

ORIGINAL ARTICLE

Detailed studies on the anticancer action of Blumeatin flavanone in human oral carcinoma cells: determining its impact on cellular autophagy, DNA damage and cell migration and invasion

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Summary

Purpose: Oral cancer is one among the devastating types of malignancies and imposes tremendous disease burden on humans. This study was undertaken to investigate the anticancer properties of a plant-derived flavanone, Blumeatin, against human oral cancer cells. Additionally, this study also attempted to unveil the molecular mechanisms responsible for the anticancer properties of this molecule.

Methods: MTT assay was used for the assessment of cell viability. Transwell and wound healing assays were used for the determination of cell invasion and migration, respectively. Comet assay was used for the determination of cell viability. Transmission electron microscopy (TEM) analysis was done to assess the induction of autophagy. The protein expression was determined by western blot analysis.

Results: Blumeatin inhibited the growth of SCC-4 oral can-

cer cells with minimal cytotoxic effects against the normal hTRET-OME cells. The flow cytometric analysis showed that Blumeatin triggers DNA damage in the SCC-4 cells. Blumeatin also activated autophagy in SCC-4 cells which was accompanied with upregulation of LC3B and Beclin 1. This molecule also increased ROS and decreased the MMP levels in human SCC-4 cells. The effects of Blumeatin were also examined on the migration and invasion of the SCC-4 cells and it was revealed that the molecule suppresses both migration and invasion of the SCC-4 oral cancer cells.

Conclusion: This study indicates that Blumeatin exhibits potent anticancer effects and points towards its use in the development of a new systemic therapy for oral cancer.

Key words: oral cancer, blumeatin, cell cycle arrest, chemotherapy, flavonoids

Introduction

Oral cancer has very high mortality rate and is considered one of the devastating malignancies of oral cavity and pharynx [1]. Globally, it is the 6th leading cancer type with approximately 0.3 million cases being detected annually across the world. Geographically, South and South-East Asian countries show very high incidence of oral cancer [2]. Additionally, estimates show that men have comparatively higher chances of developing oral

cancer relative to women. Finally, the poor 5-year survival rate of oral cancer rate (approximately 5%) further adds to the miseries caused by oral cancer [3]. Therefore, the development of efficacious chemotherapy without adverse effects is one of the major challenges for the treatment of this disease. Natural sources, especially plants and microbes, may serve as repositories for the discovery of anticancer drugs. The discovery of drugs from plant

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sources started since the times when human beings started using plant parts or their crude extracts for treating diseases and disorders [4]. The use of pure forms of plant compounds started just recently with the advent of the natural product chemistry [5]. Chemical analysis of the plant extracts has now showed that plants contain different categories of natural scaffolds including flavonoids, terpenoids and alkaloids to name a few [6]. Cancer is one of the most infamous and devastating disease responsible for huge mortality and morbidity world over [7]. In the developing countries, it is currently ranked as the second prevalent cause of mortality [8]. This has created a pressing need to discover anticancer drugs that are both effective and efficient. As for anticancer drugs, plants are unparalleled sources in terms of the structural diversity and biological activities [9]. Flavonoids have shown remarkable potential to inhibit the growth of cancer cells [10]. This study was designed to investigate the anticancer effects of Blumeatin flavanone against human oral cancer cells. It was found that Blumeatin suppressed the growth of human oral cancer cells via induction of DNA damage and autophagy. The impact of the Blumeatin flavanone was also assessed on the migration and invasion of these cells and it was observed that the molecule could efficiently block both these processes. Taken together, we strongly believe that this study will form a cornerstone in establishing Blumeatin as lead molecule for the development of a new systemic therapy for oral cancer.

Methods

Cell viability assay

The SCC-4 oral carcinoma cell line and the normal hTRET-OME cell line viability was evaluated by MTT assay. The SCC-4 cells at 1.5×10^4 cells per 0.2 mL were incubated in 96-well plates for 24 h. The cells were treated with different doses of Blumeatin ranging from 0 to 200 μM dosage. Afterward, 5 μL of MTT (10 mg/L) were added and again incubated at 37°C for 4 h. The formazan crystals formed were solubilised by the addition of 10% dimethylsulfoxide (DMSO). Finally, the absorbance was taken at 570 nm by spectrophotometer to assess the cell viability.

Transwell assay

The transwell chamber assay was used to determine the migration and invasion of SCC-4 oral cancer cells. The cell culture with about 10^5 cells per well along with 0, 5, 10, 20 μM Blumeatin were added to the upper chamber of the transwell, and the lower chamber was separated by filter paper of 5 micron size and contained only 10% fetal bovine serum (FBS). After being incubated at 37°C for 24h, the filter paper was removed. Its upper surface was wiped carefully to remove the cells. The lower side of the filter paper was washed and the cells were fixed using 70% ethanol. Staining of cells was done using 0.1% crystal violet stain and finally the cells were visualized under light microscope.

Wound healing assay

For wound healing assay the SCC-4 cells were seeded on 12-well plates and cultured till the confluence reached 70%. Thereafter, a scratch was made using

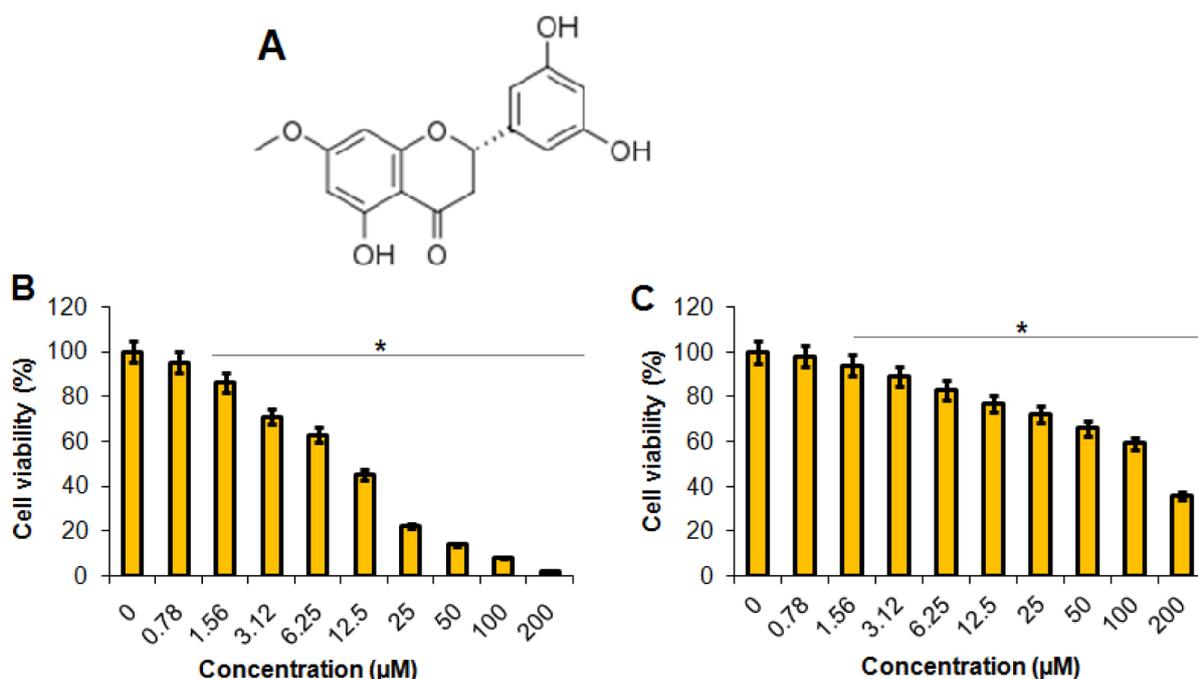


Figure 1. A: Chemical structure of Blumeatin. **B:** Inhibition of SCC-4 cell viability by Blumeatin; and **C:** Inhibition of SCC-4 cell viability by Blumeatin. The experiments were performed in triplicate and expressed as mean \pm SD (* $p < 0.05$).

a sterile pipette tip. Photographs were taken at 0 h and 24 h of incubation at 37°C.

Transmission electron microscopy

Transmission electron microscopy (TEM) was used to assess that Blumeatin -induced autophagy in human SCC-4 cells. Briefly, SCC-4 cells were treated for 24h with Blumeatin. Collection of the cells were done with trypsinization and later they were washed with PBS. Thereafter, fixation was done in glutaraldehyde (2%) in phosphate buffered saline (PBS) (0.1 M). Post fixation was done with 1.5% osmium tetroxide. After post fixation the cells were treated with ethanol embedded in resin. Then, ultramicrotome was used to cut thin sections and then the cells were subjected to TEM.

ROS and MMP determination

SCC-4 cells were seeded at a density of 2×10^5 cells/well in 6-well plates and incubated for 24 h. Cells were then treated with 0, 20, 40 and 80 μM and dihydrocurcubitacin B for 24 h at 37°C in 5% CO_2 . Cells were washed twice with PBS and resuspended in 500 μl dihydrofluorescein diacetate (10 μM) (Sigma-Aldrich, USA) for ROS estimation and DiOC6 (1 $\mu\text{mol/l}$) at

37°C in the dark for 35 min to measure the MMP flow cytometrically.

Western blot analysis

The Blumeatin-treated SCC-4 cells were harvested and the proteins were isolated in RIPA lysis buffer. After determining the concentration of the proteins with bicinchoninic acid (BCA) assay, the protein samples were separated on 10% SDS-PAGE and subsequently transferred to nitrocellulose membranes. After blocking with skim milk, the membranes were incubated with primary antibody for 50 min at 25°C and then for 12 h at 4°C. The membranes were then incubated with secondary antibody at room temperature for 2.5 h. Actin was used as internal control. Enhanced chemiluminescence reagent (Bio-Rad Lab., Hercules, California, USA) was utilised for the visualisation of the protein bands of interest.

Statistics

The experiments were done in triplicate and the values are shown as mean \pm standard deviation (SD). $P < 0.05$ was considered as significant difference. Student's t-test using Graph Pad prism 7 software was used for the statistical analysis.

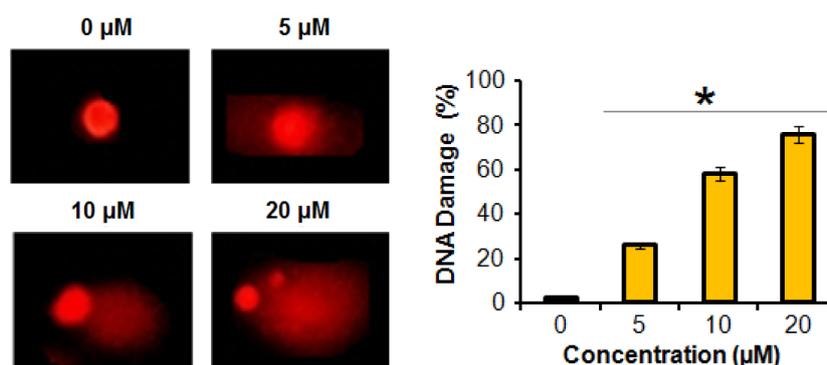


Figure 2. Comet assay showing DNA damage in SCC-4 cells. The experiments were performed in triplicate and expressed as mean \pm SD (* $p < 0.05$).

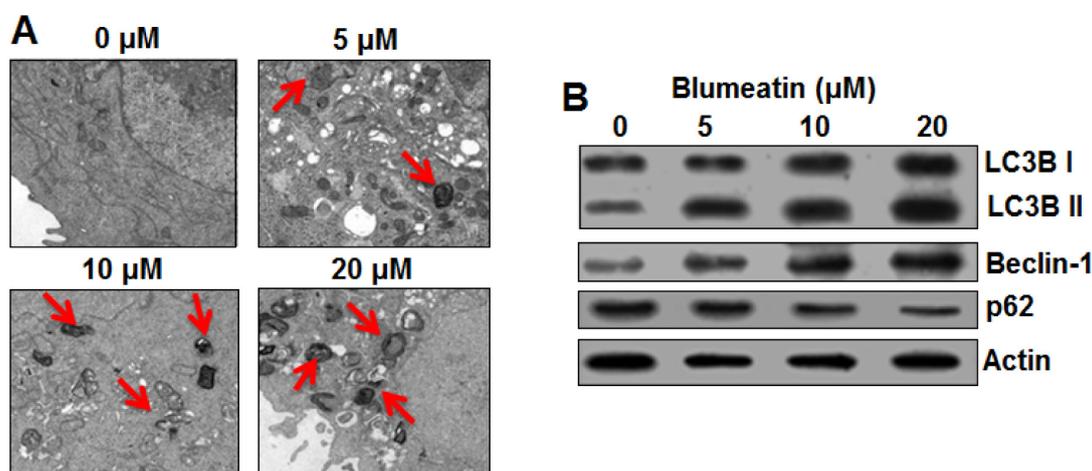


Figure 3. A: TEM analysis showing induction of autophagy in SCC-4 cells (arrows depict autophagosomes). **B:** Western blot analysis showing Blumeatin suppresses the expression of autophagy-related proteins in a dose-dependent manner. The experiments were performed in triplicate.

Results

Blumeatin exhibits anti-proliferative effects on oral cancer cells

Blumeatin's (Figure 1A) anti-proliferative effects on the human oral cancer cells was examined by MTT assay. The viability of the SCC-4 and hTRET-OME cells was determined after treatment with 0 to 200 μM dosage of Blumeatin. It was found that Blumeatin suppresses the growth of SCC-4 cells dose-dependently and exhibited an IC_{50} of 10 μM (Figure 1B). Nonetheless, the anti-proliferative effects of Blumeatin were relatively lower against the hTRET-OME cells which exhibited an IC_{50} of 110 μM (Figure 1C). The IC_{50} of Blumeatin against the hTRET-OME cells was 11-fold higher than against the SCC-4 cells suggesting oral cancer specific activity of Blumeatin.

Blumeatin induces DNA damage in SCC-4 cells

The impact of Blumeatin on DNA damage of SCC-4 cells was examined by comet assay. The SCC-4 cells were administered 0, 5, 10 and 20 μM Blumeatin which showed that the molecule activated DNA damage in the SCC-4 cells as evidenced from the development of tails in the SCC-4 cells (Figure 2). Furthermore, the percentage of DNA damage augmented with increase in the concentration of Blumeatin.

Blumeatin induces autophagy in SCC-4 cells

Next, TEM analysis was performed to examine if Blumeatin induces autophagy in the SCC-4 cells. The SCC-4 cells were treated with 0, 5, 10 and 20 μM concentrations of Blumeatin and subjected to TEM. The results revealed that Blumeatin promoted the development of autophagosomes in

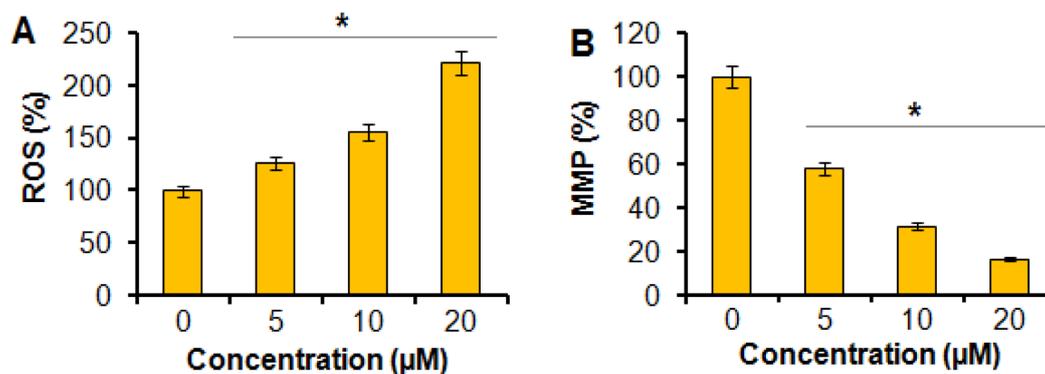


Figure 4. A: ROS levels and B: MMP levels at different doses of Blumeatin in the SCC-4 cells. The experiment were performed in triplicate and expressed as mean \pm SD (* $p < 0.05$).

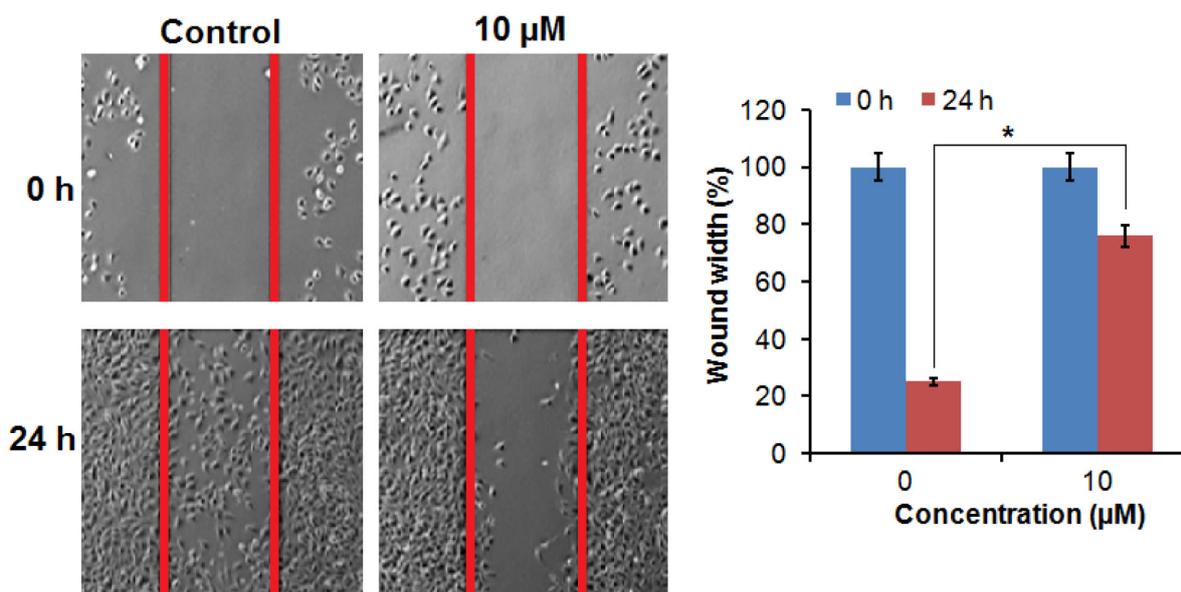


Figure 5. Wound healing assay showing the effect of Blumeatin on the migration of SCC-4 cells. The experiments were performed in triplicate and expressed as mean \pm SD (* $p < 0.05$).

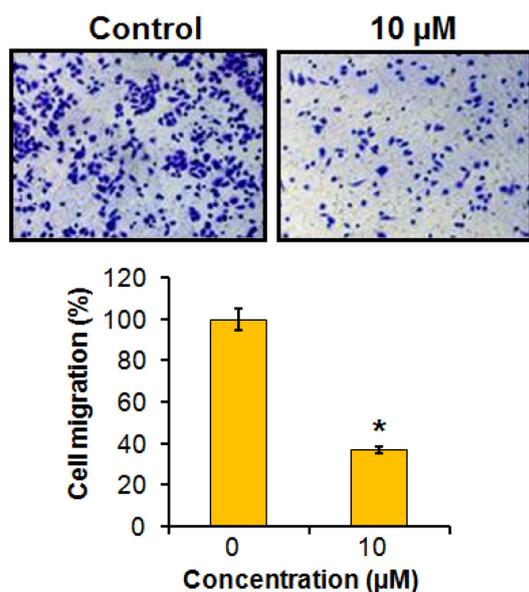


Figure 6. Transwell assay showing the effect of Blumeatin on the invasion of SCC-4 cells. The experiment were performed in triplicate and expressed as mean \pm SD (* $p < 0.05$).

the SCC-4 cells. Additionally, Blumeatin caused a remarkable increase in the LC3B II and Beclin 1 (Figure 3A and B). On the contrary, the expression of p62 was depleted dose-dependently.

Blumeatin increases ROS and decreases MMP levels in oral cancer cells

The effects of Blumeatin were examined on the ROS and MMP levels of SCC-4 cells at 0, 5, 10 and 20 μ M dosage. The results showed that the ROS levels increased dose-dependently in the SCC-4 cells. The ROS levels at 0, 5, 10 and 20 μ M Blumeatin were 100, 127, 156 and 222% (Figure 4A). On the contrary, Blumeatin caused a remarkable decrease in the MMP levels and in a dose-dependent manner. The MMP levels were 100, 58, 32 and 17% at 0, 5, 10 and 20 μ M Blumeatin (Figure 4B).

Blumeatin suppresses migration and invasion of SCC-4 cells

The effects of Blumeatin on the migration of the SCC-4 cells was examined by wound healing assay. It was found that the migration of the SCC-4 cells were significantly decreased after 24 h at 10 μ M (Figure 5). The transwell assay also showed that the invasion of the SCC-4 cells decreased significantly (Figure 6). At IC_{50} of Blumeatin, the invasion of the SCC-4 cells was decreased by 63%.

Discussion

Owing to the lack of reliable biomarkers, most of the oral cancers are diagnosed at advanced

stages and as a result the 5-year survival rate of 5% is very poor [11]. The surgical interventions and subsequent chemotherapy or radiotherapy are the currently employed standard treatments for oral cancer. However, the severe adverse effects of chemotherapy and frequent relapses hurdles the management of oral cancer [12]. Against this background this study examined the anticancer effects of an important flavanone, Blumeatin, against the human oral cancer cells. It was found that the molecule selectively suppresses the growth of the human oral cancer cells with relatively negligible antiproliferative effects against the normal oral cells. These observations are in agreement with previous investigations such as Pectolinarigenin flavanone has been shown to suppress the colony formation and growth of nasopharyngeal carcinoma cells [13]. Anticancer agents suppress the growth of the cancer cells via multiple mechanisms such as apoptosis, autophagy and cell cycle arrest to name a few [14,15]. Herein, we found that the antiproliferative effects of this molecule were mainly due to its potential to induce DNA damage of the oral cancer cells and promote the autophagic SCC-4 cell death. Several authors found that several flavonoids suppress the growth of cancer cells via induction of DNA damage and autophagy [16-18]. The generation of ROS and disruption of MMP have shown to have implications in the suppression of cancer cell proliferation [19]. The present study also revealed that the Blumeatin's antiproliferative effects were accompanied with enhancement of ROS and decrease of the MMP levels in the oral cancer cells. For cancer cells to metastasise, migration and invasion are the initial processes that take place [20]. Herein, we found that Blumeatin suppresses both migration and invasion of the SCC-4 cells. Taken together, Blumeatin may be utilised to target these pathways for the management of oral cancer.

Conclusion

To sum up, this study revealed that Blumeatin, a plant derived triterpene suppresses the growth of the human oral cancer cells via DNA damage, autophagy, ROS generation and disruption of MMP. Additionally, Blumeatin also exhibited potential suppression of the migration and invasion of the oral cancer cells suggestive of its potent anticancer effects.

Conflict of interests

The authors declare no conflict of interests.

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