Bufalin Inhibits the Proliferation of Human Esophageal Carcinoma TE13 Cells through Down-regulation of ERK

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ABSTRACT— ***The purpose of this study was to investigate the effect of bufalin on the proliferation in the human esophageal carcinoma cell lines TE13 and to determine the relevant molecular mechanism. Bufalin could be a useful agent as a novel antitumor agent to inhibit carcinoma cell proliferation through down-regulate ERK. The immunocytochemistry and western blotting analysis were applied to measure the levels of ERK in bufalin-treated TE13 cells. The results indicated that bufalin inhibited the phosphorylation of ERK. The cytometry and MTT assays showed that proliferation effects were inhibited especially in bufalin-treated esophageal carcinoma TE13 cells. Taken together, the results of the research confirmed that bufalin produced the anti-proliferative effect on TE13 cells by contributing to the down-regulation of ERK.***

Keywords--- Esophageal carcinoma; bufalin; ERK

1. INTRODUCTION

Esophageal carcinoma is the sixth frequent cause of cancer-related death worldwide, and esophageal squamous carcinoma (ESCC) accounts for 90% of the esophageal carcinoma in Asian countries. Although surgical techniques and perioperative management have progressed, the prognosis for patients with esophageal carcinoma remains poor. Finding molecular therapeutic targets for esophageal carcinoma treatment is one of the most promising avenues of research that might help to improve the survival of patients with this type of refractory cancer. Some of the genetic alterations associated with development or progression of esophageal carcinoma have been described. However, few of these genes have been demonstrated to be associated with biological or pathological features of esophageal carcinoma. Therefore, novel genes associated with a progression of esophageal carcinoma apparently need to be identified.

There are multiple signal transduction pathways involved in the occurrence and development of esophageal carcinoma, but the mitogen-activated protein kinase(MAPK) is the main pathways. Extracellular signal-regulated kinase (ERK) is a important member of mitogen-activated protein kinase(MAPK) family, and it can be activated into the state of phosphorylation (p-ERK) by variety of stressor and mitogen-activated signaling. Activated ERK regulates cell proliferation, differentiation, apoptosis and malignant transformation of cell. A lot of previous studies reported that the level of p-ERK involved in the occurrence and development of malignant tumor including esophageal cancer. ERK is the the focus of the study currently, it can provide new target for clinical therapy.

The anti-cancer activity of cardiac glycosides (the main active ingredients in toad venom) has been a subject of study since 1967 [1]. Emerging evidence now suggests that cardiac glycosides are able to inhibit cell proliferation and induce cell apoptosis in various tumor cell lines [2–5] . Bufalin, a cardioactive C-24 steroid of the major component of the traditional Chinese medicine Chan-Su obtained from the skin and parotid venom glands of the toad [6–8], has been confirmed to inhibit proliferation in a variety of cancers and has been investigated in cell cycle arrest and apoptotic effects experimentally[9–12]. However, the anti-tumor effects of bufalin have not been demonstrated in human esophageal carcinoma TE13 cells. The purpose of this study was to evaluate the anti-proliferative effect of bufalin on ESCC cell lines.

2. MATERIALS AND METHODS

2.1 Cell Lines

The human ESCC cell line TE13 originated from the American Type Culture Collection (ATCC) and were cultured in RPMI-1640 containing 10% fetal bovine serum (FBS, Sijiqing, Beijing, China) and 5% CO_2 at 37℃Unless otherwise indicated, cell culture reagents were obtained from Bio Technologies, Inc. (Shanghai, China).

2.2 *Compounds*

Bufalin [3β, 14-dihydroxy-5β, 20(22)-bufadienolide, 5β, 20(22)-bufadienolide-3β, 14-diol] was purchased from Sigma (St. Louis, MO). Compounds were dissolved in 100% DMSO (Sigma, St. Louis, MO) and diluted in RPMI 1640

to the desired concentrations, with a final DMSO concentration of 0.1% (v/v) for in vitro studies. DMSO was added to cultures at 0.1% (v/v) as a solvent control.

2.3 *Western Blotting*

The human ESCC cell line TE13 was cultured at 37°C with 5% CO2 in an air atmosphere , supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. TE13 cells at exponential growth stage were employed in all of the experiments. Two hours before the end of culture, TE13 cells were treated by bufalin on different concentration and one and half an hour later, the cells were stimulated by 15% fetal calf serum (FCS).

Then collected the cells for Western blot analysis. Briefly, the cells were lysed in RIPA buffer for 40 min on the ice. Lysates were collected after centrifuging at 12,000 rpm for 20 min at 4°C. Protein levels were quantified using Lowry method. Equivalent amounts of protein (50 μg/lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. The membranes were blocked in PBS containing 5% non-fat dry milk (w/v) for 1 h, and then incubated with primary antibodies(ERK, p-ERK) overnight at 4°C. The membranes were then incubated with HRP-conjugated secondary antibodies at room temperature for 30 min, and developed using DAB enhanced chemiluminescence reagent and calculated by semiquantitative analysis.

2.4 *Immunocytochemistry Assay*

TE13 cells were cultured on coverslip and treated by bufalin as describe above. Cells were fixed in 4 % cool formalin for 30 min at room temperature. The formalin-fixed samples were washed twice with PBS and then were blocked with blocking buffer (0.2 % Triton-100 and 5 % goat serum in PBS) for 1 h. After that, the samples were incubated with primary antibody and secondary antibody for 1h, respectively. Dyed the samples by DAB and hematoxylin reagent consecutively. Then got the results by a microscope after dehydrated alcohol gradient.

2.5 Cytometry Analysis

TE13 cells were treated by bufalin as describe above and the cells were collected and added 0.4% trypan blue reagent. The samples were counted on the ruled counting chamber. Through a microscope, live cells were counted and calculated by the formula as follows: Cell concentration = (total cellular score of four grids/4)×10^4×2 cells/mL. Data were confirmed in at least five independent experiments and calculated averaging.

2.6 MTT Assay

MTT assay was used to examine the effects of bufalin on the proliferation of TE13 cells. Briefly, the cells were seeded in 96-well plates at 1×10^5 cells/well in 100μL medium and cultured for 12h to allow attachment. Then the cells in the wells were treated with fresh medium containing bufalin. PBS was added to the wells as negative control. The cells were cultured for 24h. Four hours before the end of culture, MTT solution (0.5 mg/mL in 20μL PBS) was added to each well and incubated for 4 h at 37°C. The growth medium was then removed and replaced with DMSO (100 μL/well), and incubated for 10 min. The inhibition rate was counted by universal microplate spectrophotometer. The inhibition rate of cell proliferation was calculated as follows: inhibition rate (%) = 1−A492 (test)/A492 (control)×100%. Data were calculated from three independent experiments, each performed in triplicate.

2.7 Statistical Analysis

The data were expressed as the mean ± standard deviation (SD) from experiments performed in triplicate. Variance analysis was used to identify statistically significant differences between the experimental and control groups. The statistical analyses were performed using the SPSS 13.0 . A value < 0.05 was considered statistical significance.

3. RESULTS

3.1 Bufalin Modulate the Expression of ERK in TE13 Cells by Immunocytochemistry

To confirm that bufalin regulated the activation of ERK, we used immunocytochemistry analysis to test Bufalin inhibited the activity of ERK in the cultured esophageal carcinoma TE13 cells. The expression of ERK mainly showed in the cytoplasm of the esophageal carcinoma TE13 cells at high magnifying observation, and the phospho-ERK(p-ERK) mainly expressed in the nuclear or nuclear-plasma. The result of this assay showed obviously (Fig.1,Fig.2) . The effects of Bufalin on ERK was not variative significantly after treated with various doses of bufalin in TE13 cells. On the contrary, The number of positive cells reduced with the dose of Bufalin increase, and the brown yellow particles and density became to weaken gradually. All in all, bufalin inhibited the activity of ERK in TE13 cells in a dose dependent manner.

3.2 Uo126 Enhance the Bufalin Inhibitting Effect of ERK Phosphorylation

Bufalin alone inhibited the Ras/Raf/MEK/ERK signaling pathway in the cultured ESCC cells by down-regulating the phosphorylation of ERK in TE13 cells. Bufalin alone affected significantly in decreased the phosphorylation of ERK.

To provide further evidence, we adopted specific ERK inhibitor Uo126. Although both bufalin 10nmol/L and Uo126 20μmol/L alone inhibited the phosphorylation of ERK, the reduction in p-ERK was more pronounced in the cells treated with a combination of bufalin and Uo126 (Fig.4) . Thus, by employing ERK specific inhibitor Uo126, we could demonstrate the synergistic effects of bufalin and Uo126. And bufalin could significantly augment the inhibitory effects of Uo126 on ERK activation in esophageal carcinoma TE13 cells.

3.3 Bufalin Inhibits the Proliferation of TE13 Cells

To study the proliferative effect of bufalin on TE13 cells, we applied the cytometry assay in this research for the purpose of investigating the proliferative effect inhibited by bufalin at various times. Consequently, As shown in figure 5, the analysis was explicit. The growth of human esophageal carcinoma TE13 cells was inhibited gradually by Bufalin with the increase of concentration 10 nmol/L, 25 nmol/L, 50 nmol/L, or 100 nmol/L for 24h. Therefore, bufalin inhibited the growth of TE13 cells in a dose dependent manner.

We speculated that the proliferation viability of TE13 cells may be inhibited by bufalin, thus we applied MTT analysis to detect the proliferation. TE13 cells were treated with 10 nmol/L, 25 nmol/L, 50 nmol/L, or 100 nmol/L bufalin for 24h, 48h or 72h respectively. As is shown in figure 6, cell growth was inhibited gradually by bufalin with the dose and time increase. In sum up, MTT analysis showed that bufalin inhibited the proliferation of TE13 cells in a dose and time dependent manner as well.

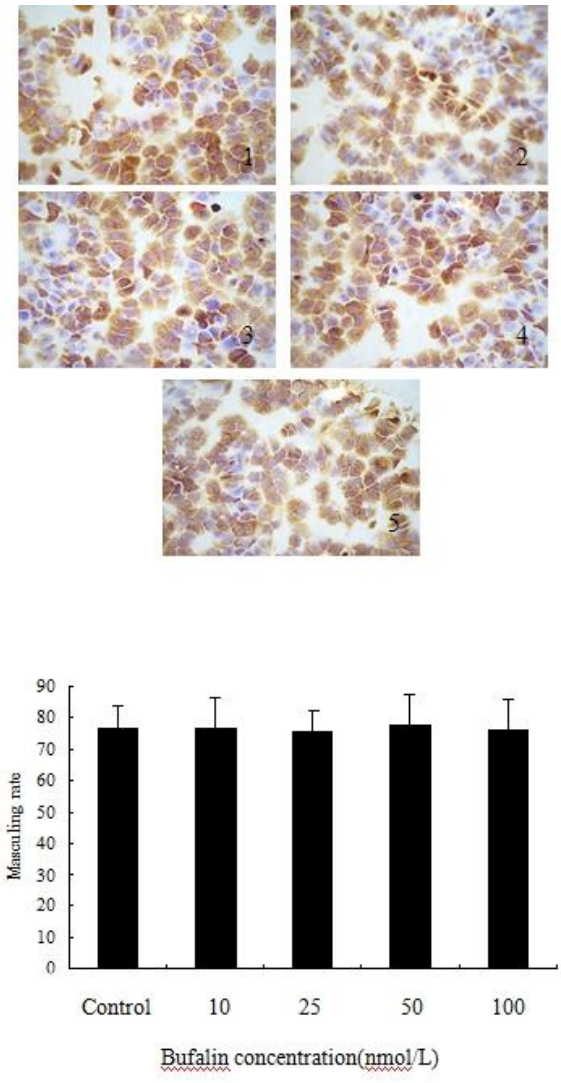

Fig.1 Bufalin regulates the expression of proliferation related protein in TE13 cells. Immunocytochemistry analysis of ERK in TE13 cells treated with 0nmol/L, 10nmol/L, 25nmol/L, 50nmol/L and 100 nmol/L bufalin for two hours. Data were derived from three independent experiments.

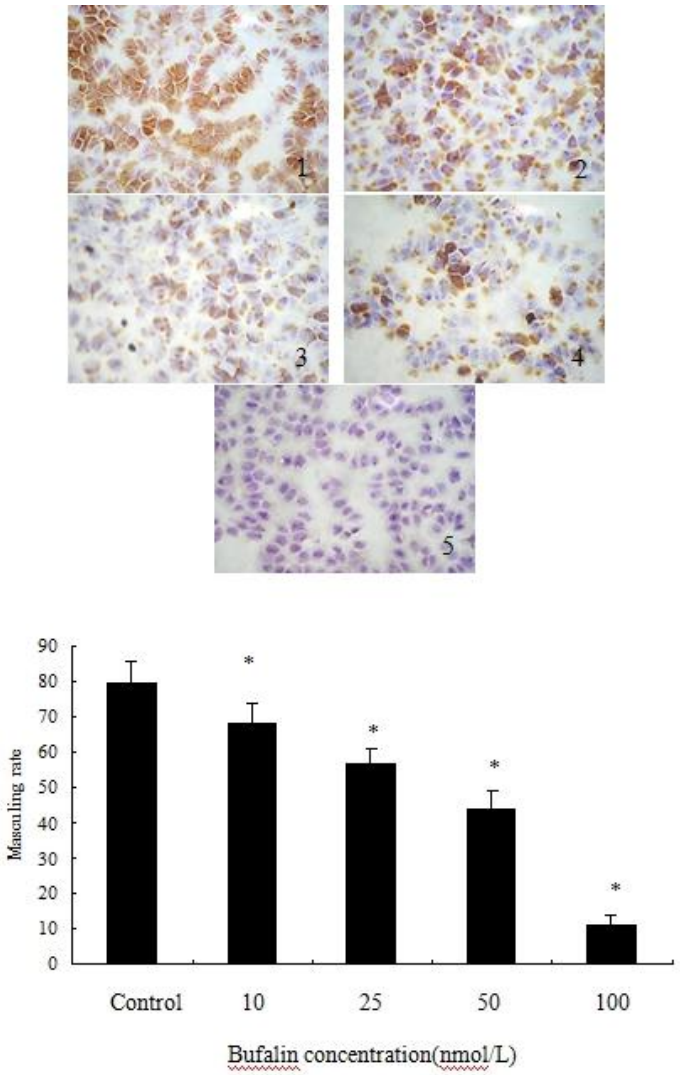

Fig.2 Bufalin regulates the expression of proliferation related protein in TE13 cells. Immunocytochemistry analysis of p-ERK in TE13 cells treated with 0nmol/L, 10nmol/L, 25nmol/L, 50nmol/L and 100 nmol/L bufalin for two hours. Data were derived from three independent experiments.

*P<0.05, vs control group

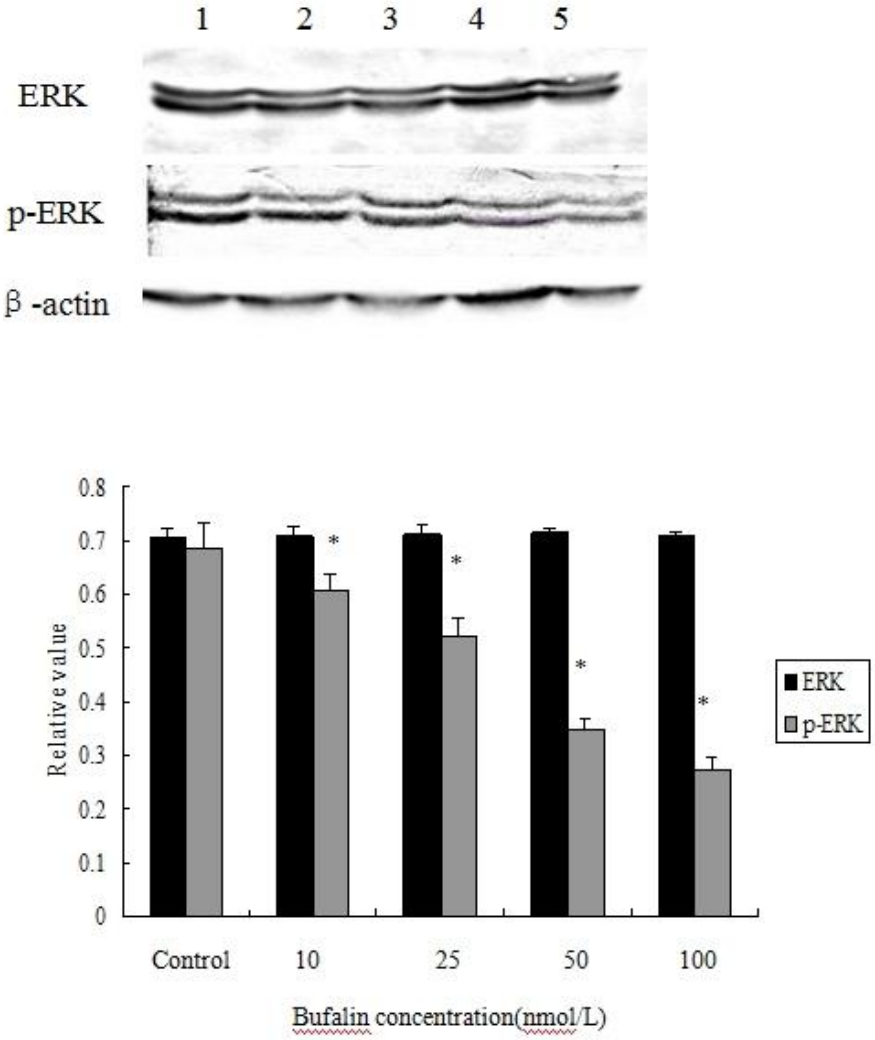

Fig.3 Bufalin regulates the expression of proliferation related protein in TE13 cells. Western blot analysis of ERK and p-ERK in TE13 cells treated with 0nmol/L, 10nmol/L, 25nmol/L, 50nmol/L and 100 nmol/L bufalin for two hours. Data were derived from three independent experiments.

*P<0.05, vs control group

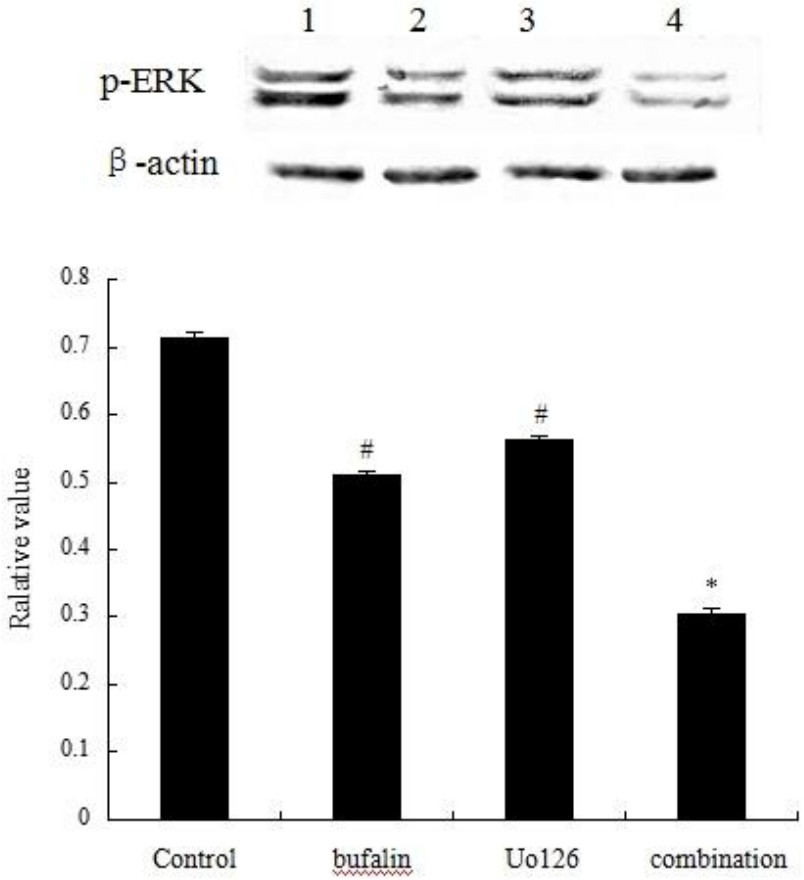

Fig.4 Both bufalin 10nmol/L and Uo126 20μmol/L alone inhibited the phosphorylation of ERK, the reduction in p-ERK was more pronounced in the cells treated with a combination of bufalin and Uo126 by Western blot. Data were derived from three independent experiments.

**P*<0.05, vs other groups #*P*<0.05,vs control group

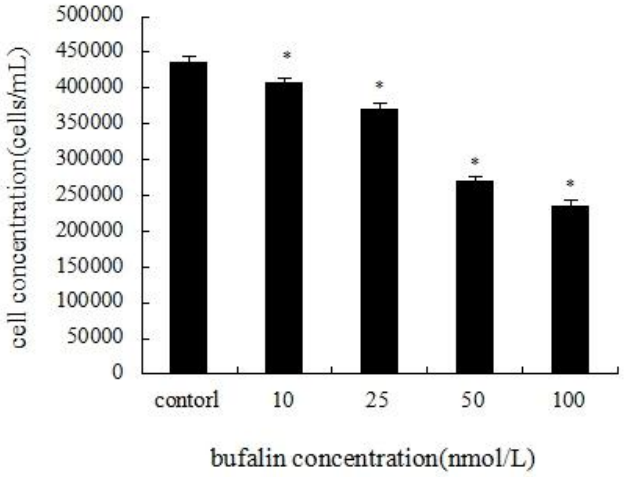

Fig.5 Bufalin inhibits the proliferation of human esophageal carcinoma TE13 cells. The cytometry assay of proliferation is treated with 0nmol/L, 10nmol/L, 25nmol/L, 50nmol/L and 100 nmol/L bufalin for two hours. Data were derived from five independent experiments.

**P*<0.05, vs control group

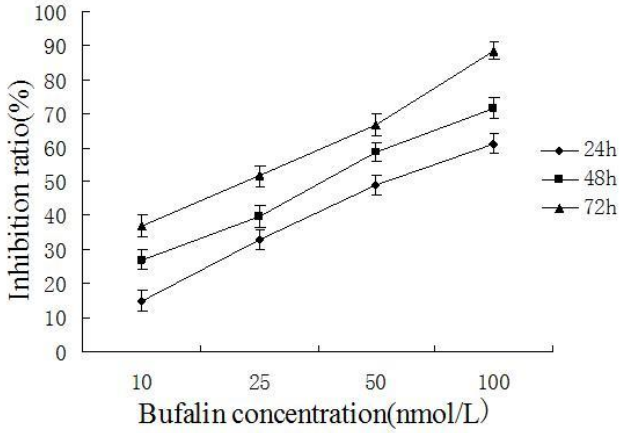

Fig.6 Bufalin inhibits the proliferation of human esophageal carcinoma TE13 cells. The MTT assay of proliferation is treated with 10nmol/L, 25nmol/L, 50nmol/L and 100 nmol/L bufalin for 24h, 48h, 72h. Data were derived from three independent experiments.

4. DISCUSSION

In ESCC, several signaling pathways have been shown to be critical players. Among these, the Raf/MEK/ERK and PI3K/AKT pathways play an important role in the proliferation and induction of esophageal carcinoma cells and could be involved in therapy cancers through molecule target of drugs. Previously, numerous studies have demonstrated that Raf/MEK/ERK signaling pathway has been activated in esophageal carcinoma and ERK is the key molecule of the ERK signaling pathway [13], which promotes the induction of cancer cells to proliferation. Hence, we investigate the effects of bufalin on ERK signaling pathways.

Just like several antitumor drugs including etoposide, adriamycin, and genistein, bufalin is also known as an inhibitor of topoisomerase II. It has been used in clinical trials for cancer treatments in China and has been demonstrated to inhibit the proliferation through induced apoptosis and autophagy of certain cancers, including colorectal carcinoma, melanoma, hepatoma, breast carcinoma, and gastric carcinoma [14-17].Many studies also have revealed the underlying molecular mechanism of antitumor effect of bufalin. Yu et al. have found that bufalin could cause apoptosis in prostate cancer cells via caspase [18]. Bufalin is also found to induce autophagy-mediated cell death through reactive oxygen

species generation and JNK activation in human colon cancer cells [19]. Combination of bufalin with other anticancer drugs is reported to enhance the antitumor efficacy in several tumor models [20, 21].

The effects of bufalin on several cancers associated with proliferation are examined by various studies. There is however, no available report on bufalin-inhibiting proliferation of esophageal carcinoma cells through ERK pathway. Just because of this, we investigate the functions of bufalin on the proliferation of human esophageal carcinoma TE13 cells and determine if bufalin could inhibit the proliferation of esophageal carcinoma in TE13 cells via regulating ERK.

In the present study, We find that bufalin significantly inhibit the proliferation of TE13 cells. We also find that bufalin significantly inhibited the activity of ERK. Bufalin reduce levels of certain proteins associated with proliferation. These results demonstrate that the anti-proliferation effect of bufalin is associated with inhibition of ERK. And furthermore, through restrain the activity of ERK bufalin inhibits the proliferation of esophageal carcinoma TE13 cells in a dose dependent manner. In addition, Uo126, as a specific ERK inhibitor generally known, has been demonstrated the synergistic effects of bufalin. Bufalin could significantly augment the inhibitory effects of Uo126 on ERK activation in esophageal carcinoma TE13 cells. So combined application of bufalin and Uo126 could enhance the antitumor efficacy in esophageal carcinoma cells.

However, further studies are needed to elucidate the mechanism by which bufalin modulates the activation of ERK/p-ERK and other signaling pathways that are crucial for esophageal carcinoma survival and chemoresistance.

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