Curcumin analog EF24 induces apoptosis and downregulates the mitogen activated protein kinase/extracellular signal-regulated signaling pathway in oral squamous cell carcinoma

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Abstract. Oral squamous cell carcinoma (OSCC) is one of the most common malignancies worldwide. Diphenyldifluoroketone (EF24) is a curcumin analog that has been demonstrated to improve anticancer activity; however, its therapeutic potential and mechanisms in oral cancer remain unknown. In the present study, the effect of EF24 on apoptosis induction and its potential underlying mechanism in the CAL-27 human OSCC cell line was investigated. To achieve this, various concentrations of cisplatin or EF24 were administrated to CAL-27 cells for 24 h, and cell viability, apoptotic DNA fragmentation, and cleaved caspase 3 and 9 levels were evaluated. To investigate the potential underlying mechanism, the levels of mitogen-activated protein kinase kinase 1 (MEK1) and extracellular signal-regulated kinase (ERK), two key proteins in the mitogen-activated protein kinase/ERK signaling pathway, were additionally examined. The results indicated that EF24 and cisplatin treatment decreased cell viability. EF24 treatment increased the levels of activated caspase 3 and 9, and decreased the phosphorylated forms of MEK1 and ERK. Sequential treatments of EF24 and 12-phorbol-13-myristate acetate, a MAPK/ERK activator, resulted in a significant increase of activated MEK1 and ERK, and reversed cell viability. These results suggested that EF24 has potent anti-tumor activity in OSCC via deactivation of the MAPK/ERK signaling pathway. Further analyses using animal models are required to confirm these findings *in vivo*.

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Introduction

Oral squamous cell carcinoma (OSCC) has an annual incidence of >300,000 cases, which makes it the sixth most common type of cancer worldwide (1). Patients exhibit frequent lymph node metastasis and local invasion, causing poor prognosis (2). Currently, chemo-radiotherapy with cisplatin or other platinum compounds is considered to be a standard treatment regimen for patients with locoregionally advanced OSCC (3). However, due to its high risk of toxicity and chemoresistancy, the application of cisplatin remains limited (4). Thus, a novel and more effective management strategy with favorable toxicity levels is required.

Diferuloylmethane (curcumin), which is derived from the rhizome of *Curcuma longa*, has been reported to have chemopreventive and antiproliferative activity in many human cancers (5,6). As one of the novel synthetic curcumin analogs, the chemotherapeutic potential of diphenyl difluoroketone (EF24) has been demonstrated in numerous types of human cancers (7-10). However, the effects of EF24 on OSCC cells remain to be studied. Further investigations are required to elucidate the underlying mechanism of EF24 treatment, in order to advance the clinical development of this agent as a therapeutic candidate.

The present study examined the effects of EF24 and cisplatin on OSCC cell survival, and the mechanism of cell death elicited by EF24 in CAL-27 human oral cancer cells. It was demonstrated that EF24 had a higher cytotoxic potency compared with cisplatin on CAL-27 cells, and that this cytotoxic effect was associated with the activation of caspases in the mitogen activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway in OSCC cells.

Materials and methods

Cell culture. The CAL-27 human oral cancer cell line was maintained in Dulbecco's modified Eagle's medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), penicillin (100 units/ml; Gibco;

Thermo Fisher Scientific, Inc.), streptomycin (100 μg/ml; Gibco; Thermo Fisher Scientific, Inc.). Cells were incubated at 37˚C in 5% CO_2 in a humid atmosphere.

Cell viability. Cell viability rate was determined by MTT assay. EF24 (Sigma Aldrich; Merck KGaA, Darmstadt, Germany) and cisplatin (Sigma Aldrich; Merck KGaA) were dissolved in dimethyl sulfoxide (DMSO) and distilled water, respectively. The final concentration of DMSO was <0.1%. CAL27 cells (density, $2x10^4$) were seeded into each well of 96-well plates and incubated for 24 h, after which the cells were administered with selected doses of EF24 (0.1-30 μM) or cisplatin as a positive control (0.1-30 μM), and the dose-dependent effects of the drugs on the cells were determined. Following a 24-h incubation, 10 ml MTT solution (5 mg/ml) was added to the medium and maintained at 37˚C for 4 h. DMSO was used for dissolving formazan crystals, and the absorbance values were measured at a wavelength of 570 nm using a Synergy H1 Hybrid Multi-Mode Microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). All experiments were performed in quintuplicate for each dose.

Quantitation of DNA fragmentation. DNA fragmentation was detected as a marker of apoptosis using a Cell Death Detection ELISA^{Plus} kit (Sigma-Aldrich; Merck KGaA), following the manufacturer's protocol. Briefly, 24 h prior to drug treatment, $2x10^4$ CAL27 cells were seeded into each well of 96-well plates. Various concentrations of EF24 or cisplatin were added to the wells followed by a 24-h incubation. Vehicle control cells were treated with an equal amount of DMSO. Absorbance was measured at a wavelength of 405 nm with a Synergy H1 Hybrid Multi-Mode Microplate reader (BioTek Instruments, Inc.).

Western blot analysis. CAL-27 cells were treated with EF24 or DMSO only for 24 h. For the MAPK/ERK activation assay, cells treated with 1 μM EF24 for 24 h, following which EF24 was removed and 50 ng/ml 12-phorbol-13-myristate acetate (PMA; Sigma Aldrich; Merck KGaA) was used for another 24 h of incubation. At the end of treatment, whole cells lysates were acquired using Pierce IP Lysis Buffer (Thermo Fisher Scientific, Inc.) and centrifuged at 13,000 x g for 10 min at 4˚C. Protein concentration of the supernatant was determined using a Pierce Bicinchoninic Acid assay kit (Thermo Fisher Scientific, Inc.). Total proteins (40 μg per lane) were subjected to 12% SDS-PAGE and subsequently transferred onto polyvinylidene difluoride membranes. After incubating with a blocking solution containing 3% bovine serum albumin (Sigma Aldrich; Merck KGaA) and 0.1% Tween-20 in PBS at room temperature for 30 min, the membranes were incubated with the desired primary antibodies at room temperature for 2 h (Table I), followed by incubation with a goat anti-rabbit horseradish peroxidase-conjugated IgG secondary antibody (cat. no. 31460; 1:20,000; Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 1 h. The immunoactive bands were detected using Pierce™ Western Blot Signal Enhancer (Thermo Fisher Scientific, Inc.), and digital chemiluminescence images were analyzed by GeneTools (version 4.1; Syngene, Frederick, MD, USA). The entire western blot analyses were repeated at least three times.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Apoptosis of CAL-27 cells was detected using the *in-situ* Cell Death Detection kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocol. Briefly, $1x10^6$ CAL-27 cells were treated with 1 μM EF24 for 24 h, or subsequently treated with 1 μM EF24 for 24 h then 50 ng/ml PMA for another 24 h. Following drug administration, cells were fixed with 4% paraformaldehyde (pH 7.4) and incubated with PBS consisting of 0.1% Triton X-100 and 0.1% sodium citrate at 4˚C for 5 min. Following this, 50 μl TUNEL reaction mixture was added to the solution, and the cells were incubated at 37˚C for 1 h. Following three washes with PBS, cells were stained with DAPI for 5 min at room temperature. Apoptotic cells were identified by blue excitation light at wavelength of 488 nm. Images were captured using a Nikon Eclipse Ti microscope (Nikon Corporation, Tokyo, Japan) at a magnification of x40.

Statistical analysis. All experiments were performed in at least triplicate. The results are presented as the mean ± standard deviation. The statistical significance was analyzed by SPSS software (version 20.0; IBM Corp., Armonk, NY, USA) using Student's t-test for the two group comparisons and one-way analysis of the variance for the multi-group comparisons. For multiple comparisons, the Student-Newman-Keuls method was used as a post-hoc test. $P<0.05$ was considered indicate a statistically significant difference.

Results

EF24 has a higher cytotoxic potency than cisplatin on CAL-27 cells. Previous studies have demonstrated the cytotoxicity of EF24 in prostate, ovarian and breast cancer cells (11-13). To determine the ability of EF24 to induce apoptosis in oral cancer, human CAL-27 cells were treated with various concentrations of EF24 for 24 h, and cisplatin was used as positive control. As presented in Fig. 1A, treatment with cisplatin at doses from 0.1 to 1 μM did not significantly affect the viability of CAL-27 cells, but cisplatin at 3, 10 and 30 μM significantly reduced CAL-27 cell viability ($P<0.05$). When the cells were treated with EF24, the growth of CAL-27 cells was inhibited ($P<0.05$) in a dose-dependent manner from 1 to 30 μM, and the viability under each condition was lower that of cisplatin. Notably, the inhibitory effects were observed at 1 μM EF24, a dose at which cisplatin had no significant effects on cell viability, indicating the higher potency of EF24. Similarly, compared with cisplatin treatment, administration of EF24 elevated the DNA fragmentation rate of CAL-27 cells in a in a dose-dependent manner from 1 to 30 μM ($P<0.05$), suggesting that EF24 is more effective at inducing the apoptosis of CAL-27 cells than cisplatin (Fig. 1B).

EF24-induced apoptosis was resulted from the activation of caspases. CAL-27 cells incubated with EF24 (1 or 10 μM) for 24 h were assessed by western blot analysis, using antibodies that recognize apoptosis-associated proteins. Protein expression levels of cytochrome c, cleaved caspase 3 and cleaved caspase 9 were significantly increased, compared with non-EF24 treated controls ($P<0.05$; Fig. 2).

Table I. Primary antibodies used in the present study.

Antigen	Catalog no.	Raised in	Dilution	Supplier
Cytochrome c	ab133504	Rabbit	1:5,000	Abcam
Caspase 3	ab2302	Rabbit	1:200	Abcam
Caspase 9	ab63488	Rabbit	1:1,000	Abcam
MEK1	8727	Rabbit	1:1,000	Cell Signaling Technology
pMEK1	9127	Rabbit	1:1,000	Cell Signaling Technology
ERK	4370	Rabbit	1:2,000	Cell Signaling Technology
pERK	5683	Rabbit	1:1,000	Cell Signaling Technology
GAPDH	ab181602	Rabbit	1:10,000	Abcam

Abcam, Cambridge, UK; Cell Signaling Technology, Inc., Danvers, MA, USA. MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; p, phosphorylated.

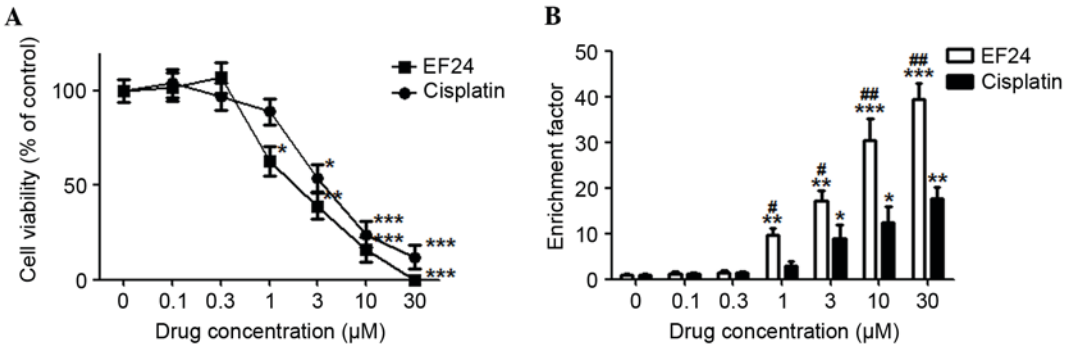

Figure 1. Concentration-dependent effects of EF24 and cisplatin on the viability of CAL-27 human oral squamous cell carcinoma cells. (A) CAL-27 cells were treated with various concentrations of EF24 (square) and cisplatin (circle) for 24 h, and cell viabilities were determined by MTT assays. (B) DNA fragmentation rate following treatment with selected doses of EF24 and cisplatin. All data were normalized to the untreated cells, which was set as 100%. Data are presented as the mean ± standard error from five replicated experiments. *P<0.05 vs. control; **P<0.01 vs. control; ***P<0.001 vs. control; $^{\#}$P<0.05 vs. cisplatin; $^{\#\#}$P<0.01 vs. cisplatin. EF24, diphenyldifluoroketone.

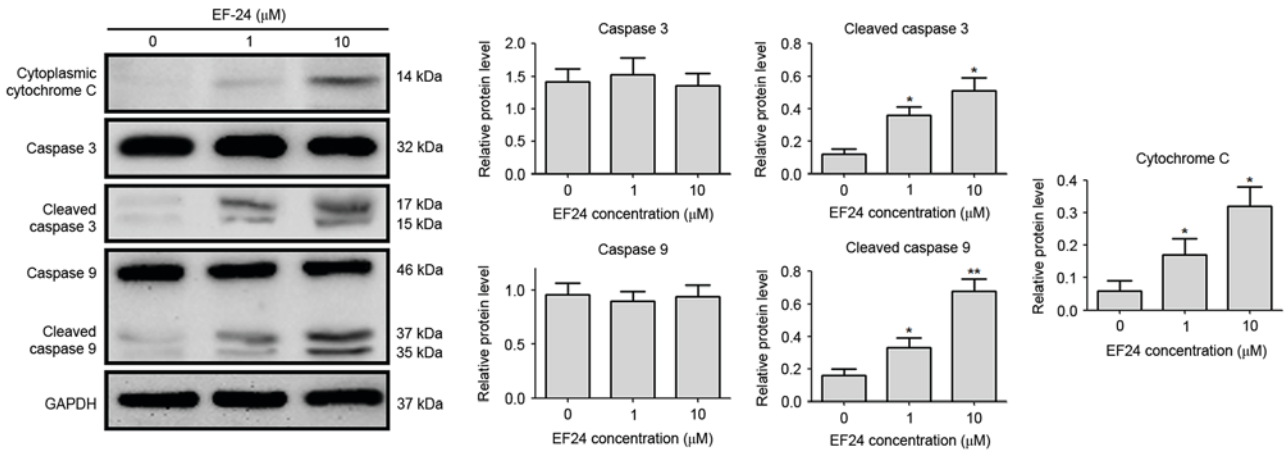

Figure 2. EF24-induced apoptosis mediated by activation of caspases. CAL-27 cells were treated with indicated concentrations of EF24 for 24 h. Representative western blot images and protein expression levels of cytoplasmic cytochrome C, caspase 3 and 9, and cleaved caspase 3 and 9. Relative protein expression levels were normalized against the expression of GAPDH. Data are presented as the mean ± standard deviation from three independent experiments. *P<0.05, **P<0.01 vs. 0 μM EF24. EF24, diphenyldifluoroketone.

Furthermore, the relative expression of cytochrome c, cleaved caspase 3 and cleaved caspase 9 was higher in 10 μM EF24-treated cells, when compared with 1 μM EF24-treated cells. This dose-dependent result indicated that apoptosis induced by EF24 was at least partly via the activation of caspases.

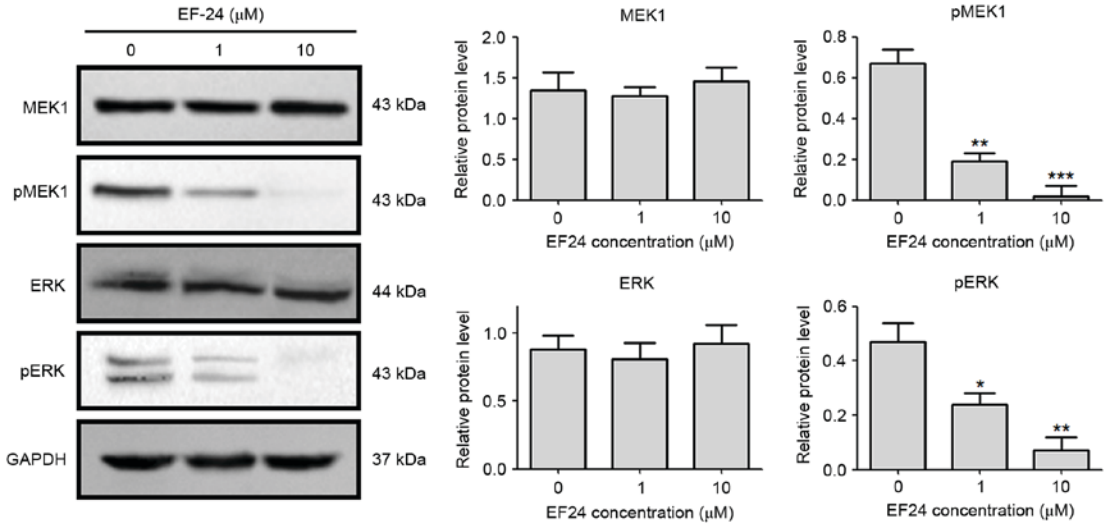

Figure 3. EF24 inhibits phosphorylation of MEK1 and ERK1/2 in CAL-27 cells. CAL-27 cells were treated with indicated concentrations of EF24 for 24 h. Lysates from cells were examined by western blotting for proteins in the mitogen-activated protein kinase/ERK signaling pathway. Relative protein levels were normalized against the expression of GAPDH. Data are presented as the mean ± standard deviation from three independent experiments. *P<0.05, **P<0.01, ***P<0.001 vs. 0 μM EF24. EF24, diphenyldifluoroketone; MEK1, mitogen-activated protein kinase kinase 1; ERK, extracellular signal-regulated kinase; p, phosphorylated.

EF24 treatment decreases activation of the MAPK/ERK signaling pathway in CAL-27 cells. Several studies have demonstrated the important role of the MAPK/ERK signaling pathway in cell cycle arrest and apoptosis in response to stress (14-16). To determine whether the MAPK/ERK signaling pathway is involved in EF24 stimulated apoptosis, the activated and inactivated forms of mitogen activated protein kinase kinase 1 (MEK1) and ERK protein were examined in CAL-27 cells treated with EF24 (1 or 10 μM) for 24 h. Representative results from each condition are presented in Fig. 3. Densitometric analysis indicated that EF24 induces deactivation of the MAPKs in a dose-dependent manner, as revealed by their downregulated phosphorylation (P<0.05). These results suggested that EF24 may inhibit the MAPK/ERK signaling pathway during initiation of oral cancer cell apoptosis.

PMA reverses EF24-induced inhibition of the MAPK signaling pathway in CAL-27 cells. PMA is a well-known tumorigen that activates protein kinase C isozymes, which are upstream regulators of MAPK signaling pathway (17). The phosphorylation level of MEK1 and ERK in CAL-27 cells in response to treatment with EF24 and PMA were studied. Western blot analysis revealed that sequential administration of EF24 and PMA significantly elevated active MEK1 and ERK levels, when compared with EF24 alone (P<0.05; Fig. 4), indicating that PMA treatment abolished the EF24-induced MAPK/ERK signaling pathway deactivation.

Activation of the MAPK/ERK signaling pathway by PMA rescues EF24-induced apoptosis in CAL-27 cells. To examine whether the MAPK/ERK activator PMA may rescue EF24-induced apoptosis, CAL-27 cells were treated with DMSO, EF24 alone or a sequential combination of EF24 and PMA. Western blotting results demonstrated that the ratios of cleaved caspase 3 and 9 to GAPDH were significantly decreased with the administration of PMA (P<0.05; Fig. 5A). Similarly, TUNEL staining indicated that cells sequentially treated with EF24 and PMA had a lower percentage of apoptotic (TUNEL-positive) cells, when compared with EF24 alone (P<0.05; Fig. 5B). These results indicated that PMA treatment effectively ameliorated the cytotoxicity of EF24, thus confirming that deactivation of the MAPK/ERK signaling pathway may represent a major underlying cellular mechanism of EF24-induced apoptosis of CAL-27.

Discussion

Concurrent chemo-radiation treatment of OSCC has already been established and validated, and cisplatin-based chemotherapy remains to be the standard treatment strategy (18). Apoptosis induced by cisplatin varies between different tumor cells and drug concentrations, and are mediated by activation of various signal transduction pathways, including calcium and death receptor signaling, in addition to activation of mitochondrial pathways. However, besides its tumoricidal property, cisplatin induces diverse side effects, such as neural and renal toxicity (19). To minimize the disadvantages of cisplatin treatment, identifying novel anticancer agents that may kill cancerous cells with minimal toxicity is critical.

Recent studies have demonstrated that some chemicals derived from herbal extracts possess antineoplastic effects (20,21), which were reported to arrest the progression of the cell cycle and to induce apoptotic cell death (22). Similarly, it has been presented by many studies that curcumin is one of the tumor suppressors that induces cell cycle arrest and apoptosis in a variety of cancer cells (23,24). As a monoketone analog of curcumin, EF24 was reported to inhibit the growth of tumor xenografts *in vivo* (25). Although EF24 may reduce cancer cell viability, the mechanisms underlying it remain unclear, and the effects of EF24 and curcumin on OSCC remain to be established. The present study examined the cytotoxic effects

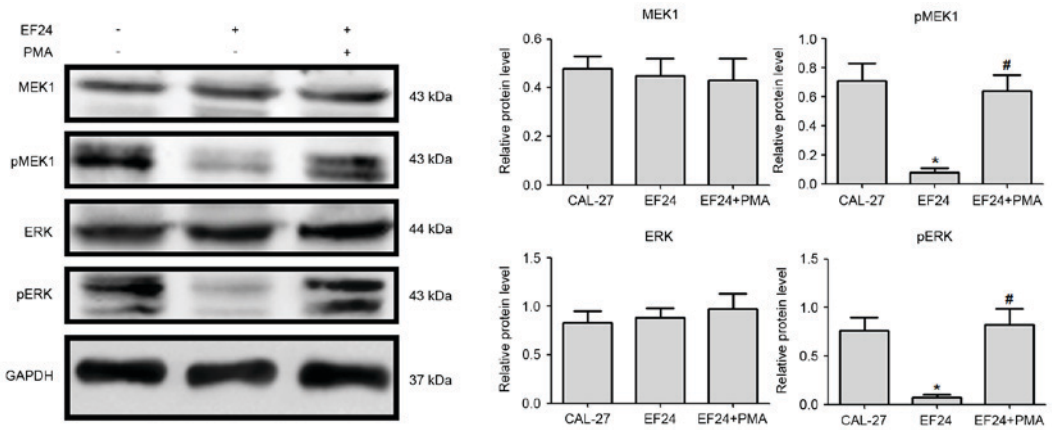

Figure 4. PMA activates MAPK/ERK in EF24-treated CAL-27 cells. CAL-27 cells were stimulated with 1 μM EF24 for 24 h, followed by the administration of 50 ng/ml PMA or equivalent volume of DMSO for another 24 h of incubation. Control cells were treated with DMSO only. Representative western blot images and quantification of MEK, pMEK1, ERK and pERK. Relative expression levels were determined following normalization to GAPDH expression. Data are presented as the mean ± standard deviation from three independent experiments. *P<0.05 vs. control; #P<0.05 vs. EF24-treated only. PMA, 12-phorbol-13-myristate acetate; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; EF24, diphenyldifluoroketone; DMSO, dimethylsulfoxide; MEK, mitogen-activated protein kinase kinase; p, phosphorylated.

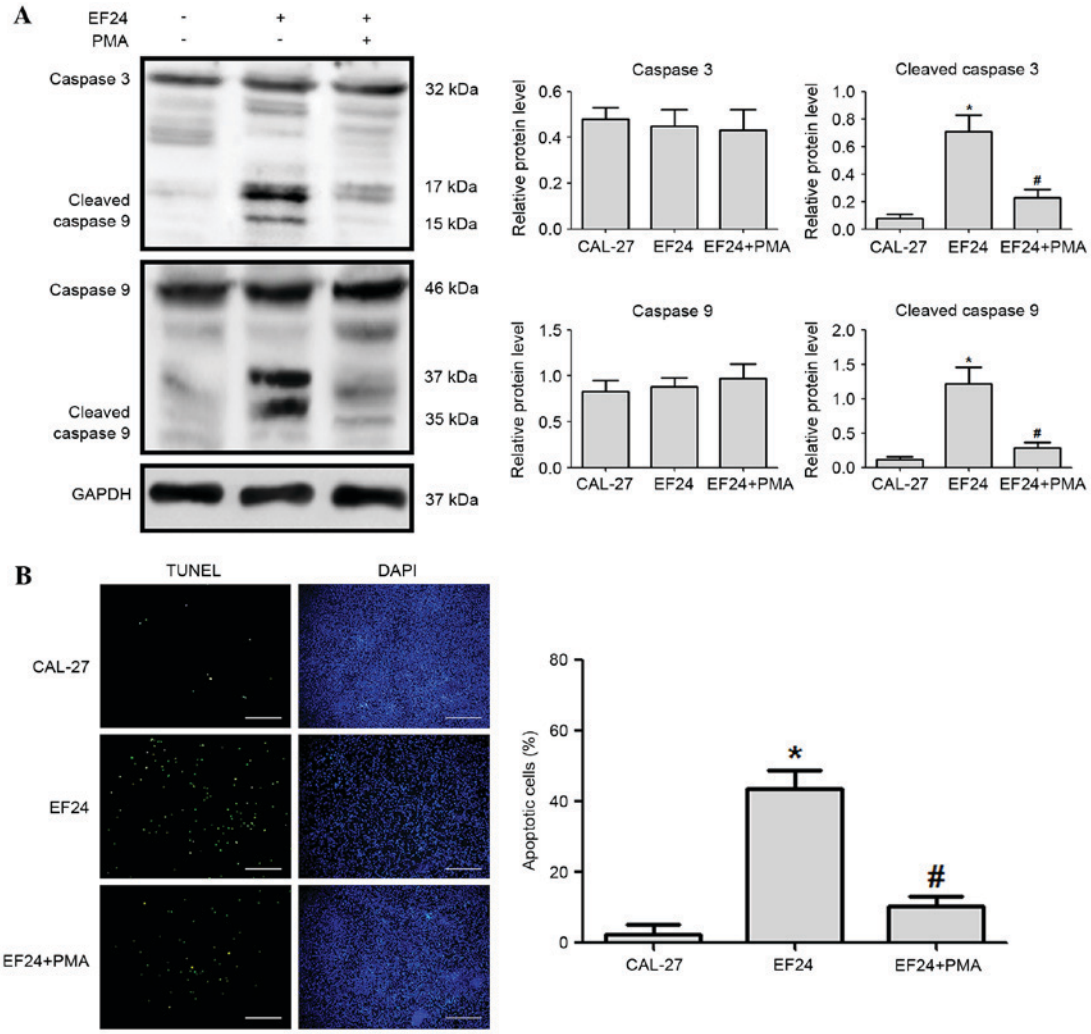

Figure 5. PMA treatment reverses apoptosis of EF24-treated CAL-27 cells. CAL-27 cells were stimulated with 1 μM EF24 for 24 h, followed by the administration of 50 ng/ml PMA or an equivalent volume of DMSO for another 24 h of incubation. Control cells were treated with DMSO only. (A) Representative western blot images and quantification of apoptosis-associated protein expression levels. Relative expression levels were determined following normalization to GAPDH expression. (B) TUNEL staining of treated cells. Left, epifluorescence images representing apoptotic cells. Right, DAPI staining representing the nucleus of the same cells. Scale bar=200 μm. Data are expressed as the mean ± standard deviation of three independent experiments. *P<0.05 vs. control; #P<0.05 vs. EF24-treated only. TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling; PMA, 12-phorbol-13-myristate acetate; EF24, diphenyldifluoroketone; DMSO, dimethylsulfoxide.

of EF24, and the potential underlying mechanism of apoptosis exhibited by EF24 in CAL27 human OSCC cells was assessed.

In the apoptotic signaling pathways, activation of caspases serves an important role in the initiation and execution of apoptosis induced by extracellular and intracellular stimuli (26). Among the caspases identified in mammalian cells, proteolytic activation of caspase-3/-9 into its cleaved forms is an indispensable step to initiate apoptotic cell death (27). The present study demonstrated that the amount of cleaved caspase-3/-9 in CAL-27 cells increased following EF-24 treatment, which is consistent with a previous study that revealed that anticancer agents, including curcumin, may cause activation of p38, c-Jun N-terminal kinase 1/2 and caspase-3, and induce similar apoptotic events (28,29). These findings suggest that EF24 possesses considerable potential as an anti-oral cancer therapeutic.

MAPK signaling, which includes activation of MEK1 and ERK, has been reported to be a major regulative pathway controlling cell cycle and induction of apoptosis in oral cancer cells (30). Various cytotoxic agents induce apoptotic cell death via deactivation of MAPK signaling and induction of caspase-3 and -9 (31). In addition, the MAPK signaling pathway triggers the release of intermediate proteins in apoptosis, including cytochrome c, in cancer cells (32). Liu *et al* (33) reported that release of cytochrome c from the mitochondria was increased in the EF24-treated group. Similarly, the current results demonstrated that, in CAL-27 cells, EF24 upregulated the level of cytoplasmic cytochrome c, which contributes to a decrease in cell viability. Phorbol esters, including PMA, are known tumor promoters that activate protein kinase C as substitutes to their physiological activators, including diacylglycerol and phosphatidylserine (34). As presented in the results, PMA treatment in CAL-27 cells activated members of MAPK family, as evident from the transient increase in their phosphorylated forms in western blotting. EF24 and PMA combined treatment indicated that EF24-induced deactivation of the MAPK signaling pathway was reversed by PMA, and the same trend of reversal was detected by the apoptotic hallmarks cleaved caspase-3 and -9. Although further inhibition of the MAPK signaling pathway by its inhibitor in CAL-27 cells remains to be investigated, double-inhibition may result in enhanced viability loss, when compared with EF24 administration alone. Extending the results from present study into an *in vivo* study may further provide valuable insights on the bioavailability of EF24 and its mechanistic role in the prevention of oral tumorigenesis.

Acknowledgements

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