Inhibition of human cervical cancer cell growth by Salviolone is mediated via autophagy induction, cell migration and cell invasion suppression, G2/M cell cycle arrest and downregulation of Nf-kB/m-TOR/PI3K/AKT pathway

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Summary

**Purpose:** Cervical cancer is one of the prevalently diagnosed cancers in women worldwide. In this study the antiproliferative and anticancer effects of Salviolone were evaluated against HeLa cervical cancer cell line along with determining the anticancer mode of action.

**Methods:** MTT assay was employed to examine the proliferation rate of HeLa cells. Transmission electron microscopy (TEM) was used for the detection of the autophagic cell death. The cell invasion and migration were investigated by transwell assay and the expression of the proteins was estimated by western blotting.

**Results:** The results revealed that Salviolone exerts anticancer effects on the HeLa cells with an IC50 of 20 µM. The effect of Salviolone on the viability of normal FR-2 cells was very low. TEM analysis showed that Salviolone triggers autophagic cell death in HeLa cells. Salviolone could also cause arrest of the HeLa cervical cancer cells in the G2/M phase of the cell cycle and suppress their ability to migrate and invade. Western blotting analysis revealed that Salviolone could inhibit the Nf-kB/m-TOR/PI3K/AKT signalling pathway in HeLa cells.

**Conclusion:** Taken all together, it is concluded that Salviolone could prove to be an important lead molecule for the treatment of cervical cancer.

**Key words:** autophagy, cell cycle arrest, cervical cancer, invasion, salviolone

Introduction

Cervical cancer is one of the prevalently diagnosed cancers in women around the globe and accounts for a significant proportion of cancer-related mortality [1]. Although cervical cancer is currently treated by radical hysterectomy and radiotherapy, it still causes high mortality [2]. Besides, for cervical cancer diagnosed at early stage, surgery is the only reasonable therapeutic option. It has also been reported that all these treatment strategies of cervical cancer have a negative influence on the patient quality of life. Therefore, research directed at identifying new drug candidates for cervical cancer is most justified [3,4]. In this context, this study was undertaken to evaluate the anticancer effects of Salviolone on a human cervical cancer cell line. Salviolone is a plant-derived natural product which has been found to exert a number of bioactivities such as anticancer, antimicrobial and antioxidant [5,6]. For instance, Salviolone present in the Salvia extracts has been reported to exert anticancer effects on several types of cancer cells [7]. In this study we observed that Salviolone reduced the cell viability of cervical cancer cells. Although Salviolone exerted its antiproliferative effects on all the...
cell lines, however, the lowest IC\textsubscript{50} of 20 µM was observed against the HeLa cervical cancer cell line. Interestingly, Salviolone showed comparatively lower cytotoxicity on the normal cervical FR2 cells with an IC\textsubscript{50} of 120 µM. Given these results, the underlying mechanism for the anticancer effects of Salviolone was investigated. It was found that Salviolone prompts autophagic cell death of the HeLa cells as shown from TEM and western blotting analysis. The investigation of HeLa cells in various phases of the cell cycle upon treatment with Salviolone revealed that this molecule caused arrest of the HeLa cells in the G2/M phase of the cell cycle. Examination of the effect of Salviolone on the invasion of the HeLa cells revealed that it inhibits the invasion and the migration of these cells. NF-kB/m-TOR/Pi3K/AKT pathway is one of the vital pathways that has been shown to be over-expressed in cancer cells and downregulation of this pathway halts the growth of these cells [8]. In this study we observed that Salviolone could inhibit the expression of the main proteins of this signalling pathway and as such Salviolone could prove beneficial in the treatment of the cervical cancer.

**Methods**

**Cell lines and culture conditions**

The HeLa cervical cancer cell line and one normal cell line (FR-2) were procured from American Type Culture Collection. Both cell lines were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS), antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin), and 2 mM glutamine. The cells were cultured in CO\textsubscript{2} incubator (Thermo Scientific, Waltham, Massachusetts, USA) at 37°C with 98% humidity and 5% CO\textsubscript{2}.

**Cell viability and colony formation assay**

The viability of HeLa cells was determined by MTT assay. In brief, the cultured cervical cancer cells were seeded at a density of 1.5×10\textsuperscript{5} in 96-well microtiter plates. This was followed by addition of MTT solution in all the wells and then the absorbance at 570 nm was taken using an ELISA plate reader. To assess the impact of Salviolone on the colony formation potential, the HeLa cells were collected at the exponential phase of growth and were then counted using a hemocytometer.

**Transmission electron microscopy (TEM)**

The samples for TEM were prepared as described previously [9]. In brief, the untreated and Salviolone-treated HeLa cells were fixed in glutaraldehyde (2.5%) in phosphate buffer for 35 min and post-fixed in 1% osmium tetroxide in the same buffer for 35 min. This was followed by dehydration of the cells in molecular graded ethanol and subsequent washing with propylene oxide and then embedded in Epon. This was followed by sectioning on a Reichert-Jung ultramicrotome at 90-nm thickness. The sections were then stained with 5% uranyl acetate and 5% lead citrate and observed on a Hitachi H7100 transmission electron microscope at 75 kV.

**Cell cycle analysis**

To investigate the distribution of the HeLa cervical cancer cells in different phases of the cell cycle, approximately 1×10\textsuperscript{5} cells in each well in 6-well plates were kept at 37°C overnight to allow the cells to adhere. This was followed by treatment with various doses of Salviolone (0, 10, 20 and 40 µM), followed by incubation at 37°C. Finally the distribution of the HeLa cells in various cell cycle phases was determined by flow cytometry.

**Cell migration and invasion assay**

The cell migration ability of HeLa cells was evaluated by transwell assay. Briefly, the cells were seeded at 2×10\textsuperscript{5} cells/mL and incubated for 24 hrs at 37°C. Thereafter, 200 ml cell suspensions were added into the upper chamber and complete medium was added into the bottom wells. After 24-h culturing, the cells in the upper chambers were removed and cells migrated through the chambers were subjected to fixation with methyl alcohol, followed by staining with crystal violet. Finally, the number of cells that migrated was determined by counting the cells under an inverted microscope. A similar procedure was employed for cell invasion assay except for Matrigel usage in this case.

**Western blotting**

The HeLa cells were harvested and lysed with lysis buffer. The protein extracts were incubated at 99°C for 15 min in the presence of loading buffer, followed by separation of cell extracts using 15% SDS-PAGE gel. The samples were then put onto polyvinylidene fluoride membranes blocked using 5% skimmed milk powder. Membrane incubation with primary antibodies was performed for 24 hrs at 4°C. The membranes were incubated with horseradish peroxidase-linked biotinylated secondary antibodies at 1:1,000 dilution for 2 hrs. Washing of the membranes with PBS was followed by visualization of the immunoreactive bands with the help of ECL-PLUS kit according to the instructions of the manufacturer. The immune complexes development was carried out using an ECL detection kit according to the manufacturer’s instructions. The bands were analyzed using GelDoc2000 imaging system.

**Statistics**

The experiments were repeated three times and presented as mean ± SD. Student’s t-test with help of
GraphPad 6 was used for statistical analysis. A p value <0.05 was considered statistically significant.

Results

Anticancer effects of Salviolone on HeLa cells

To evaluate the growth inhibitory effects of Salviolone (Figure 1) on HeLa cervical cancer cell line, the cells were subjected to treatment with Salviolone at varied doses. It was observed that Salviolone exerted considerable anti-cancer effects on these cells with an IC$_{50}$ of 20 µM. However, Salviolone showed much lower growth inhibitory effects on normal human FR-2 cells (IC$_{50}$ of 120 µM). The effect of Salviolone on the growth of HeLa cells was concentration-dependent (Figure 2). Furthermore, evaluation of the effect of the Salviolone on the colony formation potential revealed that this molecule could suppress the colony forming potential of the HeLa cells dose-dependently (Figure 3).

Salviolone triggered autophagy in HeLa cells

To decipher the mechanism by which Salviolone induces anticancer effects we carried out TEM of the untreated and Salviolone-treated HeLa cells. The results showed that treatment of these cells led to generation of autophagic vesicles in these cells (Figure 4). To further validate the autophagic potential of Salviolone we determined the expression of autophagy-associated proteins in the untreated and Salviolone-treated cells. It was observed that the expression of Beclin-1 and LC3-II proteins was significantly upregulated in the Salviolone-administered HeLa cells. Moreover, the expression of p62 was found to be significantly decreased in the Salviolone-treated HeLa cells. However, no visible alterations were observed on the expression of LC3-I and Vps34 (Figure 5).

Salviolone triggered cell cycle arrest

To examine the impact of Salviolone on the distribution of the HeLa cells in different phases of the cell cycle, the cells were treated with 0, 4, 8 and 16 µM of Salviolone for 24 hrs. It was found that the percentage of cells in G2 phase increased in a dose-dependent manner, causing cell cycle arrest (Figure 6). At 10 µM, there was slight increase in

![Figure 1. Chemical structure of Salviolone.](image1)

![Figure 2. Effect of Salviolone on the HeLa cervical cancer cells and FR-2 normal cells as depicted by MTT assay. The experiments were repeated thrice and present as mean ± SD (*p<0.05).](image2)

![Figure 3. Effect of Salviolone on the colony formation potential of HeLa cervical cancer cells. The experiments were repeated thrice. The Figure proves that Salviolone inhibits colony formation concentration-dependently.](image3)

![Figure 4. Salviolone causes autophagic cell death of the HeLa cervical cancer cells as depicted by TEM. The experiments were repeated thrice (Arrowheads depict autophagic vesicles).](image4)
G2 phase cells while at 40 µM there was a marked increase in G2 phase cells.

**Salviolone inhibited cell migration and invasion of HeLa cells**

The impact of Salviolone on cell migration and invasion of cervical cancer HeLa cells was also investigated. The results of cell invasion assay at 20 µM Salviolone for 24 hrs indicated that Salviolone reduced the migration of the HeLa cells (Figure 7). Similar results were observed in the case of cell invasion (Figure 8).

**Salviolone inhibited the Nf-kB/m-TOR/PI3K/AKT signalling pathway**

The effect of Salviolone was also evaluated on the Nf-kB/m-TOR/PI3K/AKT signalling pathways. The results showed that Salviolone significantly decreased the expression of p-mTOR, p-AKT, p-PI3K, and Nf-kB. However, comparatively lower effects were observed on the expression of mTOR, AKT and PI3K (Figure 9).

![Figure 5](image1.png)  
**Figure 5.** Effect of Salviolone on the expression of the autophagy-related proteins. The experiments were repeated thrice. The Figure depicts that Salviolone increases the expression of Beclin and LC3H and decreases the expression of P62.

![Figure 6](image2.png)  
**Figure 6.** Salviolone triggers G2/M cell cycle arrest in HeLa cancer cells as determined by flow cytometry. The experiments were repeated thrice. The Figure depicts that Salviolone induces cell cycle arrest of HeLa cells concentration-dependently.

![Figure 7](image3.png)  
**Figure 7.** Salviolone suppresses the migration of HeLa cervical cancer cells as determined by transwell assay. The experiments were repeated thrice and presented as mean ± SD (*p<0.05).

![Figure 8](image4.png)  
**Figure 8.** Salviolone suppresses the invasion of HeLa cervical cancer cells as determined by transwell assay. The experiments were repeated thrice and presented as mean ± SD (*p<0.05).
Anticancer effect of salviolone in cervical cancer

Discussion

Plants are natural chemical repositories which have tremendous capability to manufacture a huge array of secondary metabolites. These metabolites have a wide array of structures and play pivotal functions in plants [10]. Since, plants are always open to extreme environmental conditions, they have evolved to manufacture different types of secondary metabolites [11] which have been shown to prove beneficial in the treatment of human diseases [12]. Salviolone, a diterpenoid, has been reported to possess a number of bioactivities [5,6]. In this study we evaluated the anticancer activity of Salviolone on the human HeLa cervical cancer cell line. Salviolone was observed to exhibit antiproliferative effects on the cervical cancer cells used in the present study with an IC<sub>50</sub> of 20 µM. Unlike cervical cancer cells, the anticancer effects of Salviolone on the normal FR-2 cells were very low. Previous studies have also reported the anticancer effects of Salviolone-containing extracts on several types of cancers [7]. Moreover, several plant-derived diterpenoids induce autophagic cell death of cancer cells [13]. Autophagy is an essential mechanism by which harmful and unwanted cells are eliminated from the body [14]. In the current study we observed that Salviolone exerts its anticancer effects on the HeLa cancer cells by induction of autophagic cell death. The results of TEM and western blotting confirmed that Salviolone exerts its anticancer effects by triggering autophagy in cancer cells. These observations are also supported by previous studies wherein diterpenoids have been observed to trigger autophagic cell death in cancer cells. For example, oridonin, a diterpenoid, induces autophagy in cervical cancer cells [15].

Apart from autophagy, the arrest of cancer cells in different phases of cell cycle is an important mechanism by which the proliferation of cancer cells is prevented by chemotherapeutic agents [16]. In this study we observed that Salviolone induces G2/M cell cycle arrest in HeLa cancer cells and the percentage of HeLa cells in G2 phase of the cell cycle increased with increase in the concentration of Salviolone. These results are also in accordance with previous studies wherein diterpenoids have been reported to trigger cell cycle arrest of cancer cells [17].

Cell migration and invasion capacity is an important factor in the metastasis of cancer cells [18] and in this study we observed that Salviolone could inhibit the invasion of HeLa cells. This suggests that Salviolone may prove to be an important anti-metastatic agent in vivo.

We also examined the effects of Salviolone on the expression of some important proteins of NF-kB/m-TOR/PI3K/AKT pathway. Salviolone significantly decreased the expression of phosphorylated (p) -mTOR, p-PI3K, p-AKT and NF-kB. However, no apparent effects were observed on the expression of mTOR, PI3K and AKT. Previous investigations have revealed that NF-kB/m-TOR/PI3K/AKT pathway is activated in several types of cancers and it is believed that this pathway can be utilised as a target for anticancer agents [8].

Conclusion

In conclusion, the results of this study indicated that Salviolone inhibits the proliferation of HeLa human cervical cancer cells with minimal effects on normal cells. Furthermore, the anticancer effects of Salviolone were found to be due to induction of autophagy and cycle arrest. The results of this study suggest Salviolone can prove useful in the treatment of cervical cancer. However, further in vivo studies are required for in depth evaluation of Salviolone.

Conflict of interests

The authors declare no conflict of interests.
References