RESEARCH ARTICLE

Kaempferol induced apoptosis *via* endoplasmic reticulum stress and mitochondria-dependent pathway in human osteosarcoma U-2 OS cells

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Kaempferol is a natural flavonoid. Previous studies have reported that kaempferol has anti-proliferation activities and induces apoptosis in many cancer cell lines. However, there are no reports on human osteosarcoma. In this study, we investigate the anti-cancer effects and molecular mechanisms of kaempferol in human osteosarcoma cells. Our results demonstrate that kaempferol significantly reduces cell viabilities of U-2 OS, HOB and 143B cells, especially U-2 OS cells in a dose-dependent manner, but exerts low cytotoxicity on human fetal osteoblast progenitor hFOB cells. Comet assay, DAPI staining and DNA gel electrophoresis confirm the effects of DNA damage and apoptosis in U-2 OS cells. Flow cytometry detects the increase of cytoplasmic Ca^{2+} levels and the decrease of mitochondria membrane potential. Western blotting and fluorogenic enzymatic assay show that kaempferol treatment influences the time-dependent expression of proteins involved in the endoplasmic reticulum stress pathway and mitochondrial signaling pathway. In addition, pretreating cells with caspase inhibitors, BAPTA or calpeptin before exposure to kaempferol increases cell viabilities. The anti-cancer effects of kaempferol *in vivo* are evaluated in BALB/$c^{nu/nu}$ mice inoculated with U-2 OS cells, and the results indicate inhibition of tumor growth. In conclusion, kaempferol inhibits human osteosarcoma cells *in vivo* and *in vitro*.

Keywords:
Apoptosis / Endoplasmic reticulum stress / Kaempferol / Mitochondria-dependent/ U-2 OS

1 Introduction

Flavonoids are a class of plant secondary metabolites, assorted into flavones, flavonols, flavanones, isoflavones, and anthocyanidins [1]. It has been reported that intake of flavonoids is associated with many biological properties, such as antiviral [2], antitumor [3], anti-oxidative [4], anti-inflammatory [5], hepatoprotective activities [6] and the

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Abbreviations: PI, propidium iodide; **DMSO,** dimethyl sulfoxide; **FBS,** fetal bovine serum; **Z-LEHD-FMK,** z-Leu-Glu-His-Asp-fluoromethyl ketone; **Z-DEVDFMK,** z-Asp-Met-Gln-Asp-fluoromethyl ketone; **hFOB,** human fetal osteoblast; **MTT,** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **$\Delta\psi$m,** mitochondrial membrane potential; **ER,** endoplasmic reticulum; **EDTA,** ethylenediaminetetraacetic acid; **DAPI,** 4′,6-diamidino-2-phenylindole

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prevention of cardiovascular diseases [7]. Kaempferol, 3, 4', 5, 7-tetra hydroxyflavone, a natural flavonoid, has been isolated from various plant sources [8]. Kaempferol is commonly known for antioxidant activity and is used for cyto-protection agents. Previous studies have reported that kaempferol has anti-proliferation activity and induces apoptosis in various human cancer cell lines *in vitro*, such as non-small cell lung cancer [9], leukemia [10], esophageal cancer [11], prostate cancer [12], oral cavity cancer [13] and colon cancer [14], but no reports on osteosarcoma.

High-grade osteosarcoma is the most common bone malignancy, accounting for about 60% of malignant bone tumors diagnosed in the first two decades of life, with an aggressive local pattern of growth and high metastatic potential [15]. Current standard treatment is to use chemotherapy followed by surgical resection [16]. The survival rate of patients with localized osteosarcoma is about 11% with surgery alone, compared to approximately 70% when combined with chemotherapy [17]. Despite the success of frontline therapy, about 40% of patients have progression and further therapy with additional chemotherapy is palliative and toxic. It is estimated that less than 30% of patients with recurrent metastasis will be cured [18]. Chemotherapy-resistant cancer is one of the most serious obstacles. Therefore, in this study, we focus on identifying new agents to treat osteosarcoma.

Apoptosis is the process of programmed cell death that occur in multi-cellular organisms, playing an important role in normal physiology in animals [19]. However, impairment of apoptotic function has been associated with several diseases [20], such as neurodegenerative disorders and cancers [21]. The perturbation of this process is considered a crucial part of cancer prevention and therapy.

To date, one of the most effective anti-cancer strategies is through the induction of apoptosis. Although previous studies have reported that kaempferol has anti-cancer activity in various human cancer cell lines [9–14], little is known of the mechanisms exerting cytotoxicity in human osteosarcoma. Therefore, the purpose of this study is to investigate the apoptotic effects and the molecular mechanisms of kaemperol in osteosarcoma cell lines *in vitro* and *in vivo*.

2 Materials and methods

2.1 Chemicals and reagents

Kaempferol, propidium iodide (PI), Tris-HCl, calpeptin and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). BAPTA, dimethyl sulfoxide (DMSO) and potassium phosphates were purchased from Merck Co. (Darmstadt, Germany). Eagle's minimum essential medium (MEM), penicillin-streptomycin, trypsin-EDTA, fetal bovine serum (FBS) and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). Caspase activity assay kit was bought from OncoImmunin (MD, USA). Caspase-9 inhibitor z-Leu-Glu-His-Asp-fluoromethyl ketone (Z-LEHD-FMK) and caspase-3 inhibitor z-Asp-Met-Gln-Asp-fluoromethyl ketone (Z-DEVDFMK) were bought from R&D.

2.2 Human osteosarcoma cell lines (U-2 OS, HOB, 143B) and human fetal osteoblast progenitor cell line (hFOB)

Human osteosarcoma U-2 OS, HOB, 143B cells and conditionally immortalized human fetal osteoblast progenitor hFOB cells were purchased from American Type Culture Collection (ATCC). U-2 OS cells were cultured in McCoy's 5A medium (GIBCO-BRL) with 10% FBS and antibiotics (100 U/ml of penicillin G and 100 μg/ml of streptomycin) at 37°C in a humidified atmosphere of 5% CO_2/95% air. HOB cells were cultured in minimum essential medium (GIBCO-BRL) with 10% FBS and antibiotics (100 U/ml of penicillin G and 100 μg/ml of streptomycin) and 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids at 37°C in a humidified atmosphere of 5% CO_2/95% air. 143B cells were cultured in minimum essential medium (GIBCO-BRL) with 10% FBS and antibiotics (100 U/ml of penicillin G and 100 μg/ml of streptomycin) and 0.015 mg/ml 5-bromo-2′-deoxyuridine at 37°C in a humidified atmosphere of 5% CO_2/95% air. hFOB cells were cultured in DMEM/Ham's F12 medium (GIBCO-BRL) with 10% FBS and antibiotics (100 U/ml of penicillin G, 100 μg/ml of streptomycin and 300 μg/ml geneticin) at 33.5°C in a humidified atmosphere of 5% CO_2/95% air [22].

2.3 Cell viability assay

The cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay. Human osteosarcoma U-2 OS, HOB, 143B cells and conditionally immortalized human fetal osteoblast progenitor hFOB cells were cultured in 96-well culture plates and allowed to attach for hours before treated with various concentrations (0, 25 50, 100, 150 or 200 μM) of kaempferol. After cultivation for 24 h, 0.5 mg/ml of MTT was then added to each well and the mixture was incubated for 4 h at 37°C. Culture medium was then replaced with an equal volume of 0.04N HCl/isopropanol to dissolve formazan crystals. Absorbance of each well was determined at 570 nm wavelength using ELISA reader [23].

2.4 Phase-contrast microscopy of morphological changes

U-2 OS cells were plated in 24-well plates at a density of 2.5×10^5 cells/well. The 50, 100 or 150 μM of kaempferol were added, and the cells were incubated for 24 h. A phase-

contrast microscope was used for photography to determine morphological changes as described elsewhere [24].

2.5 Comet assay and DAPI staining

After treated with 50, 100 or 150 μM of kaempferol, U-2 OS cells were harvested and mixed with low melting point (LMP) agarose at 37°C. This mixture was placed on the top of previous layer of 5% agarose (normal melting point) on the slide, and then covered with a covership at 4°C until solid. Subsequently, the covership was removed gently and some agarose was added onto this slide, and then covered with the covership again. The slide was placed at 4°C until the mixture was solid, and put in chilled alkaline lysis buffer for electrophoresis. Afterwards, the slide was gently washed with neutralized buffer, and stained with DAPI [25].

2.6 Agarose gel electrophoresis

After treated with 150 μM of kaempferol, U-2 OS cells were harvested then lysed in lysis buffer (20 mM Tris, 10 mM EDTA, 0.2 % Triton X-100, pH 8.0) at 4°C for 15 min, and then the lysate was centrifuged for 13,000 rpm, 10 min at 4°C. The supernatant containing fragmented DNA was collected and incubated at 50°C overnight with proteinase K (0.1 mg/ml) to digest protein, followed by RNase A (50 μg/ml) digestion at 37°C for 30 min. After extracted with phenol/chloroform/isoamyl alcohol (25:24:1), the DNA was precipitated in 50% isopropanol with 1 μl of glycogen (20 μg/ml) at −20°C overnight. The precipitated DNA was centrifuged at 14,000 rpm for 30 min, dried, and dissolved in 10 μl H_2O. After electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5 μg/ml) in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0), the DNA in gel was resolved with UV light [26].

2.7 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

After treated with 150 and 200 μM of kaempferol, U-2 OS and hFOB cells were harvested. Evaluation of apoptosis in the U-2 OS and hFOB cells were accomplished by flow cytometry to detect cells labeled by TUNEL, using fluorescein-labeled dUTP (treatment). Controls consisted of cells incubated with fluorescein dUTP without Tdt (In Situ Death Kit, Boehringer-Mannheim Biochemicals) [27].

2.8 Intracellular Ca^{2+} levels assay

U-2 OS cells (2.5×10^5/well) in 12-well plate were treated with 150 μM of kaempferol and incubated for 0, 6, 12 or 24 h. Cells were harvested, washed twice, re-suspended in 3 μg/ml of Indo 1/AM (Calbiochem; La Jolla, CA) at 37°C for 30 min and analyzed by flow cytometry (Becton Dickinson FACS Calibur) [26].

2.9 Calpain activity assays

U-2 OS cells were prepared on 24-well plates and pretreated with BAPTA, a Ca^{2+} chelator or calpeptin and an inhibitor of calpain for 1 h. Then, cells were loaded with 40 M Suc-Leu-Leu-Val-Tyr-AMC calpain protease substrate (Biomol) and treated with 150 μM of kaempferol to the indicated time at 37°C in a humidified 5% CO_2 incubator. Proteolysis of the fluorescent probe was monitored by a fluorescent plate reading system (HTS-7000 Plus Series BioAssay, Perkin Elmer) with filter settings of 360 ± 20 nm for excitation and 460 ± 20 nm for emission [28].

2.10 Determination of mitochondrial membrane potential ($\Delta\psi_m$)

The mitochondrial membrane potential ($\Delta\psi_m$) of the U-2 OS cells was determined by flow cytometry using DiOC6 (Molecular Probes). U-2 OS cells were treated with 150 μM of kaempferol for 0, 6, 12 or 24 h to detect the changes of $\Delta\psi_m$. The cells were harvested and washed twice, re-suspended in 500 μl of DiOC6 (4 μmol/L) and incubated at 37°C for 30 min before analyzed by flow cytometry (Becton Dickinson FACSCalibur) [29].

2.11 Caspase-3, -8 and -9 activities assay

Caspase-3, -8 and -9 activities were assessed according to manufacturer's instruction of caspase colorimetric kit (R&D system Inc., MN, USA). U-2 OS cells were seeded in 12-well cell culture plates at an initial density of 5.0×10^6 cells and pretreated with caspase-3 inhibitor (Z-DEVD-FMK), caspase-8 inhibitor (Z-IETD-FMK) or caspase-9 inhibitor (Z-LEHD-FMK) for 1 h prior to treatment with 150 μM of kaempferol for 0, 6, 12 or 24 h. Cells were harvested and lysed for 10 min in 50 μl lysis buffer which contained 2 mM DTT. After centrifugation, the supernatant containing 100 μg protein were incubated with caspase-3 substrate (Ac-DEVD-pNA), caspase-8 substrate (Ac-IETD-pNA) and caspase-9 substrate (Ac-LEHD-pNA) respectively in reaction buffer. Then all samples were incubated in 96-well flat bottom microplate at 37°C for 1 h. Levels of released pNA were measured at O.D.405 nm with ELISA reader (Anthos 2001) [23].

2.12 Western blot analysis

Briefly, the cytosolic and total proteins were collected from U-2 OS cells which were treated with 150 μM of kaempferol

for 0, 6, 12 or 24 h. Protein concentrations were determined by the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, USA). Protein samples (30 μg each) were boiled with gel loading buffer for 5 min. Protein extracts were separated on 10% SDS-polyacrylamide electrophoresis gels (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking with TBST (0.05% Triton X-100 in PBS) buffer of 5% non-fat milk for 1 h, the membrane was exposed to the primary antibody: GADD153, GRP78, GRP94, ATF-6α, ATF-6β, calpain 1, calpain 2, Fas, FasL, Bax, Bcl-2, cytochrome c, Apaf-1, AIF, caspase-4, caspase-9, caspase-3, caspase-7, caspase-8, caspase-12 and β-actin, primary antibodies were diluted in PBST (0.05% Triton X-100 in PBS) buffer of 5% non-fat milk, and incubated at 4°C overnight. The secondary antibodies were coupled to horseradish peroxidase. Finally, they were detected by ECL [27].

2.13 *In vivo* tumor xenograft model

Fifteen BALB/$c^{nu/nu}$ mice eight-week-old (approximately 22–28 g) were purchased from the Laboratory Animal Center, National Taiwan University, College of Medicine (Taipei, Taiwan). U-2 OS cells (1×10^7) in culture medium were subcutaneously injected into the flank of each mouse. Mice with tumors were randomly assigned to three groups and each group contained five animals. The treatment was initiated when xenografts reached a volume of about 100 mm^3 and these mice were treated orally every day with olive oil (control vehicle), 25 mg/kg or 50 mg/kg of kaempferol in olive oil. After xenograft tumor transplantation, mice were closely monitored, counted and weighted. The tumor sizes were measured every four days using calipers and tumor volume was estimated according to the following formula: tumor volume (mm^3) = $LxW^2/2$ (L: length and W: width). At the end of the study, animals were sacrificed. Tumors were removed, measured and weighted individually [29, 30].

2.14 Densitometry and statistical analysis

All data were expressed as mean ± SEM from at least three separate experiments. Statistical calculations of the data were performed using an unpaired Student's *t*-test. Statistical significance was set at $^*P<0.05$; $^{**}P<0.01$;$^{***}P<0.001$ was taken as significant.

3 Results

3.1 Effects of kaempferol on cell viability in human osteosarcoma U-2 OS, HOS and 143B cells

We treated human osteosarcoma U-2 OS, HOB, 143B and human fetal osteoblast progenitor hFOB cells with kaempferol at different concentrations from 0 to 200 μM for 24 h. The number of viable cells was counted by MTT method. As shown in Fig. 1A, the viability was significantly decreased in the kaempferol-treated human osteosarcoma cells groups, but not in hFOB cells ($IC_{50} > 200$ μM). The IC_{50} for U-2 OS cells was 148.36 μM. This therefore indicated that kaempferol reduced the proportion of viable osteogenic cancer cells in dose-dependent manner, but with low toxicity to hFOB cells.

3.2 Effects of kaempferol on cell morphological changes, DNA damage and apoptosis in human osteosarcoma U-2 OS cells

To investigate the occurrence of morphological changes and DNA damage in human osteosarcoma cells, we predominantly focused on U-2 OS cells and treated them with kaempferol at different concentrations from 0 to 150 μM for 24 h. In Fig. 1B, morphological examinations of U-2 OS cells showed the difference between the kaempferol-treated groups and the control. In the kaempferol-treated groups, cancer cells were detached from the surface and contained some debris, whereas the control group was well spread with a flattened morphology. In Fig. 1C–D, the data showed that U-2 OS cells induced DNA fragmentation and DNA damage was determined by DAPI staining and comet assay. In Fig. 1E, in order to reconfirm the induction of DNA damage, we isolated DNA from the cells after treatment with 150 μM kaempferol for 24 h, and then they were harvested for DNA fragmentation determination in DNA gel electrophoresis. The results showed that kaempferol induced apoptosis because of the occurrence of DNA ladder. We investigated whether or not kaempferol induces U-2 OS cell death through an apoptotic mechanism. TUNEL assay was used for the detection of DNA fragmentation in apoptosis. In Fig. 1F, compared with control cells, U-2 OS cell were treated with kaempferol showed significant cell apoptosis. However, hFOB cell were showed non-significant cell apoptosis. We suggested that kaempferol represented a promising candidate as an anti-osteosarcoma drug with low toxicity to normal cells.

3.3 Effects of kaempferol on the cytoplasmic Ca^{2+} and mitochondria membrane potential ($\Delta\psi_m$) levels in human osteosarcoma U-2 OS cells

In order to elucidate the possible signaling pathways of kaempferol-induced apoptosis in U-2 OS cells, we examined intracellular Ca^{2+} levels and mitochondria membrane potential by flow cytometry analysis. As shown in Fig. 2A and Fig. 3A, U-2 OS cells were treated with 150 μM of kaempferol for 24 h and this significantly increased

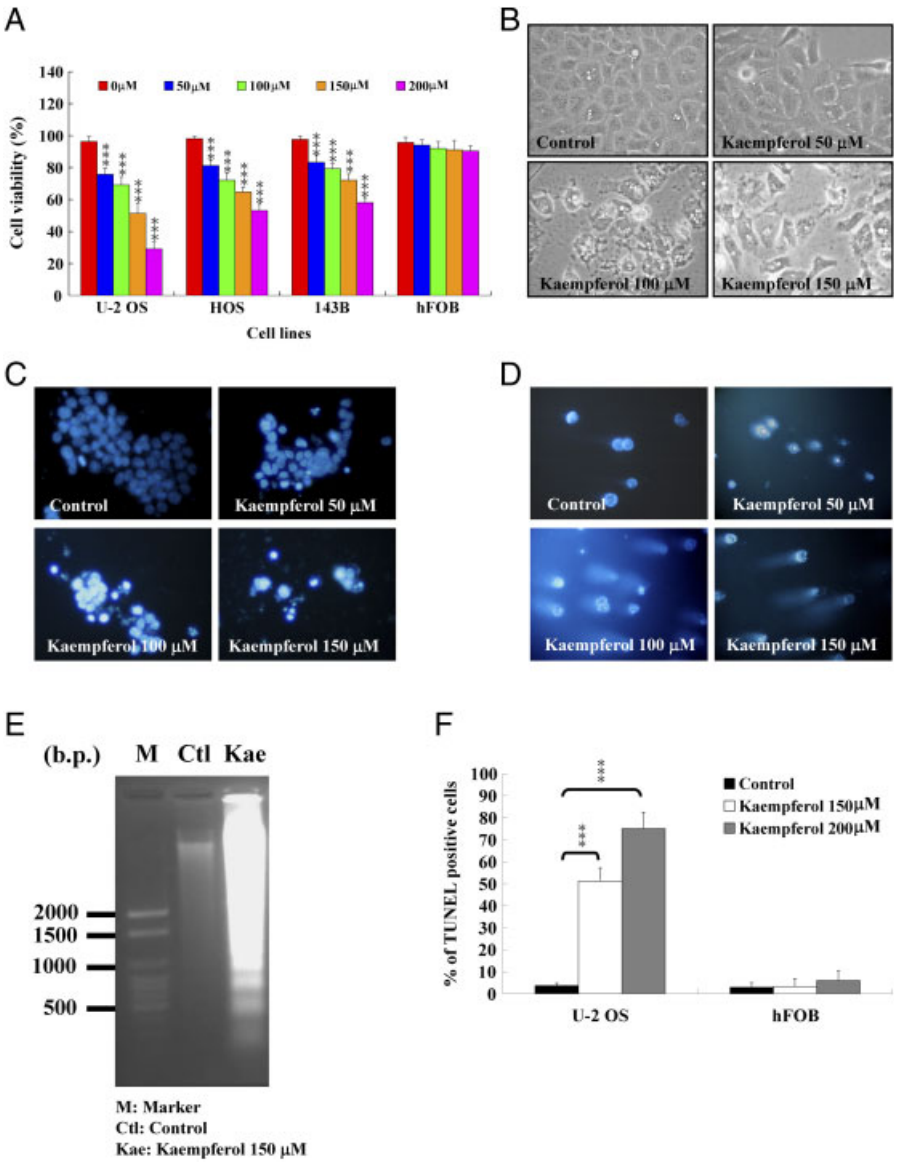

Figure 1. Effects of kaempferol on cell viability and apoptosis in osteosarcoma cell lines. After treatment with various concentrations of kaempferol for 24 h, the cell viabilities of U-2 OS, HOB, 143B osteosarcoma cell lines and the conditionally immortalized human fetal osteoblast progenitor hFOB cells are shown (A). Data represent mean ± SD of three experiments. ***$p < 0.001$. U-2 OS cells in response to various concentrations of kaempferol for 24 h showed morphological changes (B) which indicated kaempferol-induced cell death, and DAPI staining (C), comet assay (D), gel electrophoresis (E) and TUNEL assay (F) revealing kaempferol-induced DNA damage, fragmentation and apoptosis, which was another hallmark of cells undergoing apoptosis.

cytoplasmic Ca^{2+} levels and decreased $\Delta\psi_m$ in time-dependent manner. These results suggested that kaempferol-induced apoptotic response might be mediated by endoplasmic reticulum stress and mitochondrial-dependent apoptotic pathways.

3.4 Effects of kaempferol on the levels of endoplasmic reticulum stress related proteins in human osteosarcoma U-2 OS cells

To be more detail in the molecular mechanisms of endoplasmic reticulum stress pathway, we investigated these related protein levels: GADD153, GRP78, GRP94, ATF-6α, ATF-6β, caspase-4, caspase-12, calpain 1 and calpain 2 by western blotting. As shown in Fig. 2B, kaempferol increased these protein levels in time-dependent manner, but caspase-12 has no statistical influence. These results suggested that kaempferol-induced apoptosis was mediated *via* endoplasmic reticulum stress pathway.

3.5 Effects of kaempferol with BAPTA or Calpeptin on the levels of cytoplasmic Ca^{2+} and Calpain activity and cell viability in human osteosarcoma U-2 OS cells

In order to confirm that kaempferol-induced apoptosis was mediated by ER stress pathway, we pretreated U-2 OS cells with BAPTA, a Ca^{2+} chelator or calpeptin, an inhibitor of calpain, after exposure to kaempferol. As shown in

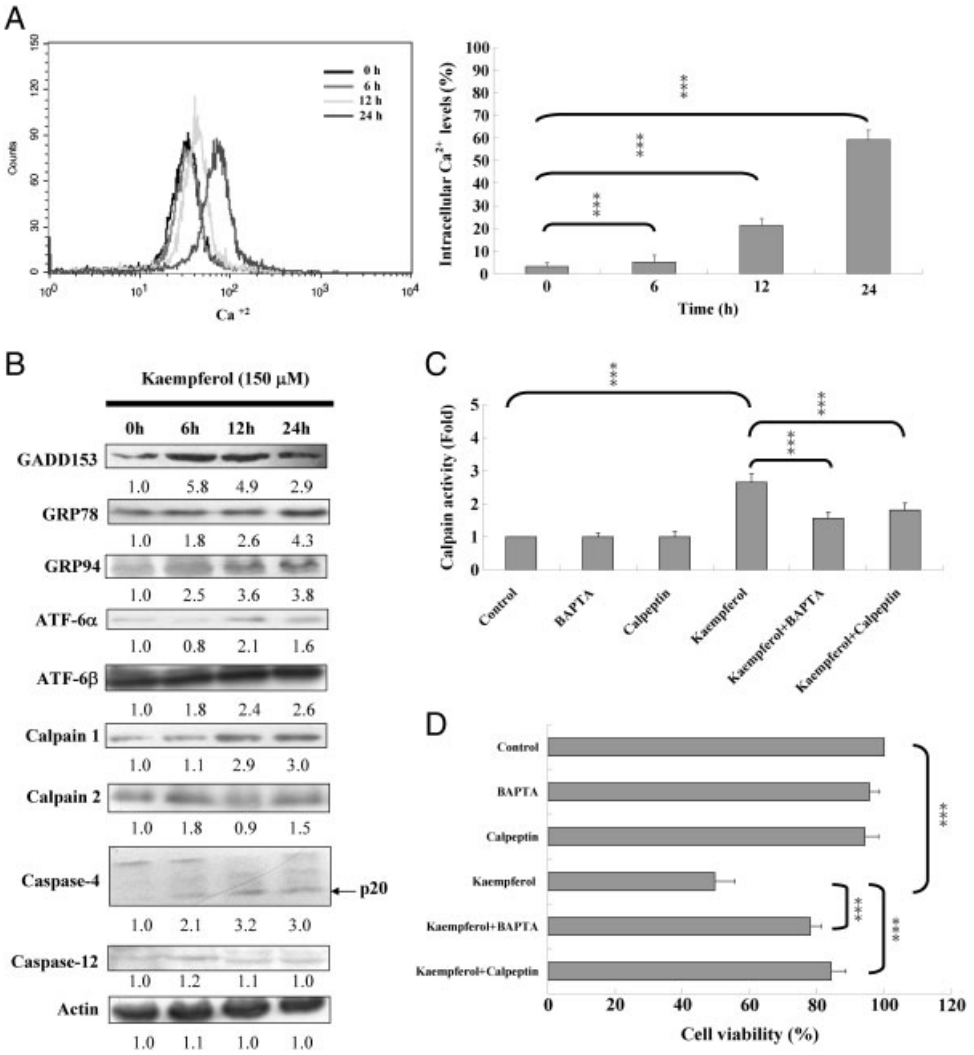

Figure 2. Effects of kaempferol on U-2 OS cells in endoplasmic reticulum stress apoptotic pathway. The intracellular Ca^{2+} levels in kaempferol-treated U-2 OS cells from each time point were measured by flow cytometric analysis (A). Cells were treated with 150 μM of kaempferol for the indicated time, cytosolic proteins or whole cell lysate were prepared, and subjected to Western blotting. The resulting blots were probed for GADD153, GRP78, GRP94, ATF-6α, ATF-6β, caspase-4, caspase-12, calpain1 and calpain2 (whole cell lysate). β-actin served as the loading control. Levels of the associated proteins in endoplasmic reticulum stress apoptotic pathway were affected (B). Cells were pretreated with BAPTA, a Ca^{2+} chelator or calpeptin, an inhibitor of calpain for 1 h after exposure to kaempferol, then incubated for 24 h. The whole-cell lysates were subjected to calpain activity assay (C) and cells were collected to determine the percentage of viable cells (D). Data from three independent experiments were presented (***$P<0.001$, as compared with control treatments).

Fig. 2C–D, the levels of calpain activity and cell viability were significantly influenced. Overall, in Fig. 2, these data demonstrated that activation of ER stress pathway played an important role in kaempferol-induced apoptosis in U-2 OS cells.

3.6 Effects of kaempferol on the levels of Bcl-2 family in human osteosarcoma U-2 OS cells

Previous studies have demonstrated that Bcl-2 and Bax locate in the mitochondrial outer-membrane and the Bcl-2/Bax ratio regulate the release of mitochondrial cytochrome *c* to cytosol [31, 32]. We investigated expression levels of Bcl-2 and Bax in kaempferol-treated U-2 OS cells by western blotting. As shown in Fig. 3B, the pro-apoptotic protein level of Bax was up-regulated, whereas the anti-apoptotic protein level of Bcl-2 was down-regulated in time-dependent manner.

3.7 Effects of kaempferol on the levels of mitochondrial caspase-dependent and caspase-independent pathway related proteins in human osteosarcoma U-2 OS cells

To be more detail in the molecular mechanisms of mitochondrial-dependent apoptotic pathway, we examined the expression levels of cytochrome *c*, Apaf-1, caspase-9, caspase-3, caspase-7 and AIF by western blotting. As shown in Fig. 3B, these protein levels were increased in time-dependent manner. Our results suggested that kaempferol-induced apoptotic response was mediated by mitochondrial-dependent cascade.

3.8 Effects of kaempferol on the caspase-9 and caspase -3 activities in human osteosarcoma U-2 OS cells

In order to confirm that kaempferol-induced apoptosis was mediated by caspase-dependent pathway, we investigated the

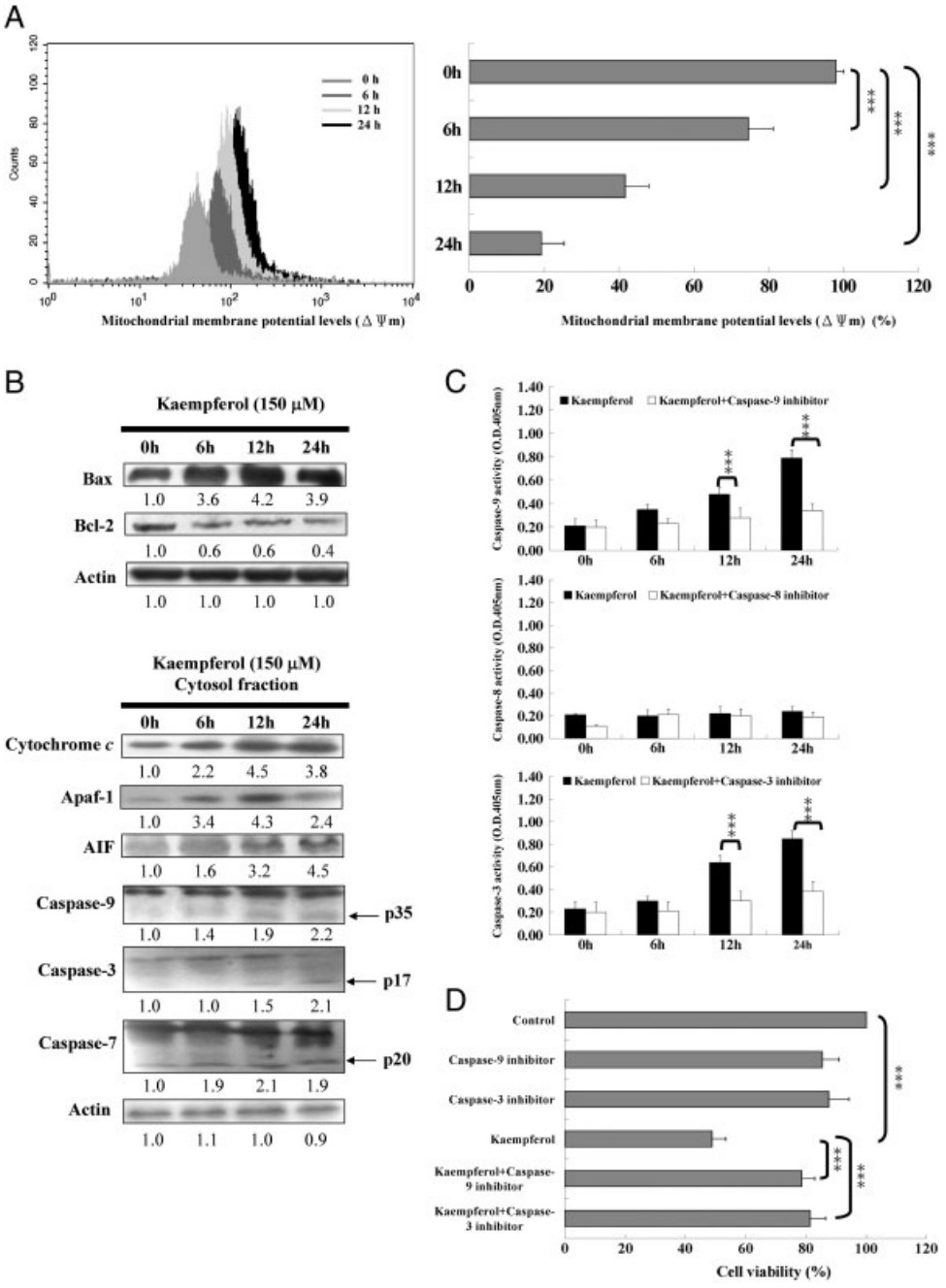

Figure 3. Effects of kaempferol on U-2 OS cells in mitochondrial-dependent apoptotic pathway. The mitochondrial membrane potential ($\Delta\psi_m$) of kaempferol-treated U-2 OS cells from each time point was measured by staining with DiOC6 (A). Cells were treated with 150 μM of kaempferol for the indicated time, cytosolic proteins or whole cell lysate were prepared, and subjected to Western blotting. The resulting blots were probed for cytochrome c, Apaf-1, AIF, caspase-9, caspase-3, caspase-7 (cytosolic proteins), and Bcl-2,Bax (whole cell lysate). β-actin served as the loading control. Levels of the associated proteins in mitochondrial-dependent apoptotic pathway were affected (B). Cells were pretreated with specific inhibitors of caspases-9 (Z-LEHD-FMK), caspase-3 (Z-DEVE-FMK) or caspase-8 inhibitor (Z-IETD-FMK) for 1 h after exposure to kaempferol, then incubated for 24 h. The whole-cell lysates were subjected to caspase activity assay (C) and cells were collected to determine the percentage of viable cells (D). Data from three independent experiments were presented (***$P<0.001$, as compared with control treatments).

caspase-9, -3 and -8 activities by fluorogenic enzymatic assay. As shown in Fig. 3C–D, both caspase-9 and caspase-3 activities were significantly increased. Moreover, pre-incubation with specific inhibitors of caspases-9 (Z-LEHD-FMK), caspase-3 (Z-DEVE-FMK) strongly reduced the caspase-9 or caspase-3 activities and increased U-2 OS cell viability. However, caspase-8 activity has no significant influence. Overall, in Fig. 3, these data demonstrated that caspase-dependent mitochondrial pathway played an important role in kaempferol-induced apoptosis in U-2 OS cells.

3.9 Effects of kaempferol on anti-proliferative activity in BALB/c$^{nu/nu}$ mice after injection with human osteosarcoma U-2 OS cells

Three groups of mice were respectively treated with DMSO control vehicle, 25 mg/kg or 50 mg/kg of kaempferol. These representative animals with tumors were shown in Fig. 4A. In Fig. 4B–C, kaempferol significantly decreased the tumor weight and tumor volume compared to the control group.

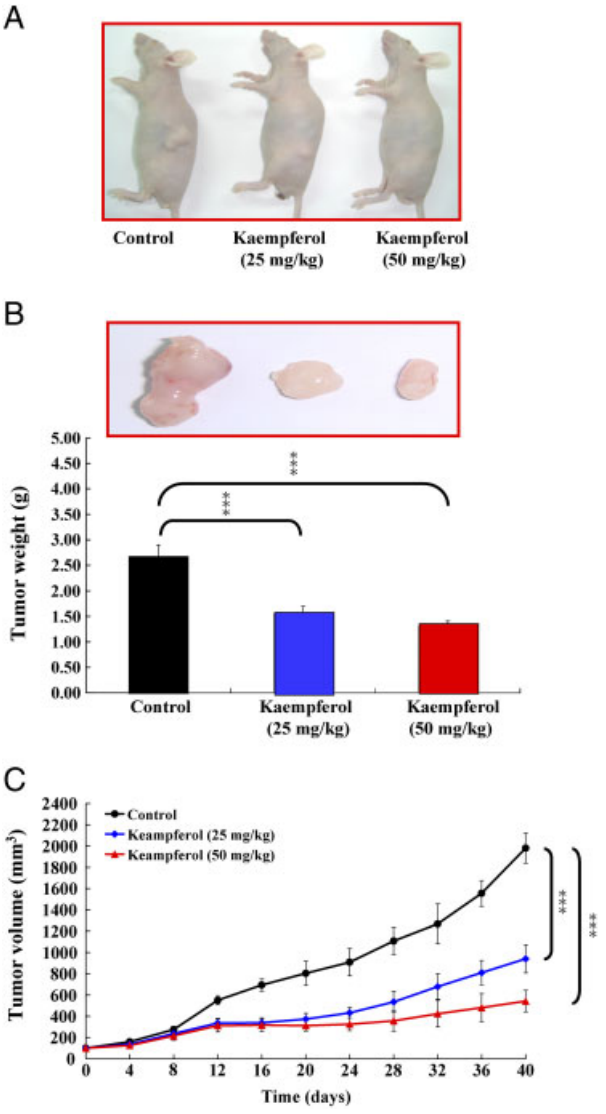

Figure 4. *In vivo* anti-tumor activity of kaempferol. BALB/c$^{nu/nu}$ mice were administered 25 and 50 mg/kg of kaempferol orally. Representative animals with tumors (A), tumor weight (B) and total tumor volume of BALB/c$^{nu/nu}$ mice (C). Data were presented (***$P<0.001$, as compared with control treatments).

4 Discussion

Kaempferol, a natural flavonoid, has been reported to induce apoptosis and inhibit proliferation in various human cancer cell lines, including non-small cell lung cancer [9], leukemia [10], esophageal cancer [11], prostate cancer [12], oral cavity cancer [33] and colon cancer [14]. Furthermore, Zhang *et al.* demonstrated that kaempferol not only effectively inhibited pancreatic cancer cell proliferation and induced apoptosis, but also may sensitized pancreatic tumor cells to chemotherapy [34]. However, little is known in human osteosarcoma cell lines. In contrast to beneficial effects, there are still some question marks about the toxic side-effects to normal tissue. Li *et al.* showed cytotoxicities of kaempferol at higher doses in human normal liver L-02 cells (IC_{50} = 57.05 μM, cultivation for 48 h) and human hepatoma HepG2 cells (IC_{50} = 84.72 μM, cultivation for 48 h) *in vitro* [35]. Soares *et al.* showed that the viability of kaempferol-treated mouse fibroblast McCoy cells was fell, without the hepatic S9 microsomal fraction; but low toxicity occurred (IC_{50} > 500 μM) when the S9 mixture metabolized these compounds [36]. In this study, we first reported that kaempferol was active against human osteosarcoma U-2 OS, HOB and 143B cell lines *in vitro* and U-2 OS *in vivo*. In Fig. 1, it was shown that kaempferol reduced the percentage of viable cancer cells in a dose-dependent manner and induced apoptotic cell death in U-2 OS cells; however, it exhibited low toxicity to human fetal osteoblast progenitor hFOB cells (IC_{50} > 200 μM).

Inducing apoptosis in cancer cells is one of the major strategies of cancer therapeutics. Three major pathways lead to apoptosis [37]. First, the death receptor pathway is triggered by the binding of extrinsic signals to surface receptors, resulting in activation of caspase-8 followed by the activation of caspase-3 and -7 [19]. Second, the mitochondrial pathway is triggered by various stimuli damage inside the cell. When an excess of pro-apoptotic over anti-apoptotic signals, it initiates mitochondrial outer membrane permeabilization and results in caspase dependent and independent apoptotic pathway [31, 32]. Kang *et al.* demonstrated that kaempferol and quercetin, components of ginkgo biloba extract, induced caspase-3-dependent apoptosis in oral cavity cancer cell lines, SCC-1483, SCC-25 and SCC-QLL1 [33]. Leung *et al.* showed that kaempferol-induced apoptosis in human lung non-small carcinoma H460 cells was through caspase-3 (caspase-dependent) and AIF (caspase-independent) pathways [9]. Zhang *et al.* reported that kaempferol exerted cytotoxic effects on OE33, a human esophageal adenocarcinoma cell line, causing G2/M arrest and inducing caspase-dependent apoptosis [11]. Furthermore, Marfe *et al.* demonstrated that kaempferol induced apoptosis in K562 and U937 leukemia cell lines *via* Akt inactivation and mitochondrial dysfunction [10]. Our results are in agreement with previous studies. In Fig. 3, these data indicated that kaempferol up-regulated the level of pro-apoptotic protein Bax and down-regulated anti-apoptotic protein Bcl-2, accompanied with the loss of $\Delta\psi_m$, and then promoting activities of caspase-9, -3, and -7, but not caspase-8. Specific inhibitors of caspase-9 and -3 which decreased caspase activities and increased the kaempferol-treated cell viability suggested that kaempferol induced apoptosis through the mitochondrial-dependent pathway in U-2 OS cells. Also, up-regulating the protein level of AIF indicated that apoptosis was also undergone via caspase-independent mitochondrial pathway. Third, the novel endoplasmic reticulum (ER)-specific apoptotic pathway, it is induced by accumulation of unfolded/misfolded protein aggregating in ER or by excessive protein traffic. Increasing the proteins level of GADD153, GRP78, GRP94 and ATF which are the hallmarks of ER stress induces a rise in intracellular Ca^{2+} level, mitochondrial membrane depolarization and

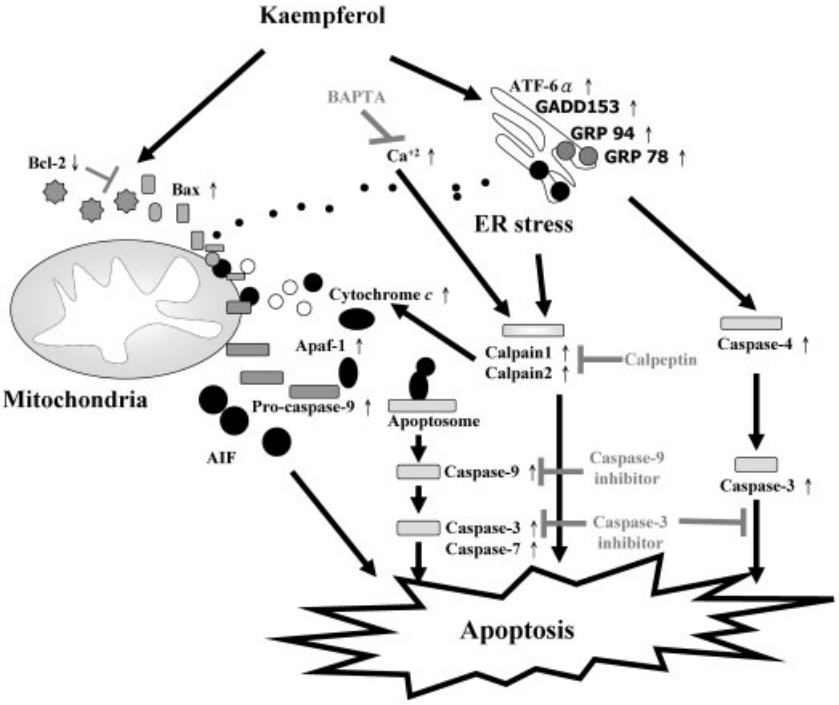

Figure 5. A proposed model illustrates the molecular mechanism and the overall possible signaling pathways of kaempferol-induced apoptosis in U-2 OS cells.

activation of calpain and caspase-12 in murine systems and/or caspase-4 in human cells [38–40]. However, there are no reports about kaempferol-induced ER stress in cancer cells. In Fig. 2, increased levels of GADD153, GRP78, GRP94, ATF-6α and ATF-6β were followed by releasing Ca^{2+} from ER, increasing calpain proteins expression and activating caspase-4, and finally leading to apoptosis. Our result was shown in fig. 2B and caspase-12 protein expression level has no significance influence. Caspase-12 has been shown to be involved ER stress-induced apoptosis pathways, but in humans, although the caspase-12 gene is transcribed into mRNA, mature caspase-12 protein would not be produced because the gene is interrupted by a frame shift and a premature stop codon. [38–40]. ER stress signaling pathway was reconfirmed by pre-treating with BAPTA, a Ca^{2+} chelator, and calpeptin, an inhibitor of calpain, in kaempferol-treated U-2 OS cells, and it showed decrease of calpain activity and increase of cell viability. These accumulating data demonstrated that the activation of ER stress pathway played an important role in kaempferol-induced apoptosis in U-2 OS cells.

Concentrated and selected accumulation of anti-cancer drugs at the tumor site is essential for the success of drug treatment *in vivo*. Previous studies have reported that flavonoid exhibits ability to inhibit human colorectal tumor formation and block rat glioma tumoral invasion and migration *in vivo* [41, 42]. Besides, two cohort studies have showed that high level of kaempferol intake significantly decreases ovarian cancer incidence, and intake of flavonol and catechin may be associated with a decreased colorectal cancer risk in normal weight women [43, 44]. In Fig. 4, our results showed that both 25 mg/kg and 50 mg/kg of kaempferol significantly reduced the tumor volume and weight in BALB/$c^{nu/nu}$ osteosarcoma mice. Additional prospective studies are needed to further evaluate these associations.

In conclusion, with this report, we now show that kaempferol exhibits direct anti-tumor activity, inducing tumor cell apoptosis and suppressing tumor cell proliferation. Moreover, kaempferol induced apoptosis through the mitochondria- dependent and ER stress pathways in human osteosarcoma U-2 OS cells. Finally, we show that kaempferol profoundly suppresses the in U-2 OS tumor xenograft-bearing mice *in vivo*. The proposed signal pathways of kaempferol-induced apoptosis in human osteosarcoma U-2 OS cells are shown in Fig. 5. Although there are still some controversy about the safety and biological effects of flavonoids, these findings provide important possible molecular mechanisms of the anti-human osteosarcoma and confirm that kaempferol may be an anti-osteosarcoma cancer drug candidate.

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5 References

[1] Hollman, P. C., Katan, M. B., Dietary flavonoids: intake, health effects and bioavailability. *Food Chem. Toxicol.* 1999, *37*, 937–942.

[2] Guo, Q., Zhao, L., You, Q., Yang, Y. *et al.*, Anti-hepatitis B virus activity of wogonin *in vitro* and *in vivo*. *Antiviral Res.* 2007, *74*, 16–24.

[3] Cardenas, M., Marder, M., Blank, V. C., Roguin, L. P., Anti-tumor activity of some natural flavonoids and synthetic derivatives on various human and murine cancer cell lines. *Bioorg. Med. Chem.* 2006, *14*, 2966–2971.

[4] Burda, S., Oleszek, W., Antioxidant and antiradical activities of flavonoids. *J. Agric. Food. Chem.* 2001, *49*, 2774–2779.

[5] Gonzalez-Gallego, J., Sanchez-Campos, S., Tunon, M. J., Anti-inflammatory properties of dietary flavonoids. *Nutr. Hosp.* 2007, *22*, 287–293.

[6] Yao, P., Nussler, A., Liu, L., Hao, L. *et al.*, Quercetin protects human hepatocytes from ethanol-derived oxidative stress by inducing heme oxygenase-1 *via* the MAPK/Nrf2 pathways. *J. Hepatol.* 2007, *47*, 253–261.

[7] Tijburg, L. B., Mattern, T., Folts, J. D., Weisgerber, U. M., Katan, M. B., Tea flavonoids and cardiovascular disease: a review. *Crit. Rev. Food. Sci. Nutr.* 1997, *37*, 771–785.

[8] Park, J. S., Rho, H. S., Kim, D. H., Chang, I. S., Enzymatic preparation of kaempferol from green tea seed and its antioxidant activity. *J. Agric. Food. Chem.* 2006, *54*, 2951–2956.

[9] Leung, H. W., Lin, C. J., Hour, M. J., Yang, W. H. *et al.*, Kaempferol induces apoptosis in human lung non-small carcinoma cells accompanied by an induction of antioxidant enzymes. *Food Chem. Toxicol.* 2007, *45*, 2005–2013.

[10] Marfe, G., Tafani, M., Indelicato, M., Sinibaldi-Salimei, P. *et al.*, Kaempferol induces apoptosis in two different cell lines *via* Akt inactivation, Bax and SIRT3 activation, and mitochondrial dysfunction. *J. Cell. Biochem.* 2009, *106*, 643–650.

[11] Zhang, Q., Zhao, X. H., Wang, Z. J., Flavones and flavonols exert cytotoxic effects on a human oesophageal adenocarcinoma cell line (OE33) by causing G2/M arrest and inducing apoptosis. *Food Chem. Toxicol.* 2008, *46*, 2042–2053.

[12] De Leo, M., Braca, A., Sanogo, R., Cardile, V. *et al.*, Antiproliferative activity of Pteleopsis suberosa leaf extract and its flavonoid components in human prostate carcinoma cells. *Planta. Med.* 2006, *72*, 604–610.

[13] Kim, K. S., Rhee, K. H., Yoon, J. H., Lee, J. G. *et al.*, *Ginkgo biloba* extract (EGb 761) induces apoptosis by the activation of caspase-3 in oral cavity cancer cells. *Oral Oncol.* 2005, *41*, 383–389.

[14] Mutoh, M., Takahashi, M., Fukuda, K., Matsushima-Hibiya, Y. *et al.*, Suppression of cyclooxygenase-2 promoter-dependent transcriptional activity in colon cancer cells by chemopreventive agents with a resorcin-type structure. *Carcinogenesis* 2000, *21*, 959–963.

[15] Arndt, C. A., Crist, W. M., Common musculoskeletal tumors of childhood and adolescence. *N. Engl. J. Med.* 1999, *341*, 342–352.

[16] Bielack, S., Carrle, D., Jost, L., Osteosarcoma: ESMO clinical recommendations for diagnosis, treatment and follow-up. *Ann. Oncol.* 2008, *19*, ii94–ii96.

[17] Siegel, H. J., Pressey, J. G., Current concepts on the surgical and medical management of osteosarcoma. *Expert Rev. Anticancer Ther.* 2008, *8*, 1257–1269.

[18] Duan, Z., Choy, E., Harmon, D., Yang, C. *et al.*, Insulin-like growth factor-I receptor tyrosine kinase inhibitor cyclolignan picropodophyllin inhibits proliferation and induces apoptosis in multidrug resistant osteosarcoma cell lines. *Mol. Cancer. Ther.* 2009, *8*, 2122–2130.

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

[20] Lockshin, R. A., Zakeri, Z., Cell death in health and disease. *J. Cell Mol. Med.* 2007, *11*, 1214–1224.

[21] Moran, J. M., Gonzalez-Polo, R. A., Ortiz-Ortiz, M. A., Niso-Santano, M. *et al.*, Identification of genes associated with paraquat-induced toxicity in SH-SY5Y cells by PCR array focused on apoptotic pathways. *J. Toxicol. Environ. Health A* 2008, *71*, 1457–1467.

[22] Hsu, S. C., Yang, J. S., Kuo, C. L., Lo, C. *et al.*, Novel quinolone CHM-1 induces apoptosis and inhibits metastasis in a human osterogenic sarcoma cell line. *J. Orthop. Res.* 2009, *27*, 1637–1644.

[23] Lin, S. S., Huang, H. P., Yang, J. S., Wu, J. Y. *et al.*, DNA damage and endoplasmic reticulum stress mediated curcumin-induced cell cycle arrest and apoptosis in human lung carcinoma A-549 cells through the activation caspases cascade- and mitochondrial-dependent pathway. *Cancer Lett.* 2008, *272*, 77–90.

[24] Ho, Y. T., Yang, J. S., Li, T. C., Lin, J. J. *et al.*, Berberine suppresses *in vitro* migration and invasion of human SCC-4 tongue squamous cancer cells through the inhibitions of FAK, IKK, NF-kappaB, u-PA and MMP-2 and -9. *Cancer Lett.* 2009, *279*, 155–162.

[25] Chou, L. C., Yang, J. S., Huang, L. J., Wu, H. C. *et al.*, The synthesized 2-(2-fluorophenyl)-6,7-methylenedioxyquinolin-4-one (CHM-1) promoted G2/M arrest through inhibition of CDK1 and induced apoptosis through the mitochondrial-dependent pathway in CT-26 murine colorectal adenocarcinoma cells. *J. Gastroenterol* 2009, *44*, 1055–1063.

[26] Yang, J. S., Chen, G. W., Hsia, T. C., Ho, H. C. *et al.*, Diallyl disulfide induces apoptosis in human colon cancer cell line (COLO 205) through the induction of reactive oxygen species, endoplasmic reticulum stress, caspases casade and mitochondrial-dependent pathways. *Food Chem. Toxicol.* 2009, *47*, 171–179.

[27] Chung, J. G., Yang, J. S., Huang, L. J., Lee, F. Y. *et al.*, Proteomic approach to studying the cytotoxicity of YC-1 on U937 leukemia cells and antileukemia activity in orthotopic model of leukemia mice. *Proteomics* 2007, *7*, 3305–3317.

[28] Lee, M. J., Kee, K. H., Suh, C. H., Lim, S. C., Oh, S. H., Capsaicin-induced apoptosis is regulated by endoplasmic reticulum stress- and calpain-mediated mitochondrial cell death pathways. *Toxicology* 2009, *264*, 205–214.

[29] Wen, Y. F., Yang, J. S., Kuo, S. C., Hwang, C. S. *et al.*, Investigation of anti-leukemia molecular mechanism of ITR-284, a carboxamide analog, in leukemia cells and its effects in WEHI-3 leukemia mice. *Biochem. Pharmacol.* 2010, *79*, 389–398.

[30] Koch, T. C., Briviba, K., Watzl, B., Fahndrich, C. *et al.*, Prevention of colon carcinogenesis by apple juice *in vivo*:

impact of juice constituents and obesity. *Mol. Nutr. Food Res.* 2009, *53*, 1289–1302.

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

[32] Chen, J. Q., Brown, T. R., Yager, J. D., Mechanisms of hormone carcinogenesis: evolution of views, role of mitochondria. *Adv. Exp. Med. Biol.* 2008, *630*, 1–18.

[33] Kang, J. W., Kim, J. H., Song, K., Kim, S. H. *et al.*, Kaempferol and quercetin, components of *Ginkgo biloba* extract (EGb 761), induce caspase-3-dependent apoptosis in oral cavity cancer cells. *Phytother. Res.* 2010, *148*, S77–82.

[34] Zhang, Y., Chen, A. Y., Li, M., Chen, C., Yao, Q., *Ginkgo biloba* extract kaempferol inhibits cell proliferation and induces apoptosis in pancreatic cancer cells. *J. Surg. Res.* 2008, *148*, 17–23.

[35] Li, N., Liu, J. H., Zhang, J., Yu, B. Y., Comparative evaluation of cytotoxicity and antioxidative activity of 20 flavonoids. *J. Agric. Food Chem.* 2008, *56*, 3876–3883.

[36] Soares, V. C., Varanda, E. A., Raddi, M. S., *In vitro* basal and metabolism-mediated cytotoxicity of flavonoids. *Food Chem. Toxicol.* 2006, *44*, 835–838.

[37] Ziegler, D. S., Kung, A. L., Therapeutic targeting of apoptosis pathways in cancer. *Curr. Opin. Oncol.* 2008, *20*, 97–103.

[38] Nieto-Miguel, T., Fonteriz, R. I., Vay, L., Gajate, C. *et al.*, Endoplasmic reticulum stress in the proapoptotic action of edelfosine in solid tumor cells. *Cancer Res.* 2007, *67*, 10368–10378.

[39] Hitomi, J., Katayama, T., Eguchi, Y., Kudo, T. *et al.*, Involvement of caspase-4 in endoplasmic reticulum stress-induced apoptosis and Abeta-induced cell death. *J. Cell Biol.* 2004, *165*, 347–356.

[40] Rao, R. V., Ellerby, H. M., Bredesen, D. E., Coupling endoplasmic reticulum stress to the cell death program. *Cell Death Differ.* 2004, *11*, 372–380.

[41] Chen, Y. C., Shen, S. C., Chow, J. M., Ko, C. H., Tseng, S. W., Flavone inhibition of tumor growth via apoptosis *in vitro* and *in vivo*. *Int. J. Oncol.* 2004, *25*, 661–670.

[42] Shen, S. C., Lin, C. W., Lee, H. M., Chien, L. L., Chen, Y. C., Lipopolysaccharide plus 12-o-tetradecanoylphorbol 13-acetate induction of migration and invasion of glioma cells *in vitro* and *in vivo*: Differential inhibitory effects of flavonoids. *Neuroscience* 2006, *140*, 477–489.

[43] Gates, M. A., Tworoger, S. S., Hecht, J. L., De Vivo, I. *et al.*, A prospective study of dietary flavonoid intake and incidence of epithelial ovarian cancer. *Int. J. Cancer* 2007, *121*, 2225–2232.

[44] Simons, C. C., Hughes, L. A., Arts, I. C., Goldbohm, R. A. *et al.*, Dietary flavonol, flavone and catechin intake and risk of colorectal cancer in the Netherlands Cohort Study. *Int. J. Cancer* 2009, *125*, 2945–2952.

