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ORIGINAL ARTICLE

Acetylshikonin inhibits *in vitro* and *in vivo* tumorigenesis in cisplatin-resistant oral cancer cells by inducing autophagy, programmed cell death and targeting m-TOR/PI3K/Akt signalling pathway

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Summary

Purpose: *Oral cancer causes considerable mortality across the globe, mainly due development of chemoresistance and lack of efficient chemotherapeutic agents. In the current study the anticancer potential of Acetylshikonin was examined against KB-R cisplatin-resistant oral cancer cells along with evaluation of in vitro and in vivo modes of action.*

Methods: *The proliferation rate of the oral cancer cells was checked by MTT assay. Autophagy was detected by electron microscopy. Apoptotic cell death was assessed by DAPI and annexin V/propidium iodide (PI) staining. Protein expression was determined by immunoblotting. Xenografted mice models were used for in vivo evaluation of Acetylshikonin.*

Results: *The results revealed that Acetylshikonin could significantly inhibit the proliferation of all the oral cancer cells with lower cytoxicity compared with the normal cells. The anticancer activity of Acetylshikonin against the KB-R cisplatin-resistant cells was found to be due to induction of autophagy and apoptosis. The Acetylshikonin prompted apoptosis and autophagy was also associated with alteration in the apoptosis and autophagy-related protein expression. Furthermore, it was observed that Acetylshikonin could inhibit the mTOR/PI3K/AKT signalling pathway in the cisplatin-resistant KB-R oral cancer cells. The effects of the Acetylshikonin were also examined in vivo in xenografted mice models and it was observed that Acetylshikonin inhibited the growth of xenografted tumors.*

Conclusions: *These results suggest that Acetylshikonin considerably suppresses the growth of cisplatin-resistant oral cancer in vitro and in vivo and may prove beneficial in the treatment of drug-resistant oral cancer.*

Key words: *autophagy, apoptosis, cell cycle, acetylshikonin, oral cancer, flow cytometry*

Introduction

Oral cancers cause considerable mortality across the globe and currently are ranked as the sixth most common type of cancer [1]. Despite the progress made in cancer, the overall survival still remains unsatisfactory. Moreover, the development of drug resistance in cancer cells creates a strong obstacle in the improvement of treatment of oral cancer [2]. The existing drugs have also a number of side effects and therefore the identification of new drug candidates in the need of the hour. Plants and microbes have provided mankind with a number of drugs for the treatment of deadly conditions and they are likely to continue to serve as source of more new important drugs [3]. Plants are specialised to produce metabolites to combat environmental stresses. Such metabolites, commonly referred to as secondary metabolites, have been employed for the treatment of several diseases including cancer [4]. For example, taxanes and campothecins are among the common anticancer

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agents of plant origin [5]. Although these secondary metabolites have been chemically classified into different groups, shikonins form an important group with impressive pharmacologic potential [6]. They have been found to exhibit a range of bioactivities such as anticancer and antimicrobial to name a few [7]. Acetylshikonin is a an important plant-derived metabolite commonly found across the plant kingdom [8]. It has been found to inhibit the growth of cancer cells, however, its antiproliferative effects have not been examined against oral cancer [9]. Herein, we report for the first time the anticancer activity of Acetylshikonin against the cisplatin-resistant oral-cancer cells. The purpose of the current study was to investigate the anticancer potential of Acetylshikonin under both *in vitro* and *in vivo* conditions against cisplatin-resistant human oral cancer cells along with evaluating its effects on cell autophagy, cell apoptosis and m-TOR/PI3K/Akt signalling pathway.

Methods

Cell lines and culture conditions

The cisplatin-resistant oral cancer cell line KB-R5 was procured from American Type Culture Collection. The cells were maintained in Dulbecco's modified Eagle's medium in incubator (Thermo Scientific, MA, USA) at 37°C with 98% humidity and 5% CO_2.

Cell viability assay

The effect of Acetylshikonin on the viability of cisplatin-resistant oral cancer cell line was evaluated by MTT assay as described previously [14]. The oral cancer cells were treated with different concentrations of Acetylshikonin and the proliferation rate was estimated by taking the absorbance at 570 nm with Envision microplate reader (Perkin Elmer, Waltham, MA, USA).

Electron microscopy

The electron microscopic analysis of Acetylshikonin-treated KB-R oral cancer cells was used to detect the induction of autophagic cell death. Briefly, the oral cancer cells were administered 0, 20, 40 and 80 µM Acetylshikonin for 24 h. The cells were then collected by trypsinization, washed, and fixed with glutaraldehyde (2%) in phosphate buffered saline (PBS) (0.1 M). The cells were then post-fixed in osmium tetroxide (1%). This was followed by the treatment of the cells with high purity ethanol and embedding in resin. Thin sections (100 nm) were then cut with the help of an ultramicrotome and subjected to electron microscopy.

Detection of apoptosis

The effects of Acetylshikonin on the initiation of the programmed cell death was assessed by DAPI staining [15]. The oral cancer cells (0.6×10^6) were grown in 6-well plates and after incubation for 12 h, they were subjected to Acetylshikonin treatment for 24 h at 37°C. Then, they were immediately centrifuged and the pellets were washed with PBS. Afterwards, the cells were DAPI-stained, centrifuged and PBS-washed. Finally, the nuclear morphology of the stained cells was examined by fluorescence microscopy. The percentage of the apoptotic cells was estimated by annexin V/PI staining as previously described [16].

Western blotting

To determine the expression of the selected proteins in the Acetylshikonin-treated cisplatin-resistant oral cancer cells, the cells were subjected to lysis with RIPA buffer and the protein content of each lysate was estimated by bicinchoninic acid BCA assay. The samples were then loaded on SDS-PAGE. The gels were then transferred to nitrocellulose membranes and subjected to treatment with primary antibody at 4°C for a period of 24 h. After this, the membranes were incubated with HRP-conjugated secondary antibody for 50 min at 25°C. Enhanced chemi-luminescence reagent was used to visualise the protein bands.

In vivo study

The anticancer effects of Acetylshikonin were also examined *in vivo* in xenografted mice models. Three-week old male BALB/c nude mice were kept in the animal facility as per the National Institutes of Health standards for the care and use of laboratory animals. The mice were injected with 5×10^6 KB-R cells sub-cutaneously at the left flank. The mice for each group (n=5) were injected intraperitoneally with DMSO-dissolved Acetylshikonin (0.1%) and diluted with 100 µL normal saline at 50 mg/kg per body weight; this time point was taken as the day one of the experiment. Acetylshikonin was given to the rats thrice a week, while the control mice were given DMSO (0.1%) in normal saline only. At the end of 6 weeks, the mice were euthanized and the tumors were removed and analyzed.

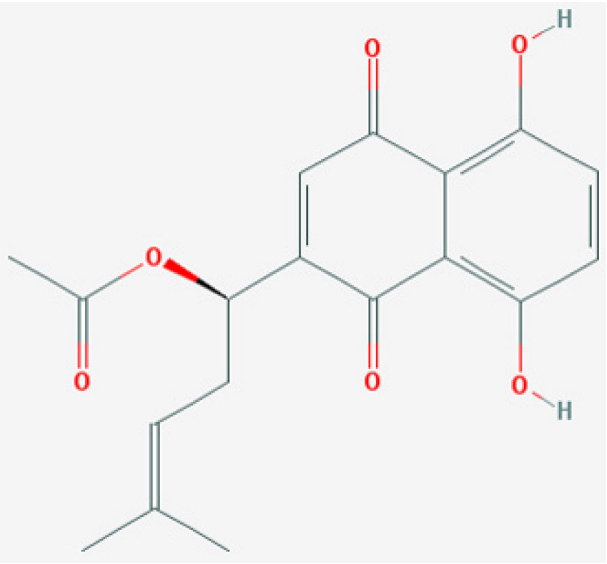

Figure 1. Chemical structure of Acetylshikonin.

Results

Acetylshikonin inhibits the growth of oral cancer cells

The effects of Acetylshikonin (Figure 1) on the proliferation of the cisplatin-resistant oral cancer cells were examined by MTT assay. It was found that that this molecule exerts antiproliferative effects on the KB-R cells with an IC_{50} of 40 μM (Figure 2). In addition, it was found that the anticancer effects of Acetylshikonin on these cells were concentration-dependent.

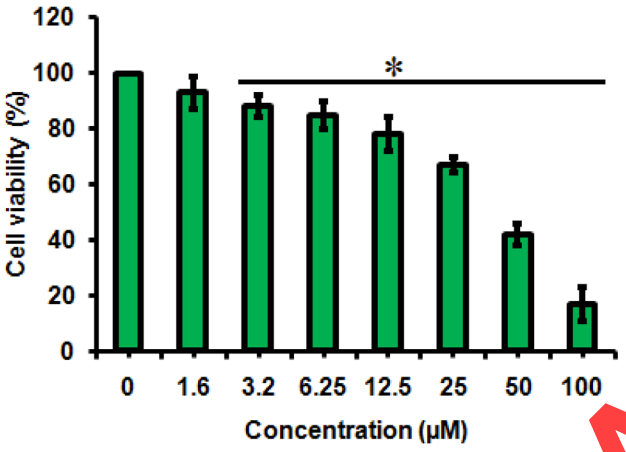

Figure 2. Effect of Acetylshikonin on the viability of the KB-R cells as determined by MTT assay. The experiments were performed in triplicate and shown as mean + SD (*$p<0.01$).

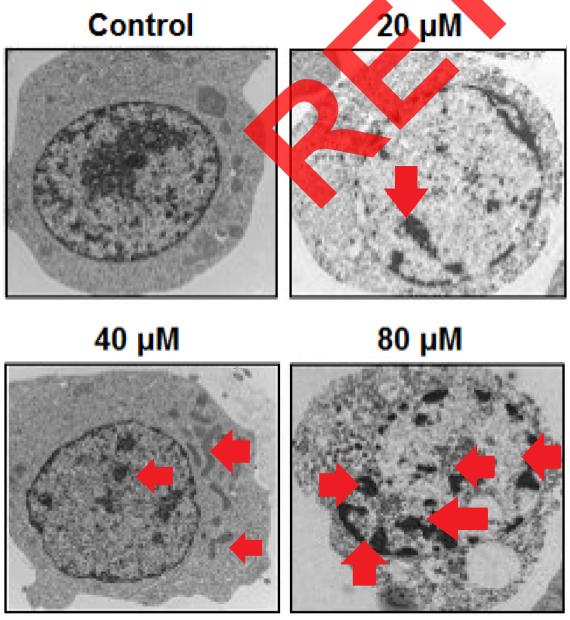

Figure 3. Electron microscopy images of Acetylshikonin treated KB-R cells showing induction of autophagy. The experiments were performed in triplicate. Red arrows indicate the onset of autophagosomes or autophagic vesicles after the treatment of cells with increasing doses of Acetylshikonin.

Acetylshikonin induces autophagy and programmed cell death in KB-R oral cancer cells

The impact of Acetylshikonin on the KB-R cisplatin-resistant cells was investigated by electron microscopy. The results revealed that this molecule caused the production of autophagosomes in KB-R cells, indicating that Acetylshikonin induces autophagy (Figure 3). Furthermore, Acetylshikonin also caused shrinkage of the nuclei of KB-R cells, suggestive of apoptosis. For the confirmation of autophagy the expression of autophagy-related proteins was investigated and it was observed that Acetylshikonin caused increase of Beclin-1 and LC3-II and suppression of p62 expression. However, no effects were found on the expression of LC3-I and Vps34 (Figure 4). The apoptosis was confirmed by DAPI staining which showed apparent changes in the nuclear morphology of the KB-R cells (Figure 5). Annexin V/PI staining revealed that the percentage of the apoptotic KB-R cells was considerably increased with increase in the concentration of Acetylshikonin (Figure 6). The apoptosis was further confirmed by the increased expression of Bax and decreased expression of the Bcl-2 in KB-R cells (Figure 7).

Acetylshikonin blocks the mTOR/PI3K/AKT signalling pathway

The effects of Acetylshikonin were also examined on the mTOR/PI3K/AKT signalling pathway of cisplatin-resistant KB-R oral cancer cells. The results showed that Acetylshikonin caused dose-

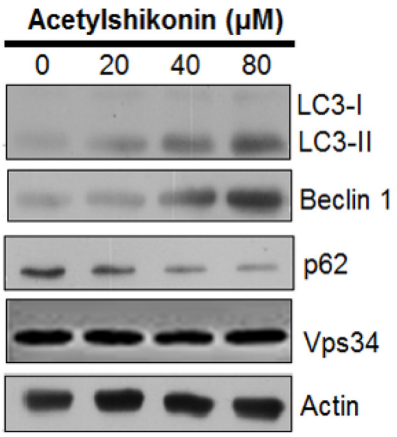

Figure 4. Effect of Acetylshikonin on the autophagy-related protein expression as revealed by western blot analysis. The Figure shows that the expression of LC3-I, LC30II and Beclin-1 increases with increasing drug doses, while the expression of p62 decreased and the expression of Vps34 remained almost unchanged. The experiments were performed in triplicate.

dependent decrease in the expression of p-mTOR, p-PI3K and p-AKT, while no visible effect was found on the expression of mTOR, PI3K and AKT (Figure 8). To sum up, the results indicate that Acetylshikonin blocks the mTOR/PI3K/AKT signalling pathway in KB-R cells.

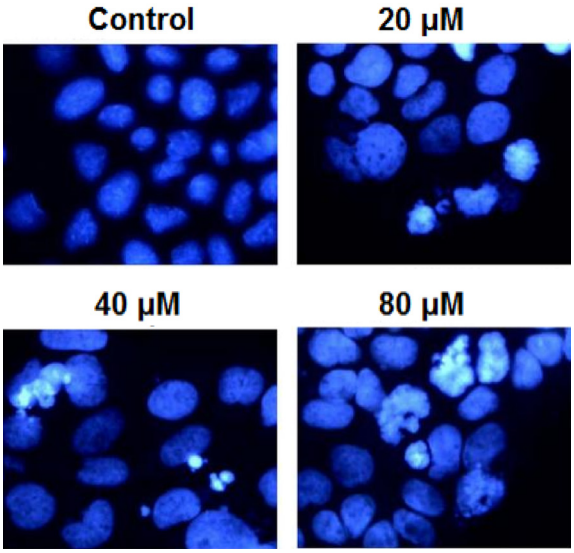

Figure 5. DAPI staining images showing induction of apoptosis by Acetylshikonin on the KB-R images. Apoptosis was confirmed by DAPI staining which showed apparent changes in the nuclear morphology (chromatin condensation and nuclear fragmentation) of the KB-R cells. The experiments were performed in triplicate.

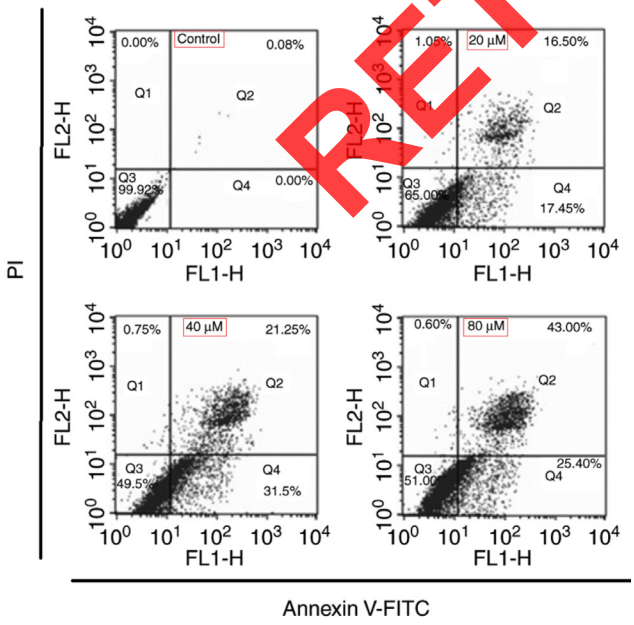

Figure 6. Estimation of the apoptotic cell populations in Acetylshikonin-treated KB-R cells as depicted by annexin V/PI staining. Anexin V/PI staining revealed that the percentage of the apoptotic KB-R cells was considerably increased with increase in the concentration of Acetylshikonin. The experiments were performed in triplicate.

Acetylshikonin inhibits tumor growth in vivo

Since Acetylshikonin was found to exert considerable anticancer effects on KB-R cells *in vitro*, we also investigated its anticancer effects *in vivo* in xenografted mice models. The results indicated that Acetylshikonin at the dosage of 50 mg/kg considerably suppressed the growth of the xenografted tumors (Figure 9A). Furthermore, Acetylshikonin inhibited the tumor weight and volume concentration-dependently (Figure 9B and C).

Discussion

Oral cancer causes considerable mortality and, due to the development of chemoresistance, it often becomes difficult to treat [17]. In addition, oral

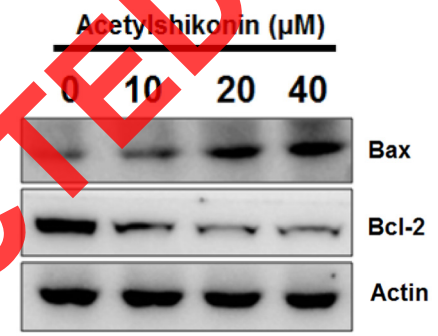

Figure 7. Effect of Acetylshikonin on the expression of Bax and Bcl-2 proteins as depicted by western blot analysis. The Figure shows that the expression of Bax increased while of Bcl-2 decreased with increased doses of Acetylshikonin. The experiments were performed in triplicate.

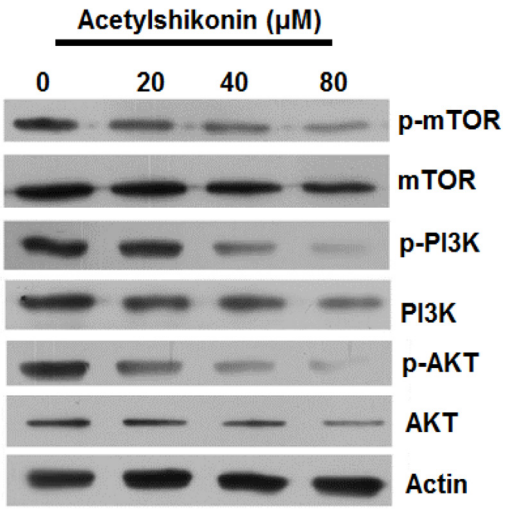

Figure 8. Effect of Acetylshikonin on the mTOR/PI3K/AKT signalling pathway as depicted by western blot analysis. Acetylshikonin caused dose-dependent decrease in the expression of p-mTOR, p-PI3K and p-AKT, while no visible effect was found on the expression of mTOR, PI3K and AKT. The experiments were performed in triplicate.

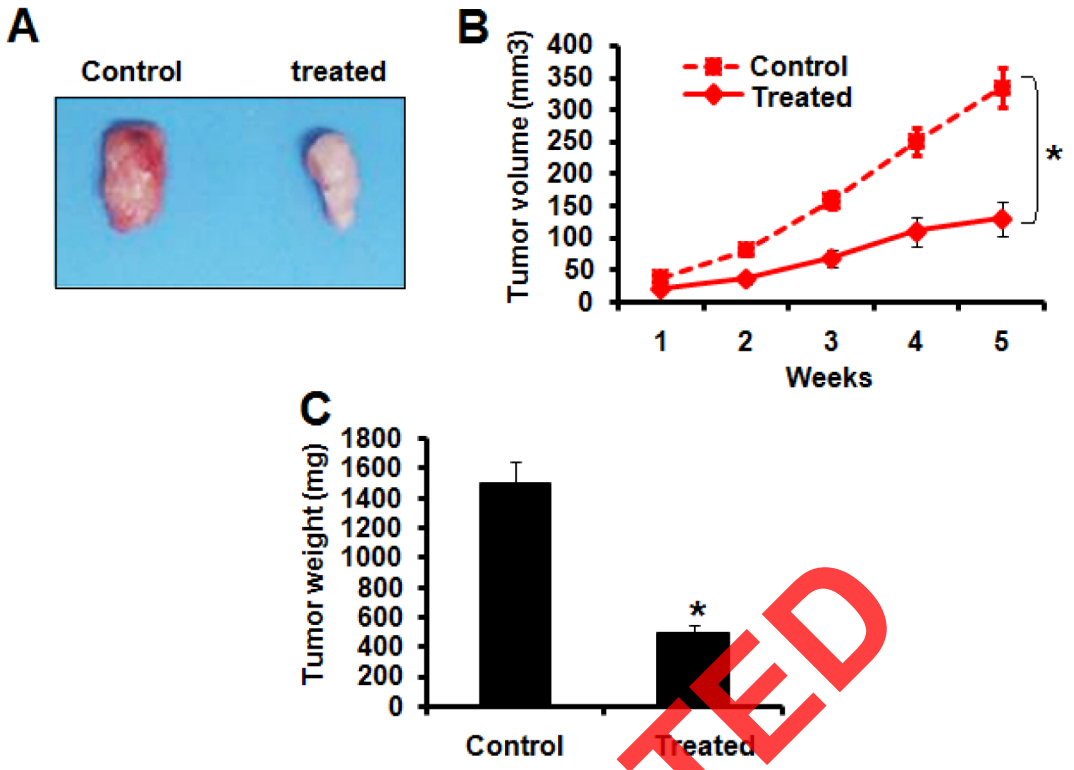

Figure 9. Effect of Acetylshikonin on tumor growth of xenografted tumor growth. **A:** Images of treated and untreated tumors. **B:** Tumor volume. **C:** Tumor weight. The experiments were performed in triplicate and shown as mean ± SD (*p<0.01).

cancer metastasis to distant body parts also adds to the problems [18]. The chemotherapeutic agents used for the management of oral cancer are mostly inefficient and show a number of adverse effects negatively impacting the overall health of patients [19]. Compounds isolated from plants have attained extraordinary attention in the recent past due to their comparatively lower toxic effects. Therefore, researchers are evaluating natural products against cancer cells to develop efficient systemic therapies for cancer in general and oral cancer in particular [20].

In this investigation, the antiproliferative effects of Acetylshikonin were investigated against cisplatin-resistant oral cancer cells and it was found that this compound lead to significant decline in the proliferation rate of these cells. In the studies carried out previously, Acetylshikonin has also been reported to inhibit the growth of gastric cancer cells by triggering apoptosis [9].

Autophagy is a vital process that triggers death of harmful cells and promotes survival of normal cells [21]. Similarly, apoptosis eliminates the harmful cells from the body of an organism [22]. Herein, the investigation of mechanism of action of Acetylshikonin showed that it induces both autophagy and apoptotic cell death of the KB-R oral cancer cells. This was also associated with changes in the expression of autophagy and apoptosis related protein expression. Previous studies have indicated that several of the anticancer molecules induce autophagy and apoptosis of cancer cells [23]. It has been found that mTOR/PI3K/AKT signalling pathway is activated in many cancer types and this plays an important role in their proliferation [10-12]. In this study we found that Acetylshikonin could inhibit the expression of p-mTOR, p-PI3K, and p-AKT in KB-R cells concentration-dependently. Because of the interesting results of the *in vitro* study, the antiproliferative effects of Acetylshikonin were also investigated *in vivo* and showed that Acetylshikonin inhibited the growth of xenografted tumors, suggestive of the potential of Acetylshikonin in the management of oral cancer.

Conclusion

Acetylshikonin suppresses the proliferation of cisplatin-resistant oral cancer cells by autophagy and apoptotic cell death. In addition, it also inhibited the tumor growth *in vivo*. Therefore, Acetylshikonin may prove a vital therapeutic agent for drug-resistant oral cancer cells and merits further evaluation.

Conflict of interests

The authors declare no conflict of interests.

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