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Anticancer effect of curcumin and its analog EF-24 in Saos2 human osteogenic sarcoma cells

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사람 골육종 Saos2 세포에서 curcumin과 curcumin 유사체 EF-24의 항암효과

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- 초 록 -

사람 골육종 Saos2 세포에서 curcumin과 curcumin 유사체 EF-24의 항암효과

양 석 진

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약용식물인 강황의 추출성분 curcumin(diferuloylmethane)은 항염증, 항세균 및 항산화 등의 효과가 있다고 보고되어져 왔으며, 특히 사람의 특정 암세포 성장을 억제시킬 수 있다고 보고되었다. 그러나 curcumin은 위장관 흡수율이 빈약하다는 단점을 갖고 있어, 더 효과적인 유사체가 요구되고 있다. EF-24(diphenyl difluoroketone)는 curcumin의 새로운 monoketone 유사체로서 항암활성을 보유하고 있다고 보고된 바 있으나, 골육종과 관련된 curcumin과 EF-24에 관한 자료는 거의 없다. 따라서 본 연구에서는 사람 골육종 세포주를 이용하여 curcumin과 EF-24의 골육종세포 성장억제에 미치는 효과와 세포성장 억제기전을 분석하였다.

본 연구에서 curcumin과 EF-24에 의한 골육종세포 성장억제와 그 기전을 조사하기 위해, 사람 골육종세포 Saos2에서 curcumin과 EF-24를 처리한 후, MTT 분석, DAPI를 이용한 세포핵 염색, immunoblotting 및 caspase 활성분석을 시행하였다.

사람 골육종세포 Saos2에서 curcumin과 EF-24는 골육종세포의 성장을 농도에 의존적으로 억제하였으며, 그 효능은 EF-24가 curcumin의 3배 이상이었다. Saos2 세포에서 curcumin과 EF-24는 핵의 응집과 파쇄를 유도하였다. Saos2 세포에 curcumin과 EF-24를 처리한 실험군에서 활성화된 caspase-3, -7 및 -9의 증가를 확인할 수 있었다. Saos2 세포에 curcumin과 EF-24를 처리한 실험군에서 caspase-3/-7의 활성화를 확인할 수 있었다.

본 연구의 결과로 curcumin과 그 유사체 EF-24는 사람 골육종세포 Saos2의 apoptosis를 유도하여 골육종세포 성장을 억제시키는 것으로 생각된다. 또한 본 연구의 결과로, curcumin과 EF-24를 이용한 골육종세포의 성장억제에 관한 하나의 방향을 제시할 수 있을 것으로 사료된다.

중심어: Curcumin, EF-24, 세포사, Apoptosis, 골육종

I. INTRODUCTION

Osteosarcoma is the most common primary bone malignant tumor, affecting primarily children and young adolescents (Zhao *et al.*, 2011; Daiy *et al.*, 2012; Thayanithy *et al.*, 2012). Despite advances in multimodality treatments, the combination of neoadjuvant chemotherapy plus surgery that dramatically increased survival rates from 20 to 65% since the 1980s, the progress has been painfully slow for the past 20 years (Fuchs and Pritchard, 2002; Gibbs *et al.*, 2002). 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 survival rates in patients with osteosarcoma (Zhao *et al.*, 2011).

In previous studies, 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 are shown to have potential values as cancer chemopreventive or therapeutic agents within the human body (Pezutto, 1997; van Poppel and van den Berg, 1997; Christou *et al.*, 2001; Mukherjee *et al.*, 2001). Most of these bioactive phytochemicals exert their cancer chemotherapeutic activity by blocking cell cycle progression and triggering apoptotic cell death (Pezutto, 1997; van Poppel and van den Berg, 1997; Christou *et al.*, 2001; Mukherjee *et al.*, 2001). Therefore, induction of apoptosis in cancer cells has become an important indicator of the cancer treatment response in employing a bioactive substance to reduce and control

human mortality due to cancer (Smets, 1994; Paschka *et al.*, 1998).

Apoptosis, which is a major way of programmed cell death, plays an important role in the regulation of tissue development and homeostasis in eukaryotes (Green and Reed, 1998; Hengartner, 2000; Kaufmann and Hengartner, 2001). Apoptosis may occur via a death receptor-dependent extrinsic or a mitochondria-dependent intrinsic pathway and apoptosis is induced by treatment of chemotherapeutic agents (Kaufmann and Earnshaw, 2000; Reed, 2001).

Curcumin (diferuloylmethane), a constituent of turmeric powder derived from the rhizome of *Curcuma longa*, has antiinflammatory, antimicrobial, antioxidative, immunomodulating and antiatherogenic properties (Mukhopadhyay *et al.*, 2001; Miquel *et al.*, 2002; Banerjee *et al.*, 2003; Sahu *et al.*, 2009). Furthermore, many studies provide the fact that curcumin has chemopreventive and antiproliferative activity in various types of human cancers (Aggarwal *et al.*, 2007; Bachmeier *et al.*, 2007; Hauser *et al.*, 2007; Shankar and Srivastava, 2007; Wahl *et al.*, 2007; Sahu *et al.*, 2009). Of particular interest is that curcumin is also pharmacologically safe as it is a naturally occurring compound (Ammon and Wahl, 1991; Goel *et al.*, 2008). Unfortunately, natural curcumin has been limited the use because of its poor intestinal absorption and low bioavailability (Anand *et al.*, 2007). However it remains an excellent compound for the design of more effective analogs. One monoketone analog in particular, EF-24 (diphenyl difluoroketone), is efficacious in anticancer screens (Adams *et al.*, 2004; Adams *et al.*, 2005). EF-24 has been shown to inhibit the growth of human breast tumor xenografts in a mouse model with relatively low toxicity and at a dose much lower than that of curcumin (Adams *et al.*, 2004; Adams *et*

al., 2005). Although it is shown to reduce cancer cell viability, its action mechanisms remain to be elucidated. In addition, the effects of EF-24 and curcumin on osteosarcoma are not clearly established.

In this study, therefore, the effects of EF-24 and curcumin on cell growth and the mechanism of cell death elicited by EF-24 and curcumin were examined in Saos2 human osteogenic sarcoma cells.

II. MATERIALS AND METHODS

1. Materials

The Saos2 human osteogenic sarcoma cells were provided by American Type Culture Collection (ATCC, Rockville, MD, USA). EF-24, curcumin (Fig. 1) and 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were supplied by Sigma (St Louis, MO, USA). Anti-cleaved caspase-3, anti-cleaved caspase-7 and anti-cleaved caspase-9 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). Other analytical reagents were purchased based on the analytical grade.

2. Cell cultures

The Saos2 human osteogenic sarcoma cells were grown in the DMEM supplemented with 10% fetal bovine serum (FBS) (Katsui *et al.*, 2004; Komatsu *et al.*, 2005). The Saos2 cells were maintained as monolayers in plastic culture plates at 37°C in the humidified atmosphere containing 5% CO_2.

3. MTT assay

The cell viability test was performed according to the previously described method with minor modifications (Lee *et al.*, 2010; Shin *et al.*, 2010). The Saos2 cells were seeded at a concentration of 5 X 10^3 cells/well in 24-well plates. After 24 hours growth, the cells were treated with EF-24 or curcumin at various concentrations for 24 hours. The cell viability was assessed using MTT assay. Six separate experiments were performed for each concentration/ exposure time combination.

4. Nuclear staining with DAPI

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

5. Immunoblotting

The Saos2 cells were treated with 0, 1 and 10 μM EF-24 or 0, 1, 10 and

30 μM curcumin for 24 hours. Immunoblotting was performed according to the previously described method with minor modifications (Kim *et al.*, 2008; Kim *et al.*, 2010). The anti-cleaved caspase-3, -7 or -9 antibodies (1:1000 dilution, Cell Signaling Technology, Inc., Danvers, MA, USA) were used as the primary antibodies.

6. Determination of caspase activation

The activity of caspase-3/-7 was determined using the cell-permeable fluorogenic substrate PhiPhiLux-G_1D_2 (OncoImmunin, Inc. Gaithersburg, MD, USA), which was used according to the manufacturer's instructions. The Saos2 cells were treated with 0 and 10 μM EF-24 or 0 and 30 μM curcumin for 24 hours and incubated with PhiPhiLux-G_1D_2. The activity of caspase-3/-7 was visualized by fluorescence microscopy (IX71, Olympus, Japan).

7. Data analysis

All experiments were performed at least four times. The results were presented as the mean ± S.E.M. The statistical significance was analyzed by using Student's t-test for the two group comparison and one way analysis of variance for the multi-group comparisons. A p value <0.05 was considered statistically significant.

Ⅲ. RESULTS

1. Cytotoxic effects of EF-24 and curcumin in Saos2 cells

To analyze and compare the effect of EF-24 and curcumin on the viability of Saos2 cells, the cells were treated with EF-24 and curcumin at various concentrations for 24 hours, and then the MTT assay was performed. As shown in Fig. 2, treatment of curcumin from 0.1 to 3 μM did not significantly affect the cell viability of Saos2 cells, but curcumin 10, 30 and 100 μM reduced Saos2 cell viability. When the Saos2 cells were treated with EF-24 for 24 hours, EF-24 inhibited the growth of Saos2 cells in a dose-dependent manner, suggesting that EF-24 induces Saos2 cell death (Fig. 2). The IC_{50} values of EF-24 and curcumin on the Saos2 cell viability after a 24 hour treatment were 2.7 ± 0.3 μM and 9.7 ± 1.4 μM, respectively. The apparent potency of EF-24 was more than 3 times that of curcumin. More importantly, the effects were observed at an EF-24 concentration less than 3 μM, a dose at which curcumin had no significant effect on cell proliferation, indicating the enhanced potency of EF-24.

2. Changes in nuclear morphology by EF-24 and curcumin

The nuclear morphological changes were assessed by DAPI staining. As shown in Fig. 3, the nuclei of the control Saos2 cells (Mock) had a normal

regular and oval shape. Treatment with 10 μM EF-24 or 30 μM curcumin for 24 hours resulted in nuclear condensation and fragmentation, which are characteristics of apoptosis.

3. Activation of caspases by EF-24 and curcumin

The levels of cleaved caspase-3, -7 and -9 were examined by immunoblotting and the activity of caspase-3/-7 was detected by fluorescence microscopy using a selective fluorogenic substrate since caspase-3, -7 and -9 are effector caspases of apoptotic cell death (Cohen, 1997; Datta *et al.*, 1997; Liu *et al.*, 1997; Hu and Kavanagh, 2003). Treatment with 1 and 10 μM EF-24 or 1, 10 and 30 μM curcumin for 24 hours significantly promoted the proteolytic cleavages of procaspase-3 in the Saos2 cells, with the increases in the amount of cleaved caspase-3 (Fig. 4). Either EF-24 (1 and 10 μM) or curcumin (1, 10 and 30 μM) for 24 hours also promoted the proteolytic cleavages of procaspase-7, with the increases in the amount of cleaved caspase-7 (Fig. 5). Treatment with EF-24 (1 and 10 μM) or curcumin (1, 10 and 30 μM) promoted the proteolytic cleavages of procaspase-9 in the Saos2 cells (Fig. 6). In addition, activation of caspase-3/-7 in EF-24 or curcumin treated Saos2 cells was confirmed by fluorescence microscopy using a fluorogenic substrate. As shown in Fig. 7, either the EF-24 or curcumin treatment led to activate the caspase-3/-7 in the living Saos2 cells.

Ⅳ. DISCUSSION

Recent studies have shown that such chemicals derived from natural materials have been identified to elicit chemopreventive and therapeutic effects (Cheng *et al.*, 2005; Park *et al.*, 2005; Tan *et al.*, 2005). It has been reported that this effect alters various factors associated with the cell cycle and thereby induce the apoptotic cell death (Hoshino *et al.*, 1991; Cheng *et al.*, 2005; Park *et al.*, 2005; Tan *et al.*, 2005; Tian *et al.*, 2006). There is a need to find new anti-cancer agents that can kill cancerous cells with minimal toxicity. 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 various cancer cells (Aggarwal *et al.*, 2007; Bachmeier *et al.*, 2007; Hauser *et al.*, 2007; Shankar and Srivastava, 2007; Wahl *et al.*, 2007; Sahu *et al.*, 2009). However, it is needed the design of more effective analogs because of curcumin's poor intestinal absorption and low bioavailability (Anand *et al.*, 2007). EF-24, the monoketone analog of curcumin, is efficacious in anticancer screens and has been shown to inhibit the growth of human breast tumor xenografts in a mouse model (Adams *et al.*, 2004; Adams *et al.*, 2005). Although EF-24 can reduce cancer cell viability, the mechanisms of action remain to be elucidated, and the effects of EF-24 and curcumin on osteosarcoma are not clearly established. Osteosarcoma is the most common type of malignant bone tumor in which the neoplastic mesenchymal cells shows evidence of osteoid production (Kim *et al.*, 2006). In this study, therefore, the cytotoxic effect of EF-24 and the mechanism of cell death exhibited by EF-24 were examined in Saos2 human osteogenic sarcoma cells. The results of this

study indicate that EF24, a novel curcumin analog, possesses profound promise as an anti osteosarcoma therapeutic.

In MTT assay, the treatment of curcumin from 0.1 to 3 μM did not significantly affect the cell viability of Saos2 cells, but curcumin 10, 30 and 100 μM reduced Saos2 cell viability (Fig. 2). EF-24 inhibited growth of Saos2 cells in a concentration-dependent manner (Fig. 2). This corresponded with the results of EF-24 and curcumin that have anti-cancer effects via the suppression of cancer cell growth in various types of cancer cells (Aggarwal *et al.*, 2007; Bachmeier *et al.*, 2007; Hauser *et al.*, 2007; Shankar and Srivastava, 2007; Wahl *et al.*, 2007; Sahu *et al.*, 2009). Also, the apparent potency of EF-24 was > 3-fold that of curcumin. These results speculate that EF-24 and curcumin have cytotoxicity to osteosarcoma cells with EF-24 having enhanced potency. In addition, these results indicate that it has potential value for anti-cancer drug discovery.

Apoptosis is an important way to maintain cellular homeostasis between cell division and cell death (Green and Reed, 1998; Hengartner, 2000; Kaufmann and Hengartner, 2001). The induction of apoptosis in cancer cells is one of useful strategies for anti-cancer drug development (Kim *et al.*, 2008). So, many studies were performed for screening of apoptosis from plant-derived compounds (Pezutto, 1997; van Poppel and van den Berg, 1997; Christou *et al.*, 2001; Mukherjee *et al.*, 2001). In this study, treatment with EF-24 and curcumin induced nuclear condensation and fragmentation in Saos2 cells, suggesting apoptotic cell death (Fig. 3). These results indicate that EF-24 and curcumin inhibit the growth of Saos2 cells by activating cell apoptosis.

The activation of a family of intracellular cysteine proteases, called

caspases, is known to play an important role in the initiation and execution of apoptosis induced by various stimuli (Datta *et al.*, 1997; Liu *et al.*, 1997). Among the caspases identified in mammalian cells, caspase-3, caspase-7 and caspase-9 may serve as effector caspases of apoptotic cell death (Cohen, 1997; Datta *et al.*, 1997; Liu *et al.*, 1997). Caspase-3, caspase-7 and caspase-9 are synthesized as inactive proenzymes (of sizes 32 kDa, 35 kDa and 47 kDa, respectively), which require proteolytic activation to cleaved enzymes (of sizes 19 kDa, 20 kDa and 37 kDa, respectively) (Cohen, 1997; Datta *et al.*, 1997; Liu *et al.*, 1997). The results of this study revealed that low levels of cleaved capase-3, -7 and -9 were present in EF-24- or curcumin-untreated Saos2 cells, and the amount of cleaved enzymes was increased after the EF-24 or curcumin treatment in Saos2 cells (Fig. 4, 5 and 6). In addition, the activity of caspase-3/-7 was increased by EF-24 or curcumin treatment in living Saos2 cells compared to DMSO treatment as a control (Fig. 7). These results suggested that EF-24 and curcumin induce apoptotic cell death through caspase-3-, -7- and -9-dependent processing in the Saos2 cells. However, the mechanisms of apoptosis induced by EF-24 and curcumin in Saos2 cells are not yet completely understood. Further studies will be needed to reveal the precise cellular and molecular mechanisms of apoptosis induced by EF-24 and curcumin.

In conclusion, these *in vitro* results indicate that the EF-24 and curcumin inhibit cell proliferation and induce apoptosis in Saos2 human osteogenic sarcoma cells. Moreover, the potency of EF-24 was more than 3 times that of curcumin. Therefore, EF-24 could be a model compound for the further development of natural product-derived anti osteosarcoma agents.

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FIGURE LEGENDS

Fig. 1. Chemical structures of EF-24 (diphenyl difluoroketone) and curcumin (diferuloylmethane).

Fig. 2. 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 hours. The cell viabilities were determined by the MTT assays. The percentage of cell viability was calculated as a ratio of A570 nms of 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).

Fig. 3. Changes in nuclear morphology by EF-24 and curcumin. The cells were treated with 10 μM EF-24 or 30 μM curcumin for 24 hours. Representative fluorescence photomicrographs show the nuclei morphology of Saos2 cells. Arrows indicate chromatin condensation, reduced nuclear size and nuclear fragmentation typically observed in apoptotic cells.

Fig. 4. Proteolytic cleavage of caspase-3 by EF-24 or curcumin treatment in Saos2 cells. Activity of cleaved caspase-3 by EF-24 (A) or curcumin (B) 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 hours. The cell lysate was prepared and analyzed by immunoblotting as described in "MATERIALS AND METHODS". The lower panels show the quantitative data for upper panels analyzed by using Imagegauge 3.12 software after β-actin normalization.

Fig. 5. Proteolytic cleavage of caspase-7 by EF-24 or curcumin treatment in Saos2 cells. Activity of cleaved caspase-7 by EF-24 (A) or curcumin (B) was measured in Saos2 cells. Other legends are the same as in Fig. 4.

Fig. 6. Proteolytic cleavage of caspase-9 by EF-24 or curcumin treatment in Saos2 cells. Activity of cleaved caspase-9 by EF-24 (A) or curcumin (B) was measured in Saos2 cells. Other legends are the same as in Fig. 4.

Fig. 7. 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 hours and added specific cell-permeable substrate Phiphilux G1D2. Active of caspase-3/-7 was visualized by fluorescence microscopy.

FIGURES

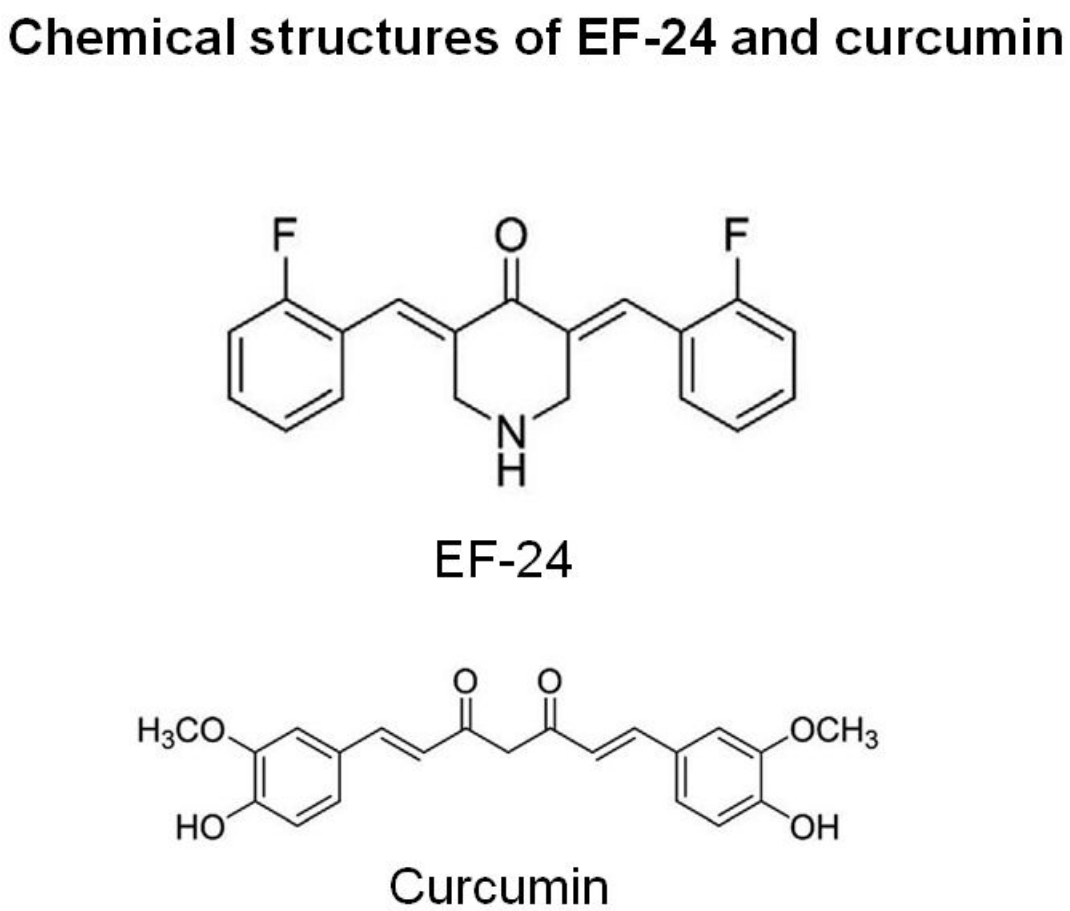

Fig. 1. Chemical structures of EF-24 (diphenyl difluoroketone) and curcumin (diferuloylmethane).

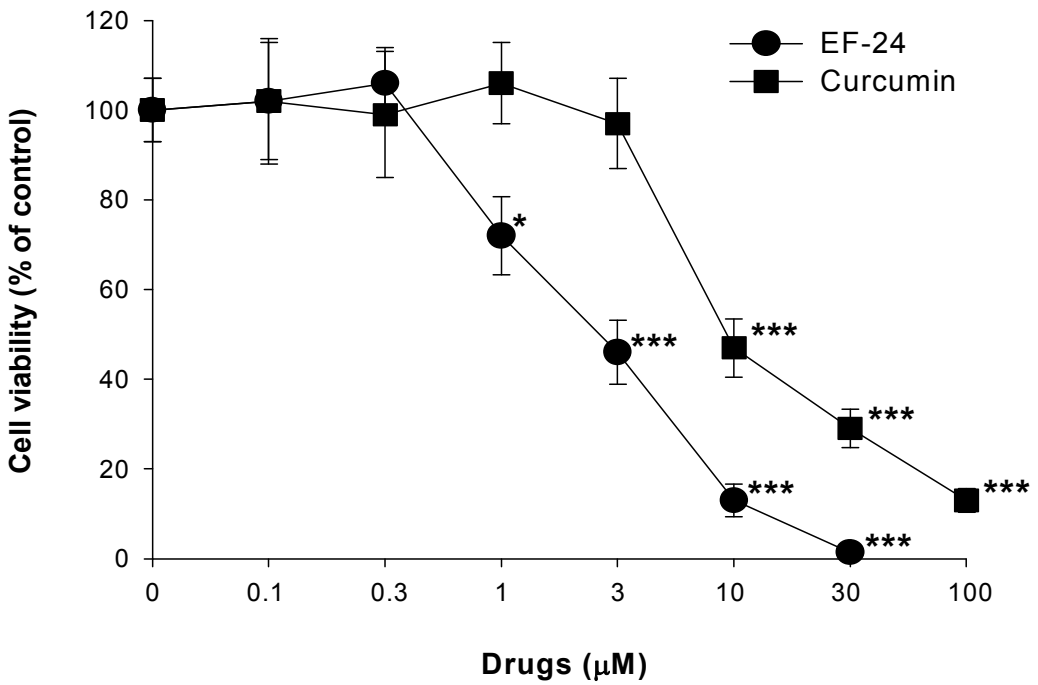

Fig. 2. Concentration-dependent effects of EF-24 and curcumin on the cell viability in Saos2 human osteogenic sarcoma cells.

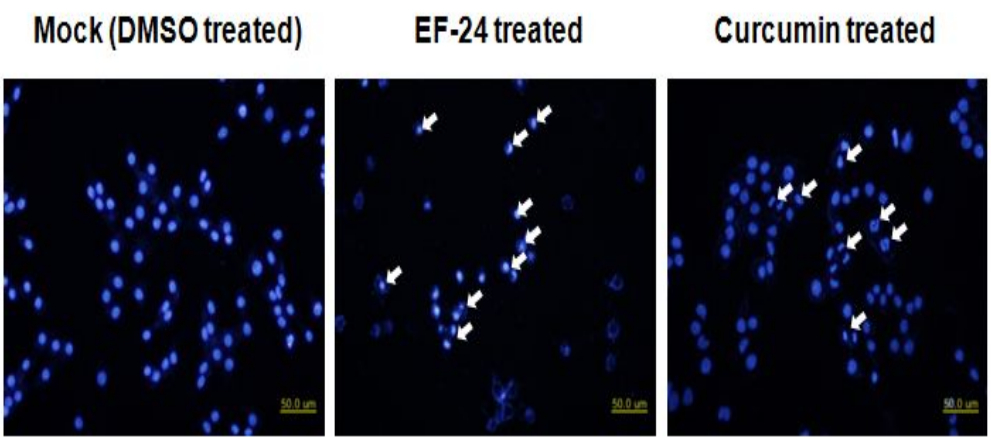

Fig. 3. Changes in nuclear morphology by EF-24 and curcumin.

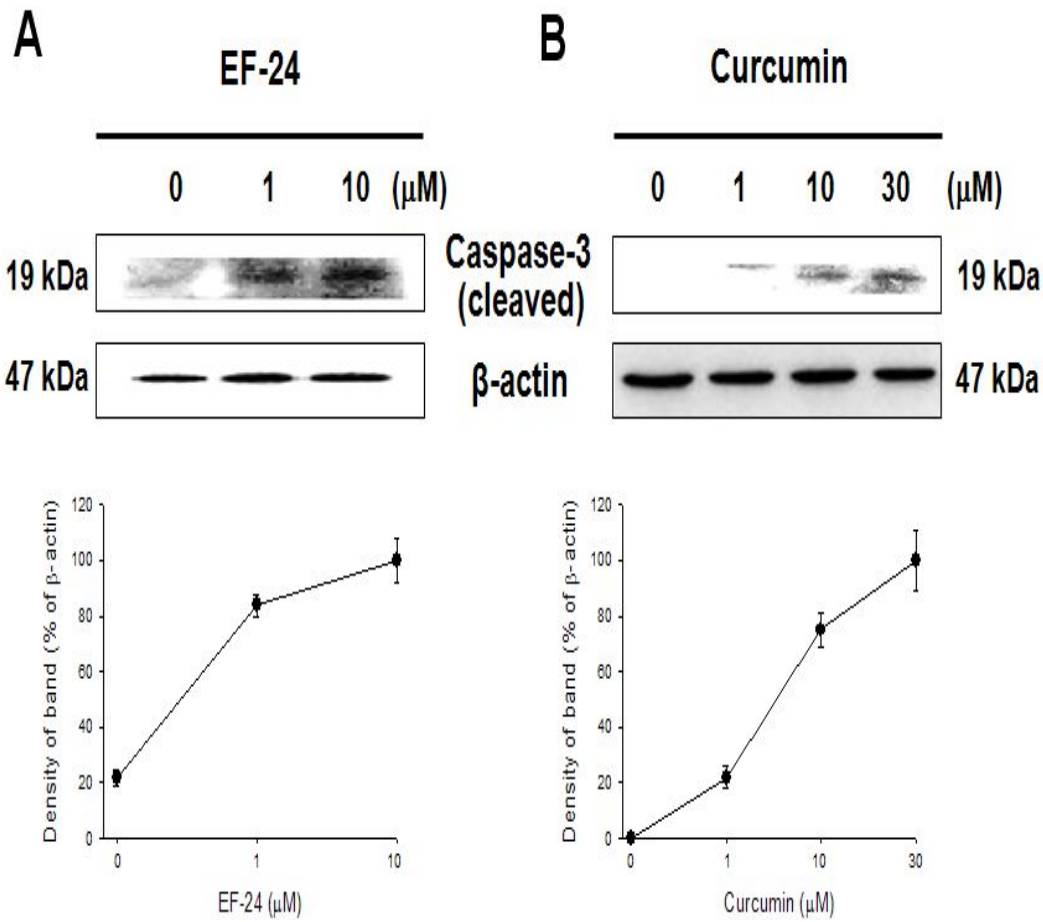

Fig. 4. Proteolytic cleavage of caspase-3 by EF-24 or curcumin treatment in Saos2 cells.

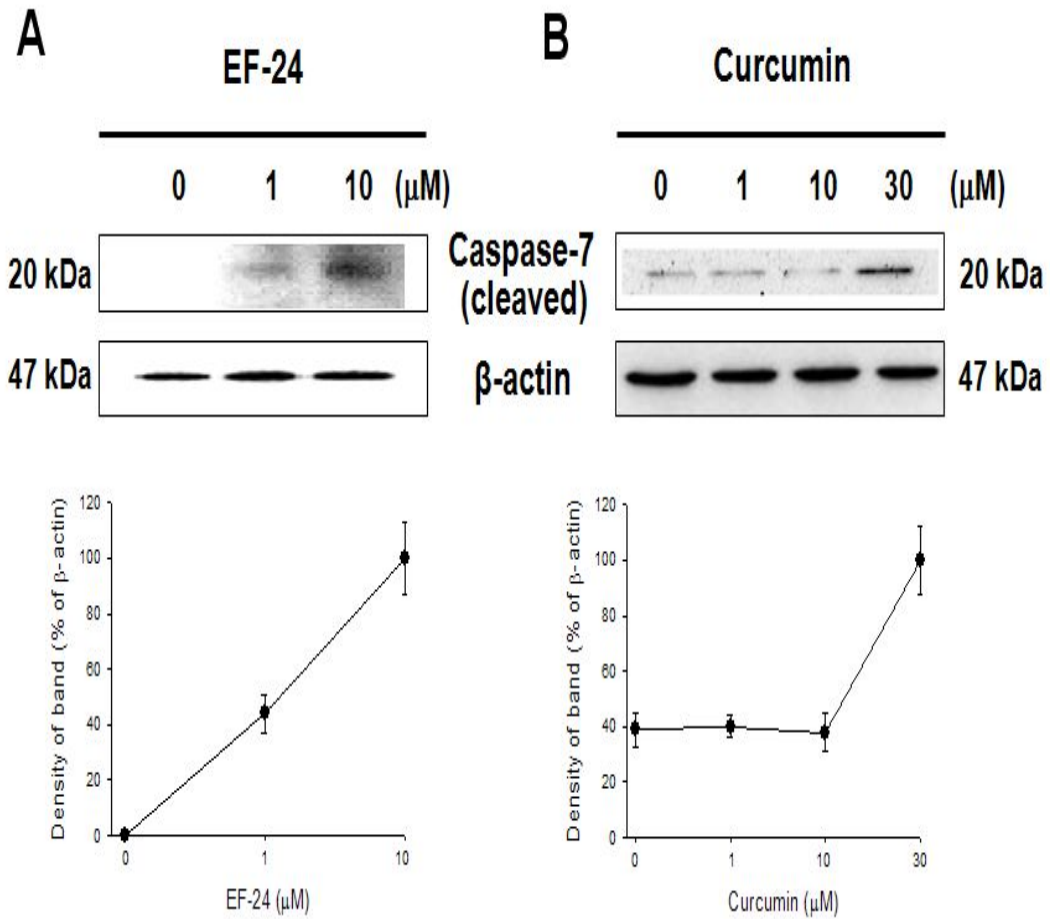

Fig. 5. Proteolytic cleavage of caspase-7 by EF-24 or curcumin treatment in Saos2 cells.

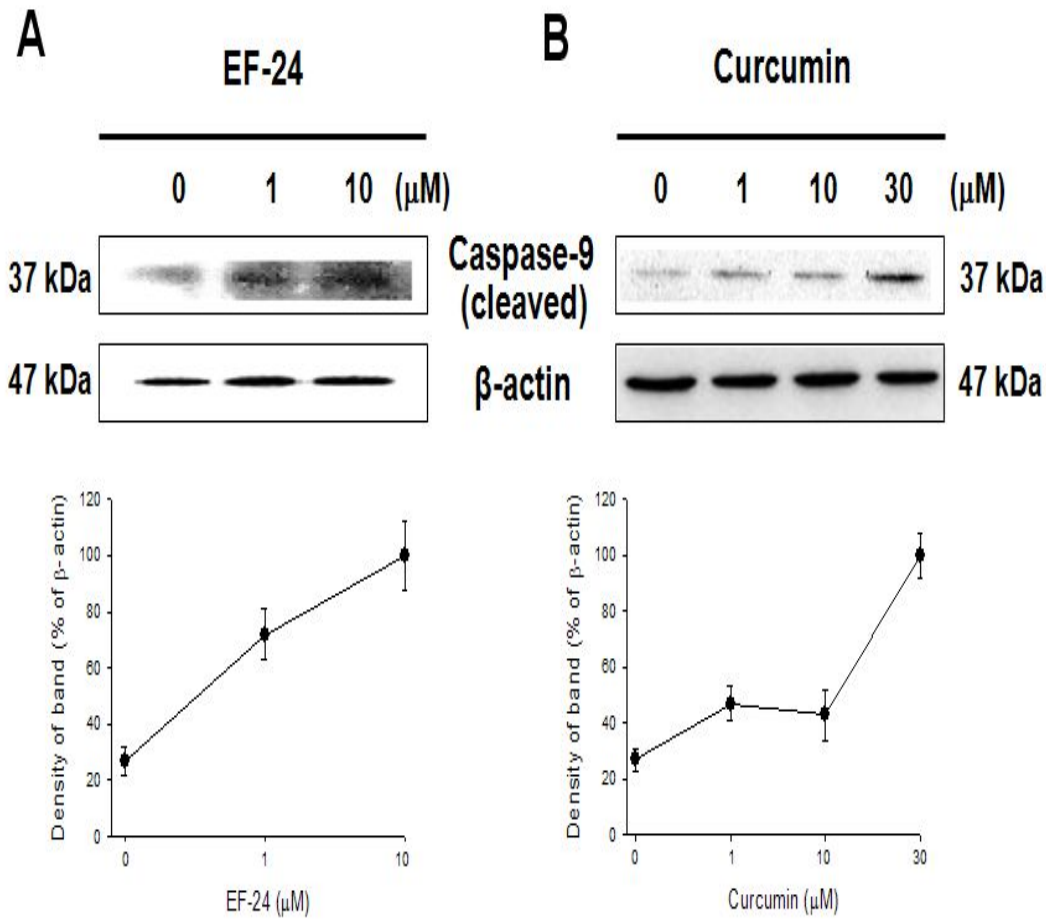

Fig. 6. Proteolytic cleavage of caspase-9 by EF-24 or curcumin treatment in Saos2 cells.

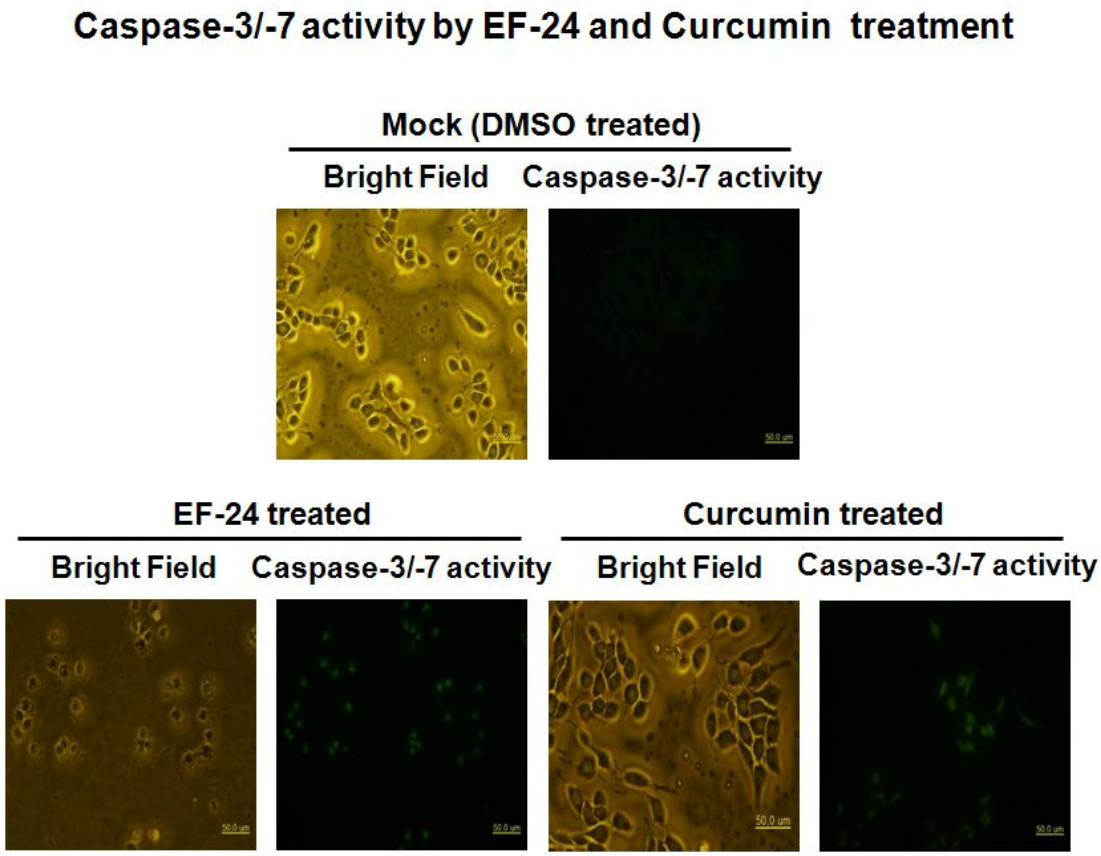

Fig. 7. Activation of caspase-3/-7 by EF-24 or curcumin treatment in living Saos2 cells.