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*Original Article*

# Phenethyl isothiocyanate potentiates anti-tumor effects of cisplatin through Nrf2/keap1 signaling pathways

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**Abstract:** Oral squamous cell carcinoma (OSCC) is the most commonly diagnosed malignancy in head and neck cancers, commonly developing therapeutic resistance. Phenethyl isothiocyanate (PEITC) is found in cruciferous vegetables, such as watercress, and is responsible for anti-cancer activities on different types of cancers. However, the underlying mechanisms of PEITC, alone and in combination with cisplatin, regarding OSCC development have not been fully elucidated. Present results showed that PEITC inhibited proliferation and induced autophagy in Cal-27. In addition, it was demonstrated that PEITC, either alone or in combination with cisplatin, inhibited Cal-27 cell proliferation through the activation of Nrf2/Keap1 signaling. PEITC enhanced cisplatin killing ability in Cal-27 cells. Collectively, this study concludes that PEITC acts via multiple molecular targets to elicit anti-carcinogenic activity. PEITC/cisplatin combination therapy may be a new potential strategy, benefitting patients with OSCC.

**Keywords:** PEITC, autophagy, oral squamous carcinoma, Nrf2/Keap1 signaling

## Introduction

Oral squamous cell carcinoma (OSCC) is the most commonly diagnosed malignancy, accounting for more than 90% of malignant tumors of the oral cavity [1, 2]. It has an annual worldwide incidence of over 300,000 cases. It has a mortality rate of 48% [3] and commonly develops therapeutic resistance [4]. Treatment modalities for nonmetastatic OSCC are radical surgery followed by adjuvant chemoradiotherapy and definitive chemoradiotherapy. Unfortunately, the five-year disease-free and overall survival (OS) rates for OSCC have remained at 50-55% for the past several decades [5]. Resistance to therapeutic regimens is a major problem for cancer therapy, as it precludes complete ablation of the tumor and enables local and distant tumor relapse, the main cause of cancer mortality. Consequently, new strategies to prevent this event and to improve survival rates are critically needed.

Cisplatin is a platinum-based compound used to treat a wide spectrum of solid neoplasms [6, 7]. It is a widely used as a first-line therapeutic agent for treatment of OSCC and remains one of the most effective modalities [8, 9]. Because cisplatin toxicity increases generation of reactive oxygen species (ROS), there is growing evidence suggesting that cytoplasmic cisplatin exhibits antitumor activity via tilting the reducing-oxidizing (redox) balance toward oxidative stress [10, 11]. Chemotherapy is accompanied by severe side effects due to its tendency to destroy both normal and tumor cells.

Identification of new drugs from plants and vegetables has a long and successful history. Dietary intake of cruciferous vegetables may be protective against different types of malignancies [12]. Phenethyl isothiocyanate (PEITC) is one of the most extensively studied members of the isothiocyanate family, found in cruciferous vegetables, such as watercress. It is

responsible for anti-cancer activities. In addition, it has shown to inhibit migration and invasion of many types of human cancer cells [13]. PEITC selectively kills cancer cells, but not normal cells. It generates reactive oxygen species (ROS) to trigger signal transduction, leading to cell cycle arrest, autophagy, and/or apoptosis [14]. However, the molecular mechanisms by which PEITC acts as a growth inhibitor and autophagy and/or apoptosis inducer in cancer cells have not been fully investigated.

Thus, the present study aimed to assess the anti-tumor properties of PEITC, alone and in combination with cisplatin, in oral squamous cell carcinoma (OSCC) cell line, Cal-27. In addition, this study proposes that the molecular mechanisms of PEITC anti-tumor activity are through Nrf2-Keap1 signaling pathways.

## Materials and methods

### *Cell lines and cell culture*

Human tongue squamous cell carcinoma (Cal-27) cell line was provided by Professor Wantao Chen (Department of Oral and Maxillofacial Surgery, Ninth People's Hospital, College of Stomatology, Shanghai Jiao Tong University, Shanghai, China). Cells were plated onto 75 $cm^2$ tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM, HyClone), supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin and 2 mML-glutamine. They were grown at 37°C under a humidified 5% $CO_2$ and 95% air at one atmosphere. The medium was changed every two days.

### *CCK-8 assay*

Effects of various concentrations of PEITC (Sigma Chemical Co., St. Louis, MO, USA) on Cal-27 proliferation was measured using a cell counting kit-8 assay (CCK-8, Dojindo). Cal-27 was seeded into 96-well culture plates (Eppendorf) with a concentration of $1\times10^4$ cells/well. After 24 hours of incubation at 37°C with 5% $CO_2$, the plates were treated with 0, 2.5, 5, 10, 15, 20, and 25 μM PEITC for 3, 6, 9, 12, 18, and 24 hours. CCK-8 was mixed with serum-free DMEM medium at a proportion of 1:10 in advance. After removal of the complete DMEM medium, the 110 μL mixture was added to each well and incubated at 37°C for 2 hours, until the media turned yellow. Groups without cells were used as zero setting. Cell viability was assessed by absorbance values in each well, measured with a spectrophotometer (Thermo, Finland) at a wavelength of 450 nm. Data were calculated using averages of three wells and untreated Cal-27 was considered as the control group. Concentrations of 20 μM PEITC were chosen for subsequent experiments.

### *Transmission electron microscopy*

Transmission electron microscopy (TEM) was performed according to acknowledged procedures. Briefly, Cal-27 cells were incubated with DMEM (Control) and 20 μM PEITC for 24 hours. Cells were then fixed in ice-cold 2.5% electron microscopy grade glutaraldehyde (Beijing Chemical Industry Group, Co., Ltd., Beijing, China), post-fixed in 1% osmium tetroxide (Beijing Chemical Industry Group, Co., Ltd.), dissolved in 0.1 M cacodylate buffer (Beijing Chemical Industry Group, Co., Ltd.), dehydrated through a graded series of ethanol (30-90%) (Beijing Chemical Industry Group, Co., Ltd.), and embedded in Epon (Beijing Chemical Industry Group, Co., Ltd.). Ultrathin sections (65 nm) were cut, stained with 4% uranyl acetate (Beijing Chemical Industry Group, Co., Ltd.), and detected with the JEM-1200EX Transmission Electron Microscope (JEOL, Ltd., Tokyo, Japan).

### *Quantitative real-time polymerase chain reaction (RT-PCR)*

After reaching 70-80% culture confluency from Cal-27 seeded in 6-well plates at a density of $1\times10^5$ cells/well, cells were incubated under the DMEM (Control), 20 μM PEITC, 20 μM PEITC+5 mmol/L 3-MA (Sigma Chemical Co., St. Louis, MO, USA), 3 mg/L cisplatin (Sigma Chemical Co., St. Louis, MO, USA), and 20 μM PEITC+3 mg/L cisplatin conditions, respectively, for 12 hours. After treatment, the cells were lysed using TRIzol Reagent (Takara Biotechnology, Co., Ltd., Dalian, China) to isolate total RNAs, according to manufacturer instructions. Single-stranded complementary DNA (cDNA) was obtained by reverse transcribing 1 μg of total RNA from each sample using a PrimeScript™ RT reagent kit (Takara Biotechnology, Co., Ltd., Dalian, China). Real-time PCR was conducted with a Roche Light Cycler 480 device (Roche Applied Science, Germany) in a total volume of 20 μl reacting system. Cycling parameters used were: 95°C for 30 seconds,

followed by 40 cycles of 95°C for 5 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, with a dissociation program of 95°C for 15 seconds, 60°C for 30 seconds, and 95°C for 15 seconds. GAPDH was used as the reference gene for normalization and mRNA expression levels were quantified using the threshold cycle method. Three independent experiments were performed in triplicate.

*Western blot analysis*

After incubation under different conditions, cells were rinsed 3 times with cold phosphate-buffered saline (PBS) for 5 minutes and lysed on ice in RIPA buffer (Shennong Bocai Biotechnology Co., Ltd., Shanghai, China). After incubation on ice for 30 minutes, the lysates were cleared by centrifugation at 14,000 g for 15 minutes at 4°C. Protein concentrations were determined using BCA protein assay kits (Beyotime Institute of Biotechnology, Shanghai, China), in accordance with manufacturer instructions, and bovine serum albumin was used as a standard. Equal amounts of total protein were collected and electrophoresed using 10-15% SDS-PAGE gels (Shennong Bocai Biotechnology Co., Ltd., Shanghai, China) at 100 V, then transferred to a polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA). The membranes were blocked in a 5% non-fat milk-TBST solution for at least 60 minutes while shaking. Next, the membranes were washed 6 times for 5 minutes each time with TBS-0.05% Tween-20 (TBST) at room temperature. Subsequently, the membranes were incubated with primary antibodies anti-LC3B antibody, anti-Atg5 antibody, anti-p62 antibody, anti-Nrf2 antibody, and anti-Keap1 antibody (Abcam, Cambridge, MA, USA) overnight at 4°C with shaking. To ensure equal protein loading, β-actin (1:1000 dilution, ZSJB-BIO, China) was detected on the same membrane and used as a loading control. Thereafter, membranes were incubated with secondary antibodies labeled with HRP for 60 minutes. After washing three times with TBST, the proteins were visualized using an Alpha Imager 2200 system (Alpha Innotech Corporation, San Leandro, CA, USA). Band density was calculated with Image J software packages.

*Immunofluorescence staining*

A total of $2\times10^5$ Cal-27 cells were seeded in 12-well plates with 14 mm-diameter coverslips and incubated with DMEM (control) and 20 μM PEITC for 24 hours. Cells were then washed 3 times with PBS for 5 minutes each time, fixed with 4% paraformaldehyde (Beijing ComWin Biotech Co., Ltd., Beijing, China) for 20 minutes at room temperature, and permeabilized with 0.2% Triton X-100 (Beijing ComWin Biotech Co., Ltd., Beijing, China) for 10 minutes. Consequently, after washing three times with PBS, the slices were blocked with goat serum for 1 hour at room temperature. They were then incubated with rabbit polyclonal anti-LC3 antibody (1:100 diluted in PBS) at 4°C overnight. The next day, the cells were washed three times with PBS and incubated for 1 hour with goat anti-rabbit immunoglobulin antibody, conjugated with fluorescein isothiocyanate (FITC; Beijing ComWin Biotech Co., Ltd., Beijing, China) (1:400 diluted in PBS) for 1 hour at room temperature in the dark. The nuclei were counterstained with DAPI (Beijing ComWin Biotech Co., LTD., China) for 10 minutes. The specimen was observed and photographed to show representative cells using a fluorescent microscope (TE2000; Nikon Corporation, Tokyo, Japan). Images were recorded using RSImage software.

*Statistical analysis*

Statistical analyses were performed using SPSS 20.0 software (IBM SPSS, Armonk, NY, USA). Differences between groups were analyzed using Student's t-test. Differences in measured variables were assessed with one-way analysis of variance (ANOVA). Data are presented as the mean ± standard deviation from the three independent experiments. $P<0.05$ indicates statistically significant differences.

## Results

*PEITC inhibits Cal-27 proliferation*

As shown in **Figure 1A**, PEITC at 2.5 to 25 μM significantly inhibited the proliferation of Cal-27 cells in a dose-dependent manner, compared with the vehicle control. Cal-27 cells were not inhibited obviously. The inhibition was most pronounced in 12 hours (**Figure 1B**) ($P<0.05$, n=10). As shown in **Figure 1C** and **1D**, Cal-27 cells became round, in shape, under a light microscope, compared with the vehicle control. Apoptotic cells were observed intensively after the 20 μM PEITC treatment.

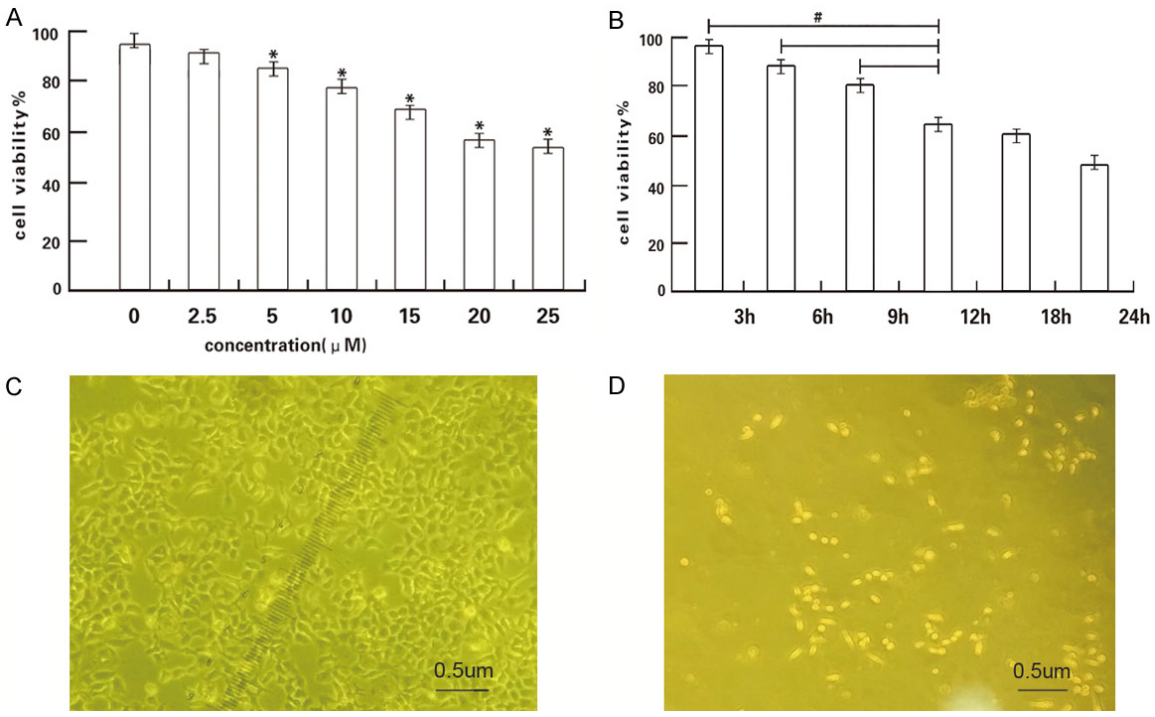


**Figure 1.** PEITC inhibited the proliferation of Cal-27 cells. A. Cells were treated with PEITC (0, 2.5, 5, 10, 15, 20, 25 μM) for 12 hours. B. Cells were treated with 20 μM PEITC for 3, 6, 9, 12, 18, and 24 hours. Final concentrations of the compounds are shown. Cell viability was determined by CCK8 assay (mean ± SD). All data were obtained from ten independent experiments performed in triplicate. *P<0.05 versus control vehicle (0 μM). #P<0.05 versus other time points. C. Control group of Cal-27 cells under light microscope. D. 20 μM PEITC group of Cal-27 cells under light microscope.

*PEITC induces autophagy in Cal-27 cells*

Several researchers have demonstrated the anti-cancer abilities of isothiocyanates in various cancer types [15-17]. To investigate whether autophagy could be induced by PEITC in OSCC cell lines, Cal-27 cells were incubated with 20 μM PEITC and 5 mmol/L 3-MA for 12 hours. This study measured expression of LC3, Atg5, and P62 at the protein levels by Western blot analyses. LC3-II acts as a biochemical marker for autophagy [18]. In addition, levels of P62 are an important measurement for evaluating autophagy pathways [19]. As shown in **Figure 2A** and **2B**, compared to the control group, LC3-II, Atg5, and P62 levels were significantly increased in the 20 μM PEITC group and obviously decreased after treatment with 3-MA, an autophagy inhibitor, indicating that autophagic flux was blocked. Present results suggest that PEITC might induce autophagy in Cal-27 cells.

This study further examined Cal-27 cells treated with PEITC using Immunofluorescence staining. Data showed that the number of LC3 punctate dots formed per cell significantly increased in cells treated with PEITC, compared to the control group (**Figure 3A**). In addition, this study examined Cal-27 cells treated with PEITC using transmission electron microscopy to observe the formation of autophagosome. Compared to the control group, there was an increased number of autophagosomes in the PEITC group (**Figure 3B**). Results further indicate that PEITC induces autophagy in Cal-27 cells.

*PEITC enhances cisplatin-induced inhibition of Cal-27 cell proliferation through activating autophagy*

To evaluate the effects of PEITC on cisplatin-induced inhibition of Cal-27 cell proliferation, this study treated Cal-27 cells with 20 μM PEITC and 3 mg/L cisplatin and measured cell viability using CCK8 assay. Cal-27 cells treated with PEITC and cisplatin were significantly inhibited, compared with the vehicle control and the cisplatin group, while Cal-27 cells were not inhibited obviously (**Figure 4A**). These results suggest that PEITC enhances the inhibitory effects of cisplatin on proliferation of Cal-27.

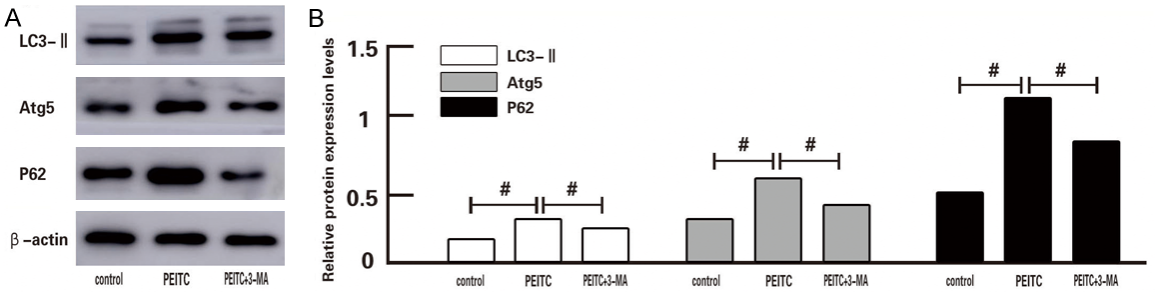


**Figure 2.** PEITC induces autophagy in Cal-27 cells. Cal-27 cells were incubated with 20 μM PEITC and 5 mmol/L 3-MA for 12 hours. Western blot analysis of the protein expression of LC3-II, Atg5, and P62 between the control, PEITC, and PEITC+3-MA group. β-actin was used as an internal control. #P<0.05. All data are the mean ± SD of three independent experiments.

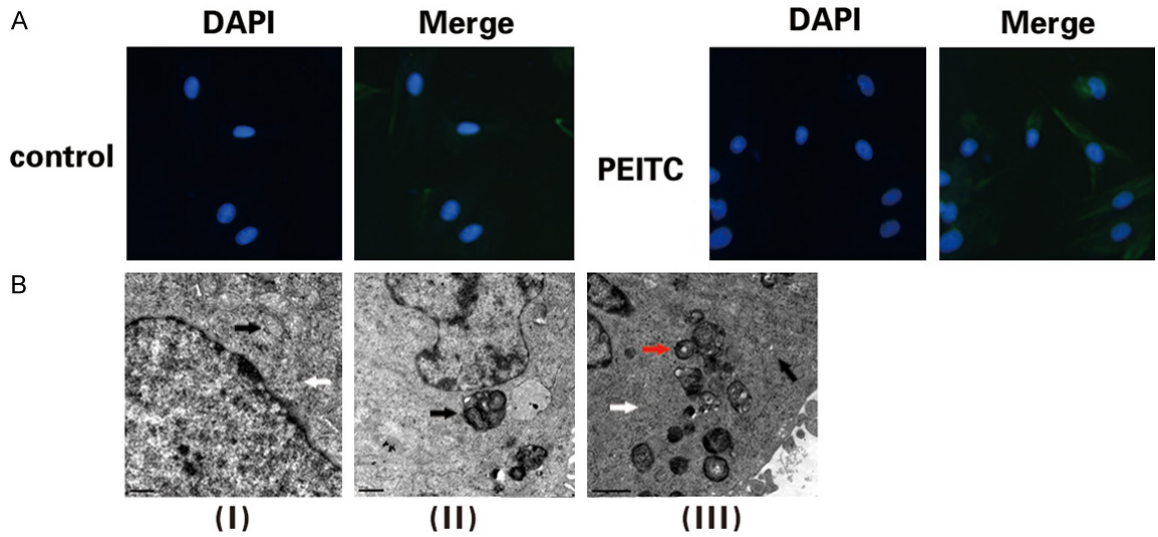


**Figure 3.** PEITC induces autophagy in Cal-27 cells. A. LC3 punctate dots were visualized by fluorescence microscopy. The LC3 puncta were quantified by randomly counting 10 cells for each group. B. The effects of PEITC on the formation of autophagosome using transmission electron microscopy. Cal-27 cells were treated with the indicated conditions. (I) Normal cell ultrastructure. Black arrows denote the mitochondria, and the white arrow denotes the endoplasmic reticulum. (II) The PEITC group. Black arrow denotes an autolysosome. (III) The PEITC group. Red arrow denotes a large-scale accumulation of autophagosomes. Black arrows denote a mitochondrial vacuolization and the white arrow denotes endoplasmic reticulum disorganization. Representative results are shown from more than three repeats.

As shown in **Figure 4B**, compared to the control group and cisplatin group, LC3-II, Atg5, and P62 levels were significantly increased in the PEITC + cisplatin group. Results suggest that PEITC enhances cisplatin-induced inhibition of Cal-27 cell proliferation through activating autophagy.

*Nrf2/Keap1 signal pathways involved in PEITC-induced autophagy and PEITC-enhanced cisplatin killing ability in Cal-27 cells*

As demonstrated above that PEITC treatment increased autophagy levels in Cal-27 cells, this study next explored mechanisms involved in this process. Previous studies have shown that p62 is a transcriptional target of Nrf2 [20] and that Nrf2/Keap1 signal pathways are the major regulator of cytoprotective responses to endogenous and exogenous stresses caused by reactive oxygen species (ROS) and cytotoxic chemotherapeutic agents [21]. Thus, this study investigated whether Nrf2/Keap1 signal pathways are involved in PEITC-induced autophagy in Cal-27 cells. Cal-27 cells were incubated under PEITC, PEITC+3-MA, cisplatin, and PEITC + cisplatin conditions for 12 hours. Total RNA and proteins were harvested to perform RT-PCR

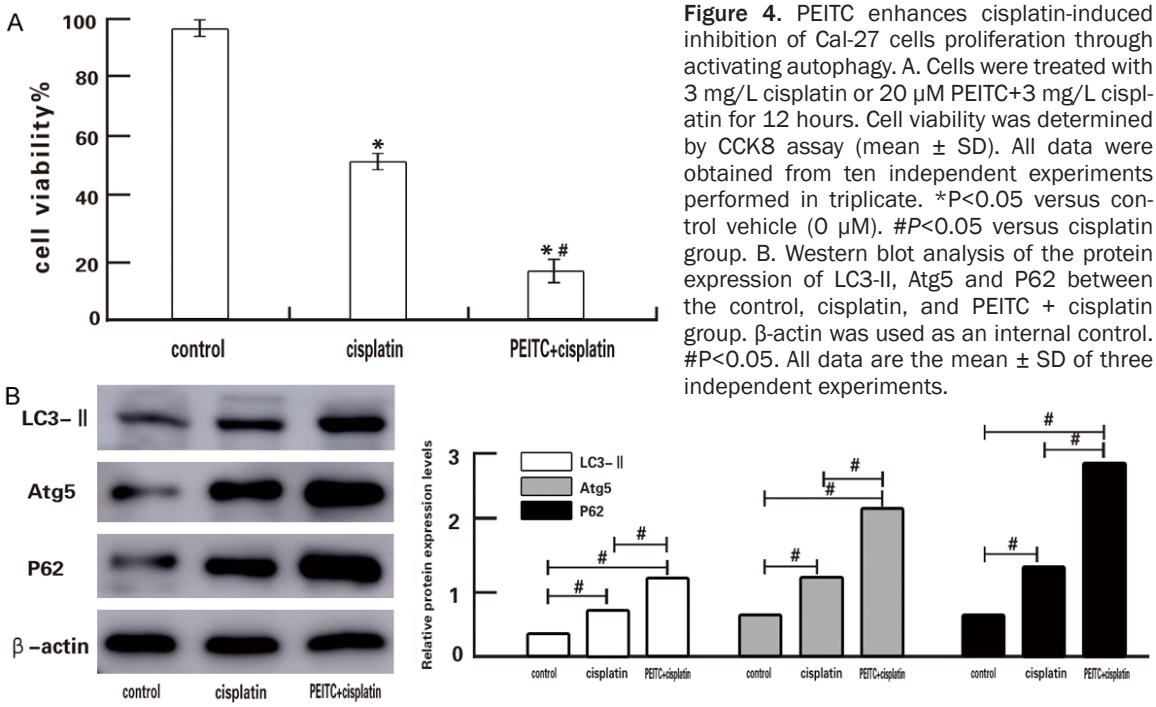


**Figure 4.** PEITC enhances cisplatin-induced inhibition of Cal-27 cells proliferation through activating autophagy. A. Cells were treated with 3 mg/L cisplatin or 20 μM PEITC+3 mg/L cisplatin for 12 hours. Cell viability was determined by CCK8 assay (mean ± SD). All data were obtained from ten independent experiments performed in triplicate. *P<0.05 versus control vehicle (0 μM). #*P*<0.05 versus cisplatin group. B. Western blot analysis of the protein expression of LC3-II, Atg5 and P62 between the control, cisplatin, and PEITC + cisplatin group. β-actin was used as an internal control. #P<0.05. All data are the mean ± SD of three independent experiments.

and Western blot analysis. Results showed that the mRNA and protein expression of p62 and Nrf2 increased significantly in the PEITC, cisplatin, and PEITC + cisplatin groups. However, mRNA and protein expression of Keap1 decreased (**Figure 5**). In conclusion, present data revealed that the effects of PEITC on autophagy induction are, at least partly, due to regulation of Nrf2/Keap1 signal pathways (**Figure 6**).

## Discussion

Natural products used to prevent or treat various ailments, including cancer, have elicited considerable interest [22]. Previous studies have shown that PEITC and other isothiocyanates exert anti-proliferative and anti-tumor activity on different types of cancers [23-26]. However, the underlying mechanisms of PEITC in OSCC development have not been fully elucidated. Present results showed that PEITC significantly inhibited the proliferation of Cal-27 cells in a dose-dependent manner and induced autophagy, which might lead to autophagic cell death in Cal-27.

Cisplatin, a broad-spectrum anticancer agent, has been used widely in the clinical management of various cancers of different tissue types. However, some side effects, including drug resistance, have greatly limited its clinical application [27-29]. Thus, developing new strategies to overcome such drug resistance are highly important [30-32]. Tang et al. reported that PEITC could overcome Dox chemo-resistance in bladder cancer [33]. In that way, does PEITC increase cisplatin sensitivity in OSCC? Thus, this study incubated Cal-27 cells with 20 μM PEITC and 3 mg/L cisplatin. Interestingly, the combination of the highest most effective dose of PEITC with showed significant inhibition of cell viability that exceeded each drug alone. These results were further reinforced by the dose-dependent cytotoxic activity of PEITC treatment in Cal-27 cells, as indicated by a CCK8 assay. In addition, current results showed that PEITC induced autophagy in Cal-27 cells and, compared to the PEITC group and cisplatin group, the autophagy levels of the PEITC/cisplatin combination treatment were significantly increased. Therefore, this combination treatment gave an optimum reduction of cellular viability through activating autophagy.

To further explore the underlying mechanisms, RT-PCR and Western blot analysis in Cal-27 were used to investigate the effects of PEITC, either alone or in combination with cisplatin, on Nrf2/Keap1 signal pathways. The Nrf2/Keap1 signal pathway is a major regulator of cytopro-

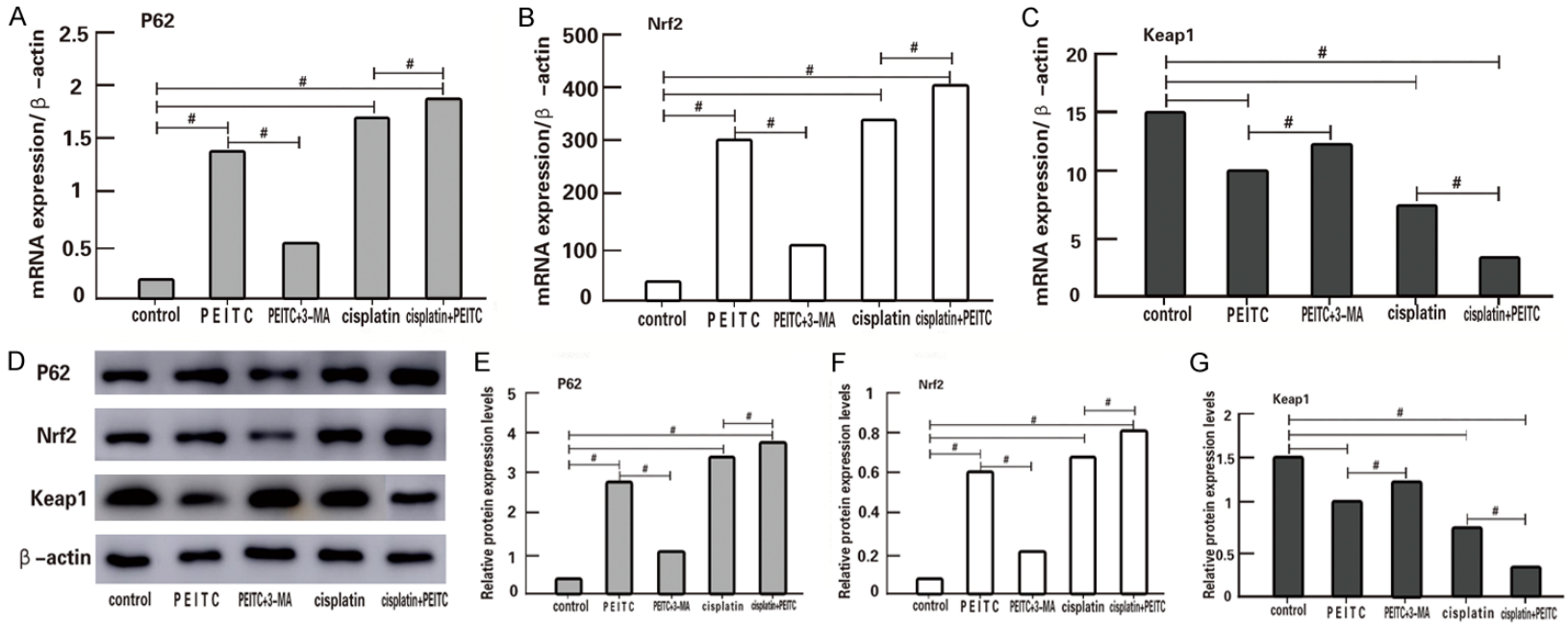


**Figure 5.** Regulation of the Nrf2/Keap1 signal pathway under PEITC conditions in Cal-27 cells. mRNA (A-C) and protein (D-G) expression of Nrf2, Keap1, and p62 was detected by Western blot and RT-PCR, respectively. #$P<0.05$, n=3.

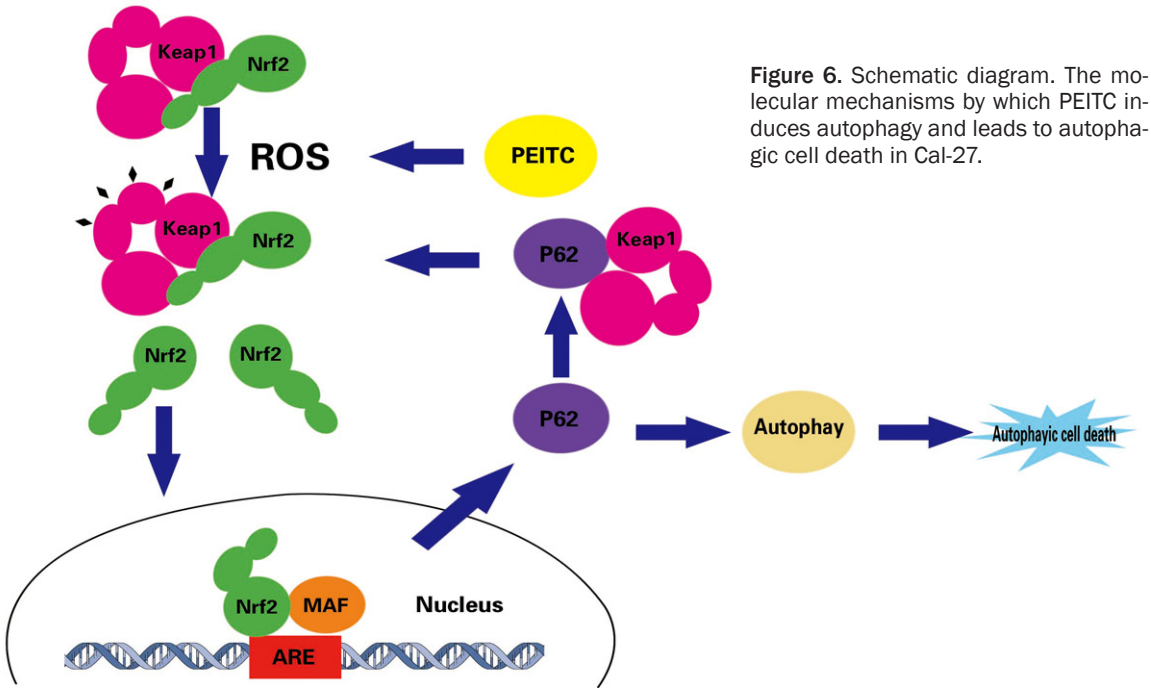


**Figure 6.** Schematic diagram. The molecular mechanisms by which PEITC induces autophagy and leads to autophagic cell death in Cal-27.

tective response during excessive oxidative and electrophilic conditions [34, 35]. Nrf2, nuclear factor erythroid-2 related factor 2, is the key signaling protein within the pathway. It binds together with small Maf proteins to the antioxidant response element (ARE) in the regulatory regions of target genes. Keap1, Kelch ECH associating protein 1, is a very cysteine-rich protein [36] that regulates Nrf2 activity by binding and consequently mediating its polyubiquitination and subsequent proteasomal degradation under non-stressed conditions. Under stressful conditions, Nrf2 is released and accumulates in the nucleus where it binds to ARE sequence and induces expression of cytoprotective antioxidant genes [37]. P62, also known as sequestosome-1, can compete with Nrf2 to bind with Keap1. The association of p62 with Keap1 allows Nrf2 to activate antioxidant gene expression [38]. This leads to a positive feedback loop where p62 activates Nrf2 and contributes to the autophagic death of cancer cells [39-41]. Present results showed that mRNA and protein expression of p62 and Nrf2 increased significantly in the PEITC/cisplatin combination treatment group. However, mRNA and protein expression of Keap1 decreased. This study shows that PEITC-induced autophagy degrades Keap1 and induces Nrf2 activation. These might identify PEITC as a Nrf2 activator that enhances the chemotherapeutic efficacy of cisplatin. Thus, results suggest that PEITC, either alone or in a combination with cisplatin, may have inhibited Cal-27 cell proliferation through the activation of Nrf2/Keap1 signaling (**Figure 6**).

In conclusion, the present study revealed that PEITC suppressed the growth of Cal-27 cells *in vitro* and activated autophagy, playing an important role in the anti-tumor effects of PEITC. Moreover, PEITC used in combination with cisplatin was able to enhance the killing ability caused by cisplatin treatment alone. Nrf2/Keap1 signal pathways may be critical in modulating sustained PEITC-induced autophagy in Cal-27 cells. Consequently, PEITC/cisplatin combination treatment might be used as a potential novel strategy, benefitting patients with head and neck cancers. However, further research, using different cell lines on the genetic level and *in vivo* testing of animals, is necessary to fully understand the inhibitory effects of PEITC and/or cisplatin on the treatment of head and neck malignancies before consideration for clinical trials.

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## Disclosure of conflict of interest

None.

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