Induction of apoptosis by diphenyldifluoroketone in osteogenic sarcoma cells is associated with activation of caspases

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Abstract. The aim of the present study was to investigate and compare the effects of diferuloylmethane (curcumin) and diphenyldifluoroketone (EF-24) on cell growth and apoptosis induction in human osteogenic sarcoma cells. This was examined by MTT assay, nuclear DAPI staining, caspase-activation assay, flow cytometry analysis and immunoblotting in Saos2 human osteogenic sarcoma cells. Curcumin and EF-24 inhibited the growth of Saos2 cells in a dose-dependent manner. The apparent potency of EF-24 was more than 3-fold higher that of curcumin. Treatment with curcumin or EF-24 resulted in nuclear condensation and fragmentation in the cells. The caspase-3/-7 activities were detected in living cells treated with curcumin or EF-24. Flow cytometry showed that the rate of apoptosis was increased by curcumin and EF-24 compared to the control. Curcumin and EF-24 promoted the proteolytic cleavages of procaspase-3/-7/-8/-9 with increases in the amount of cleaved caspase-3/-7/-8/-9. The curcumin- or EF-24-induced apoptosis in the Saos2 cells was mediated by the expression of Fas and activation of caspase-8, caspase-3 and poly(ADP-ribose) polymerase. Immunoblotting revealed the Bid and Bcl-2 proteins to be downregulated, and truncated-Bid, Bax and p53 proteins to be upregulated by curcumin and EF-24. Curcumin and EF-24 increased the Bax/Bcl-2 ratio significantly. These results suggest that the curcumin and EF-24 inhibit cell proliferation and induce apoptotic cell death in Saos2 human osteogenic sarcoma cells via both the mitochondria-mediated intrinsic pathway and the death receptor-mediated extrinsic pathway, and may have potential properties for anti-osteosarcoma drug discovery.

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Introduction

Osteosarcoma is the most common primary bone malignant tumor, affecting primarily children and young adolescents (1). Despite the advances in multimodality treatments, the combination of neoadjuvant chemotherapy plus surgery have increased the survival rates markedly from 20 to 65% since the 1980s, but the progress has been considerably slow over the past 20 years (2,3). Therefore, an understanding of the molecular mechanisms of osteosarcoma is one of the most important issues for treatment. New therapeutic strategies are necessary to increase the survival rates in patients with osteosarcoma (4).

In recent years, there has been a global trend toward the importance of naturally occurring phytochemicals in plants for the prevention and treatment of human diseases. Several of these phytochemicals have shown potential as cancer chemo-preventive or therapeutic agents in the human body (5). Most of these bioactive phytochemicals exert their cancer chemo-therapeutic activity by blocking cell cycle progression and triggering apoptotic cell death (5). Therefore, the induction of apoptosis in cancer cells is an important indicator of the cancer treatment response when employing a bioactive substance to reduce and control human mortality due to cancer (6,7).

Apoptosis, which is a major form of programmed cell death, plays an important role in regulating tissue development and homeostasis in eukaryotes (8). Apoptosis can occur via a death receptor-dependent extrinsic or a mitochondria-dependent intrinsic pathway, and apoptosis is induced by a treatment with chemotherapeutic agents (9,10).

Diferuloylmethane (curcumin), which is a constituent of turmeric powder derived from the rhizome of *Curcuma longa*, has anti-inflammatory, antimicrobial, anti-oxidative, immunomodulating and anti-atherogenic properties (11,12). Furthermore, many studies have reported that curcumin has chemopreventive and antiproliferative activity in many human cancers (12,13). Of particular interest is that curcumin is also pharmacologically safe as it is a naturally occurring

compound (14). However, natural curcumin has limited use due to its poor intestinal absorption and low bioavailability (15). On the other hand, it is an excellent compound for the design of more effective analogs. One monoketone analog in particular, diphenyldifluoroketone (EF-24), is effective in anticancer screens (16). EF-24 was reported to inhibit the growth of human breast tumor xenografts in a mouse model with relatively low toxicity and at a much lower dose than that of curcumin (16). Although it was shown to reduce the cancer cell viability, its action mechanisms remain to be determined. In addition, the effects of EF-24 and curcumin on osteosarcoma are unclear.

The present study examined the effects of EF-24 and curcumin on cell growth and the mechanism of cell death elicited by EF-24 and curcumin in Saos2 human osteogenic sarcoma cells. The study reports for the first time that the induction of apoptotic cell death by EF-24 is associated with the activation of caspases in osteogenic sarcoma cells.

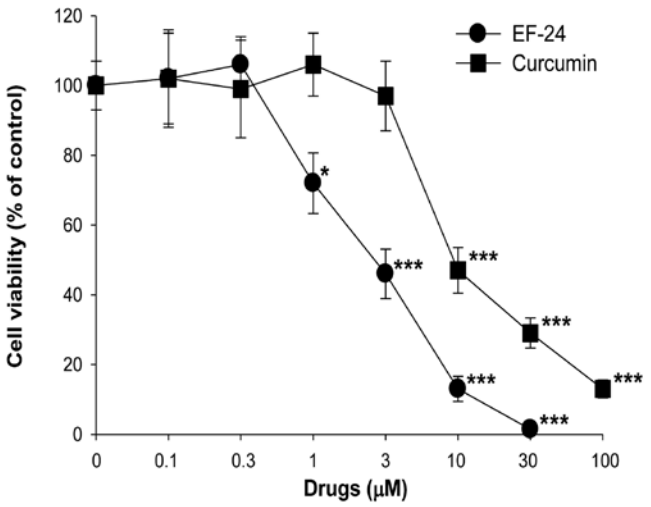

Figure 1. Concentration-dependent effects of EF-24 and curcumin on the cell viability in Saos2 human osteogenic sarcoma cells. The Saos2 cells were treated with various concentrations of EF-24 (circle) and curcumin (square) or without EF-24 and curcumin for 24 h. The cell viabilities were determined by MTT assays. The percentage of cell viability was calculated as a ratio of A570 nm of the EF-24- or curcumin-treated cells and untreated control cells. Each data point represents the mean ± SEM of six experiments. *P<0.05 vs. control and ***P<0.001 vs. control (the control cells measured in the absence of EF-24 or curcumin).

Materials and methods

Materials. Saos2 human osteogenic sarcoma cells were provided by the American Type Culture Collection (ATCC, Rockville, MD, USA). EF-24 and curcumin were supplied by Sigma (St. Louis, MO, USA). Anti-cleaved caspase-3/-7/-8/-9, poly(ADP-ribose) polymerase (PARP), Fas, Bid, Bax, Bcl-2, p53 and β-actin antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The cell-permeable fluorogenic substrate PhiPhiLux-G_1D_2 was purchased from OncoImmunin, Inc. (Gaithersburg, MD, USA). All other analytical reagents purchased were of analytical grade.

Cell cultures. The Saos2 human osteogenic sarcoma cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS) (17). The cells were maintained as monolayers in plastic culture plates at 37˚C in a humidified atmosphere containing 5% CO_2.

MTT assay. The Saos2 cells were seeded at a concentration of $5x10^3$ cells/well in 24-well plates. After 24-h growth, the cells were treated with EF-24 or curcumin at various concentrations for 24 h. The cell viability was assessed using an MTT assay.

Nuclear staining with DAPI. Nuclear staining with DAPI (40,60-diamidino-2-phenylindole) was performed to examine the level of apoptosis. The Saos2 cells were cultured in 24-well plates at a seeding density of $5x10^3$ cells/well. After 24-h growth, the cells were treated with 10 μM EF-24 or 30 μM curcumin for 24 h. The treated Saos2 cells were fixed with 1% paraformaldehyde for 30 min at room temperature and washed twice with PBS. The cells were permeated with ice-cold ethanol for 5 min at room temperature and washed twice with PBS. The fixed cells were stained with DAPI (300 nM) for 5 min at room temperature in the dark, washed twice with PBS and examined by fluorescent inverted microscopy (IX71; Olympus, Tokyo, Japan).

Determination of caspase activation. The activity of caspase-3/-7 was determined using the cell-permeable fluorogenic substrate, PhiPhiLux-G_1D_2, according to the manufacturer's instructions. The cells were treated with 0 and 10 μM EF-24 or 0 and 30 μM curcumin for 24 h and incubated with PhiPhiLux-G_1D_2. The activity of caspase-3/-7 was examined by fluorescence microscopy (IX71; Olympus).

Annexin V-fluorescein isothiocyanate (V-FITC), propidium iodide (PI) and flow cytometric analysis. The Saos2 cells were cultured in 100-mm tissue-culture dishes at a density of $1x10^6$ cells/dish for 24 h and were treated with 10 μM EF-24 or 30 μM curcumin for 24 h. The cells were washed twice in phosphate-buffered saline and resuspended in the binding buffer (BD Biosciences, San Diego, CA, USA). Annexin V-FITC and 7-amino-actinomycin D (BD Biosciences) were added to the cells, which were then incubated in the dark for 15 min, and resuspended in 400 μl of binding buffer. The cells were analyzed using a fluorescence activated cell sorting Calibur flow cytometer (Becton-Dickinson, San Jose, CA, USA). Data analysis was performed using standard CellQuest software (Becton-Dickinson).

Immunoblotting. The Saos2 cells were treated with EF-24 or curcumin for 24 h. Immunoblotting was performed using a minor modification of the method described elsewhere (18). Anti-cleaved caspase-3/-7/-8/-9, PARP, Fas, Bid, tBid, Bax, Bcl-2 and p53 antibodies (Cell Signaling Technology) were used as the primary antibodies.

Data analysis. All experiments were performed at least in triplicate. The results are presented as the mean ± SEM. The statistical significance was analyzed using a Student's t-test for the two group comparison and one-way analysis of the variance for the multi-group comparisons. A P-value <0.05 was considered to indicate a statistically significant difference.

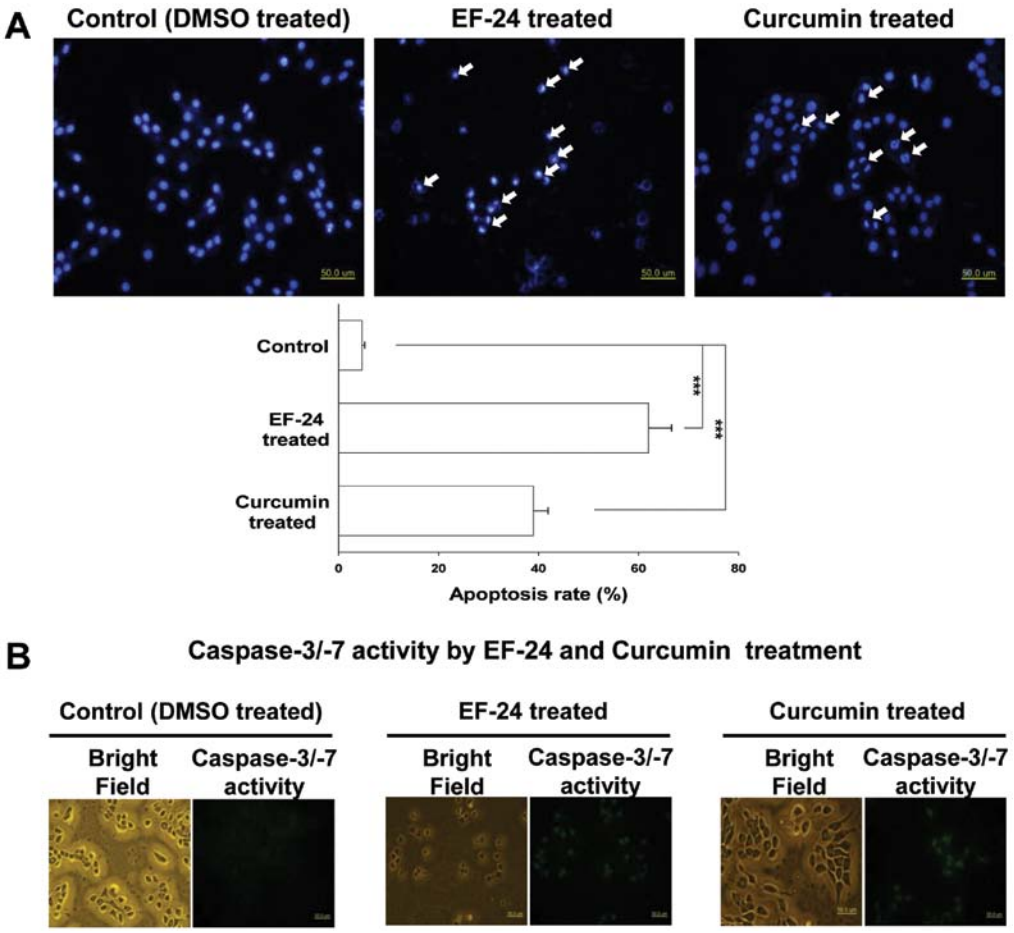

Figure 2. Induction of apoptosis by EF-24 and curcumin in Saos2 cells. (A) Changes in nuclear morphology by EF-24 and curcumin. The cells were treated with 10 μM EF-24 or 30 μM curcumin for 24 h. Representative fluorescence photomicrographs show the nuclei morphology of Saos2 cells. The arrows indicate chromatin condensation, reduced nuclear size and nuclear fragmentation typically observed in apoptotic cells. The lower panel shows that the percentage of apoptotic cells was calculated as the ratio of apoptotic cells to total cells. ***P<0.001 vs. control (the control cells measured in the absence of EF-24 or curcumin). (B) Activation of caspase-3/-7 by EF-24 or curcumin treatment in living Saos2 cells. The cells were treated with 10 μM EF-24 or 30 μM curcumin for 24 h and the specific cell-permeable substrate PhiPhiLux G_1D_2 was then added. Active caspase-3/-7 was visualized by fluorescence microscopy.

Results

Cytotoxic effects of EF-24 and curcumin on Saos2 cells. The Saos2 cells were treated with EF-24 and curcumin at various concentrations for 24 h, and were analyzed by MTT assay. As shown in Fig. 1, treatment of curcumin at doses from 0.1 to 3 μM did not significantly affect the cell viability of the Saos2 cells, but curcumin at 10, 30 and 100 μM reduced Saos2 cell viability. When the cells were treated with EF-24 for 24 h, EF-24 inhibited the growth of cells in a dose-dependent manner, suggesting that EF-24 induces Saos2 cell death (Fig. 1). The IC_{50} values of EF-24 and curcumin on Saos2 cell viability after a 24-h treatment were 2.7±0.3 and 9.7±1.4 μM, respectively. The apparent potency of EF-24 was >3-fold higher that of curcumin. More importantly, the effects were observed at an EF-24 concentration <3 μM, a dose at which curcumin had no significant effects on cell proliferation, indicating the higher potency of EF-24.

Changes in the nuclear morphology by EF-24 and curcumin. The nuclear morphological changes were assessed by DAPI staining. As shown in Fig. 2A, the nuclei of the control Saos2 cells (control) had a normal regular and oval shape. Treatment with 10 μM EF-24 or 30 μM curcumin for 24 h resulted in nuclear condensation and fragmentation, which are the characteristics of apoptosis (Fig. 2A, upper panel). As quantified in Fig. 2A (lower panel), EF-24 and curcumin increased the apoptotic rate of Saos2 cells significantly to 62.0±4.6 and 39.1±2.9%, respectively.

Activation of caspase-3/-7 by EF-24 and curcumin. The activation of caspase-3/-7 in the EF-24- or curcumin-treated Saos2 cells was confirmed by fluorescence microscopy using a fluorogenic substrate. As shown in Fig. 2B, both the EF-24 and curcumin treatment led to the activation of caspase-3/-7 in the living Saos2 cells.

Apoptosis induction of Saos2 cells by EF-24 and curcumin. To determine if EF-24- or curcumin-induced cell death is associated with the induction of apoptosis, the Saos2 cells were stimulated with 10 μM EF-24 or 30 μM curcumin for 24 h and then co-stained with Annexin V-FITC, an apoptotic marker, and PI, a necrotic marker. The percentage of Annexin V-FITC-positive cells at the stage of apoptosis was increased to 64.1 and 43.9% by EF-24 and curcumin, respectively, compared to the control (Fig. 3).

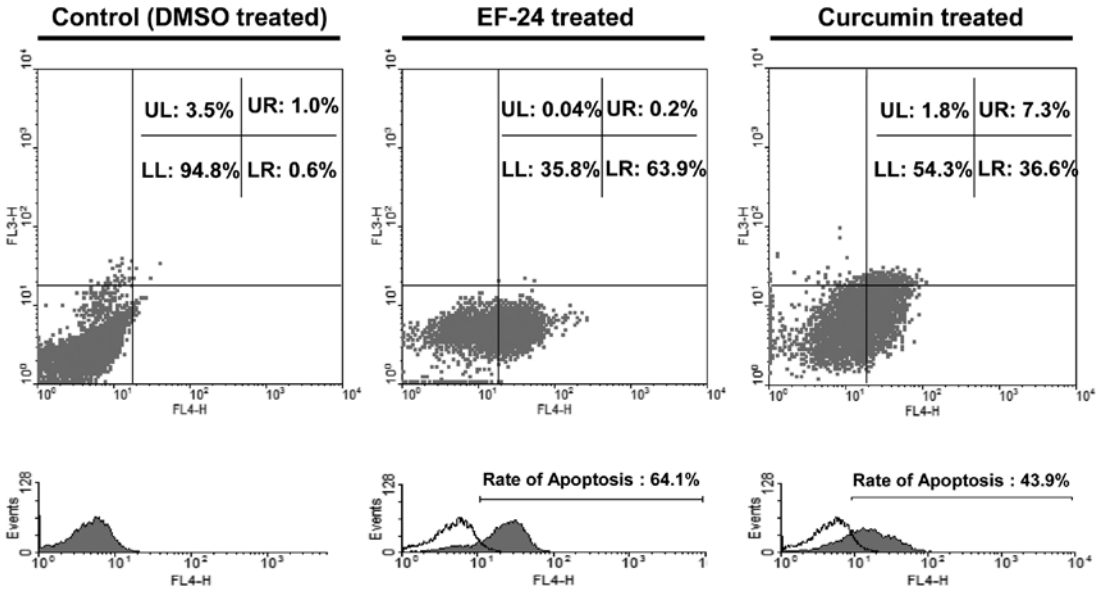

Figure 3. Apoptotic population induction of Saos2 cells by EF-24 and curcumin. To identify the EF-24- or curcumin-induced Saos2 cell apoptosis, FACS analysis was performed by Annexin V and PI staining. The Saos2 cells were cultured in complete medium, and stimulated with 10 μM EF-24 or 30 μM curcumin for 24 h. After stimulation, the cells were analyzed by flow cytometry. LL, lower left (normal); UL, upper left (necrosis); LR, lower right (early phase of apoptosis); UR, upper right (late phase of apoptosis).

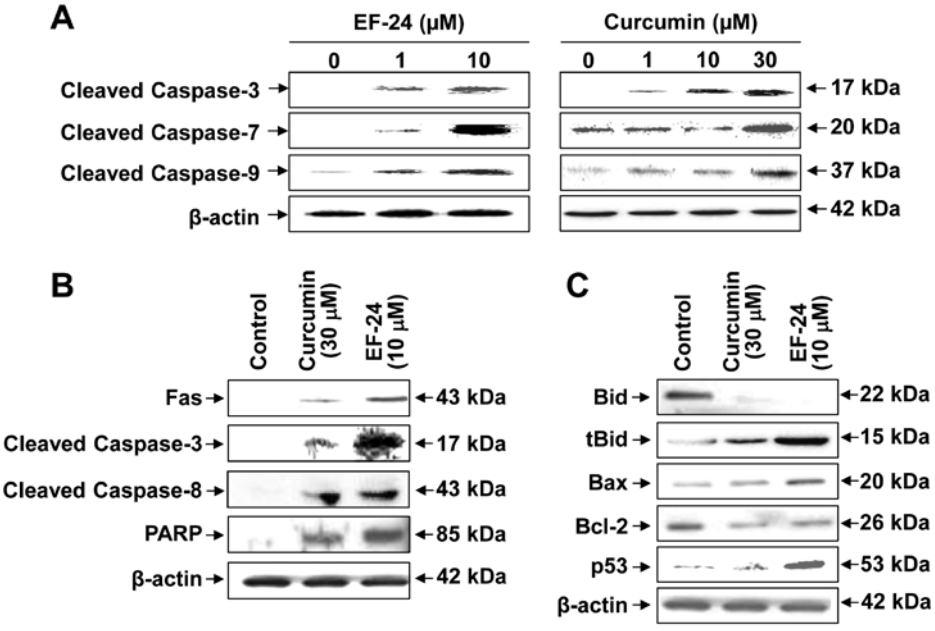

Figure 4. EF-24 or curcumin-induced apoptosis mediated by expression and activation of caspases in Saos2 cells. (A) Proteolytic cleavage of caspase-3/-7/-9 by EF-24 or curcumin treatment. The activity of cleaved caspase-3/-7/-9 by EF-24 or curcumin was measured in Saos2 cells. The cells were treated with 0, 1 and 10 μM EF-24 or 0, 1, 10 and 30 μM curcumin for 24 h. The cell lysate was prepared and analyzed by immunoblotting as described in Materials and methods. (B) Activation of the extrinsic apoptosis signaling pathway by EF-24 and curcumin. The activity of extrinsic apoptosis signaling pathway in Saos2 cells by EF-24 or curcumin was measured. The Saos2 cells were stimulated with 0 and 10 μM EF-24 or 0 and 30 μM curcumin for 24 h, harvested, and lysed using a cell lysis buffer. (C) Activation of p53 and regulation of Bid, tBid, Bax and Bcl-2 levels by EF-24 and curcumin. The activities of p53, Bid, tBid, Bax and Bcl-2 by EF-24 or curcumin were measured in Saos2 cells.

Activation of caspases by EF-24 and curcumin. The levels of cleaved caspase-3/-7/-9 were examined by immunoblotting as caspase-3/-7/-9 are effector caspases of apoptotic cell death (19). Treatment with 1 and 10 μM EF-24 or 1, 10 and 30 μM curcumin for 24 h promoted the proteolytic cleavage of procaspase-3/-7/-9 in Saos2 cells, with increases in the amount of cleaved caspase-3/-7/-9 (Fig. 4A).

Apoptosis mediated via Fas/PARP axis by EF-24 and curcumin. To determine how EF-24 or curcumin induce the apoptosis of Saos2 cells, immunoblotting was performed to measure the expression of the apoptotic genes at the protein level. As shown in Fig. 4B, Fas, which is an apoptotic ligand that triggers the extrinsic apoptotic pathway in Saos2 cells (20,21), was induced significantly by either EF-24 or

curcumin. Subsequently, the cleaved caspase-3 and -8, which are the downstream targets of Fas, were induced by EF-24 or curcumin. Cleaved PARP was increased by EF-24 or curcumin compared to the control.

Apoptosis-related signal pathways by EF-24 and curcumin. The level of several proteins that are highly relevant to understanding the apoptotic signaling pathways in Saos2 cells by EF-24 or curcumin was measured by immunoblot analysis. The level of Bid and Bcl-2 protein expressions in Saos2 cells stimulated with 10 μM EF-24 or 30 μM curcumin for 24 h decreased (Fig. 4C). On the other hand, the treatment of Saos2 cells with EF-24 or curcumin increased the level of truncated-Bid (tBid), Bax and p53 protein expression (Fig. 4C).

Discussion

Recent studies have shown that the chemicals derived from natural materials can elicit chemopreventive and therapeutic effects (5,22). This effect was reported to alter many factors associated with the cell cycle and to induce apoptotic cell death (5,23). Finding new anticancer agents that can kill cancerous cells with minimal toxicity is critical. Curcumin has been extracted from the dried ground rhizome of the perennial herb, *Curcuma longa*. Several studies have suggested that curcumin induces cell cycle arrest and apoptosis in a variety of cancer cells (12,13). On the other hand, the design of more effective analogs is needed due to the poor intestinal absorption and low bioavailability of curcumin (15). EF-24, a monoketone analog of curcumin, is efficacious in anticancer screens and was reported to inhibit the growth of human breast tumor xenografts in a mouse model (16). Although EF-24 can reduce cancer cell viability, the mechanisms of action are unclear, and the effects of EF-24 and curcumin on osteosarcoma have not been established. Osteosarcoma is the most common type of malignant bone tumor, in which the neoplastic mesenchymal cells show evidence of osteoid production (18). The present study examined the cytotoxic effects of EF-24, and the mechanism of cell death exhibited by EF-24 in Saos2 human osteogenic sarcoma cells was assessed. The results suggest that EF-24, a novel curcumin analog, possesses considerable promise as an anti-osteosarcoma therapeutic.

An MTT assay showed that the treatment of curcumin from 0.1 to 3 μM did not significantly affect the cell viability of Saos2 cells, but curcumin at 10, 30 and 100 μM reduced Saos2 cell viability (Fig. 1). EF-24 inhibited the growth of Saos2 cells in a concentration-dependent manner (Fig. 1). This corresponded to the results of EF-24 and curcumin, which have anticancer effects via the suppression of cancer cell growth in many types of cancer cells (12,13). In addition, the apparent potency of EF-24 was >3-fold higher that of curcumin. This suggests that EF-24 and curcumin are cytotoxic to osteosarcoma cells with EF-24 having higher potency. In addition, these results suggest that it has potential value for anticancer drug discovery.

Apoptosis is an important way of maintaining cellular homeostasis between cell division and cell death (8). The induction of apoptosis in cancer cells is a useful strategy for anticancer drug development (24). Therefore, many studies have screened plant-derived compounds for their effects on apoptosis (5). In the present study, treatment with EF-24 and curcumin induced nuclear condensation and fragmentation and the activation of the caspase-3/-7 in living Saos2 cells, suggesting apoptotic cell death (Fig. 2). These results indicate that EF-24 and curcumin inhibit the growth of Saos2 cells by activating cell apoptosis.

Curcumin and EF-24 were evaluated for their apoptosis-inducing activities by the flow cytometry of Saos2 cells stained with Annexin V-FITC and PI. Exposure of the membrane phospholipid, phosphatidylserine, to the external cellular environment is one of the earliest markers of apoptotic cell death (25,26). Annexin V is a calcium-dependent phospholipid binding protein with high affinity to phosphatidylserine expressed on the cell surface (25). PI does not enter a cell with an intact cell-membrane and has been used to differentiate among early apoptotic (Annexin V-positive, PI-negative), late apoptotic (Annexin V-, PI-double positive) and necrotic cell death (Annexin V-negative, PI-positive) (25). In the present study, the ratio of early apoptotic Saos2 cells (lower right) was increased after a treatment with 10 μM EF-24 (from 0.6 to 63.9%) or 30 μM curcumin (from 0.6 to 36.6%) (Fig. 3). These results showed that most of the cytotoxic activity of curcumin and EF-24 against Saos2 cells is due to the induction of apoptotic cell death.

The activation of a family of intracellular cysteine proteases, known as caspases, plays an important role in the initiation and execution of apoptosis induced by a range of stimuli (27). Among the caspases identified in mammalian cells, caspase-3/-7/-8/-9 can serve as the effector caspases of apoptotic cell death (27). Caspase-3/-7/-8/-9 are synthesized as inactive proenzymes, which require proteolytic activation to cleaved enzymes (of sizes 17, 20, 43 and 37 kDa, respectively) (27). The results of the present study showed that the amount of cleaved caspase-3/-7/-8/-9 in Saos2 cells was increased after the EF-24 or curcumin treatment (Fig. 4A and B). These results suggest that EF-24 and curcumin induce apoptotic cell death through both the mitochondria-mediated intrinsic pathway and death receptor-mediated extrinsic pathway by the activation of caspases-3/-7/-8/-9 in Saos2 cells.

The factor associated suicide ligand (Fas), which is an important regulatory factor of apoptosis, initiates the death receptor-mediated extrinsic apoptotic pathway through the activation of caspase-8, -3 and PARP, sequentially, after binding with the receptor FasR spanned on the surface of the target cells (20,21). In the present study, the expression of Fas was upregulated significantly by EF-24 or curcumin in Saos2 cells (Fig. 4B). Subsequently, Fas upregulated by EF-24 or curcumin triggers a caspase cascade, which then results in the activation of apoptotic factors, including cleaved caspase-8 and -3. Finally, activated caspase-3 cleaved its major substrate, PARP, leading to consequent apoptosis. Therefore, these results suggest that EF-24- or curcumin-induced apoptosis in Saos2 cells is mediated by the death receptor-mediated extrinsic apoptotic pathway via the Fas/PARP axis.

Next, we assessed the effects of EF-24 and curcumin on the expression of p53, Bid, tBid, Bax and Bcl-2 in Saos2 cells. The molecular biological pathways underlying the inhibition of cancerous growth typically involve tumor suppressors, such as p53 (8,28-30). The level of p53 is consistent in normal

cells and the protein becomes phosphorylated during cellular stress, which then interacts with mouse double minute 2 (MDM2), resulting in apoptosis (8,28-31). In the present study, the level of p53 was higher in the Saos2 cells stimulated with EF-24 or curcumin than in the control (Fig. 4C), indicating that p53 may mediate the EF-24- or curcumin-induced apoptosis of Saos2 cells. The pro-apoptotic proteins, Bid, tBid and Bax, and the anti-apoptotic mitochondrial protein, Bcl-2, are important regulators of cytochrome *c* release from the mitochondria (32,33). The Bcl-2 family is localized to the mitochondrial membrane and modulates apoptosis by permeabilizing the mitochondrial membrane, leading to the release of cytochrome *c* (34). In the present study, treatment of Saos2 cells with EF-24 or curcumin decreased the level of Bid and increased the level of tBid (Fig. 4C). Caspase-8 was reported to truncate Bid, and tBid could activate the mitochondrial pathway (35). In addition, treatment of Saos2 cells with EF-24 or curcumin decreased the level of Bcl-2 but increased the level of Bax (Fig. 4C). The Bax/Bcl-2 ratio is one of the indices of the intrinsic mechanism of apoptosis in the mitochondria (36). EF-24 or curcumin-induced apoptosis appears to involve Bax/Bcl-2 signal transduction since EF-24 and curcumin increased this ratio in Saos2 cells. Therefore, EF-24 and curcumin are suggested to induce apoptosis in Saos2 cells involving both the mitochondrial- and death receptor-signal transduction pathways. On the other hand, the mechanisms of apoptosis induced by EF-24 and curcumin in Saos2 cells are not fully understood. Further studies are required to examine the precise cellular and molecular mechanisms of apoptosis induced by EF-24 and curcumin.

In conclusion, these *in vitro* results suggest that EF-24 and curcumin inhibit cell proliferation and induce apoptotic cell death in Saos2 human osteogenic sarcoma cells through both the mitochondria-mediated intrinsic pathway and the death receptor-mediated extrinsic pathway. Moreover, EF-24 was >3-fold more potent than curcumin. Overall, EF-24 can be a model compound for the further development of natural product-derived anti-osteosarcoma agents. On the other hand, to elaborate on this nascent possibility, a further study of its activity including *in vivo* and the purification of bioactive compounds is currently being conducted.

Acknowledgements

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