Application of eupatilin in the treatment of osteosarcoma

YAN-YAN LI[1], HAO WU[2], YI-GUO DONG[3], BO LIN[3], GANG XU[3] and YU-BO MA[3]

[1]Department of Neurology, The Second People's Hospital of Mudanjiang, Mudanjiang, Heilongjiang 157013; [2]Department of Orthopedics, Sengong Hospital, Harbin, Heilongjiang 150040; [3]Department of Orthopedics, Hongqi Hospital, Mudanjiang Medical University, Mudanjiang, Heilongjiang 157011, P.R. China

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Abstract. 5,7-dihydroxy-3',4',6-trimethoxyflavone, commonly known as eupatilin, is a traditional Asian medicinal plant, which is mainly used for the treatment of gastritis, as well as its use as an anti-inflammatory agent. Eupatilin is a bioactive compound; however, its effects on osteosarcoma (OS) have remained to be elucidated. Therefore, the present study aimed to investigate the effects of eupatilin on this malignant bone tumor, using the U-2 OS cell line. The experimental results revealed that eupatilin inhibited U-2 OS cell growth in a concentration-dependent manner and induced G2/M phase cell cycle arrest and apoptosis. Additionally, western blot analysis indicated that eupatilin was able to trigger the mitochondrial apoptotic pathway, demonstrated by the enhanced Bax/B cell lymphoma-2 ratio, decrease in mitochondrial membrane potential, release of cytochrome *c*, caspase-3 and -9 activation and poly(ADP-ribose)polymerase cleavage detected in the U-2 OS cells. These results indicated that eupatilin was able to inhibit U-2 OS cancer cell proliferation by the induction of apoptosis via the mitochondrial intrinsic pathway. Eupatilin may therefore represent a novel anticancer drug for use in the treatment of osteosarcoma.

Introduction

Osteosarcoma (OS) is considered to be one of the most common and aggressive primary bone tumors of the musculoskeletal system, and predominantly occurs in childhood and adolescence (1,2). There are various potential treatments for OS, including radiotherapy, surgery and chemotherapy; however, the results of these available treatments remain unsatisfactory (3). Significant nephrotoxic and cardiotoxic side-effects are induced by chemopreventative medicines, thereby limiting the efficacy of their use in the treatment of OS (4). Manipulation of apoptosis is one of the major targets in the treatment of cancer. Apoptosis describes genetically-dependent programmed cell death type I, and is characterized by cell shrinkage, signal transduction (5), nucleic condensation (6,7) and DNA and cellular protein degradation (8). Previous studies have revealed that few therapeutic treatments exist that result in an enhanced ability of human tumor cell lines to undergo apoptosis (9,10). Therefore, the development of novel agents to induce or increase the phenomenon of apoptosis present a promising approach for the development of cancer treatments. Novel inducers of apoptosis may provide alternative and efficacious therapeutic anticancer strategies.

Flavonoids are associated with multiple biological effects, including antitumor, anti-oxidation, anti-inflammation, antiviral and hepatoprotective activities, as well as in the prevention of cardiovascular diseases (11-16). Eupatilin (5,7-dihydroxy-3',4',6-trimethoxyflavone) is extracted from *Artemisia asiatica (A. asiatica)* Nakai, and this isolated flavonoid contains pharmacologically active ingredients. Eupatilin has been demonstrated to exert anticancer, anti-oxidative and anti-inflammatory effects (17). A previous report indicated that Stillen™ (DA-9601), produced from the ethanol extract of *A. asiatica,* contained the pharmacologically active flavonoid compound eupatilin (17). DA-9601 demonstrated cytoprotective effects against gastric mucosal damage and ulcerative proctitis. Eupatilin has exhibited positive effects in the treatment of oxidant-dependent gastric disorders (18). Eupatilin, apigenin, wogonin and baicalein are all members of the same family of flavonoids. Although the flavones, apigenin, wogonin and baicalein, have previously been used in the treatment of OS (19-21), the molecular mechanisms underlying eupatilin-mediated apoptosis of the U-2 OS cell line have remained to be elucidated. Therefore, the present study aimed to aid the elucidation of the underlying mechanism involved in eupatilin-induced apoptosis of U-2 OS cells. This was achieved via cytotoxicity experiments, apoptosis studies and the analysis of changes in protein expression associated with apoptotic cell death.

Materials and methods

Reagents. Eupatilin was provided by Dong-A Pharmaceutical Co. Ltd (Cheoin-gu, South Korea). Antibodies against PARP, Bax, Bcl-2 and cytochrome *c* were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Caspase inhibitors Z-DEVE-FMK, Z-IETD-FMK and Z-LEHD-FMK for

Correspondence to: Mr. Yu-Bo Ma, Department of Orthopedics, Hongqi Hospital, Mudanjiang Medical University, 5 Tongxiang Road, Mudanjiang, Heilongjiang 157011, P.R. China
E-mail: mayubo198@gmail.com

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caspase-3, caspase-8 and caspase-9 were obtained from R&D Systems (Minneapolis, MP, USA). These inhibitors were dissolved in dimethyl sulfoxide (DMSO; Xi'an Chemical Co., Ltd., Boaji, China) and diluted prior to use in cell culture. The present study was approved by the ethics committee board of Mudanjiang Medical University (Mudanjiang, China). The remaining reagents and solvents used were of analytical grade and purchased from Xi'an Chemical Co., Ltd.

Cells and culture. The U-2 OS human OS cells were purchased from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were maintained in RPMI-1640 medium supplemented with fetal calf serum (FCS; 10%), glutamine (2 mM), penicillin (100 U/ml) and streptomycin (10 μg/ml) in a humidified atmosphere at 37˚C with 5% CO_2.

MTT assay. The viability of U-2 OS cells was determined using an MTT assay. U-2 OS cells were seeded at a density of $5x10^4$ cells/well in 96-well culture plates. Each well contained 100 μl medium supplemented with 10% FCS, and the cells were incubated at 37˚C for 24 h prior to treatment with various concentrations of eupatilin. Following 24 h of incubation, the medium was removed and replaced with 10% FCS containing various concentrations of eupatilin (0, 50, 100, 200 and 400 μg/ml) and incubated at 37˚C for 96 h. Subsequently, 50 μl MTT solution was added to each well and incubated for a further 4 h as described previously (22). The formazan crystals obtained were dissolved in 100 μl DMSO and the absorbance was recorded using an ELISA microplate reader (model 550; Bio-Rad, Laboratories, Inc., Hercules, CA, USA) at a wavelength of 570 nm.

Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) double staining. Cell apoptosis was detected using Annexin V-FITC/PI double staining. In brief, $1x10^5$ untreated (control) or treated cells were harvested following trypsinization and centrifugation (500 x g for 5 min). The cells were then washed twice with ice-cold phosphate-buffered saline (PBS) and stained with an Annexin V-FITC/PI apoptosis kit (BD Pharmingen, San Diego, CA, USA), according to the manufacturer's instructions. Subsequently, the samples were analyzed using the FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Double-staining was used for the quantification of the apoptosis of U-2 OS cells following treatment with eupatilin, by measurement of phosphatidylserine expression on the outer surface of the plasma membrane identified by Annexin V-FITC binding. The results of the assay were analyzed with the exclusion of PI, the plasma membrane integrity probe (23).

Flow cytometry and cell cycle analysis. Eupatilin-treated cells were harvested using 0.25% trypsin and then washed with PBS twice prior to fixation with 70% ethanol for ~30 min at 4˚C. The cells were pelleted by centrifuging at 500 x g for 5 min and resuspended in 1 ml PBS containing 100 μl RNase (10 mg/ml) and 100 μl PI (0.5 mg/ml) for ~20 min at 37˚C for cytoplasmic or nuclear DNA staining. The stained cells were then analyzed for DNA content using the FACS Calibur as previously described (24). Annexin V- and PI-positive cells were considered to be necrotic type cells, whereas Annexin-V positive and PI-negative cells were considered to be apoptotic cells.

Mitochondrial membrane potential ($\Delta\Psi$m) determination. The $\Delta\Psi$m of the U-2 OS cells was analyzed by flow cytometry, using rhodamine 123 (Rh123), a fluorescent dye which has been shown to be selectively accumulated within the mitochondria of live cells (25). In brief, the eupatilin-treated and untreated cells were incubated with Rh123 dye (1 mg/ml in DMSO) for 30 min at 37˚C in 5% CO_2. Subsequently, the cells were washed twice with PBS, resuspended in PBS, stained with 2 μg/ml PI and immediately subjected to flow cytometric analysis. The loss of $\Delta\Psi$m was calculated as a percentage using CellQuest™ software (version 5.1; BD Biosciences).

Caspase-3, -8 and -9 activities. The activities of caspase-3, -8 and -9 were evaluated using a caspase colorimetric assay kit (BioTek Instruments, Inc., Winooski, VT, USA) according to the manufacturer's instructions. U-2 OS cells were seeded in 12-well culture plates at a density of $2x10^5$ cells/well prior to incubation with eupatilin for 48 h. The cells were then harvested, lysed with lysis buffer for ~5 min in an ice bath and then centrifuged at 10,000 x g for 10 min. Subsequently, the reaction buffer was added to the supernatant solutions containing the proteins (100 μg). Caspase-3, -8 and -9 colorimetric substrates (5 μl each) were then added for 2 h at 37˚C in a CO_2 incubator, prior to quantification of the optical density (OD) of the mixture at 405 nm using a spectrophotometer. Their respective activities were expressed relative to the theoretical OD value, calculated using the Sigma-Aldrich Caspase 9 Assay Kit, Colorimetric Technical Bulletin (www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Bulletin/casp-8cbul.pdf).

Western blot analysis. Cells were subjected to eupatilin treatment, and cell proteins were subsequently obtained by incubation for 1 h in 200 μl lysis buffer containing NaCl (300 mM), Tris HCl (50 mM; pH 7.6), Triton X-100 (0.5%), phenylmethanesulfonyl fluoride (2 mM), aprotinin (2 μl/ml) and leupeptin (2 μl/ml) at 4˚C. A bicinchoninic acid protein assay kit (Pierce Biotechnology, Inc.; Thermo Fisher Scientific, Rockford, IL, USA) was used to quantify the protein expression levels, according to the manufacturer's instructions. Equal quantities of each protein (20 μg) were separated by 12% SDS-PAGE and then electrotransferred onto polyvinylidene difluoride membranes. The membranes were then incubated for 1 h with PBS containing 5% non-fat milk as a blocking solution at room temperature, prior to incubation with polyclonal rabbit anti-mouse poly(ADP-ribose)polymerase (PARP; 1:1,000 dilution; cat no. 9542), B cell lymphoma-2 (1:1,000 dilution; cat no. 2876), Bcl-2-like protein 4 (Bax; 1:1,000 dilution; cat no. 2772) and cytochrome *c* (1:1,000 dilution; cat no. 4272) antibodies (all from Cell Signaling Technology, Inc., Danvers, MA, USA) diluted with blocking solution at 4˚C overnight. The membranes were subsequently incubated with horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody (1:2,000-5,000) for ~2 h at

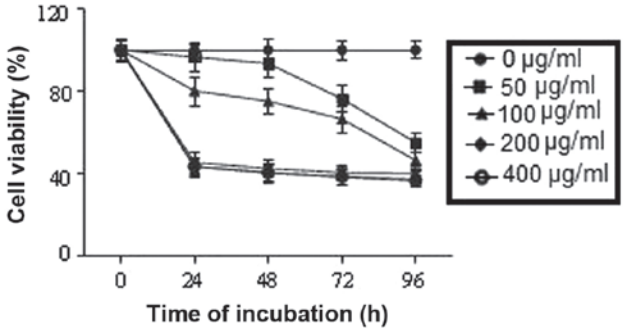

Figure 1. Effects of eupatilin on the growth of U-2 osteosarcoma cells. The data are presented as the mean ± standard deviation of triplicate experiments.

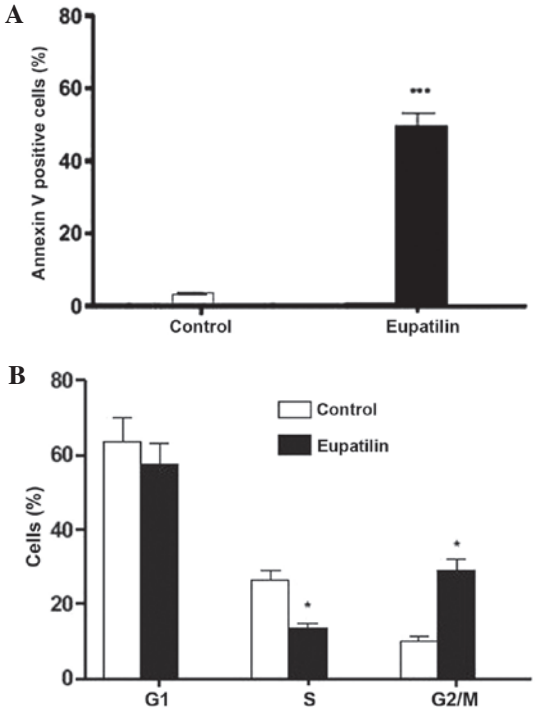

Figure 2. Eupatilin alters the cell cycle distribution and induces apoptosis of U-2 OS cells. (A) Eupatilin (100 μg/ml) induced significant apoptosis of U-2 OS cells. (B) Eupatilin (100 μg/ml) induced alterations in cell cycle phase distribution in U-2 OS cells. Data are presented as the mean ± standard deviation of triplicate experiments. *P<0.05, ***P<0.001 vs. control (untreated cells). OS, osteosarcoma.

room temperature. The developed immunoblots were then visualized with an enhanced chemiluminescence detecting system (GE Healthcare Life Sciences, Chalfont, UK). Expression was analyzed relatively, based on the ratio between the target protein and that of polyclonal rabbit β-actin (1:1,000 dilution; cat no. 4967; Cell Signaling Technology, Inc.).

Statistical analysis. All the quantitative results obtained are presented as the mean ± standard deviation. Student's t-test was used to calculate statistical differences between the control and eupatilin-treated cells, using GraphPad Prism 3.03 software (GraphPad Software, Inc., La Jolla, CA, USA) and P<0.05 was considered to indicate a statistically significant difference. All the studies were performed at least in triplicate.

Results

Eupatilin inhibits growth of U-2 OS cells. The effects of eupatilin on U-2 OS cell proliferation were evaluated by MTT assay. The cells were treated with various concentrations of eupatilin (0, 50, 100, 200 and 400 μg/ml) for various time-periods (0, 24, 48, 72 and 96 h) and cell proliferation under these conditions was evaluated. The results revealed a dose-dependent inhibitory effect of eupatilin on U-2 OS cell proliferation 24 h post-treatment (Fig. 1). The inhibitory effect of eupatilin was demonstrated to be significant at a concentration of 100 μg/ml, whereas the most marked inhibition was observed in cells which were treated with eupatilin at concentrations of 200 and 400 μg/ml.

These results confirmed the antiproliferative effect of eupatilin on U-2 OS cells, and in order to elucidate the mechanism underlying this effect, eupatilin at a concentration of 100 μg/ml was selected for use in the subsequent experiments.

Eupatilin alters the cell cycle distribution and induces apoptosis of U-2 OS cells. To ascertain whether eupatilin inhibited U-2 OS cell proliferation via induction of apoptosis, the cells were treated with eupatilin and then subjected to flow cytometric analysis. The results revealed a significant increase in Annexin V-FITC cell binding following 100 μg/ml eupatilin treatment, compared with that of the control cells, indicating the initiation of apoptosis. Fig. 2A indicates that the apoptotic cell proportion was significantly increased from 3.23% in untreated U-2 OS cells to 49.75% in eupatilin-treated U-2 OS cells. These results confirmed that the cell death observed in U-2 OS cells following eupatilin treatment occurred via the induction of apoptosis. In addition, flow cytometric analysis revealed that eupatilin treatment for 24 h significantly reduced the proportion of cells in S phase, compared with that of the control cells (Fig. 2B). Furthermore, elevated accumulation of cells in the G2/M apoptotic phase was observed, with respect to control cells, while the cell population in G1 phase demonstrated a slight decrease. These results revealed that the eupatilin responses of U-2 OS cells are correlated with apoptotic cell death.

Eupatilin induces mitochondrial dysfunction and cytochrome c release. In order to confirm whether mitochondrial dysfunction was involved in eupatilin-induced apoptosis, changes in $\Delta\Psi$m were evaluated using Rh123 fluorescent dye. Rh123 was used as it is able to cross the mitochondrial membrane and thus accumulates in the mitochondrial matrix. However, this accumulation only occurs when the transmembrane potential is maintained (26). As indicated in Fig. 3A, following exposure of U-2 OS cells to 100 μg/ml eupatilin for 24 h, a significant reduction in $\Delta\Psi$m was observed. The release of cytochrome *c* from the mitochondria to the cytosol is typically associated with $\Delta\Psi$m depolarization (27). In addition, cytochrome *c* has been demonstrated to have a vital role in apoptosis (28,29). Therefore, the expression of cytochrome *c* was analyzed by western blotting. Fig. 3B indicates an increase in cytosolic cytochrome *c* and a decrease in mitochondrial cytochrome *c* in eupatilin-treated cells, when compared with control cells. These results indicated that

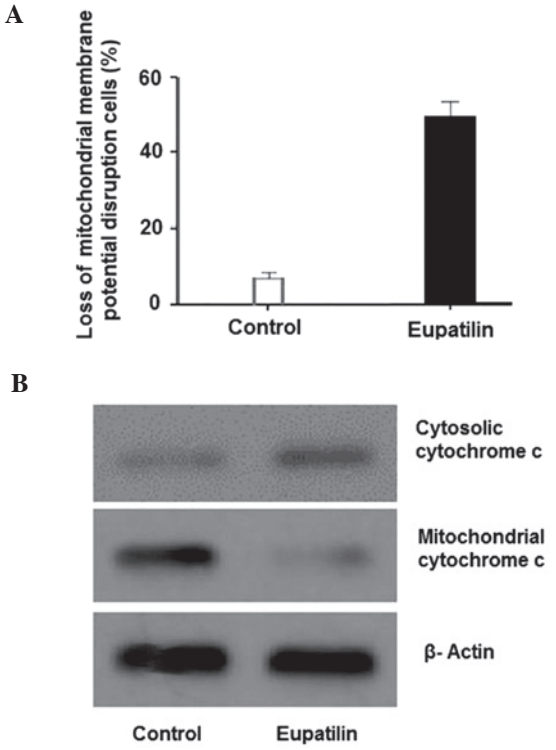

Figure 3. Eupatilin induces mitochondrial dysfunction and cytochrome *c* release. (A) Eupatilin (100 μg/ml)-induced a significant reduction in the mitochondrial membrane potential in U-2 OS cells. (B) Eupatilin (100 μg/ml)-induced release of cytochrome *c* into the cytosol of U-2 OS cells. Data are presented as the mean ± standard deviation of triplicate experiments. *P<0.05 vs. control. OS, osteosarcoma.

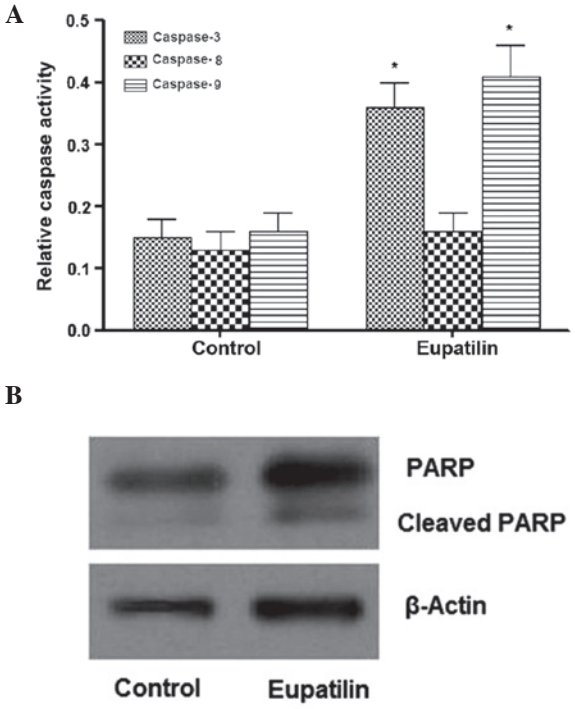

Figure 4. Eupatilin induces caspase activation and PARP cleavage in U-2 OS cells. (A) Eupatilin (100 μg/ml) enhanced the activation of caspase-8 and -9 but not caspase-3. (B) Eupatilin induced PARP cleavage in U-2 OS cells. Data are presented as the mean ± standard deviation of triplicate experiments. *P<0.05 vs. control. OS, osteosarcoma.

eupatilin-induced apoptosis involves mitochondrial dysfunction, associated with a loss of ΔΨm and cytosolic cytochrome *c* release.

Eupatilin induces caspase activation and PARP cleavage in U-2 OS cells. Subsequently, the effects of eupatilin on caspase-3, -8 and -9 activation and PARP cleavage were evaluated, in order to determine the death receptor involvement and mitochondrial pathways in eupatilin-induced apoptosis. The results revealed that eupatilin treatment of U-2 OS cells for 24 h induced activation of caspase-3 and -9, but not caspase-8 (Fig. 4A). Furthermore, as indicated in Fig. 4B, an increase in PARP cleavage was detected following eupatilin treatment. To further investigate the role of caspase activation in eupatilin-induced apoptosis, the effects of caspase inhibitors z-DEVE-FMK, Z-IETD-FMK, and Z-LEHD-FMK for caspase-3, -8 and -9 on apoptosis in U-2 OS cells were evaluated. Significant inhibition of eupatilin-induced apoptosis was observed following pretreatment with caspase-3 and -9 inhibitors, whereas the levels of early apoptosis remained unchanged with respect to caspase-8 inhibition (data not shown). These observations suggested that eupatilin induced caspase-dependent apoptosis in U-2 OS cells via a mitochondrial-dependent pathway.

Eupatilin upregulates Bax and downregulates Bcl-2 in U-2 OS cells. The effects of eupatilin on Bax and Bcl-2 expression in U-2 OS cells were subsequently examined via analysis of Bax and Bcl-2 protein expression levels following 24 h of treatment. Western blot analysis indicated a marked increase in Bax

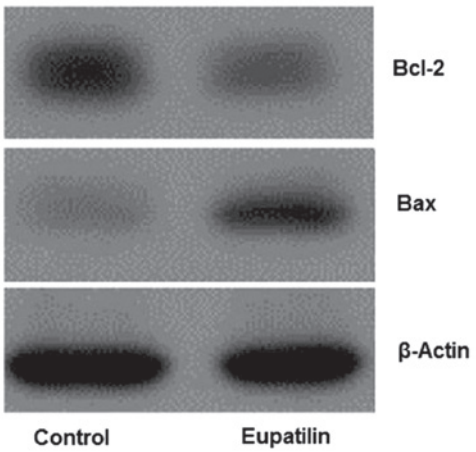

Figure 5. Eupatilin-mediated upregulation of Bax and downregulation of Bcl-2 in U-2 osteosarcoma cells identified by western blotting assay. Data are presented as the mean ± standard deviation of triplicate experiments. *P<0.05 vs. control.

expression levels in eupatilin-treated cells, whereas a significant decrease was observed in Bcl-2 expression (Fig. 5). This high ratio of Bax/Bcl-2 may contribute to the induction of apoptosis by eupatilin via the mitochondrial-dependent pathway.

Discussion

Apoptosis is a genetically mediated mechanism of type 1 programmed cell death. Shrinkage of cells, plasma membrane blebbing and chromatin condensation associated with DNA

cleavage into ladders comprise the major characteristics of apoptosis (30,31).

Accumulating evidence has indicated that the antitumor effects of a wide variety of compounds and herbal medicines obtained from natural products, which exhibit anticancer effects, are able to induce apoptosis in numerous human tumor cell lines (32,33). Previous studies have indicated a cytoprotective effect of *A. asiatica* ethanol extract against gastric mucosa damage and ulcerative colitis. *A. asiatica* is also known to be effective in the treatment of oxidant-dependent gastric disease (18,34). In the present study, the anticancer efficacy of eupatilin and its underlying mechanism in human osteosarcoma U-2 OS cells, *in vitro*, was evaluated. The MTT assay results demonstrated that eupatilin effectively suppressed the proliferation of U-2 OS cells in a concentration- and time-dependent response. The results of FACS analysis indicated that eupatilin induced apoptosis in U-2 OS cells and also increased the proportion of cells at G2/M phase. These results demonstrated that the eupatilin was able to potently trigger apoptosis in U-2 OS cells. Subsequently, the present study aimed to identify the apoptotic mechanism the effects of eupatilin in U-2 OS cells. Mitochondrial integrity disruption is one of the most common and earliest intracellular events to occur following apoptosis initiation (35). Increasing evidence suggests that this mitochondrial dysfunction may activate particular cell signaling pathways, resulting in the induction of apoptosis, as well as the reduction in $\Delta\Psi m$ associated with mitochondrial dysfunction. For this reason, the loss of $\Delta\Psi m$ is significant during mitochondrial-dependent apoptosis (36-38), as, in turn, it induces the efflux of cytochrome *c* into the cytosol from mitochondria. Following release into the cytosol, cytochrome *c* is able to initiate caspase activation, which aids termination of the cells by apoptosis. The results of the present study indicated that eupatilin exposure induced $\Delta\Psi m$ loss and an increase in cytochrome *c* release to the cytosol from the mitochondria in U-2OS cells, which indicated that eupatilin-induced cell death potentially occurred via the mitochondrial apoptotic pathway.

Previous studies have indicated that the mitochondria-mediated pathway for apoptosis is regulated by proteins of the Bcl-2 family (39,40). It was suggested that the balance between Bax and Bcl-2 is significant in conferring cell susceptibility to apoptosis (41). In order to further elucidate the mechanisms underlying the anticancer effects of eupatilin, the expression levels of two major apoptotic signaling proteins, Bax and Bcl-2, were evaluated. An increase in Bax:Bcl-2 ratio was observed in the eupatilin-treated cells, suggesting that eupatilin-induced apoptosis was associated with alterations in Bax and Bcl-2 expression. Apoptosis is controlled by cell suicide mechanisms induced by specific external and internal signals. Currently, two major pathways associated with the induction of apoptosis are known: The mitochondrial signaling pathway and the cell-suicide receptor pathway, controlled by caspase-9 and -8, respectively (42). Accumulating evidence has revealed the essential roles of caspase action in the apoptotic cascade. In the mitochondrial pathway (the intrinsic pathway), cytochrome *c* is released from the mitochondria to the cytosol, and is then able to bind with Apaf-1 and activate caspase-9. Activated caspase-9 subsequently activates the downstream caspases, caspase-3 and/or -7, which in turn aids the cleavage or degradation of various cellular substances, including PARP, inducing apoptosis (43-48). In the cell suicide pathway (the extrinsic pathway), the death receptors that are present on the cell surface (Fas/FasL) are activated, triggering caspase-8 activation (49,50). To elucidate which of these signaling pathways was involved in eupatilin-induced apoptosis, the apoptosis-associated protein expression of casapases-3, -8, -9 and PARP were investigated in U-2 OS cells. The results indicated that apoptosis was induced by caspase-3 and -9 activation, but not caspase-8 activation. Furthermore, the identification of PARP cleavage confirmed the participation of caspase-3 in the induction of apoptosis in the eupatilin-treated cells. In addition, apoptosis was significantly attenuated in the presence of Z-DEVE-FMK and Z-LEHD-FMK inhibitors of caspase-3 and -9, respectively. By contrast, the number of eupatilin-induced early apoptotic U-2 OS cells remained unchanged with respect to caspase-8 inhibitor response. These results revealed that eupatilin-induced apoptosis in U-2 OS cells occurred via the intrinsic pathway, associated with caspase-3 and -9 activation and PARP cleavage.

In conclusion, the results of the present study indicated that eupatilin perturbed U-2 OS cell growth in a dose-dependent fashion. The decrease in cell viability occurred as a result of G2/M phase cell cycle arrest and the induction of apoptosis, hallmarks of the intrinsic apoptotic pathway in U-2 OS cells. Furthermore, eupatilin triggered apoptosis via the mitochondria-mediated pathway, which involved the inhibition of Bcl-2 expression and the induction of Bax expression for the degradation of the outer mitochondrial membrane and release of cytochrome *c*. Eupatilin also induced caspase-3 and -9 activation, but not caspase-8 activation. Finally, eupatilin induced PARP cleavage, which is the substrate for caspase-3 activation following eupatilin treatment. *In vivo* studies of eupatilin effects on U-2 OS xenografts in nude tumor mice are currently underway. The results of the present study aid the elucidation of the molecular mechanisms involved in the antitumor effects of eupatilin in OS, and confirmed that eupatilin may be effective as a drug for use in the treatment of OS.

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