apoptosis. (A) Eupafolin induced the activations of caspases-8 and -9 in HeLa cells. Cells were treated with eupafolin (10–60 μM for 48 h) and then harvested. Total cell lysates were prepared and separated by SDS-PAGE, transferred to PVDF membranes, and blotted with caspases-8 and -9 antibodies. β-actin was used as an internal control. (B) Eupafolin-induced activations of caspases-3, -6, and -7, and the cleavages of PARP and lamin A/C. (C) The enzymatic activities of caspases-3, -8, and -9 were induced by eupafolin in HeLa cells. Cells were treated with eupafolin (10–60 μM for 48 h) and then harvested. The enzymatic activities of caspases induced by eupafolin were assayed using a colorimetric assay. Data are presented means ±SD of results obtained from three independent experiments. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ by the Student's *t*-test. (D) Cells were pretreated with 50 μM *z*-VAD-fmk, 100 μM *z*-DEVD-fmk, 100 μM *z*-IETD-fmk, or 100 μM *z*-LEHD-fmk for 1 h, and then treated with or without eupafolin (30 μM for 48 h). Cells were co-stained with PI and FITC-conjugated annexin V, and the translocation of PS was detected using flow cytometry after eupafolin treatment. (E) Cells were pretreated with 50 μM *z*-VAD-fmk for 1 h, and then treated with or without eupafolin (30 μM for 48 h). DNA fragmentation was observed by DAPI staining, as described in Methods.

the kinetics of the activations of caspases-3, -8, and -9. As shown in Fig. 3, caspase-9 activation was detected at 3 h after eupafolin treatment and peaked at 48 h, whereas caspase-3 activation was evident at 12 h and caspase-8 activation was visible only at 24–48 h. These findings suggest that eupafolin stimulates the activation of caspase-9 initially, and this results in downstream activation of caspase-3, which then initiates caspase-8 activation.

3.3 Eupafolin induced mitochondrial membrane depolarization and modulated the expressions of Bcl-2 family proteins

The activation of caspase-9 by eupafolin suggested that the mitochondrial apoptotic pathway is involved in eupafolin-induced apoptosis in HeLa cells. Mitochondria plays an essential role in apoptosis induction. In particular, transition pore opening in the mitochondrial membrane is known to induce a collapse of $\Delta\Psi_m$, which results in the rapid release of caspase activators from mitochondria [18]. Therefore, we investigated the effects of eupafolin on $\Delta\Psi_m$ using $DiOC_6$, a mitochondria-specific voltage-dependent dye, and the protonophore CCCP was used to induce mitochondrial membrane depolarization. As shown in Fig. 4A and B, mitochondrial membrane depolarization was visible at 3 h after treatment with eupafolin and this increased at 48 h. Furthermore, pretreatment with CsA, an inhibitor of mitochondrial permeability transition, attenuated eupafolin-induced loss of the $\Delta\Psi_m$, which suggests that eupafolin-induced caspase-9 activation is mediated by loss of the $\Delta\Psi_m$ in HeLa cells.

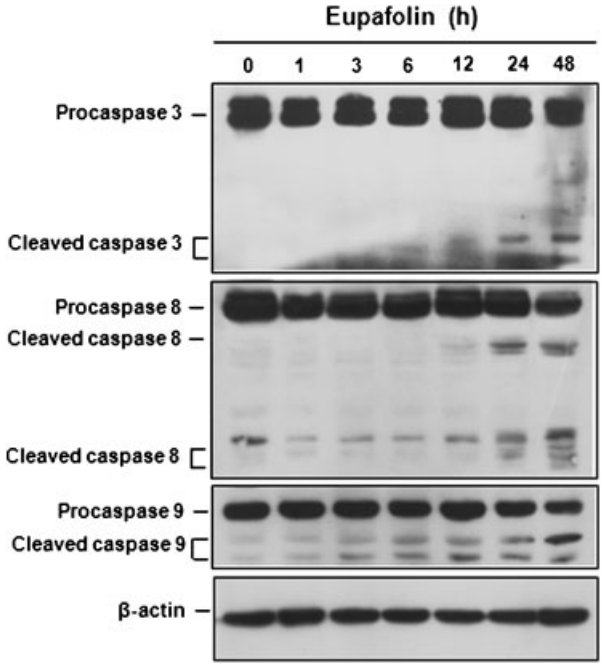

Figure 3. Effects of eupafolin on the kinetics of the activations of caspases -3, -8, and -9. Cells were treated with eupafolin for the indicated times and then harvested. Total cell lysates were prepared, separated by SDS-PAGE, transferred to PVDF membranes, and blotted with caspases-3, -8, and -9 specific antibodies. β-actin was used as an internal control.

Disruption of $\Delta\Psi_m$ is usually associated with the diffusion of cytochrome *c* into the cytosol, which normally locates in the space between the inner and outer mitochondrial membranes [19]. Cytosolic cytochrome *c* activates procaspase-9 by binding to Apaf-1, which leads to caspase-9 activation and the subsequent activations of downstream executioner caspases (caspases-3, -6, and -7) [4]. As shown in Fig. 4C, levels of cytosolic cytochrome *c* and Apaf-1 were elevated by eupafolin in HeLa cells, whereas XIAP levels decreased, and thus suggesting the involvement of the mitochondrial pathway in eupafolin-induced apoptosis.

Since Bcl-2 family proteins are known to control apoptosis that occurs *via* the mitochondrial pathway by maintaining a balance between pro- and anti-apoptotic members, we examined the effects of eupafolin on levels of Bcl-2 family proteins in HeLa cells. Our results showed that eupafolin decreased the cytosolic levels of pro-apoptotic Bid, but increased tBid mitochondrial levels. However, we did not find any changes in Bax levels in mitochondria or cytosol. On the other hand, levels of Bcl-2 were reduced after eupafolin treatment (Fig. 5A). These findings suggest that eupafolin modulates the protein levels of Bid and Bcl-2, and this results in $\Delta\Psi_m$ loss and the release of cytochrome *c* from mitochondria. To confirm whether eupafolin-induced apoptosis is mediated by the Bcl-2-dependent mitochondrial pathway, we investigated the pro-apoptotic activity of eupafolin in Bcl-2-transfected HeLa cells. As shown in Fig. 5B, loss of $\Delta\Psi_m$ in response to 30 μM of eupafolin was detected in empty vector-transfected HeLa cells, whereas no significant change in $\Delta\Psi_m$ was observed in Bcl-2-transfected HeLa cells. In agreement with these findings, eupafolin induced apoptosis (60.46% annexin V-positive cells) in empty vector-transfected HeLa cells, whereas Bcl-2 overexpression markedly abolished the apoptosis induction by eupafolin (Fig. 5C).

3.4 Caspase-8 activation by eupafolin in HeLa cells occurred *via* a Bcl-2-dependent pathway

Although no significant increase was observed in the protein expression levels of death domains, death receptors, or their ligands following eupafolin treatment (data not shown), the results mentioned above suggest that eupafolin-induced apoptosis involves the activation of caspase-8 in HeLa cells. Numerous reports have suggested that caspase-8 can be activated in a death-receptor pathway-independent manner [20, 21]. Furthermore, several studies have demonstrated that caspase-8 activation can be triggered by a caspase-3-mediated feedback amplification loop [22, 23]. Therefore, we further examined whether caspase-3 activation is involved in the activation of caspase-8 in eupafolin-treated HeLa cells. It was found that *z*-DVED-fmk (a caspase-3 inhibitor) pretreatment significantly abolished the eupafolin-stimulated activation of caspase-8 (Fig. 6A).

Considering that Bcl-2 overexpression prevented eupafolin-induced apoptosis, we further evaluated caspase-8 activation after treatment with eupafolin in Bcl-2-transfected HeLa cells. As shown in Fig. 6B, eupafolin-induced caspase-8 activation was observed in empty vector-transfected cells, but not in Bcl-2-transfected HeLa cells. These results clearly indicate that the Bcl-2-dependent mitochondrial apoptotic pathway, including caspase-3 activation, is required for the sequential activation of caspase-8 during the eupafolin-induced apoptosis of HeLa cells.

4 Discussion

As an important resource of anti-cancer agent, many plant-derived substances have been shown to have various bioactivities. For example, *Artemisia* species are widely used in traditional medicine in East Asia, and have been reported to show anti-mutagenic and anti-inflammatory effects [24, 25]. Moreover, the flavonoids, eupatilin, jaceosidin, and eupafolin are the most well known pharmacologically active principles in *Artemisia* species, and many reports have been issued on the pharmacological activities of eupatilin [26, 27] and jaceocidin [28–30]. Although eupafolin, eupatilin, and jaceocidin have similar structures, relatively few reports have been issued on the anti-proliferative effects of eupafolin [11], and the molecular mechanism involved in eupafolin-induced apoptosis was poorly understood. Interestingly,

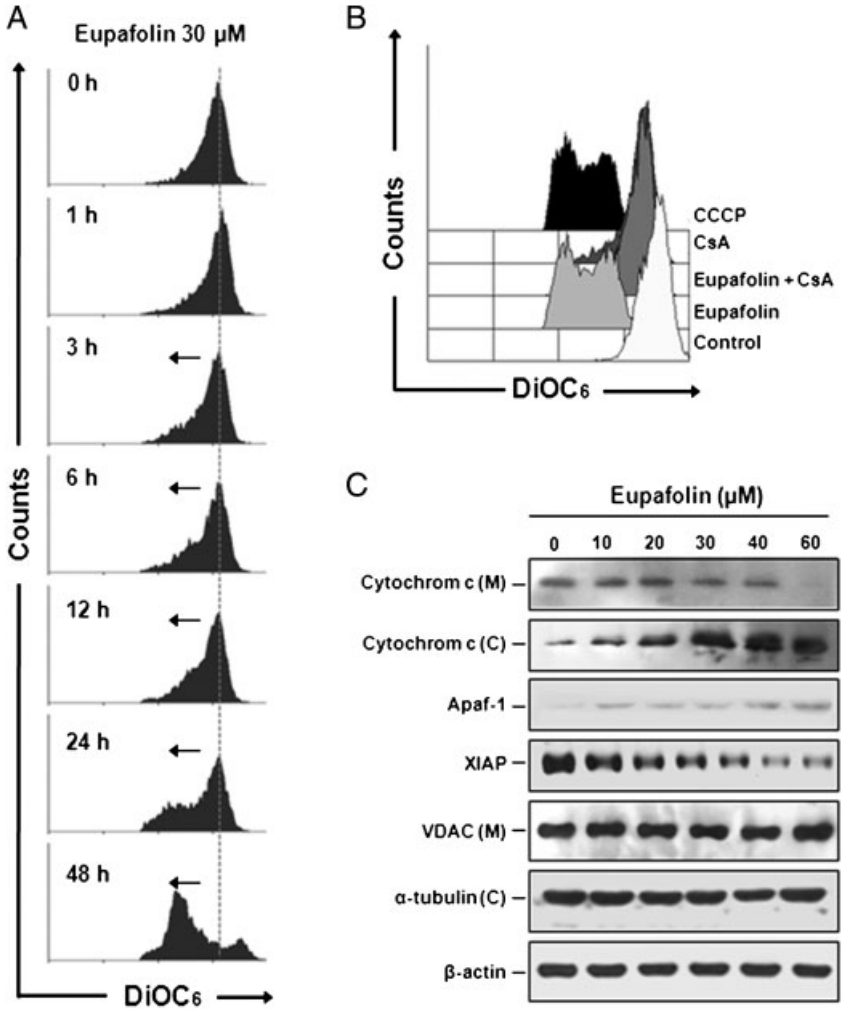

Figure 4. Disruption of $\Delta\Psi_m$ and the modulation of cytochrome *c*, Apaf-1, and XIAP by eupafolin in HeLa cells. (A) Cells were treated with 30 μM of eupafolin for the indicated times, stained with $DiOC_6$, and analyzed by flow cytometry, as described in Methods. (B) Cells were treated with 30 μM of eupafolin for 48 h, stained with $DiOC_6$, and analyzed by flow cytometry, as described in Methods. CCCP (100 μM) was used as a positive control. Cells were also pretreated with CsA (5 μM) for 30 min, treated with eupafolin (30 μM) for 48 h, stained with $DiOC_6$, and analyzed by flow cytometry. (C) Cells were treated with eupafolin (10–60 μM, 48 h) and then harvested. Total cell lysates and mitochondrial (M) and cytosolic (C) fractions were prepared as described in Methods. Equal amounts of protein were separated by 12% SDS-PAGE, transferred to PVDF membranes, and blotted with cytochrome *c*, Apaf-1, and XIAP specific antibodies. VDAC, α-tubulin, and β-actin were used as internal controls.

it has been reported that flavonoids sensitize cancer cells to apoptosis induced by anti-cancer agents [31–33]. In this study, we tested the pro-apoptotic effects of eupafolin, a flavonoid isolated from *Artemisia princeps*, in HeLa cells (human cervical adenocarcinoma cells). Despite bioavailability concern of flavone compounds, natural or synthetic flavones compounds have often been evaluated as a potential therapeutics for several cancers. For example, the FDA recently granted a fast-track designation for the use of the flavonoid, phenoxodiol for the treatment of recurrent ovarian cancer resistant to platins and taxanes. In this study, we found that eupafolin is potent when compared with cisplatin in HeLa cells, which encourages further study on its pro-apoptotic effects *in vivo* model of cervical cancer. Furthermore, due to the several promising reports issued recently on combination therapies, we are now investigating the anti-cancer effects of eupafolin in combination with other anti-cancer drugs.

The caspases are a family of aspartate-specific cysteine proteases, and play important roles in the apoptosis triggered by various pro-apoptotic signals [34, 35]. In general, caspases can be categorized as initiator and downstream effector caspases. [36]. The initiator caspases, such as, caspase-8, -9, and -10, contain a long pro-domain that is used to recruit enzymes to high molecular weight activation platforms, such as, apoptosomes in the mitochondrial pathway or death-induced signaling complex in the death receptor pathway [37, 38]. Effector caspases, such as, caspase-3, -6 and -7, in contrast, generally contain only a small pro-domain and cleave diverse cellular substrates, such as, PARP and lamin A/C, which also leads to apoptosis.

In this study, we found that eupafolin activates caspases-3, -6, -7, -8, and -9, and increases the cleavages of their substrates (PARP and lamin A/C). In addition, *z*-VAD-fmk (a broad caspase inhibitor), *z*-DEVD-fmk (a caspase-3 inhibitor), *z*-IETD-fmk (a caspase-8 inhibitor), and *z*-LEHD-fmk (a caspase-9 inhibitor) potently prevented eupafolin-induced apoptosis, indicating that eupafolin induces caspase-dependent apoptosis in HeLa cells. Caspase-8 is a key mediator of the apoptotic signals triggered by death receptors, such as, CD95/Fas/Apo1, TNFR1, 2, and DR 3–6 [39]. Furthermore,

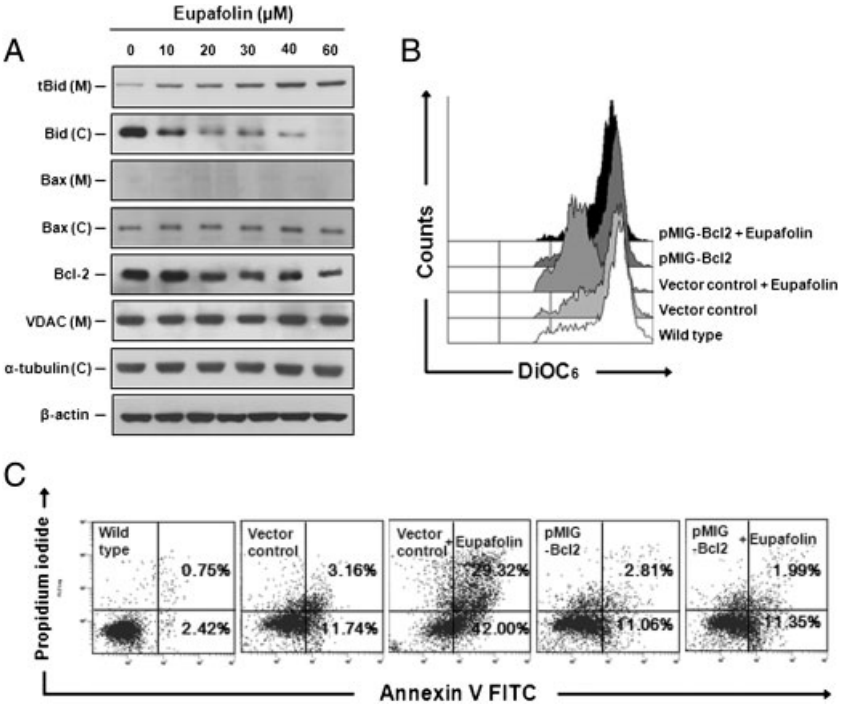

Figure 5. Effects of eupafolin on the levels of Bcl-2 family proteins in HeLa cells. (A) Cells were treated with eupafolin for 48 h and then harvested. Total cell lysates, and mitochondrial (M) and cytosolic (C) fractions were prepared as described in Methods. Equal amounts of protein were separated by 12% SDS-PAGE, transferred to PVDF membranes, and blotted with Bid, Bax, and Bcl-2 specific antibodies. VDAC, α-tubulin, and β-actin were used as internal controls. (B) Control vector or Bcl-2-transfected cells were treated with eupafolin for 48 h. Cells were then processed to determine for apoptosis by flow cytometry after PI and FITC-conjugated annexin V staining. (C) $\Delta\Psi_m$ levels were determined using $DiOC_6$ and flow cytometry, as described in Methods.

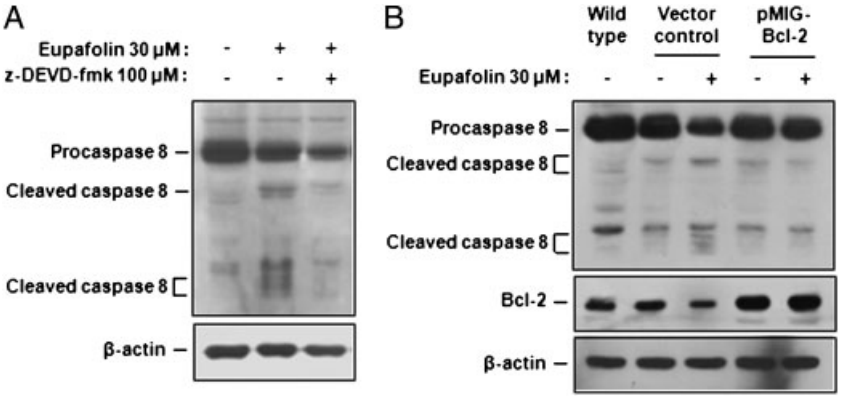

Figure 6. Effects of caspase-3 and Bcl-2 overexpression on eupafolin-induced caspase-8 activation. (A) Cells were pretreated with 100 μM *z*-DEVD-fmk for 1 h, then eupafolin (30 μM, for 48 h), and harvested. Total cell lysates were prepared and separated by SDS-PAGE, transferred to PVDF membranes, and blotted with caspase-8 specific antibody. β-Actin was used as an internal control. (B) Control vector or Bcl-2-transfected cells were treated with eupafolin for 48 h. Cell lysates were then prepared and separated by SDS-PAGE. Proteins were transferred to PVDF membranes, and blotted with caspase-8 and Bcl-2 specific antibodies. β-actin was used as the internal control.

in the case of the TRAIL receptors and CD95/Fas/Apo1, caspase-8 is directly recruited to death-induced signaling complex by the adapter protein FADD [40, 41]. On the other hand, several reports have claimed that caspase-8 can be activated in a death receptor-independent manner [23, 42, 43]. In earlier studies, caspase-8 activation was found to be triggered by a caspase-3-mediated feedback amplification loop, and caspase-8 was proposed to act as an executioner caspase, rather than as an initiator caspase, in the death receptor-independent activation pathway. Considering that eupafolin was found to induce the activation of caspase-8 without provoking death receptor signaling (data not shown), we hypothesized that caspase-3 is required for the activation of caspase-8 during eupafolin-induced apoptosis. We found that *z*-DVED-fmk (a caspase-3 inhibitor) pretreatment and the overexpression of Bcl-2 inhibited eupafolin-induced caspase-8 activation. Furthermore, caspase-8 became active after the activations of caspases-3 and -9 in HeLa cells treated with eupafolin. Altogether, these findings indicate that eupafolin induces the death receptor-independent activation of caspase-8 *via* the sequential activations of caspases-9 and -3.

Bcl-2 family proteins are critical regulators of the mitochondrial apoptotic pathway. In humans, more than 20 members of this family have been identified including proteins that suppress apoptosis (*e.g.* Bcl-2, Bcl-xL, and Bcl-1) and proteins that promote apoptosis (*e.g.* Bax, Bak, Bid) [44]. These pro- and anti-apoptotic proteins may control apoptosis by the formation of heterodimers among them [45], which results in the mutual neutralization of bound pro- and anti-apoptotic proteins. Therefore, the balance between the expression levels of pro- and anti-apoptotic proteins is critical for cell survival or cell death. In this study, a decrease in Bcl-2 expression and an increase in tBid expression were observed after treating HeLa cells with eupafolin. Furthermore, the overexpression of Bcl-2 not only inhibited eupafolin-induced cell death but also prevented the $\Delta\Psi_m$ changes induced by eupafolin exposure. This finding demonstrates that eupafolin-induced apoptosis is mediated *via* the mitochondrial death pathway and this mediation is regulated by Bcl-2.

The permeabilization of mitochondrial membranes facilitates cytochrome *c* release from mitochondria, which interacts with Apaf-1 and caspase-9 to initiate a protease cascade [46]. Our investigations show that the dissipation of $\Delta\Psi_m$ occurred at 3 h after eupafolin treatment and this coincided with caspase-9 activation. In addition, increased levels of cytosolic cytochrome *c* and Apaf-1 were observed in HeLa cells undergoing eupafolin-induced apoptosis. These results also suggest that eupafolin-induced apoptosis is a consequence of the activation of the mitochondrial apoptotic pathway.

Defects in the apoptotic process promote tumor progression, immune evasion by neo-plastic cells, and resistance to chemotherapy and irradiation [47]. Several genes have been identified that are critically required for the regulation of apoptosis. XIAP, a member of the IAP family, is believed to act as a key determinant of apoptosis resistance by effectively inhibiting the activations of caspases-3, -7, and -9 [48]. Furthermore, high expressions of XIAP have been reported in many malignant tumor types, such as, in carcinomas of the breast, ovaries, lung, pancreas, cervix, and prostate [49], and interestingly, in this study, we also found that treatment with eupafolin down regulated XIAP in HeLa cells. In summary, our data provide experimental evidence that eupafolin mediates the apoptosis of HeLa cells by downregulating Bcl-2, reducing $\Delta\Psi_m$, inducing mitochondrial cytochrome *c* release, and triggering the activations of caspases-9, -3, and -8. Based on these findings, we suggest that eupafolin should be considered as a potential therapeutic and chemopreventive for the treatment of human cervical adenocarcinoma.

This research was supported by a grant from the GangHwa County for the Investigation of Biological Active Components and Evaluation of Pharmacological Efficacy in GangHwa Indigeous Crops.

The authors have declared no conflict of interest.

5 References

[1] Ghobrial, I. M., Witzig, T. E., Adjei, A. A., Targeting apoptosis pathways in cancer therapy. *CA Cancer J. Clin.* 2005, *55*, 178–194.

[2] Ashkenazi, A., Dixit, V. M., Death receptors: signaling and modulation. *Science* 1998, *281*, 1305–1308.

[3] Thorburn, A., Death receptor-induced cell killing. *Cell Signal.* 2004, *16*, 139–144.

[4] 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.

[5] Zou, H., Li, Y., Liu, X., Wang, X., An APAF-1, cytochrome *c* multimeric complex is a functional apoptosome that activates procaspase-9. *J. Biol. Chem.* 1999, *274*, 11549–11556.

[6] Mueller, T., Voigt, W., Simon, H., Fruehauf, A. *et al.*, Failure of activation of caspase-9 induces a higher threshold for apoptosis and cisplatin resistance in testicular cancer. *Cancer Res.* 2003, *63*, 513–521.

[7] Tan, R. X., Zheng, W. F., Tang, H. Q., Biologically active substances from the genus *Artemisia*. *Planta Med.* 1998, *64*, 295–302.

[8] Dabaghi-Barbosa, P., Mariante Rocha, A., Franco da Cruz Lima, A., Heleno de Oliveira, B. *et al.*, Hispidulin: antioxidant properties and effect on mitochondrial energy metabolism. *Free Radic. Res.* 2005, *39*, 1305–1315.

[9] Kim, S. R., Park, M. J., Lee, M. K., Sung, S. H. *et al.*, Flavonoids of Inula britannica protect cultured cortical cells from necrotic cell death induced by glutamate. *Free Radic. Biol. Med.* 2002, *32*, 596–604.

[10] Sanz, M. J., Ferrandiz, M. L., Cejudo, M., Terencio, M. C. *et al.*, Influence of a series of natural flavonoids on free radical generating systems and oxidative stress. *Xenobiotica* 1994, *24*, 689–699.

[11] Abe, F., Nagao, T., Okabe, H., Antiproliferative constituents in plants 9, Aerial parts of Lippia dulcis and Lippia canescens. *Biol. Pharm. Bull.* 2002, *25*, 920–922.

[12] Tattersall, M. H., Chemotherapy in cervical cancer: is cisplatin alone still the standard of care? *Ann. Oncol.* 2001, *12*, 883.

[13] Pozarowski, P., Grabarek, J., Darzynkiewicz, Z., Flow cytometry of apoptosis. *Curr. Protoc. Cell Biol.* 2004, Chapter 18, Unit 18 18.

[14] Moore, A., Donahue, C. J., Bauer, K. D., Mather, J. P., Simultaneous measurement of cell cycle and apoptotic cell death. *Methods Cell Biol.* 1998, *57*, 265–278.

[15] Bellosillo, B., Dalmau, M., Colomer, D., Gil, J., Involvement of CED-3/ICE proteases in the apoptosis of B-chronic lymphocytic leukemia cells. *Blood* 1997, *89*, 3378–3384.

[16] Koning, A. J., Lum, P. Y., Williams, J. M., Wright, R., DiOC6 staining reveals organelle structure and dynamics in living yeast cells. *Cell Motil. Cytoskeleton* 1993, *25*, 111–128.

[17] Gupta, S., Molecular steps of death receptor and mitochondrial pathways of apoptosis. *Life Sci.* 2001, *69*, 2957–2964.

[18] Zamzami, N., Marchetti, P., Castedo, M., Zanin, C. *et al.*, Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death *in vivo*. *J. Exp. Med.* 1995, *181*, 1661–1672.

[19] Von Ahsen, O., Waterhouse, N. J., Kuwana, T., Newmeyer, D. D. *et al.*, The 'harmless' release of cytochrome *c*. *Cell Death Differ.* 2000, *7*, 1192–1199.

[20] Wesselborg, S., Engels, I. H., Rossmann, E., Los, M. *et al.*, Anticancer drugs induce caspase-8/FLICE activation and apoptosis in the absence of CD95 receptor/ligand interaction. *Blood* 1999, *93*, 3053–3063.

[21] Sohn, D., Schulze-Osthoff, K., Janicke, R. U., Caspase-8 can be activated by interchain proteolysis without receptor-triggered dimerization during drug-induced apoptosis. *J. Biol. Chem.* 2005, *280*, 5267–5273.

[22] Yang, S., Thor, A. D., Edgerton, S., Yang, X., Caspase-3 mediated feedback activation of apical caspases in doxorubicin and TNF-alpha induced apoptosis. *Apoptosis* 2006, *11*, 1987–1997.

[23] von Haefen, C., Wieder, T., Essmann, F., Schulze-Osthoff, K. *et al.*, Paclitaxel-induced apoptosis in BJAB cells proceeds *via* a death receptor-independent, caspases-3/-8-driven mitochondrial amplification loop. *Oncogene* 2003, *22*, 2236–2247.

[24] Nakasugi, T., Nakashima, M., Komai, K., Antimutagens in gaiyou (*Artemisia argyi* levl. et Vant.). *J. Agric. Food Chem.* 2000, *48*, 3256–3266.

[25] Seo, H. J., Park, K. K., Han, S. S., Chung, W. Y. *et al.*, Inhibitory effects of the standardized extract (DA-9601) of *Artemisia asiatica* Nakai on phorbol ester-induced ornithine decarboxylase activity, papilloma formation, cyclooxygenase-2 expression, inducible nitric oxide synthase expression and nuclear transcription factor kappa B activation in mouse skin. *Int. J. Cancer* 2002, *100*, 456–462.

[26] Koshihara, Y., Neichi, T., Murota, S., Lao, A. *et al.*, Selective inhibition of 5-lipoxygenase by natural compounds isolated from Chinese plants, *Artemisia rubripes* Nakai. *FEBS Lett.* 1983, *158*, 41–44.

[27] Seo, H. J., Surh, Y. J., Eupatilin, a pharmacologically active flavone derived from *Artemisia* plants, induces apoptosis in human promyelocytic leukemia cells. *Mutat. Res.* 2001, *496*, 191–198.

[28] Lv, W., Sheng, X., Chen, T., Xu, Q. *et al.*, Jaceosidin induces apoptosis in human ovary cancer cells through mitochondrial pathway. *J. Biomed. Biotechnol.* 2008, 394802.

[29] Kim, M. J., Kim, D. H., Lee, K. W., Yoon, D. Y., Surh, Y. J., Jaceosidin induces apoptosis in ras-transformed human breast epithelial cells through generation of reactive oxygen species. *Ann. N. Y. Acad. Sci.* 2007, *1095*, 483–495.

[30] Jeong, M. A., Lee, K. W., Yoon, D. Y., Lee, H. J., Jaceosidin, a pharmacologically active flavone derived from *Artemisia argyi*, inhibits phorbol-ester-induced upregulation of COX-2 and MMP-9 by blocking phosphorylation of ERK-1 and -2 in cultured human mammary epithelial cells. *Ann. N. Y. Acad. Sci.* 2007, *1095*, 458–466.

[31] Horinaka, M., Yoshida, T., Shiraishi, T., Nakata, S. *et al.*, Luteolin induces apoptosis *via* death receptor 5 upregulation in human malignant tumor cells. *Oncogene* 2005, *24*, 7180–7189.

[32] Kim, Y. H., Lee, Y. J., TRAIL apoptosis is enhanced by quercetin through Akt dephosphorylation. *J. Cell Biochem.* 2007, *100*, 998–1009.

[33] Pan, M. H., Lai, C. S., Hsu, P. C., Wang, Y. J., Acacetin induces apoptosis in human gastric carcinoma cells accompanied by activation of caspase cascades and production of reactive oxygen species. *J. Agric. Food Chem.* 2005, *53*, 620–630.

[34] Thornberry, N. A., Lazebnik, Y., Caspases: enemies within. *Science* 1998, *281*, 1312–1316.

[35] Nunez, G., Benedict, M. A., Hu, Y., Inohara, N., Caspases: the proteases of the apoptotic pathway. *Oncogene* 1998, *17*, 3237–3245.

[36] Fischer, U., Janicke, R. U., Schulze-Osthoff, K., Many cuts to ruin: a comprehensive update of caspase substrates. *Cell Death Differ.* 2003, *10*, 76–100.

[37] Green, D. R., Apoptotic pathways: paper wraps stone blunts scissors. *Cell* 2000, *102*, 1–4.

[38] Jiang, X., Wang, X., Cytochrome C-mediated apoptosis. *Annu. Rev. Biochem.* 2004, *73*, 87–106.

[39] Degterev, A., Boyce, M., Yuan, J., A decade of caspases. *Oncogene* 2003, *22*, 8543–8567.

[40] Schulze-Osthoff, K., Ferrari, D., Los, M., Wesselborg, S., Peter, M. E., Apoptosis signaling by death receptors. *Eur. J. Biochem.* 1998, *254*, 439–459.

[41] Peter, M. E., Krammer, P. H., The CD95(APO-1/Fas) DISC and beyond. *Cell Death Differ.* 2003, *10*, 26–35.

[42] Wieder, T., Essmann, F., Prokop, A., Schmelz, K. *et al.*, Activation of caspase-8 in drug-induced apoptosis of B-lymphoid cells is independent of CD95/Fas receptor-ligand interaction and occurs downstream of caspase-3. *Blood* 2001, *97*, 1378–1387.

[43] 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.

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

[45] Reed, J. C., Double identity for proteins of the Bcl-2 family. *Nature* 1997, *387*, 773–776.

[46] Green, D. R., Reed, J. C., Mitochondria and apoptosis. *Science* 1998, *281*, 1309–1312.

[47] Igney, F. H., Krammer, P. H., Death and anti-death: tumour resistance to apoptosis. *Nat. Rev. Cancer* 2002, *2*, 277–288.

[48] Huang, Y., Park, Y. C., Rich, R. L., Segal, D. *et al.*, Structural basis of caspase inhibition by XIAP: differential roles of the linker versus the BIR domain. *Cell* 2001, *104*, 781–790.

[49] Hofmann, H. S., Simm, A., Hammer, A., Silber, R. E. *et al.*, Expression of inhibitors of apoptosis (IAP) proteins in non-small cell human lung cancer. *J. Cancer Res. Clin. Oncol.* 2002, *128*, 554–560.

