Alantolactone exhibits selective antitumor effects in HELA human cervical cancer cells by inhibiting cell migration and invasion, G2/M cell cycle arrest, mitochondrial mediated apoptosis and targeting Nf-kB signalling pathway

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Summary

Purpose: Cervical cancer is responsible for significant mortality and morbidity across the globe. Owing to the adverse effects of the currently used chemotherapy, research is directed to develop effective and safer chemotherapy for cervical cancer. This study was therefore designed to examine the effects of Alantolactone (AL) against a panel of human cervical cancer cells (HeLa, C4-1, MEK-180, C33A) and a normal cell line (H CerEPiC).

Methods: Cell viability was examined by WST-1 assay. Cell migration and invasion were examined by transwell assay. Cell cycle analysis was performed by flow cytometry. Apoptosis was detected by annexin V/propidium iodide (PI) and DAPI staining. Western blot analysis was used to examine protein expression.

Results: The results revealed that AL inhibits the growth of all the cervical cancer cells in a concentration-dependent manner and exhibited the lowest IC₅₀ of 15 µM against the HeLa cervical cancer cells. The anticancer effects of AL were due to induction of mitochondrial mediated apoptosis. AL caused enhancement in the expression of apoptosis regulatory proteins such as caspase 3 and Bax in cervical cancer cells. AL not only prompted the accumulation of the cervical cancer cells in the G2/M phase of the cell cycle but also inhibited the expressions of various cyclins. Transwell assay revealed that AL suppresses the migration and invasion of cervical cancer cells. Moreover AL could also block the NF-kB signalling pathway concentration-dependently.

Conclusions: It is concluded that AL may serve as an important lead molecule for the development of therapy for cervical cancer.

Key words: alantolactone, apoptosis, cervical cancer, flow cytometry, cell cycle

Introduction

Cervical cancer is the third most commonly diagnosed malignancy in women with approximately 0.5 million women being diagnosed annually, accounting around 9% of all newly diagnosed cancers [1]. Cervical cancer is generally treated with hysterectomy, radiotherapy and chemotherapy, and causes around 0.5 million deaths annually [2]. Although surgery may prove beneficial at early-stage cervical cancer, most of the cases are diagnosed when the disease has already metastasized. Radiotherapy employed for the treatment of advanced cervical cancer causes adverse effects such as skin reactions, pain and lymphodeama to name a few [3] that impair the quality of the patient’s life [4]. Owing to
Alantolactone has anticancer properties against HeLa cervical cancer cells

all these factors, the development of efficient therapy for cervical cancer is the need of the hour. Naturally occurring compounds with anticancer effects have attained attention due to their lower toxicity [5]. Among natural products, plants have served as an amazing repository of anticancer drugs. Based on the structure of the plant metabolites (generally referred to as secondary metabolites), they are classified into different categories [6]. Sesquiterpene lactones form a very diverse group of plant secondary metabolites abundantly reported from the family asteraceae [7]. These sesquiterpene lactones have been shown to exhibit a diversity of bioactivities including anticancer activity [8]. Because of the potent anticancer effects some of the sesquiterpene lactones have even made it to clinical trials [9]. This has further reinforced more researches that focus on evaluating the anticancer effects of novel sesquiterpene lactones. Alantolactone (AL) is an important sesquiterpene lactone prevalently found in the plants belonging to family asteraceae [10]. Although AL has been shown to suppress the growth of different types of cancer cells [11], there is hardly any report on the anticancer effects of AL on the cervical cancer cells. The main purpose of the current study was to investigate the anticancer effects of AL in HeLa human cervical cancer cells along with evaluating its effects on cell migration and invasion, cell cycle phase distribution, cell apoptosis, and NF-kB signalling pathway.

Methods

WST-1 cell viability and colony formation assay

The anticancer effect of AL was assessed on metastatic cervical cancer cells by WST-1 assay. In brief, the various cervical cancer cells and HCerEpiC normal cells were cultured at a density of 2.5×10^5 cells/well in 96-well plates and subjected to treatment with varied concentrations of AL. This was followed by incubation of the HeLa cells with WST-1 for 3 h at 37°C and the proliferation rate was determined by evaluating the absorbance at 450 nm by using ELISA reader.

4′,6-diamidino-2-phenylindole (DAPI) and annexin V/PI staining for apoptosis

The HeLa cervical cancer cells (0.6×10^6) were grown in 6-well plates. Following an incubation period of around 12 h, HeLa cells were subjected to AL treatment for 24 h at 37°C. As the cells sloughed off, 25 μl cell cultures were put onto glass slides and subjected to staining with DAPI. The slides were covered with a cover slip and examined with a fluorescent microscope. Annexin V/PI staining was performed as previously described [12].

Cell cycle analysis

After incubating the cervical HeLa cells with varied concentrations of AL (0, 4, 8 and 16 μM) for 24 h, the cells were washed with phosphate buffered saline (PBS). Afterwards, the HeLa cells were stained with PI and the distribution of the cells in cell cycle phases was assessed by FACS flow cytometer.

Cell migration and invasion assay

The migration and invasion HeLa cancer cells were examined by transwell chamber assay. In brief, 1×10^4 HeLa cells were seeded in the upper chamber of the transwell (8 μm pore size polycarbonate filters). The upper chambers were filled only with medium and the bottom chambers with nearly 200 ml of cell culture were incubated for 24 h. Swabbing was performed to remove the non-invaded cells from the upper surface. The invaded cells on the lower surface were subjected to fixation with methanol for about 35 min, followed by staining with crystal violet (0.5%) for about 50 min, subjected to washing with PBS and finally counted under light microscope (5 fields).

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Cell line</th>
<th>IC_{50}</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>C33A</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>MEK-180</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>C4-1</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>HeLa</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>HCerEpiC (normal cells)</td>
<td>86</td>
</tr>
</tbody>
</table>

Figure 1. A: Chemical structure of Alantolactone (AL). B: WST-1 assay showing the effects of AL on the viability of the HeLa and normal HCerEpiC cells. The values are the mean±SD of three experiments (p<0.05).
Western blotting

The HeLa cells were firstly subjected to washing with ice-cold PBS, suspended in a lysis buffer at 4°C and then shifted to 95°C. Thereafter, the protein content of each cell extract was checked by Bradford assay. About, 40 μg of protein were loaded from each sample and separated by SDS-PAGE before being shifted to polyvinylidene fluoride membrane. The membranes were then subjected to treatment with tris-buffered saline (TBS) and exposed to primary antibodies at 4°C. Thereafter, the cells were treated with appropriate secondary antibodies and the proteins of interest were visualised by enhanced chemiluminescence reagent.

Statistics

All the experimental data is stated as mean ± SD. One-way analysis of variance (ANOVA) for analysing data and Student’s-Newman-Keuls test for multiple comparisons were used. Statistically significant difference was set at p<0.05.

Results

AL selectively inhibits the growth of cervical cancer cells

The growth inhibitory effects of AL (Figure 1A) on the cervical cancer cells were assessed on HeLa cervical cancer cell line and normal cell line (HCerEPiC) by WST-1 assay. It was found that that AL triggers anti-proliferative effects on these cell lines (HeLa, C4-1, MEK-180, C33A) (Table 1). The maximum anti-proliferative effects were observed against the HeLa cells with an IC50 of 15 μM (Figure 1B). Nonetheless, the IC50 of AL was found to be comparatively higher against the normal HCerEPiC cervical cells (IC50 > 86 μM) (Figure 1B). In addition, it was found that the anticancer effects of AL on the cervical cancer cells were concentration-dependent.

AL induces apoptosis in cervical cancer cells

To examine if AL induces apoptosis in the HeLa cancer cells, DAPI staining was performed which showed remarkable changes in the nuclear morphology and membrane blebbing of these cells (Figure 2). Annexin V/PI staining of HeLa cells carried out at varied concentrations of AL showed that the apoptotic cell populations increased from 1.26% in the control to around 27.55% at 30 μM of AL (p<0.05) (Figure 3). The apoptosis was further

![Figure 2](image)

**Figure 2.** DAPI staining showing nuclear morphology of the AL-treated HeLa cells. The experiments were performed in triplicate. AL led to cell apoptosis as indicated by its tendency to induce cell shrinkage, chromatin condensation and nuclear fragmentation.

![Figure 3](image)

**Figure 3.** Percentage of the apoptotic HeLa cells at indicated concentrations of AL as depicted by annexin V/PI staining. The results indicate that AL increased the percentage of apoptotic cells dose-dependently. The experiments were performed in triplicate.

![Figure 4](image)

**Figure 4.** Effect of AL on the expression of Caspase-3, Bax and Bcl-2 in HeLa cells as depicted by western blot analysis. The results indicate that AL led to caspase-3 and Bax activation but downregulated the expression of Bcl-2. The experiments were performed in triplicate.
confirmed by the increased expression of caspase 3, cleaved caspase-3 and Bax and decreased expression of the Bcl-2 in HeLa cells (Figure 4).

**AL causes the G2/M arrest of cervical cancer cells**

The effects of AL on the distribution of HeLa cells in various cell cycle phases were assessed by flow cytometry. It was found that AL caused remarkable increase in the percentage of the HeLa cells in the G2 phase of the cell cycle. The percentage of HeLa cells in the G2 phase increased from 15.2 to 51.8% upon treatment with 30 μM AL (p<0.05) (Figure 5). These results clearly indicate that AL induces G2/M cell cycle arrest of the cervical cancer cells.

**AL inhibits cell migration and invasion of HeLa cancer cells**

Next, the effect of AL on the migration and invasion of the HeLa cancer cells was investigated by wound healing and transwell assays. The results
showed that at IC_{50}, AL could inhibit the migration of the HeLa cancer cells (Figure 6). Similar trend was observed in the case of cell invasion (Figure 7).

**AL inhibits the NF-kB signalling pathway**

Next, we sought to know the effects of AL on the NF-kB signalling pathway of HeLa cells. It was revealed that AL caused significant decline in the expression of the NF-kB and these inhibitory effects of AL exhibited a dose-dependent trend (Figure 8).

**Discussion**

Cervical cancer has been reported to be the second most common type of cancer in women across the world [13]. Since the clinical outcomes are far from satisfactory and the treatment options have a number of side effects, the identification of novel anticancer molecules and subsequent development of efficient and safer treatment regimens for clinical cancer are required [2,3]. Herein, we examined the anticancer potential of AL and found that AL exerted growth inhibitory effects on all the cervical cancer cell lines. Nonetheless, the cytotoxic effects of AL were comparatively negligible against the normal cervical cells, indicating the cancer cell specific activity of AL. Studies carried out previously have also reported that AL inhibits the proliferation of breast cancer cells [14]. Next, we sought to know the probable mechanism for the anticancer activity of AL and therefore we carried out DAPI staining which clearly showed membrane blebbing and induction of apoptotic cell death. The annexin V/PI staining showed that the apoptotic cell populations increased concentration-dependently. The apoptotic cell death of HeLa cervical cancer cells was confirmed by examining the expression of marker proteins of apoptosis. It was found that AL treatment prompted increase in the expression of Bax and cleavage of caspase 3. Moreover, the expression of Bcl-2 was considerably downregulated. These results are also in concordance with previous studies carried out on AL, which have shown that AL induces apoptosis in the MCF-7 breast cancer cells [15]. Apoptosis is a vital process that removes the defective cells from the body and maintains tissue homeostasis. It also prevents the development of chemoresistance in cancer cells [16]. AL was also found to block the HeLa cervical cancer cells at the G2/M check point. Previously, several plant-derived sesquiterpene lactones have been shown to cause cell cycle arrest of cancer cells, for example, AL has been shown to cause cell arrest of the SK-MES-1 lung cancer cells, further validating our results [17]. Next, the anti-metastatic potential of AL was examined by the cell invasion assay. The results showed that AL inhibits the migration of HeLa cells concentration-dependently, indicating that AL may prove beneficial against metastatic cancers. NF-kB signal transduction pathway is considered an important pathway that regulates the proliferation and tumorigenesis of several types of cancers [18]. Herein we found that it inhibits the expression of NF-kB in HeLa cells concentration-dependently, which is also supported by a previous study wherein AL has been reported to inhibit the proliferation of breast cancer cells by modulating the expression of NF-kB [19]

**Conclusion**

Alantolactone inhibits the proliferation of cervical cancer cells by triggering cell cycle arrest and apoptosis. It also inhibits the migration and invasion of cervical cancer cells by modulating the NF-kB signalling pathway. As such Alantolactone may prove beneficial for the development of cervical cancer therapy and warrants further studies.

**Conflict of interests**

The authors declare no conflict of interests.

**References**

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