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Research article

Alpha-hederin induces the apoptosis of oral cancer SCC-25 cells by regulating PI3K/Akt/mTOR signaling pathway

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ABSTRACT

Background: Oral cancer is one of the common malignant tumors of the head and neck. However, current treatments have numerous side effects, and drugs from natural sources may have better therapeutic potential. This research investigated the induction of apoptosis by α-hederin (α-HN), a constituent of *Pulsatilla chinensis* (Bunge) Regel, in the oral cancer cell line SCC-25 and its underlying mechanism.
Results: SCC-25 cells were treated with 50, 100, and 200 μmol/L α-HN. Cell proliferation; extent of apoptosis; activities of caspases-3, 8, and 9; and the expression of Bcl-2, Bax, phosphorylated (p)-phosphoinositide 3-kinase (PI3K), p-Akt, and p-mammalian target of rapamycin (mTOR) proteins were determined using the 3-(4,5)-2-thiazole-(2,5)-diphenyl tetrazolium bromide, flow cytometry, caspase activity detection kits, and western blot assays, respectively. The results showed that the proliferation of SCC-25 cells in the α-HN-treated groups decreased significantly, and the inhibitory effect was time and concentration dependent. Compared with cells in the control group, the extent of apoptosis increased significantly, caspase-3 and -9 activities were significantly enhanced, and the Bcl-2 level was lowered and the Bax level was elevated significantly in SCC-25 cells treated with α-HN for 48 h ($P < 0.05$). The expression of p-PI3K, p-Akt, and p-mTOR was also significantly lower in SCC-25 cells treated with α-HN than that in the control group ($P < 0.05$).
Conclusion: These results indicate that α-HN can inhibit proliferation and induce apoptosis of SCC-25 cells and may exert these effects by inhibiting the PI3K/Akt/mTOR signaling pathway.
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1. Introduction

Oral cancer is one of the common malignant tumors of the head and neck. In recent years, the incidence of oral cancer has increased significantly, and the population with oral cancer has become younger [1]. Surgery is the most effective means for treating oral cancer. However, most patients are in the advanced stage and thus beyond the best time for surgical treatment when they are diagnosed because early symptoms are not evident and easy to be ignored [2]. In addition, surgery of oral cancer can cause facial deformity of patients and also seriously affect their language and masticatory functions, thereby greatly lowering their quality of life [3].

Chemotherapy plays an important role in the treatment of oral cancer. Chemotherapeutic drugs commonly used for treating this disease are methotrexate, bleomycin, cisplatin, and fluorouracil. However, these drugs also kill a certain number of normal cells while killing tumor cells, thus exerting strong toxic and side effects [4,5]. Therefore, identifying drugs from natural plants that can kill oral tumor cells specifically by inducing apoptosis to enhance the efficacy of oral tumor therapy has become an important topic in oral medicine research [6].

A variety of chemical constituents with antitumor effects have been isolated from natural products. These include alkaloids (sophocarpidine and vinblastine), flavonoids (quercetin and isoflavones), saponins (ginsenoside), polysaccharides (astragalus and glycyrrhiza), and terpenes (triptolide and elemene) [7,8,9,10,11]. Research on the chemical constituents of the natural medicine *Pulsatilla chinensis* (Bunge) Regel found that all 28 saponins isolated showed cytotoxic activity in varying degrees, of which the antitumor activity of α-hederin

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(α-HN) from the Hederagenin series was the strongest [12]. Studies have shown that α-HN has therapeutic effects on the lung, colon, and breast cancers [13,14,15], but its effects on oral cancer cells are rarely reported. In this study, SCC-25 oral cancer cells were treated with α-HN, and its effects on proliferation and apoptosis were observed. In addition, the effect of α-HN on the phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway of SCC-25 cells was investigated because of its important role in the occurrence and development of malignant tumors [16].

2. Materials and methods

2.1. Materials

α-HN (purity ≥98%; chemical formula, $C_{41}H_{66}O_{12}$; catalog number, 111733) was purchased from The National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Agarose gel, 3-(4,5)-2-thiazole-(2,5)-diphenyl tetrazolium bromide (MTT), penicillin/streptomycin, dimethyl sulfoxide (DMSO), and propidium iodide (PI) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was purchased from Beckman Coulter (Brea, CA, USA). Caspase-3, -8, and -9 activity assay kits were purchased from R&D Systems (Minneapolis, MN, USA). RPMI 1640 medium and fetal bovine serum were purchased from Gibco-BRL (Gaithersburg, MD, USA). Bax, Bcl-2, phosphorylated (p)-PI3K Tyr458, p-Akt Ser473, p-mTOR Ser2448, and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-labeled secondary antibodies were purchased from Wuhan Bohai Biotechnology (Wuhan, China).

2.2. Cell culture

The human oral cancer cell line SCC-25 was purchased from the Cell Bank of the Shanghai Institute of Cell Biology. SCC-25 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin and passaged every 2–3 d under culture conditions of 37°C in 5% CO_2. Cells in the logarithmic growth phase were used for subsequent experiments.

2.3. Cell viability assay

The viability of SCC-25 cells was estimated by the MTT assay. In a previous study, we studied the inhibition of SCC25 cell proliferation under different concentrations of α-HN. The results showed that the inhibitory effects of 50, 100, and 200 μmol/L on the proliferation of SCC25 cells were more evident than those of other concentrations tested (40, 80, and 160 μmol/L and 30, 60, and 120 μmol/L). SCC-25 cells in the logarithmic growth phase were seeded in 96-well plates and treated with 50, 100, and 200 μmol/L α-HN for 24, 48, or 72 h. DMSO was used as the control. At the end of each incubation period, 20 μL of a 5 mg/mL MTT solution was added into each well. The cells were then incubated at 37°C in 5% CO_2 for 4 h. After the medium was removed, 150 μL of DMSO (final concentration 0.1%) was added into each well to terminate the reaction and dissolve the formazan. Absorbance was detected at 570 nm to calculate cell viability compared to the control.

2.4. Apoptosis detection

Cells treated with 50, 100, and 200 μmol/L α-HN for 48 h were washed with phosphate-buffered saline and then stained according to the instructions of the annexin V-FITC/PI apoptosis detection kit. Approximately 10,000 cells were analyzed in each group, and the extent of apoptosis was measured using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Apoptosis is expressed as the percentage of Annexin V^+/PI^- cells in total cells.

2.5. Caspase activity assay

Colorimetric assay kits were used to determine the activities of caspases-3, -8, and -9. After treatment with 50, 100, and 200 μmol/L α-HN for 48 h, SCC-25 cells were collected and centrifuged (10,000*g*, 4°C). After freeze-thawing, the supernatants collected were considered to be cytoplasmic extracts. Five microliters of caspase-3, -8, or -9 chromogenic substrate was added to the supernatants, which were then placed in a CO_2 incubator for 4 h. Absorbance values were measured at 405 nm using a microplate reader, and caspase activities were calculated according to the kit instructions.

2.6. Western blot analysis

Lysates from control and α-HN-treated cells were extracted, and the protein concentration of each sample was measured by the Lowry method. Twenty micrograms of protein from each sample was loaded onto 12% sodium dodecyl sulfate polyacrylamide gels, electrophoresed, and transferred to nitrocellulose membranes. Skim milk powder (5%) was used for blocking for 1 h at room temperature. Membranes were washed with phosphate-buffered saline three times and then incubated with Bax, p-PI3K Tyr458, p-Akt Ser473, Bcl-2, p-mTOR Ser2448, and β-actin antibodies at 4°C overnight. Horseradish peroxidase-coupled secondary antibodies were added onto the membranes and incubated at room temperature for 30 min, and an enhanced chemiluminescence kit was used for visualization. The optical density of each band was analyzed using IPP 5.0 software (Intel, Santa Clara, CA, USA).

2.7. Statistical analyses

Data were analyzed using SPSS 18.0 software (IBM, Chicago, IL, USA). Each experiment was repeated five times. Data are expressed as mean ± SD. One-way analysis of variance was used for repeated comparisons among multiple groups and Student's *t* test for comparison between two groups. $P < 0.05$ and $P < 0.01$ were considered statistically significant.

3. Results

3.1. Effect of α-HN on the proliferation of SCC-25 cells

The proliferation of SCC-25 cells treated with 50, 100, and 200 μmol/L α-HN for 24, 48, and 72 h was detected by the MTT assay. Compared with the control group, the proliferation of α-HN-treated SCC-25 cells decreased significantly in both time- and concentration-dependent manners (Fig. 1). The proliferation of SCC-25 cells treated with 200 μmol/L α-HN for 72 h was the lowest.

3.2. Effect of α-HN on the apoptosis of SCC-25 cells

Apoptosis of SCC-25 cells treated with 50, 100, and 200 μmol/L α-HN for 48 h was detected by annexin V-FITC/PI staining. As shown in Fig. 2A and B, compared with the control group, the extent of apoptosis of SCC-25 cells in the α-HN-treated groups increased significantly in a concentration-dependent manner. Apoptosis was highest in SCC-25 cells treated with 200 μmol/L α-HN.

3.3. Effects of α-HN on caspase activities in SCC-25 cells

Caspase activity kits were used to detect the activities of caspases-3, -8, and -9 in SCC-25 cells treated with 50, 100, and 200 μmol/L α-HN for 48 h. Compared with the control group, caspases-3 (Fig. 2C) and -9 (Fig. 2D) activities increased significantly in a concentration-dependent manner in the α-HN-treated groups ($P < 0.01$). In contrast, caspase-8 activity increased slightly but not significantly ($P > 0.05$).

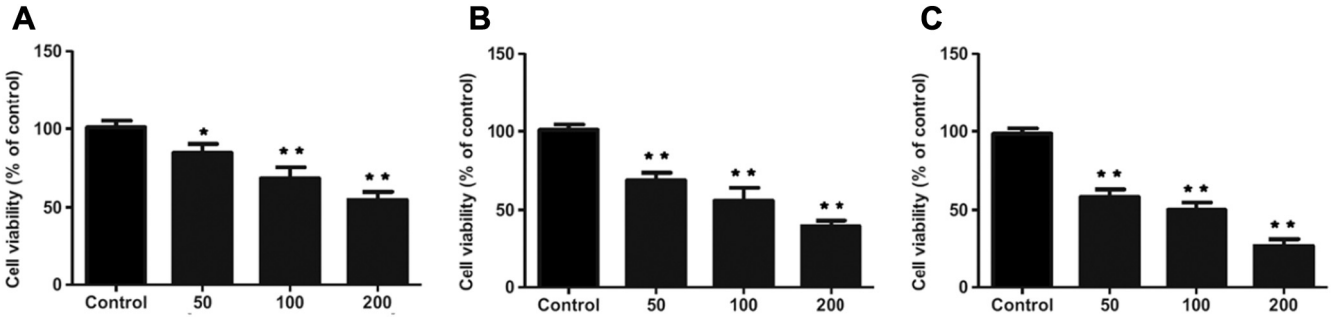

Fig. 1. Effects of 50, 100, and 200 μmol/L α-hederin (α-HN) on the proliferation of SCC-25 cells. A: 24 h, B: 48 h, C: 72 h. Values are expressed as mean ± SD ($n = 5$). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group treated with DMSO.

3.4. Effects of α-HN on the expression of apoptosis-related proteins in SCC-25 cells

The expression of Bcl-2 and Bax in SCC-25 cells treated with 50, 100, and 200 μmol/L α-HN for 48 h was detected by western blotting. The results showed that, compared with the control group, levels of the antiapoptotic protein Bcl-2 decreased significantly, whereas levels of the proapoptotic protein Bax increased significantly in α-HN-treated groups. The Bcl-2 level was the lowest and the Bax level was the highest in the 200 μmol/L α-HN group (Fig. 3).

3.5. Effects of α-HN on PI3K/AKT/mTOR signaling pathway-related proteins

Western blots were used to detect the expression of the major components of the PI3K/AKT/mTOR signaling pathway (p-PI3K, p-Akt, and p-mTOR) in SCC-25 cells treated with 50, 100, and 200 μmol/L

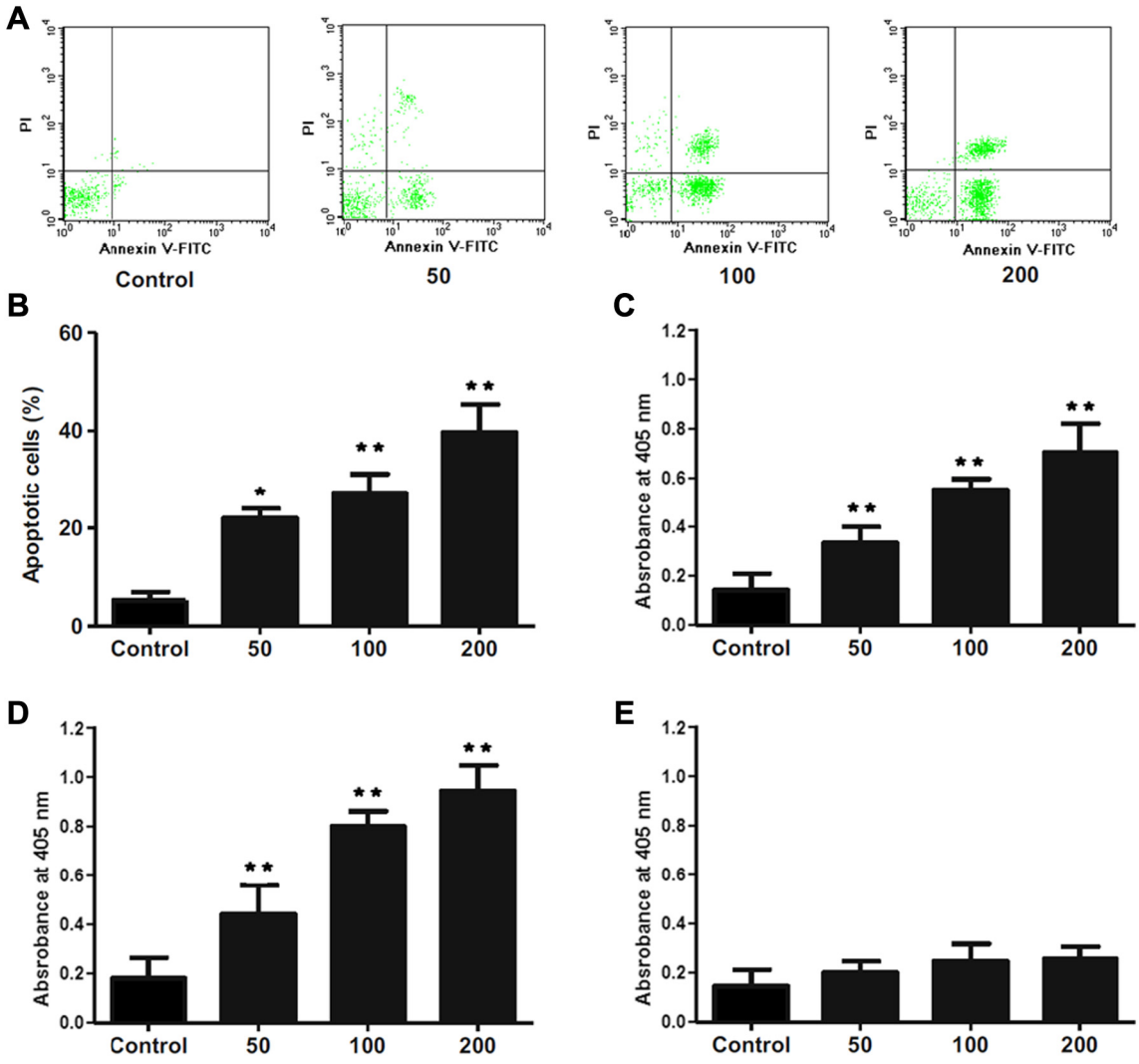

Fig. 2. Effects of 50, 100, and 200 μmol/L α-hederin (α-HN) on apoptosis and caspase activities of SCC-25 cells after a 48-h treatment. A: Flow cytometry analyses. B: Quantitation of apoptosis. C: Caspase-3 activity. D: Caspase-9 activity. E: caspase-8 activity. Values are expressed as mean ± SD ($n = 5$). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group treated with DMSO.

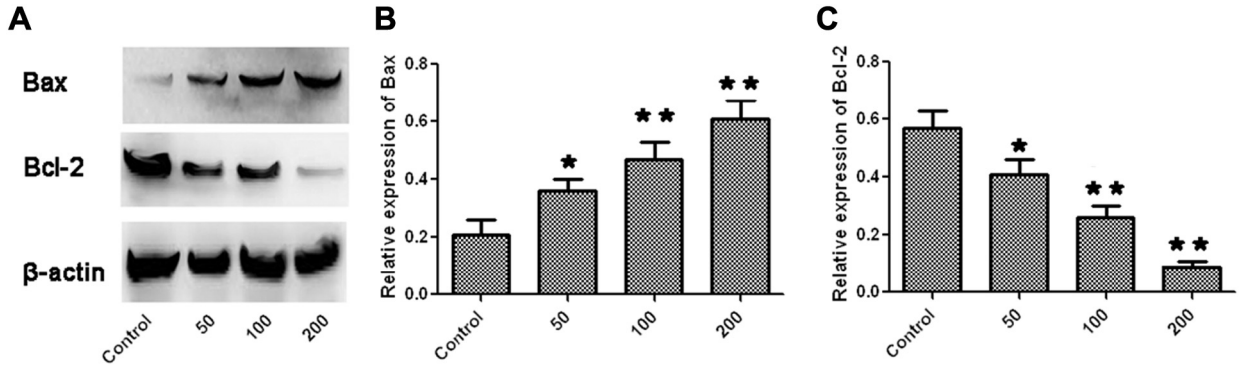

Fig. 3. Effects of 50, 100, and 200 μmol/L α-hederin (α-HN) for 48 h on the expression of apoptosis-related proteins in SCC-25 cells. A: Representative western blots of Bax and Bcl-2. β-Actin was used as the loading control. B: Relative expression of Bax. C: Relative expression of Bcl-2. Values are expressed as mean ± SD ($n = 5$). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group treated with DMSO.

α-HN for 48 h. Compared with the control group, p-PI3K, p-Akt, and p-mTOR levels in the α-HN-treated groups decreased significantly (Fig. 4). Levels of these proteins in the 200 μmol/L α-HN group were the lowest.

4. Discussion

Increasing evidence has shown that α-HN has desirable potential for the treatment of a variety of tumors. Ding et al. [17] isolated four saponins from Clematis, of which α-HN showed a strong inhibitory effect on breast cancer MCF-7 and MDA-MB-231 cells. Charles et al. [18] investigated the cytotoxicity of 35 saponins on lung cancer A549 cells and colorectal cancer DLD-1 cells and found that the IC50 values of α-HN on these two cell types were 33 and 60 μmol/L, respectively. This suggested that α-HN exerts cytotoxic effects on various tumor cells, although currently there is no research on the effect of α-HN on oral cancer cells. In addition, related reports demonstrated that α-HN inhibited the proliferation of tumor cells through a series of biological pathways including cell cycle regulation, adhesion and invasion, and inhibition of a cell proliferation process that was often accompanied by apoptosis [19,20].

In the current study, SCC-25 cells treated with α-HN showed some morphological changes indicative of apoptosis by microscopy, such as cytoplasmic vacuoles, nuclear pyknosis, and nuclear chromatin aggregation. Thus, the apoptotic effect of α-HN in SCC-25 cells was further evaluated by flow cytometry, caspase activity measurements,

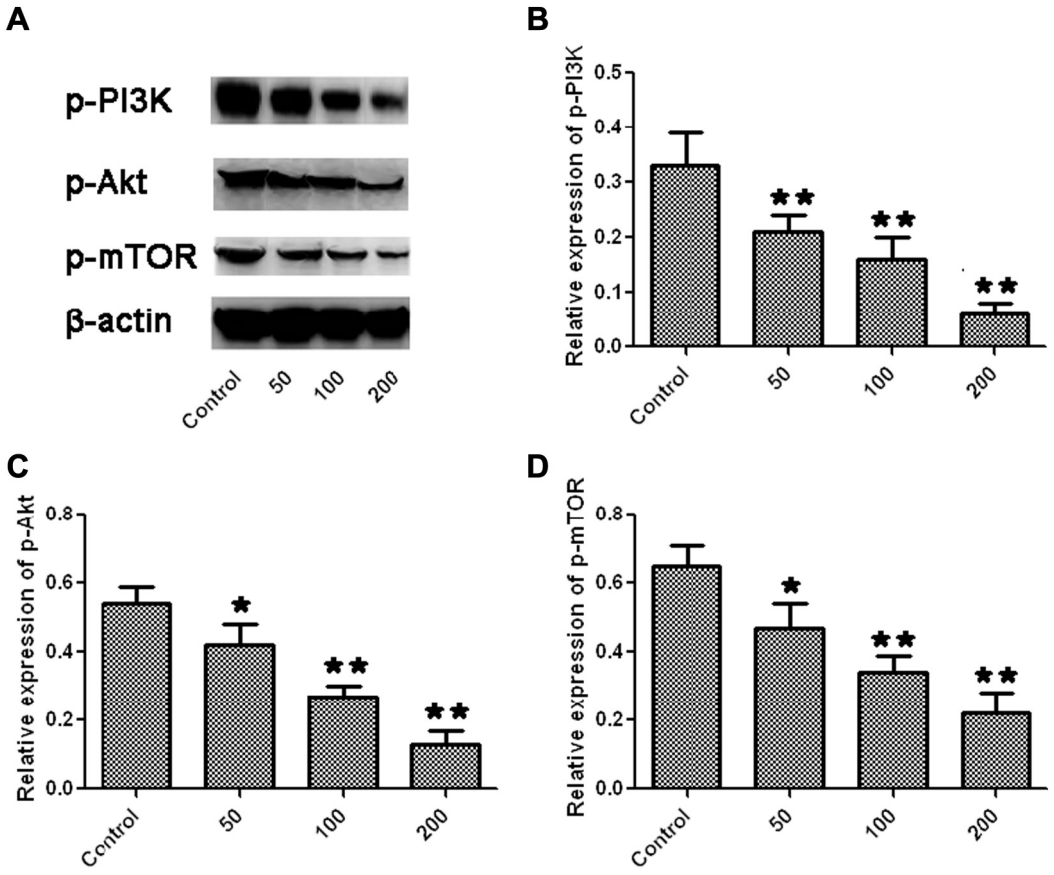

Fig. 4. Effects of 50, 100, and 200 μmol/L α-hederin (α-HN) for 48 h on the expression of phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway-related proteins. β-Actin was used as the loading control. A: Representative western blots of phosphorylated (p)-PI3K, p-Akt, and p-mTOR. B: Relative expression of p-PI3K. C: Relative expression of p-Akt. D: Relative expression of p-mTOR. Values are expressed as mean ± *SD* ($n = 5$). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group treated with DMSO.

and an assessment of Bax and Bcl-2 protein expression. These studies showed that the extent of apoptosis of SCC-25 cells treated with α-HN increased. Furthermore, significant changes in the levels of both Bax and Bcl-2, key apoptotic regulation factors of the Bcl-2 protein family, were consistent with those occurring in apoptosis, and the activities of caspases-3 and -9, apoptotic effector members of the caspase family, were enhanced. Overall, these results suggest that α-HN can promote the apoptosis of SCC-25 cells.

The PI3K/Akt/mTOR signaling pathway is composed of multiple proteins including PI3K, Akt, and mTOR. Activation of this pathway can inhibit the apoptosis of various tumor cells and promote cell cycle progression, thereby enhancing the survival and proliferation of tumor cells. At the same time, the PI3K/Akt/mTOR signaling pathway may also participate in vascularization, thereby playing an important role in the occurrence, development, and drug resistance of malignant tumors [21, 22]. Studies have verified that the PI3K/Akt/mTOR signaling pathway is involved in the invasion and metastasis of tumors [23,24]. Akt is located at the core of this signaling pathway, and activated Akt can promote cell proliferation, inhibit apoptosis, regulate the cell cycle, and facilitate angiogenesis and resistance to chemotherapeutics by activating downstream substrates, ultimately accelerating the occurrence and development of tumors [25].

mTOR is a downstream target of PI3K/Akt signaling and is mainly activated through this pathway [26]. Activated mTOR promotes the proliferation and inhibits the apoptosis of cells through the activation of its downstream proteins [25]. The PI3K/Akt/mTOR signaling pathway is activated in a variety of malignant tumors, and the targeted inhibition of this pathway, which is involved in regulating the proliferation and apoptosis of cells, has become a potential means of treating oral cancer [27,28]. In the current study, we found that p-PI3K, p-Akt, and p-mTOR levels were all decreased in SCC-25 cells treated with α-HN, thus suggesting that this compound can inhibit the activation of the PI3K/Akt/mTOR signaling pathway in SCC-25 cells. Nevertheless, our study only examined the inhibitory effect of α-HN on SCC-25 cells in vitro. Further studies determining the effect of α-HN in tumor models in vivo are needed to confirm its potential therapeutic utility.

5. Conclusion

α-HN can inhibit the proliferation and induce the apoptosis of SCC-25 cells. These cytotoxic effects appear to be mediated by inhibiting the PI3K/Akt/mTOR pathway. The current results indicate that α-HN is a promising candidate for the treatment of oral cancer.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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