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ORIGINAL ARTICLE

Arglabin is a plant sesquiterpene lactone that exerts potent anticancer effects on human oral squamous cancer cells via mitochondrial apoptosis and downregulation of the mTOR/PI3K/Akt signaling pathway to inhibit tumor growth *in vivo*

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Summary

Purpose: *To evaluate the anticancer effects and the underlying mechanism of arglabin on oral squamous cell carcinoma (OSCC) cells.*

Methods: *4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) and annexin V/propidium iodide (PI) staining were performed to evaluate apoptosis. Reactive oxygen species (ROS) levels and mitochondrial membrane potential (MMP) were examined by flow cytometry. Protein expression was assessed by western blot analysis. To examine the anticancer activity of arglabin in vivo, subcutaneous xenografts in nude mice were evaluated.*

Results: *Arglabin exhibited an IC_{50} of 10 μM in OSCC cells and induced apoptosis by inhibiting MMP and enhancing intracellular ROS levels. DAPI and annexin V/PI staining indicated apoptosis of OSCC cells induced by arglabin. Arglabin also downregulated the expression of key proteins in the mTOR/PI3K/Akt signaling pathway. In vivo evaluation showed that arglabin reduced the average tumor volumes and growth of xenografted tumors, indicative of its anticancer activity.*

Conclusions: *Arglabin showed selective in vitro and in vivo anticancer activities against OSCC cells and is therefore a potential therapeutic agent for the management of OSCC.*

Key words: *apoptosis, arglabin, mTOR, oral squamous cell carcinoma, reactive oxygen species*

Introduction

Oral cancer is the fourth most frequent cancer worldwide and the cause of more than 0.13 million cancer-related deaths [1]. Moreover, 90% of oral malignant tumors are oral squamous cell carcinoma (OSCC) [2]. OSCC generally affects males at approximately 40 years of age who are exposed to tobacco or alcohol or deficient in micronutrients. Recently, OSCC has been reported even in younger patients who had not been exposed to any cancer-inducing factors. OSCC originates mainly at the base of the tongue or oropharynx and is often accompanied by human papilloma viral infection. The main treatments are surgical intervention and irradiation of the tumor followed by chemotherapy. However, these approaches have low clinical efficacy and severe side effects that influence the quality of life of patients [3].

Plant-derived natural products have been used in different health care systems and evaluated scientifically for a diversity of bioactivities [4-7]. Due to side effects associated with synthetic drugs, drugs of natural origin with fewer side effects are

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gaining increasing attention [5-8]. Arglabin is one such compound, which is isolated from different plant species such as *Artemisia glabella.* Arglabin is a sesquiterpene gamma lactone with extensive pharmacological potential. This molecule has several uses, including antimicrobial, neuroprotective and anticancer activities [8]. Although the anticancer activity of arglabin has been evaluated in several cancer cells lines, the underlying mechanism has not been reported. The present study therefore evaluated the anticancer activity and underlying mechanism of Arglabin.

Methods

Chemicals and reagents

Arglabin, RPMI-1640, streptomycin, penicillin G, 3-(4,5-dimethylthiazole-2yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), tannic acid, Rhodamine-123, and 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) were procured from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was procured from Gibco (Gaithersburg, MD, USA). All antibodies, beta-actin, and annexin V/PI were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture, growth conditions and treatment

A panel of five cancer cell lines (LS-180 colorectal adenocarcinoma, SCC-4 human oral cancer, MCF-7 human breast adenocarcinoma, Hs 683 human brain glioma, and HL-60 human promyelocytic leukemia cells) were acquired from the European Collection of Cell Culture (Salisbury, UK) and used for the initial assay. The cells were cultured in RPMI medium supplemented with 10% FBS, streptomycin (100 mg/L), penicillin G (70 mg/L), and $NaHCO_3$ (3.7 g/L) and maintained in a CO_2 incubator at 37°C, 5% CO_2, and 98% humidity. Cells were treated with arglabin dissolved in DMSO, and the control was treated with vehicle only (<0.2% DMSO).

Cell viability assay

The MTT assay was used to evaluate the effect of arglabin on cancer cell viability. The SCC-4 cells were grown at 1×10^6/well in 96-well plates for 12 hrs and then exposed to 0, 10, 20, 40, 100, 150, and 200 μM arglabin for 48 hrs. MTT solution (20 μL) was added to each well. Prior to the addition of 500 μL DMSO, the medium was completely removed. To solubilize MTT formazan crystals, 500 μL DMSO were added. An enzyme-linked immunosorbent assay plate reader was used to determine the optical density. Because arglabin showed the lowest half maximal inhibitory concentration (IC_{50}) against SCC-4 cells, SCC-4 cells were treated with arglabin at 0, 5, 10, and 20 μM.

Detection of apoptosis and determination of colony-forming potential

SCC-4 cells were seeded into 6-well plates and treated with arglabin (0, 5, 10, and 20 μM) for 48 hrs. Treated cells were collected and washed twice with phosphate-buffered saline (PBS), and then the cells were resuspended in 500 μL binding buffer with annexin antibody in the dark for 20 min according to the manufacturer's instructions. The apoptotic cell population was analyzed by flow cytometry. For the clonogenic assay, SCC-4 cells in the exponential growth phase were harvested and counted with a hemocytometer. SCC-4 cells were seeded at 200/well, incubated for 48 hrs to allow the cells to attach, and then incubated with different doses (0, 5, 10, and 20 μM) of arglabin. After treatment, the cells were kept in the incubator for 6 days, washed with PBS, fixed with methanol, and stained with Crystal Violet for 30 min before counting under a light microscope.

Quantitation of ROS levels and mitochondrial membrane potential

SCC-4 cells were seeded at a density of 2×10^5/well in a 6-well plate and incubated for 24 hrs and then treated with different concentrations (0, 5, 10, and 20 μM) of arglabin for 72 hrs at 37°C, 5% CO_2, and 95% air. Thereafter, the cells from all samples were collected, washed twice with PBS, and resuspended in 500 μL DCFH-DA (10 μM) for quantitation of ROS levels and in 3,3'-dihexyloxacarbocyanine iodide (1 μmol/L) for quantitation of MMP in the dark for 30 min at 37°C. The samples were then immediately examined by flow cytometry as described previously [9,10].

Determination of the cell cycle distribution of SCC-4 cells

Cells seeded in 6-well plates (2×10^5/well) were incubated for 24 hrs with 0, 5, 10, and 20 μM arglabin. DMSO was used as control. To quantitate DNA content, the cells were washed with PBS, then fixed in ethanol at −20°C, followed by resuspension in PBS containing 40 μg/mL PI, RNase A (0.1 mg/mL,), and Triton X-100 (0.1%) for 30 min in the dark at 37°C. Flow cytometry was then performed as reported previously [9].

Cell migration assay

A Boyden chamber assay was used for the cell migration assay with some modifications. Cells (5×10^4/well) were suspended in 2% FBS-containing medium and placed in the upper chamber of 8 μm pore size Transwell® chambers. Medium supplemented with 10% FBS was then added to the lower chamber, followed by incubation for 48 hrs. On the upper surface of the membrane, the non-migrated cells were removed, while on the lower surface of the membrane, cells were fixed in methanol (100%) and stained with Giemsa. The cell migration was estimated by counting the number of migrated cells under a microscope. The *in vitro* cell invasion assay was performed using Transwell® chambers with an 8 μm pore size coated with Matrigel, using the same protocol as that of the cell migration assay described previously.

In vivo xenograft study

The experiments were performed in accordance with the standard guidelines of the animal committee and approved by the Institutional Animal Care and Use Committee of the institute. Six-week-old female immune-

deficient nude mice, obtained from the Animal Center of our institute, were used. Two to three mice were housed per cage with sterilized stainless steel covers and with bedding, under a 12-h light/dark cycle at 22°±2°C and 40–60% relative humidity. Mice were subcutaneously injected with 5×10^6 SCC cells in the left flank. When tumors became apparent (~3 mm after approximately 12 days), the mice (n=10 per group) were injected intraperitoneally with DMSO (0.1%) and dissolved arglabin and then diluted with 100 µL normal saline at 40 µg/g body weight, which was considered day 1 of the experiment. The dosage was given three times a week. The control mice received DMSO (0.1%) in normal saline only. Every week, the tumor sizes were determined using calipers, and tumor volumes were calculated according to a standard formula. After 4 weeks, the mice were sacrificed by deep anesthesia with isoflurane, and the organs were collected for evaluation of tumor growth and other parameters.

Western blot analysis

The arglabin-treated cells were harvested and lysed. The protein concentrations of the lysates were quantitated by BCA assay. Equal amounts of protein from each sample were loaded onto a 12% denaturing SDS gel and separated by electrophoresis. The resolved proteins were then electroblotted onto polyvinylidene difluoride membranes (0.45 µm pore size) and identified using specific antibodies. β-actin was used as control.

Statistics

Statistical analyses were performed using Student's *t*-test with GraphPad 7 software (GraphPad, San Diego, CA, USA). The results are representatives of three biological replicates and expressed as means ± standard deviation. The differences were considered significant at *p <0.01, **p <0.001, and ***p <0.000.

Results

Arglabin inhibits the proliferation of SCC-4 cells

The initial cytotoxicity of arglabin in various cancer cell lines was determined by MTT assay (Table 1). Different concentrations (0, 5, 10, and 20 µM) of arglabin for 48 hrs decreased the number of viable cells in a concentration-dependent manner (Figure 1A). Because the SCC-4 cells were most sensitive to arglabin half maximal inhibitory concentration (IC_{50}) of 10 µM, this cell line was used in the subsequent studies.

Table 1. IC_{50} of arglabin against different cancer cell lines as determined by MTT assay

Cell line	*IC_{50} (µM)*
Colorectal adenocarcinoma LS-180	20
Oral cancer cell line SSC-4	10
Cervical cancer HeLa	20
Human brain glioma Hs 683	30
Breast MCF-7	20
Human promyelocytic leukemia cells HL-60	50

Arglabin induces apoptosis of SCC-4 cells

To determine whether arglabin induced apoptosis, SCC-4 cells were treated with arglabin in a concentration-dependent manner and then stained with DAPI to identify apoptotic cells. Untreated cells exhibited uniformly bright nuclei, and treated cells showed apoptotic bodies (Figure 1B), indicating that arglabin induced apoptotic death in a concentration-dependent manner. To confirm apoptosis, cells were stained with annexin V/PI, which showed that the percentage apoptotic cells was increased to 5.9%, 39.20%, and 76.25% at 5, 10, and 20 µM of arglabin as compared with the untreated control (0.91%) in a concentration-dependent manner (Figure 2). These results indicated that arglabin induces apoptosis in a dose-dependent manner. Moreover, arglabin also reduced the colony-forming potential of SCC-4 cells (Figure 3).

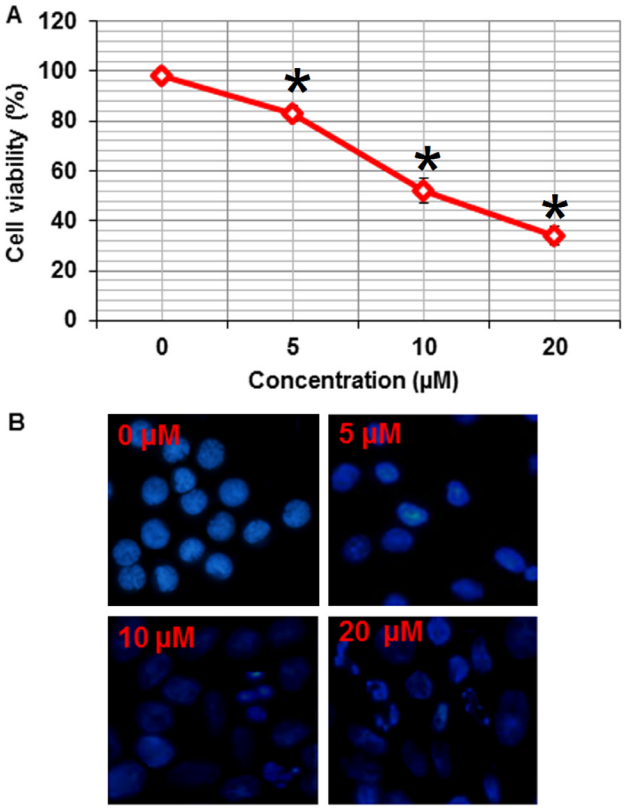


Figure 1. (A) Inhibition of SCC-4 cell viability by arglabin treatment. **(B)** Induction of apoptosis in SCC-4 cells at the indicated doses of arglabin, assessed using 4',6-diamidino-2-phenylindole dihydrochloride staining and fluorescence microscopy. All experiments are shown as mean ± standard deviation of three biological replicates (*p<0.01).

ROS generation and loss of mitochondrial membrane potential in SCC-4 cells

We used the fluorescent DCFH-DA probe to characterize the generation of ROS by arglabin as a possible mechanism for the induction of apoptosis in SCC-4 cells assay. Arglabin increased intracellular ROS levels by approximately 200% at an arglabin concentration of 20 μM compared with the control (Figure 4A), indicating that arglabin generated ROS in a concentration-dependent manner to induce apoptosis. Mitochondria play important roles in the progression of apoptosis and loss of MMP during the early phase of apoptosis. To investigate the effect of arglabin on MMP, we analyzed MMP using Rhodamine-123. Our results showed that fluorescence was quenched after treatment with 0, 5, 10, and 20 μM arglabin (Figure 4B), confirming that arglabin induced apoptosis by ROS-mediated alterations in MMP.

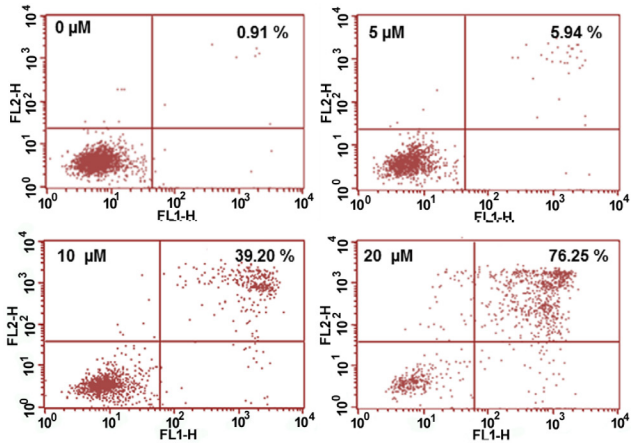


Figure 2. Induction of apoptosis in SCC-4 cells by arglabin at the indicated doses, as determined by flow cytometry and annexin V staining. The experiments were performed in triplicate.

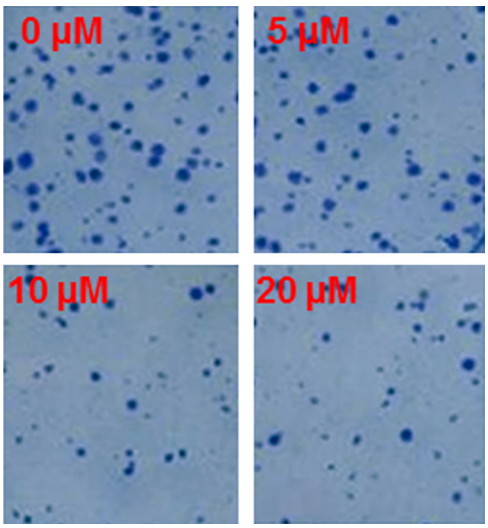


Figure 3. The effect of the indicated doses of arglabin on the colony formation of SCC-4 cells. The Figure shows that arglabin inhibits the colony formation in a concentration-dependent manner. The experiments were performed in triplicate.

Arglabin alters the cell cycle distribution of SCC-4 cells

The percentage of SCC-4 cells in the G1 phase was significantly increased at concentrations of 0–20 μM arglabin, suggesting G1 arrest (Figure 5). Additionally the population of SCC-4 cells in the sub-G1 phase was marginally increased at 5 μM, reasonably increased at 10 μM, and dramatically increased at 20 μM arglabin. Arglabin therefore induced an increase in the proportion of sub-G1 SCC-4 cancer cells in a dose-dependent manner.

Arglabin inhibits cell migration and invasion

The migration of SCC-4 cells *in vitro* was assessed using the modified Boyden chamber assay, which revealed that the area of migrated cells was decreased after arglabin treatment for 48 hrs (Figure 6A), and arglabin treatment inhibited the migration of SCC-4 cells at IC_{50} (10 μM) arglabin (Figure 6B).

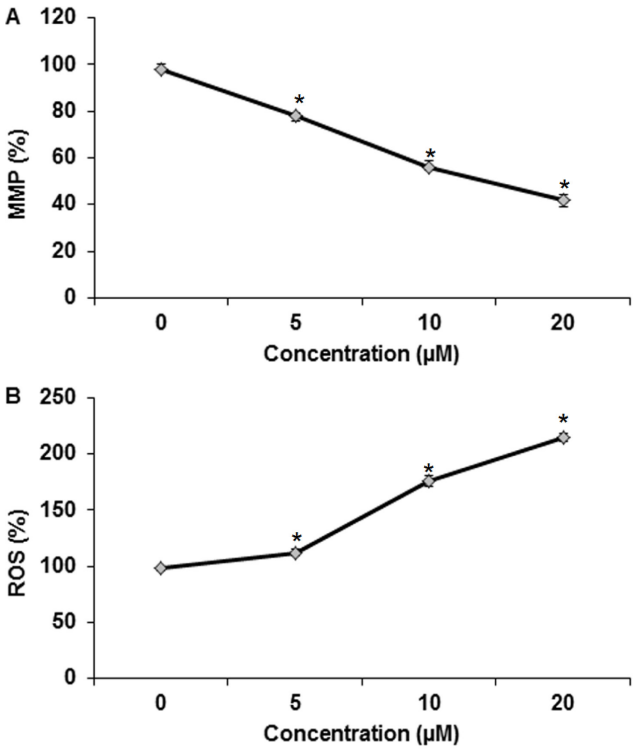
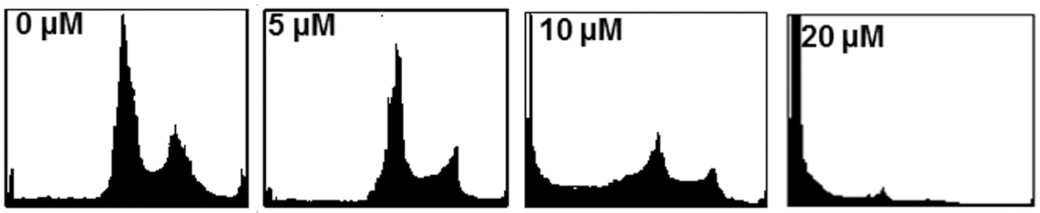


Figure 4. The effect of arglabin on reactive oxygen species generation and mitochondrial membrane potential in SCC-4 cells. After treatment of SCC-4 cells with the indicated doses of arglabin for 48 hrs, cells were assessed for **(A)** mitochondrial membrane potential and **(B)** reactive oxygen species production. The experiments were performed in triplicate and expressed as mean ± SD (*p <0.01).



SubG1	01.9 ± 0.2	02.7 ± 0.3	33.3 ± 0.3	85.2 ± 0.4
G1	40.1 ± 0.2	47.2 ± 0.2	29.7 ± 0.1	05.2 ± 0.1
S	22.4 ± 0.6	23.4 ± 0.4	14.2 ± 0.1	03.8 ± 0.2
G2/M	35.4 ± 0.4	26.6 ± 0.3	22.6 ± 0.3	06.6 ± 0.2

Figure 5. The effect of the indicated doses of arglabin on cell cycle distribution in SCC-4 cells, as determined by flow cytometry using propidium iodide staining. The Figure depicts that arglabin induces cell cycle arrest in a concentration-dependent manner. The experiments were performed in triplicate.

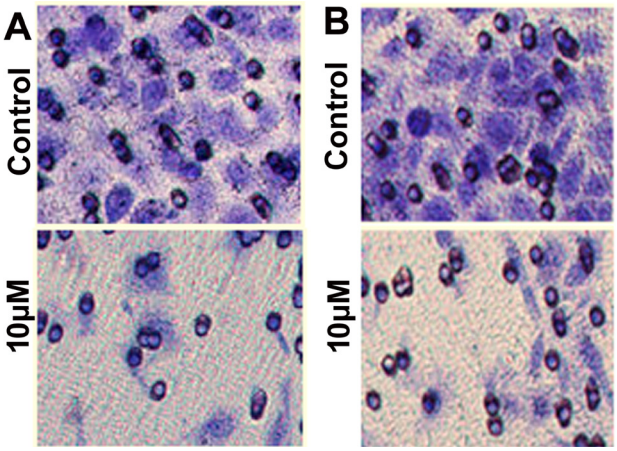


Figure 6. The effect of the IC_{50} concentration of arglabin on cell **(A)** migration and **(B)** invasion. The Figure depicts that arglabin inhibits cell invasion in a concentration-dependent manner. The experiments were performed in triplicate.

Arglabin targets the mTOR/PI3K/Akt signaling pathway

Our results indicated that arglabin affected the protein levels of mTOR/PI3K/Akt signaling pathway proteins. Compared with untreated control cells, arglabin treatment showed a concentration-dependent downregulation of mTOR and phosphorylated (p)-mammalian target of rapamycin (mTOR) proteins, as well as PI3K and Akt proteins (Figure 7). Together, the results strongly suggest that arglabin induces anticancer and apoptotic effects via the mTOR/PI3K/Akt signaling pathway.

Arglabin inhibits tumor growth in vivo

To examine the anticancer activity of arglabin *in vivo* we evaluated subcutaneous xenografts in nude mice. SCC-4 tumor growth was significantly inhibited by arglabin treatment compared with the untreated control group. After 4 weeks of arglabin treatment, the average tumor volumes were significantly higher in the untreated control group than in the arglabin-treated groups (Figure 8A–C). Additionally, the protein level of Ki-67 was downregulated and that of cleaved caspase 3 upregulated in xenografted tumors treated with arglabin (Figure 8D–E).

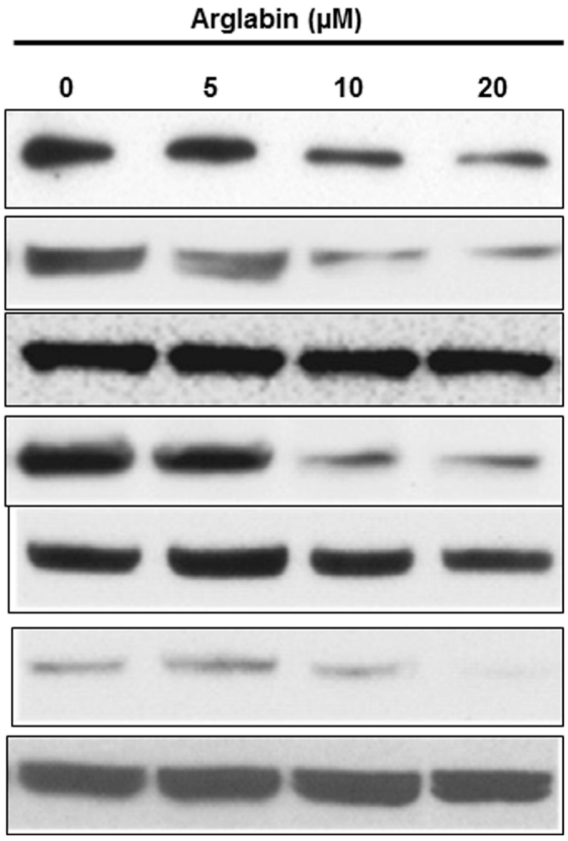


Figure 7. Western blot analysis of the levels of mTOR/P13K/Akt pathway proteins showing that arglabin caused dose-dependent inhibition of p-mTOR, p-PI3K and p-AKT proteins. The experiments were performed in triplicate.

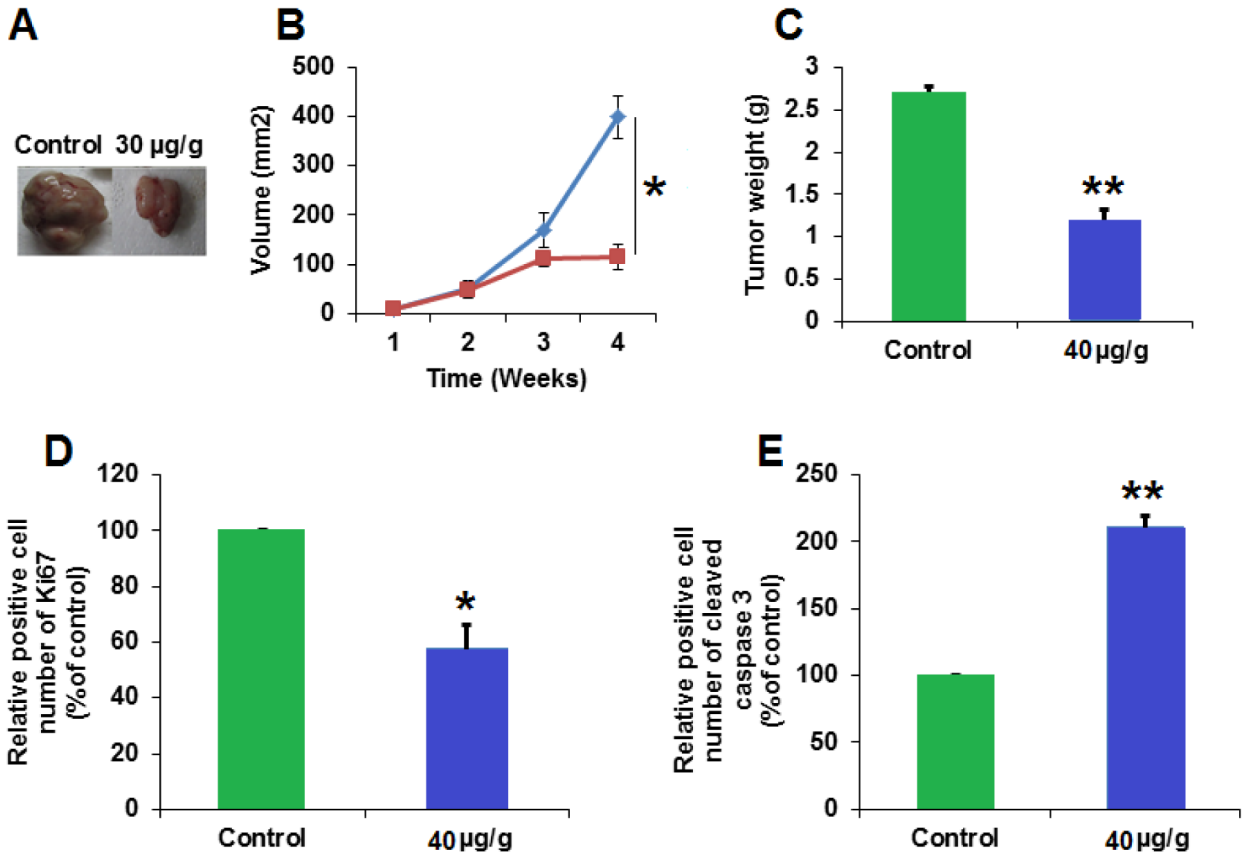


Figure 8. Arglabin inhibits oral tumor growth *in vivo*. **(A)** Representative tumor-bearing nude mice and tumors isolated from the control and arglabin-treated groups. **(B)** Tumor volumes. **(C)** Tumor weights at the indicated time intervals and dosages. **(D)** Relative percentages of Ki67, and **(E)** cleaved caspase 3-positive cells. Results are shown as mean ± standard deviation of three biological replicates. The differences were considered significant at *p<0.01, and **p<0.001.

Discussion

Oral cancer is one of the most frequent malignancies worldwide, and the limited treatment options for oral cancer are accompanied by severe side effects [1]. Natural products are attracting the attention of many scientists because of their anticancer activities [5,6]. Unlike synthetic drugs, natural products are considered safer because of their decreased side effects [7,8]. Arglabin is a natural product of plant origin with known anticancer activities. As increasing studies have reported the therapeutic potential of arglabin against various cancers, interest in this plant product has increased [11]. Herein, we report for the first time that arglabin induced growth arrest and apoptosis in SCC-4 cells *in vitro*. DAPI staining showed a dose-dependent increase in apoptotic parameters, such as bleb formation, chromosomal condensation, and fragmentation of SCC-4 cells after treatment with arglabin. Arglabin-induced apoptosis was confirmed by annexin V/PI staining. An increase in the arglabin concentration caused increase in ROS production, which was accompanied by a greater loss of MMP in SCC-4 cells. Arglabin therefore induced apoptosis in SCC-4 cells by increasing ROS production, which is responsible for inducing cancer cell apoptosis by generating transition pore openings in the mitochondrial membranes. Our results were consistent with previous studies, showing that many drugs exhibit antiproliferative effects via induction of apoptosis. For example, several chemotherapeutic drugs, such as cisplatin, taxol and 5-fluorouracil, have been reported to alter specific apoptotic pathways [12-17]. Induction of apoptosis by arglabin in SCC-4 cells was also associated with inhibition of colony formation, cell migration, and invasion of SCC-4 cells. These results suggest the apoptosis-inducing properties of arglabin. The effects of arglabin on the expression levels of various proteins including mTOR, pmTOR, PI3K, p-PI3K, and Akt were also evaluating by western blot analysis. Arglabin-treated cells showed a concentration-dependent downregulation of mTOR, pmTOR, and PI3K/Akt protein expression. Taken together, the results suggest that arglabin is a potential anticancer molecule for the treatment of OSCC. In the xenograft mouse model, arglabin sig-

nificantly inhibited OSCC tumor growth compared with the untreated control group, with no visible toxicity (Figure 7D). Ki67 and cleaved caspase 3 are cellular markers of proliferation and apoptosis, respectively [18,19]. The significant reduction in Ki67-positive cells and significant upregulation of cleaved caspase 3 levels suggest that arglabin inhibits OSCC cells growth *in vivo*.

Conclusions

The results of the present study showed that arglabin is a potent anticancer molecule. It selectively inhibited the growth of SCC-4 cells via induction of mitochondrial-mediated apoptosis, suppression of cancer cell migration, and invasion, and inhibition of the mTOR/PI3K/Akt pathway. Moreover, arglabin also inhibited tumor growth *in vivo* and may prove to be an important molecule for the development of effective anticancer treatments.

Conflict of interests

The authors declare no conflict of interests.

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