

ORIGINAL ARTICLE

Anticancer activity of Voacangine against human oral cancer cells is due to G2/M cell cycle arrest, ROS-mediated cell death and inhibition of PI3K/AKT signalling pathway

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Summary

Purpose: Oral cancer is the 6th most prevalent type of cancer and is responsible for high human morbidity and mortality. The present study was designed to investigate the anticancer effects of Voacangine against human oral cancer and to decipher the underlying molecular mechanisms responsible for its anticancer properties.

Methods: SCC-1 oral cancer cell line and normal hTRET-OME cell line were used in this study. Cell viability was determined by MTT assay. Acridine orange (AO)/ ethidium bromide (EB) and annexin V/propidium iodide (PI) assay were used for assessment of apoptosis. Cell cycle analysis and reactive oxygen species (ROS) determination was done by flow cytometry. The protein expression was determined by western blot analysis.

Results: The results showed that Voacangine caused a remarkable decline in proliferation of SCC-1 human oral

cancer cells with negligible toxic effects on the normal human hTRET-OME cells. The IC₅₀ of Voacangine was 9 μ M against SCC-1 cells relative to IC₅₀ of 100 μ M against normal hTRET-OME cells. The reduction of the proliferative rates was attributed to the induction of ROS triggered apoptosis which was associated with activation of Caspase-3, upregulation of Bax and suppression of Bcl-2. Voacangine induced G2/M cell cycle arrest in a dose-dependent manner. Additionally, the anticancer effects of Voacangine on oral cancer cells were exerted through the inhibition of PI3K/AKT signaling cascade.

Conclusion: Taken all together, we conclude that Voacangine is a potent anticancer molecule and may be utilized for the development of systemic therapy for oral cancer.

Key words: Voacangine, oral cancer, anticancer, apoptosis, cell cycle arrest, flow cytometry

Introduction

Despite advancements in science and technology, the current strategies employed for oral cancer management are still far from descent. As such, oral cancer is still at 6th rank in terms of prevalence scores, globally [1]. The survival rates of oral cancer are comparatively lower than breast cancer, where the overall 5-year survival rate of the former is only 62% against 99% for breast cancer, respectively [2]. Another factor that is somewhat worrying is the problem of recurrence of human oral cancer [3].

Hence, there is pressing need to look for the alternative approaches for the management of oral cancer. Hence, the researchers are actively engaged in exploring the cellular targets for the management of oral cancer and to develop more potent anticancer agents against this malignancy. In this regard, the recent research studies have laid lot of focus on the characterization of natural compounds for their anticancer effects against the growth and proliferation of cancer cells [4]. The natural compounds possess

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health beneficial effects on human body and they act as vital source for the development of effective drug molecules [5]. These compounds possess antioxidant and anticancer properties [6]. Alkaloids have shown potent anticancer properties against different types of malignancies [7]. Consistently, the present study aimed at the exploration of anticancer effects of an alkaloid, Voacangine, against human oral cancer cells.

Methods

Cell lines and culture

The human oral SCC-1 cancer cell line as well as the normal oral hTRET-OME cell line were procured from the ATCC collection center, USA. The cell lines were cultured using the Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Thermo Scientific, Waltham, MASS, USA). The cell lines were maintained in a humidified CO₂ incubator at 37°C with 5% CO₂/95% air concentration.

MTT cell viability assay

The seeding of the SCC-1 cells was done in 96-well plates with 4500 cells per well. The SCC-1 cells were then treated from 0 to 200 µM of Voacangine for 24 h. After replacing the medium, the cells were treated with 10 µL of 5 mg/mL MTT solution at 37°C for 4 h. Finally, 100 µL of DMSO was added and absorbance was measured at 570 nm to determine cell viability.

AO/EB dual staining

The SCC-1 cancer cells treated with 0, 9, 18 and 36 µM Voacangine for 24h were centrifuged and the collected cell pellets were washed with phosphate buffered saline (PBS), and fixed using 70% ethanol. The cells were then stained with AO and EB (AO/EB) dual staining, mixed and analyzed for the morphological changes under fluorescence microscope.

Annexin V- FITC/PI fluorescent staining

The annexin V-FITC/PI staining assay was performed to assess the level of cell apoptosis under 0, 9, 18 and 36 µM Voacangine concentrations for 24h. The SCC-1 cancer cells were fixed using methanol and stained with dual annexin V-FITC/PI staining solution. Then, the cells were examined for nuclear morphology under fluorescent microscope and the percentage of apoptotic cells was determined.

Reactive oxygen species (ROS) determination

The presence of ROS in the SCC-1 cells was estimated using 2',7'-dichlorofluorescein diacetate (DCFH-DA). Briefly, the cell cultures were centrifuged at 25000 rpm for 7 min and PBS-washed. Then, the SCC-1 cells were again suspended in fresh medium and treated with 10 mM DCFH-DA at 37°C for 25-min incubation. These SCC-1 cells were then rinsed thrice in PBS and the ROS levels were estimated by flow cytometry.

Cell cycle analysis

For studying the cell cycle phase distribution, the SCC-1 oral cancer cells were treated with 0, 9, 18 and 36 µM Voacangine for 24 h. Centrifugation was used to harvest the treated cells which were then washed with PBS three times, fixed using 4% formaldehyde and stained with Annexin V/PI solution. The cells were then examined using flow cytometer for the analysis of mitosis.

Western blot analysis

To extract the total proteins the SCC-1 cells were treated with RIPA lysis buffer (Thermo Scientific, Waltham, MASS, USA). The Bradford method was used to determine the total protein concentrations of the cell lysates. Equal protein concentrations were loaded on 8% SDS-PAGE gel. The page gel was then blotted to transfer the contents to nylon membranes which were exposed to primary and secondary antibodies designed for respective proteins of interest which were then visualized and their expression levels assessed by the chemiluminescence method.

Statistics

The experiments were performed in triplicate. The values are shown as mean ± SD. Student's t-test and one way ANOVA were used for statistical analysis by utilizing GraphPad prism 7 software. The statistically significant difference was considered at p<0.05.

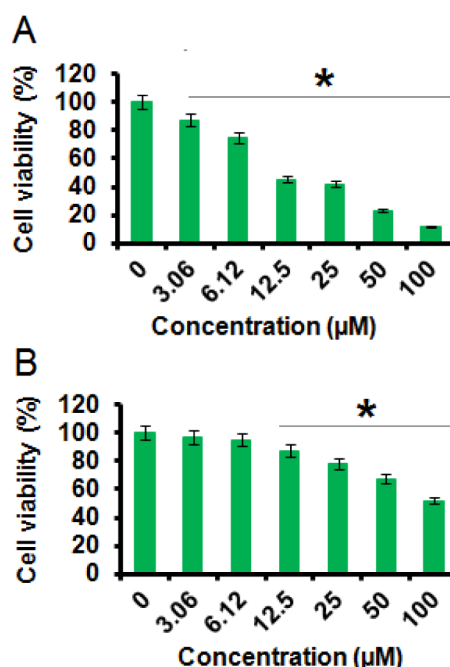


Figure 1. Voacangine selectively inhibits the proliferation of oral cancer cells. Assessment of proliferation of (A) SCC-1 cancer cells and (B) HTRET-OME normal oral cell lines by MTT assay under Voacangine administration showing concentration-dependent decrease in cell viability. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

Results

Voacangine suppresses oral cancer growth

The viability of the Voacangine-treated SCC-1 cancer and hTRET-OME normal oral cells were examined by MTT assay which showed that the molecule suppressed the proliferation of both SCC-1 and hTRET-OME oral cells. However, the antiproliferative effects were significantly more pronounced on the SCC-1 cancer cells (Figure 1A). The IC_{50} of Voacangine for SCC-1 cancer cells was 18 μ M vs 100 μ M for hTRET-OME oral cells (Figure 1B) which suggests that Voacangine selectively targets the oral cancer cells.

Voacangine induces apoptosis in SCC-1 cells

Voacangine-induced apoptosis in the oral SCC-1 cancer cells was examined by AO/EB staining assay. It was observed that the Voacangine treatment led to nuclear deformation of oral cancer cells and the effects were more prominent at higher concentrations. As evidenced from the AO/EB staining, the percentage of the orange and red color cells increased with increase in the dosage of Voacangine, suggestive of apoptosis (Figure 2). The results were further confirmed by the annexin V-FITC/PI dual staining procedure. The percentage of apoptotic cells was seen to increase with increasing doses of Voacangine treatment (Figure 3). The western blot analysis showed that Voacangine activated caspase-3, increased the expression of Bax and decreased the expression of Bcl-2 in a dose-dependent manner (Figure 4). Next, the ROS levels

were also examined in Voacangine-treated SCC-1 cells by flow cytometry which showed that ROS levels increased to 170% at 36 μ M concentration relative to control (Figure 5).

Voacangine caused G2/M cell cycle arrest of oral cancer cells

The analysis of oral cancer SCC-1 cell mitosis treated with 0, 9, 18 and 36 μ M Voacangine for 24

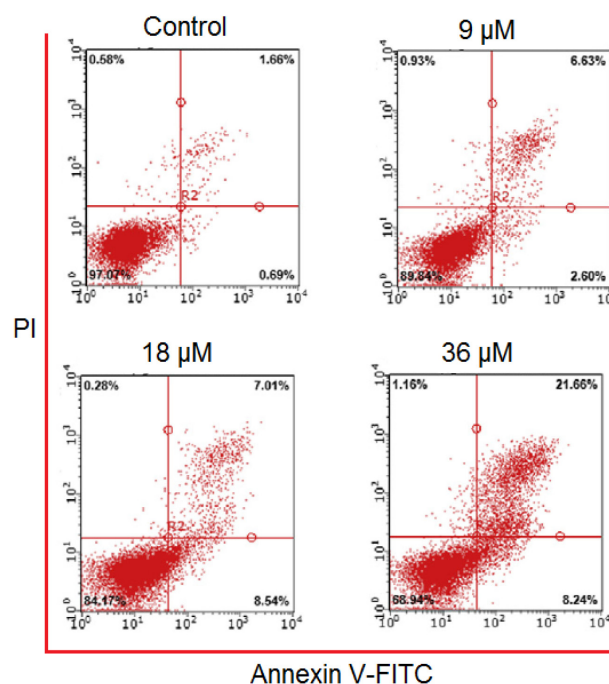


Figure 3. Annexin V/PI staining for apoptotic analysis of SCC-1 cancer cells treated with Voacangine. The Figure shows dose-dependent increase in the percentage of apoptotic SCC-1 upon Voacangine treatment. The experiments were performed in triplicate.

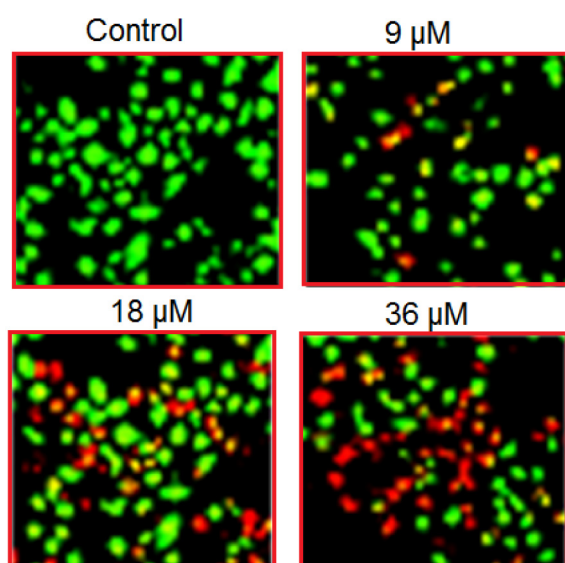


Figure 2. AO/EB staining showing induction of apoptosis of SCC-1 cancer cells treated with Voacangine in a dose-dependent manner. The experiments were performed in triplicate.

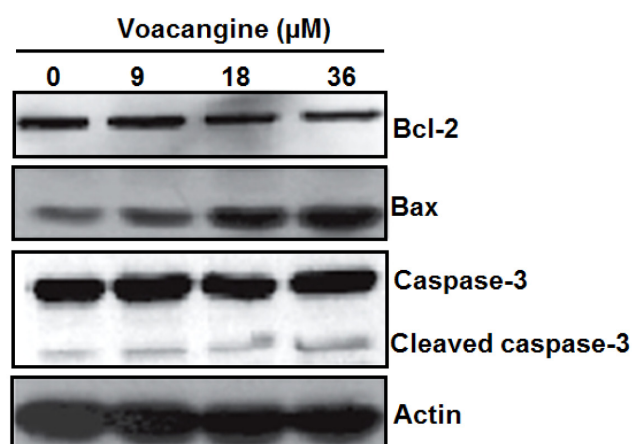


Figure 4. Western blotting for Caspase-3, Bax and Bcl-2 protein expression analysis of SCC-1 cancer cells treated without/with Voacangine showing the changing in the expression of apoptosis-related proteins. The experiments were performed in triplicate.

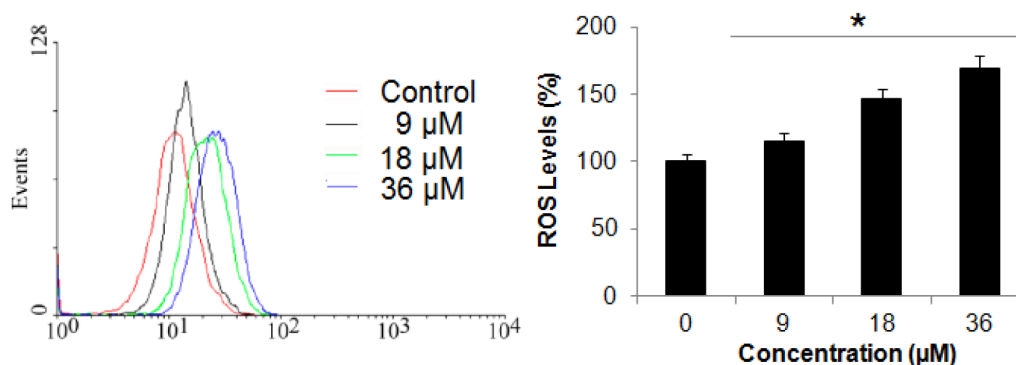


Figure 5. Flow cytometric examination showing dose-dependent increase in the ROS levels of SCC-1 cancer cells treated with Voacangine. The experiments were performed in triplicate and expressed as mean \pm SD (* p <0.05).

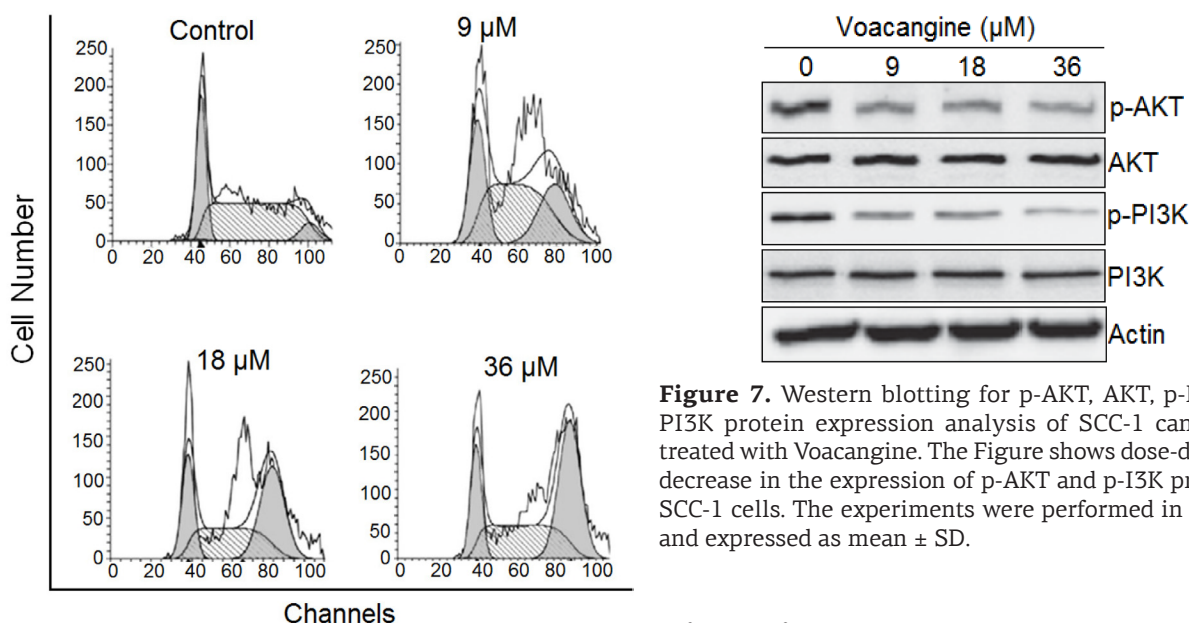


Figure 6. Flow cytometric examination for cell cycle phase distribution of SCC-1 cancer cells treated with different Voacangine doses. The Figure shows dose-dependent increase in the G2/M phase of SCC-1 cells upon Voacangine treatment. The experiments were performed in triplicate and expressed as mean \pm SD (p <0.05).

h revealed that Voacangine-treated cells exhibited higher cell percentage at G2 phase and the percentage of G2 phase cells increased in a concentration-dependent manner (Figure 6). The percentage of G2 oral cancer cells was 65.4 at 36 μM Voacangine concentration against only 11.3 under control conditions.

Voacangine targets PI3K/AKT pathway in oral cancer cells

The western blotting studies carried out for the expression analysis of PI3K and AKT proteins revealed that the protein concentration of p-PI3K and p-AKT decreased dose-dependently (Figure 7). Nonetheless, total PI3K and AKT remained constant at all doses.

Figure 7. Western blotting for p-AKT, AKT, p-PI3K and PI3K protein expression analysis of SCC-1 cancer cells treated with Voacangine. The Figure shows dose-dependent decrease in the expression of p-AKT and p-I3K proteins in SCC-1 cells. The experiments were performed in triplicate and expressed as mean \pm SD.

Discussion

Alkaloids include a diverse group of plant metabolites with well reported anticancer properties. These compounds have been shown to exert anticancer effects via diverse molecular mechanisms such as apoptosis, autophagy and cell cycle arrest to name a few [9]. Herein, we investigated the anticancer effects of Voacangine against human oral cancer cells. Human oral cancer causes a significant number of deaths across the globe and there is a pressing need to identify and develop treatment strategies for its efficient management [10]. In the current study, Voacangine exhibited selective proliferative inhibition against the human oral cancer cells and its effects were limited against the normal oral cells. Such selective inhibitory potential has been noticed for other natural compounds also [11,12]. The inhibitory effects of Voacangine on the proliferation of oral cancer cells were due to induction of apoptosis. This finding is in conformity with previous studies wherein several plant-derived molecules have been shown to

suppress the growth of cancer cells via induction of apoptosis. For example, cycloartane-type triterpenoid from *Commiphora myrrha* has been shown to induce apoptotic death of cancer cells [11-13]. Several of the natural products have been shown to promote apoptosis in cancer cells via accretion of ROS. For example, withaferin-A triggers apoptosis in osteosarcoma via production of ROS [14]. The results also revealed that this molecule induced G2/M arrest of oral cancer cells in a dose-dependent manner which is in agreement with previous studies wherein 23,24-Dihydrocucurbitacin B has been observed to induced G2/M arrest in breast cancer cells [15]. The PI3K/AKT pathway has been shown to act as a vital target in cancer management and it has been reported that molecules may be designed to target this pathway to effectively inhibit the cancer progression [16]. Herein, we found that Voacangine blocks this pathway effectively, suggestive of its anticancer potential.

Conclusion

The results of the present study indicate that Voacangine effectively inhibits the growth of oral cancer cells by inducing apoptosis, cell cycle arrest and modulation of PI3K/AKT pathway. Taken all together, these results clearly show that Voacangine may prove an essential lead molecule in the management of oral cancer.

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Conflict of interests

The authors declare no conflict of interests.

References

- Manikandan M, Rao AK, Arun Kumar G et al. Oral squamous cell carcinoma: microRNA expression profiling and integrative analyses for elucidation of tumourigenesis mechanism. *Mol Cancer* 2016;15:28-32.
- Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin* 2018;68:7-30.
- Cancer Research Campaign. Cancer Stats. Oral cancer – UK. UK: CRC; April 2005.
- Newman DJ, Cragg GM. Natural products as sources of new drugs over the last 25 years. *J Nat Prod* 2007;70:461-77.
- Reddy LA, Odhav B, Bhoola KD. Natural products for cancer prevention: a global perspective. *Pharmacol Therapeut* 2003;99:1-3.
- Rodriguez E, Towers GH, Mitchell JC: Biological activities of sesquiterpene lactones. *Phytochemistry* 1976;15:1573-80.
- Lu JJ, Bao JL, Chen XP, Huang M, Wang YT. Alkaloids isolated from natural herbs as the anticancer agents. *Evidence-Based Complement Altern Med* 2012;2:16-20.
- Engelman JA. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. *Nat Rev Cancer* 2009;9:550-62.
- Evidente A, Kornienko A. Anticancer evaluation of structurally diverse Amaryllidaceae alkaloids and their synthetic derivatives. *Phytochem Rev* 2009;8:449.
- Conway D, Macpherson LMD, Warnakulasuriya KAAS, Ogden G, Stockton DL. Incidence of oral and oropharyngeal cancer in the United Kingdom (1990-1999) – current status, recent trends and intercountry comparisons. *Oral Oncol* 2006;42:586-92.
- Hsieh YJ, Tseng SP, Kuo YH, Cheng TL, Chiang CY, Tzeng YM, Tsai WC. Ovatodiolide of anisomeles indica exerts the anticancer potential on pancreatic cancer cell lines through STAT3 and NF-κB regulation. *Evidence-Based Complement Alternat Med* 2016;2016.
- Zou J, Lin J, Li C et al. Ursolic Acid in Cancer Treatment and Metastatic Chemoprevention: From Synthesized Derivatives to Nanoformulations in Preclinical Studies. *Curr Cancer Drug Targets* 2019;19:245-56.
- Gao W, Su X, Dong X, Cycloartan-24-ene-1α, 2α, 3β-triol, a cycloartane-type triterpenoid from the resinous exudates of *Commiphora myrrha*, induces apoptosis in human prostatic cancer. PC-3 cells. *Oncol Rep* 2015;33:1107-14.
- Li AX, Sun M, Li X. Withaferin-A induces apoptosis in osteosarcoma U2OS cell line via generation of ROS and disruption of mitochondrial membrane potential. *Eur Rev Med Pharmacol Sci* 2017; 21:1368-74.
- Yang L, Wu S, Zhang Q, Liu F, Wu P. 23,24-Dihydrocucurbitacin B induces G 2/M cell-cycle arrest and mitochondria-dependent apoptosis in human breast cancer cells (Bcap37). *Cancer Lett* 2007;256:267-78.
- Caporali S, Alvino E, Lacal PM et al. Targeting the PI3K/AKT/mTOR pathway overcomes the stimulating effect of dabrafenib on the invasive behavior of melanoma cells with acquired resistance to the BRAF inhibitor. *Int J Oncol* 2016;49:1164-74.