

Alantolactone Inhibits A2780 Cell Growth through Glycolysis Inhibition and Ros-Induced Apoptosis and Overcomes Cisplatin Resistance

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ABSTRACT

Alantolactone (ALT) a biologically active sesquiterpene lactone compound has been shown to exhibit anticancer activity against a series of human cancers through multiple mechanism. However, the anticancer effects of ALT against ovarian cancer remains unknown. Here, we report that ALT inhibits growth and induces apoptosis in parental and cisplatin-resistant ovarian cancer cells. Growth inhibitory effects of ALT are evident from CCK-8, Edu, and colonogenic assays. The molecular mechanism associated with induction of apoptosis includes ROS generation, G2/M phase arrest and inhibition of aerobic glycolysis. Inhibition of glycolysis was found to be linked with down-regulation of lactate dehydrogenase A (LDHA) and glucose transporter-1 (GLUT1). Finally, ALT effectively suppressed growth and induced apoptosis in cisplatin resistant A2780/CR cells. Taken together, our findings suggest that ALT could be developed into a lead for effective treatment of ovarian cancer.

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Authors' Contribution

LC, WT, CX conducted the experimental work and wrote the manuscript. KM and LY designed and approved study.

Key words

Alantolactone, Ovarian cancer, Apoptosis, G2/M phase arrest, Glycolysis

INTRODUCTION

Ovarian cancer is considered as one of the most frequent and deadliest gynecological cancers worldwide with approximate 0.239 million new cases and 0.152 million deaths annually. It is ranked 2nd among all gynecological cancers (Liu *et al.*, 2019; Lheureux *et al.*, 2019; Momenimovahed *et al.*, 2019). Among all types of gynecological cancers, epithelial ovarian cancer is the most frequently diagnosed cancer with highest mortality rate. It has been reported that about 22,000 women died only because of epithelial ovarian cancer in China per year

(Li *et al.*, 2019). Although ovarian cancer is less frequent than breast cancer, its mortality rate is significantly higher compared to breast cancer even in developed countries like Canada and USA (Lheureux *et al.*, 2019).

At present, cytoreductive surgery followed by chemotherapy (Cisplatin and Taxol) is the systematic treatment of advanced stage ovarian cancer (Liu *et al.*, 2019; Lheureux *et al.*, 2019). However, the prognosis of ovarian cancer has not been improved since last decade with 5 years survival rate about 47% in developed countries like Canada and USA where the 5 years survival rate of breast cancer is about 85% (Lheureux *et al.*, 2019). The poor prognosis of ovarian cancer is attributed to the high recurrence rate and development of chemoresistance (Kim *et al.*, 2018; Li *et al.*, 2019). Therefore, it is important to explore novel bioactive molecules which, on one hand, could improve the chemosensitivity of clinical drugs and, on the other hand, could kill the cancer cells through a novel mechanism. Exploring such bioactive molecules with dual effects could be proved effective in overcoming drug resistance and improving the overall survival rate of ovarian cancer.

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Alantolactone (ALT), a natural bioactive molecule of sesquiterpene lactone family has been shown to exhibit anticancer effects in a wide range of human cancers through multiple mechanisms (Cai *et al.*, 2021). Apart from anticancer activity as a single agent, ALT has been reported to improve the efficacy of various clinical drugs via its chemosensitizing effects in different cancers (Maryam *et al.*, 2017; Cao *et al.*, 2019; Wang *et al.*, 2019; Zheng *et al.*, 2019). Despite impressive anti-tumor potential of ALT in various human cancers, the effect of ALT on ovarian cancer remains unexplored. Here in this study, we have evaluated the anticancer activity of ALT in ovarian cancer cells as a single agent as well as in combination therapy. Moreover, we have explored the effect of ALT on cancer metabolism which have never been reported previously.

MATERIALS AND METHODS

Chemicals and antibodies

Alantolactone (Catalog No. S8318, purity, 99.61%) and Cisplatin (Catalog No. S1166, purity, 99.11%) were purchased from Selleck (Selleck Chemicals, China). Alantolactone were dissolved in DMSO and kept in -20°C. Cisplatin were dissolved in DMF before use.

Paraformaldehyde (GLUT1), Lactate dehydrogenase A (LDHA), Hexokinase II (HK2) and β -Actin were obtained from Proteintech. Cleaved-caspase3, Cleaved-PARP (poly ADP-ribose polymerase) and γ -H2AX (phosphorylated histone H2AX) were purchased from CST. Secondary antibodies were obtained from CST (cell signaling technology).

Cell line and establishment of cisplatin-resistant cell line

Human ovarian cancer A2780 cells were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS) in CO₂ incubator. The cisplatin-resistant cell line A2780/CR was generated as described previously (Maryam *et al.*, 2017).

Cell viability assay

Cell counting kit (CCK-8) assay was employed to determine cell viability as described previously (Li *et al.*, 2019). Briefly, A2780 and A2780/CR cells (2x10³ cells per well) were seeded into 96-well culture plates in triplicate and incubated with 0, 1, 2, 5, 10 and 20 μ M ALT for 72 h. Then, 10 μ L CCK-8 (Dalian Meilun Biotechnology, China) solution was added for 2h. Microplate reader (Hangzhou Allsheng Instruments Co., LTD) was used to measure absorbance at a wavelength of 450 nm.

Colony forming assay

A2780 and A2780/CR cells were plated in 6cm

culture dishes (200 cells per dish). 12h after plating, cells were exposed to ALT (0, 1, 2, 5, 10 μ M) for 24 h. After washing, the cells were grown in ALT-free medium for 10-12 days. After fixing with 4% PFA (paraformaldehyde), the colonies were stained with crystal violet (0.5%) solution. Colonies with >50 cells were counted and photographed.

Glucose and lactate detection

A2780 cells were incubated with ALT (0, 10, 15 μ M) in 6 well plates for 24h. Culture media was collected for the measurement of glucose and lactate levels using the glucose assay and lactate assay kits (Nanjing Jiancheng Bioengineering Institute, China), respectively.

Western blot analysis

Assays were performed as we described previously (Li *et al.*, 2019).

Cell cycle analysis

To analyze the effect of ALT on cell cycle profile of A2780 and A2780/CR cells, we exposed the cells to 15 μ M ALT for 24h. The cells were then harvested and fixed with 75% ethanol. Cell pellets collected by centrifugation were washed and resuspended in a solution containing 50 μ g/mL PI and 100 μ g/mL RNase A at room temperature for 20 minutes in the dark. BD Accuri C6 plus Flow Cytometer was used to analyze samples.

EdU assay

Cells were incubated with 25 mM of 5-Ethynyl-2-deoxyuridine (EdU) (RiboBio) for 2 h at 37°C, and then fixed with 4% PFA for 15 min. After treating with Triton-X (0.5%), cells were incubated with Apollo reaction cocktail (RiboBio) and Hoechst 33342 for 30 min at room temperature. Finally, images were taken via fluorescence microscopy (Leica, Germany).

Apoptosis assay

Assays were performed as previously described (Li *et al.*, 2019).

Reactive oxygen species (ROS) detection

A2780 cells were treated with ALT (0, 10, 20 μ M) for 6h. After treatment, intracellular ROS was detected using DCFDA/H2DCFDA-Cellular ROS assay kit (Abcam). After washing the cells, images were captured using a Leica DMI8 microscope. ROS intensity was analyzed using ImageJ (NIH) software.

Analysis of glycolysis

XF24 extracellular analyzer (Seahorse Bioscience, Agilent Technologies) was used to measure extracellular acidification rate (ECAR) as described by others

previously (Uittenbogaard *et al.*, 2019). Briefly, a total of 2×10^4 cells/well were seeded into a 24-well tissue culture plate and incubated with ALT (0.15 μ M) at 37 °C for 24 h in DMEM medium. The DMEM was then replaced with XF base medium. The XF base medium contained 0% phenol red, 2 mM glutamine, 10 mM glucose, 1 mM pyruvate, and 5 mM HEPES. ECAR was detected before and after sequential injections of 0.5 μ M rotenone/antimycin A and 50 mM 2-deoxyglucose (2-DG). Each point represents the average measurement of triplicate experiments. The values were normalized to cell numbers and plotted as ECAR (mpH/min/cell $\pm$ S.D.) and PER.

Statistical analysis

Values are presented as Mean $\pm$ S.D as noted in the figure legends from three independent experiments. Student t-test was used to determine level of significance.

RESULTS

ALT suppresses growth of A2780 and A2780/CR ovarian cancer cells

To evaluate the growth inhibitory effect of ALT and cisplatin on parental (A2780) and cisplatin resistant (A2780/CR) ovarian cancer cells, we performed CCK-8 assay. The data revealed that ALT and cisplatin suppressed the growth of both parental and cisplatin-resistant ovarian cancer cells dose-dependently (Fig. 1B), however; the growth suppressive effect of ALT and cisplatin was found to be more profound in A2780 parental cells compared to cisplatin resistant A2780/CR cells. These growth inhibitory effects were further confirmed using colony forming assay and Edu cell proliferation assay. The anti-proliferative effects of ALT obtained from colonogenic assay (Fig. 1C) and Edu cell proliferating assay (Fig. 2B) were parallel to CCK-8 assay results. Collective data from CCK-8, colonogenic and Edu cell proliferating assays clearly describes the growth suppressive effects of ALT in A2780 ovarian cancer cells.

ALT induces G2/M phase arrest in A2780 and A2780/CR cells

Cell cycle arrest at a particular checkpoint and cell death are the two main causes of cell growth inhibition (Khan *et al.*, 2012). Therefore, we analyzed cell cycle profile after exposing cells to ALT for 24 h. The data demonstrated that ALT induced the cell cycle arrest at G2/M phase in both A2780 and A2780/CR cells (Fig. 2A).

ALT inhibits consumption of glucose and production of lactate in A2780 cells

Aerobic glycolysis is considered one of the major hallmarks of cancer cells and this altered energy metabolism

has been recognized as a potential drug target for the development of effective cancer therapeutics (Akins *et al.*, 2018; Shi *et al.*, 2018). Therefore, we sought to know about the effect of ALT on cancer cell glycolysis. First of all, we measured the level of glucose and lactic acid in culture media. We found that glucose consumption in cells treated with ALT was significantly lower as compared to control cells (Fig. 3A). Next, we determined the level of lactic acid in culture media. As expected, the level of lactic acid was significantly lower in treatment group compared to control group (Fig. 3B).

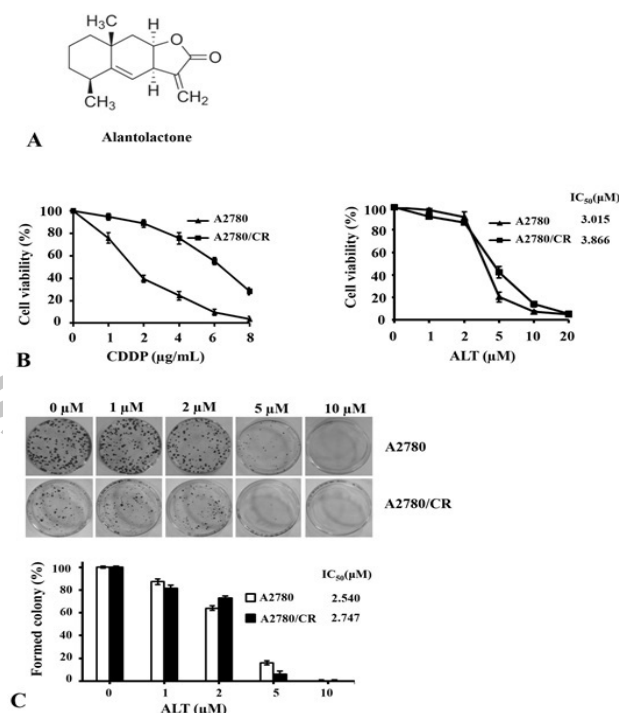


Fig. 1. Effect of alantolactone on proliferation and growth of cisplatin sensitive (A2780) and cisplatin resistant (A2780/CR) ovarian cancer cells. (A) Chemical structure of alantolactone. (B) A2780 and A2780/CR cells were cultured and treated in 96 well plates in triplicates for 72 h. The cell proliferation was measured using CCK-8 assay kit. (C) A2780 and A2780/CR cells were plated in 6 well cell culture plates for 12 h. After treating the cells with indicated dose of ALT for 24 h, medium containing drug was removed and cells were washed. Drug-free medium was added and cells were cultured for 12 days. At the end, colonies were stained and quantified.

Next, we employed immunoblotting to measure the expression of various proteins implicated in glycolytic metabolism. The data demonstrated that ALT decreased the expression of GLUT1 and LDHA dose-dependently; however, the expression of hexokinase (HK) remained unaffected (Fig. 3C).

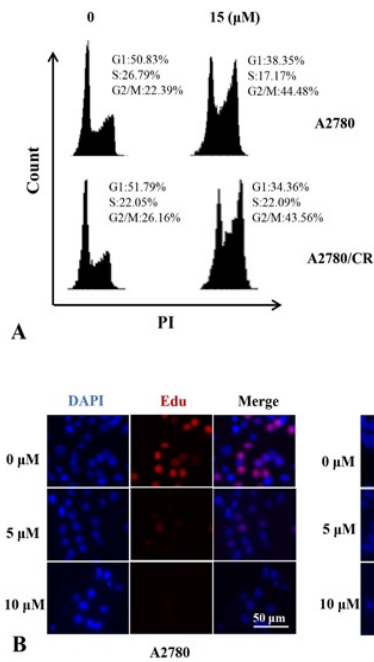


Fig. 2. Effect of ALT on cell cycle progression and proliferation. (A) A2780 and A2780/CR cells were cultured in the presence or absence of ALT for 24 h. The cells were harvested and incubated with RNase A and PI. The cells were analyzed for cell cycle profile using flow cytometer. (B) Cells proliferation rates were analyzed by Edu cell proliferation assay. Blue cells stained with DAPI (left) indicate total number of cells, Red cells (middle) labelled with Edu are proliferating cells. In merge cell population, pink cells are proliferating cells.

ALT inhibits glycolysis in A2780 cells

To further support the above findings, we measured glycolysis by measuring ECAR using Seahorse an XF24 extracellular flux analyzer. Extracellular acidification is sum of glycolytic acidification derived from lactate + H^+ produced by glycolysis and exported into assay medium and respiratory acidification derived from CO_2 produced during aerobic respiration.

To analyze the effect of ALT on glycolysis, briefly cells were treated and ECAR was detected as described in materials and methods. ECAR was detected before and after sequential injections of rotenone/antimycin A (ETC inhibitors) to inhibit mitochondrial CO_2 -derived protons and 2-deoxyglucose to inhibit glycolysis. Figure 4A represents the scheme of Seahorse XF24 glycolytic assay. The inhibitory effect of ALT on glycolysis in A2780 cells under different conditions has been shown in Figure 4B and C. The data clearly showed that ALT significantly inhibited basal glycolysis, compensatory glycolysis and mitochondrial acidification in A2780 cells.

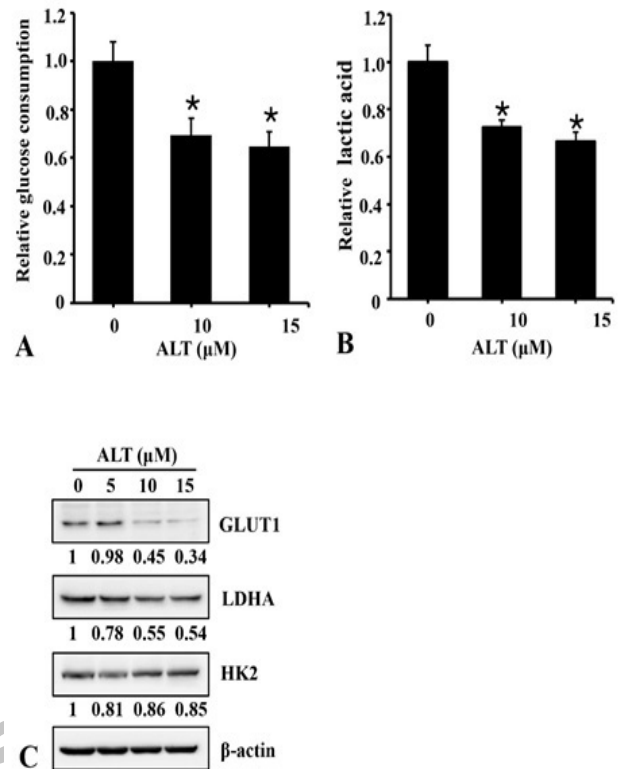


Fig. 3. Effect of ALT on glucose and lactate level. A2780 cells were cultured and treated with ALT in 6 well plates for 24h. The culture media was collected and level of glucose (A) and lactate (B) was measured using commercially available kits. (C). A2780 cells were treated with ALT in a dose-dependent fashion for 24 h and expression of GLUT1, LDHA, HK2 and B-actin was measured from total cell lysate using Western blot. The numerical values below the immunoblots show the relative density of the band normalized to Beta actin from 3 repeated experiments.

ALT induces apoptosis in A2780 and A2780/CR cells

Since ALT suppressed growth and induced cell cycle arrest at G2/M phase, we asked if ALT could also induce apoptosis. Our flow cytometry data revealed that ALT could potentially induce apoptosis in both A2780 parental and A2780/CR cisplatin resistant cancer cells dose-dependently (Fig. 5A). However, it is important to note that A2780 parental cells were highly sensitive to ALT compared to A2780/CR cisplatin resistant cancer cells. To further verify apoptosis, we determined the cleavage of caspase-3 and cleavage of PARP which are the classical markers of apoptotic cell death (Wang *et al.*, 2018). Our immunoblotting data showed that ALT remarkably increased the expression of cleaved caspase-3 and cleaved PARP in both A2780 parental and A2780/CR cisplatin resistant cancer cells dose-dependently (Fig. 5B).

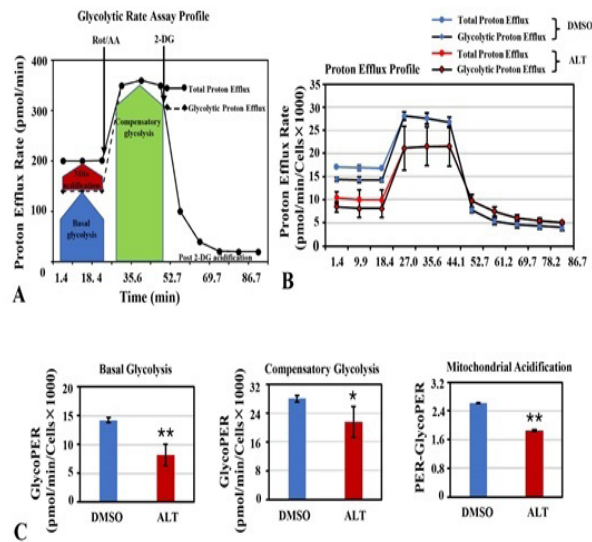


Fig. 4. Analysis of glycolysis. (A) A schematic diagram of assay workflow showing proton efflux rate (PER) measured with glycolytic rate assay. (B) Comparison of total proton efflux rate and glycolytic proton efflux rates between DMSO treated and ALT treated A2780 cells. (C) Basal glycolysis, compensatory glycolysis and mitochondrial acidification was quantified as described in methodology.

ALT induces ROS-dependent apoptosis

Since induction of apoptosis via ROS generation remains the major mechanism of anticancer activity of sesquiterpene lactone (Mehmood *et al.*, 2017; Khan *et al.*, 2012, 2020). Because sesquiterpene lactones induces apoptosis primarily by promoting ROS generation, therefore; we exposed the cells to ALT for 6 h (short time) and subsequently evaluated ROS level. The data demonstrates a dose-dependent increase in ROS generation in A2780 cells (Fig. 6A). To further probe if ALT-induced ROS production is really involved in apoptosis, we exposed cells to ALT in the presence or absence of NAC, a broad-spectrum ROS scavenger and observed cell morphological changes. As shown in Figure 6B, cells exposed to ALT exhibits severe morphological changes while pretreatment with NAC completely abolished ALT-mediated morphological alteration reflecting a crystal-clear role of ROS production in ALT-induced cytotoxicity. To further clarify the role of ROS in ALT mediated cytotoxicity, we determined rate of apoptosis in cells pretreated with or without 3mM NAC. As expected, NAC completely abrogated the apoptotic effect of ALT in A2780 cells (Fig. 6C), which clearly depicts that ALT mainly induces apoptosis by inducing ROS generation in A2780 cells.

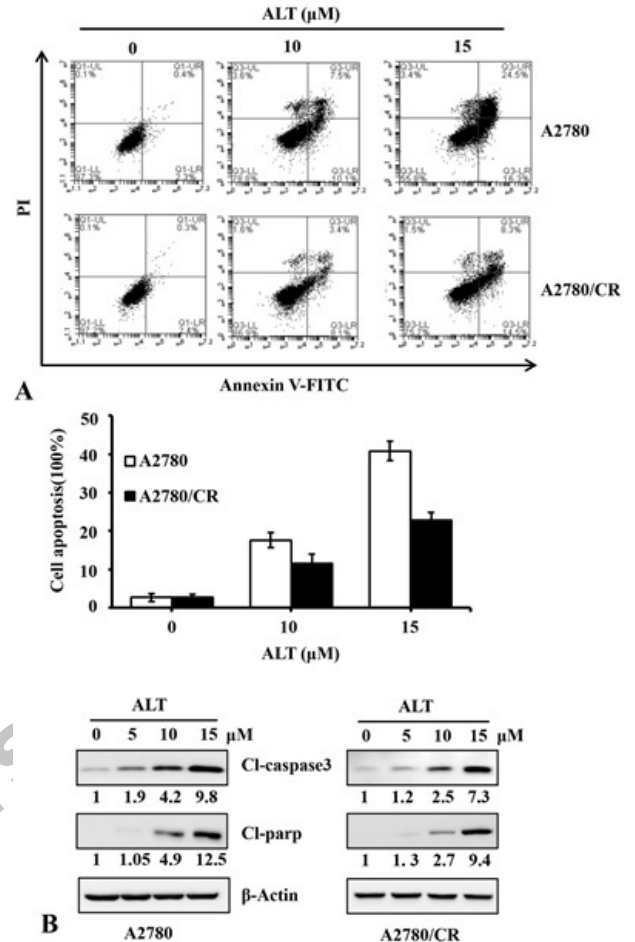


Fig. 5. ALT promotes apoptosis in A2780 (parental) and A2780/CR (Cisplatin resistant) cells. (A) A2780 and A2780/CR cells were cultured and exposed to ALT for 24 h. Apoptosis rates were determined using flow cytometry. (B) A2780 and A2780/CR cells were treated with ALT as shown in figure. The cellular extracts were subjected to immunoblotting for the expression of caspase-3, cleaved PARP, γH2AX and B-actin. The numerical values below the immunoblots show the relative density of the band normalized to Beta actin from 3 repeated experiments.

DISCUSSION

Drug resistance against chemotherapy has been considered the major challenge in successful intervention of chemotherapy. Cancer cells are considered the most advanced eukaryotic cells with evolutionary point of view which have their own well defined sets of characteristic features such as cancer heterogeneity, survival mutations and activation of alternative survival pathways in response to cancer therapy, which dictate tumor progression (Vasan *et al.*, 2019). It is well accepted now that cancer cells quickly

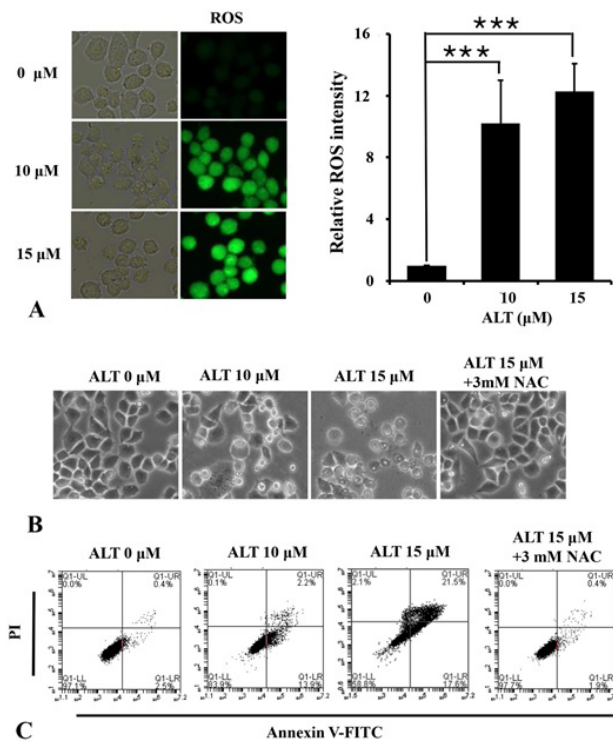


Fig. 6. ALT induces apoptosis via ROS generation. (A) A2780 cells were stained with DCFHDA for 40 min following 6 h drug treatment as shown in figure. DCF fluorescence was checked using *Leica* DMi8 fluorescence microscope and images were captured. ROS intensity was analyzed using ImageJ (NIH) software. (B and C) A2780 cells were treated with ALT either pre-treated with NAC or un-treated with NAC and cell morphological changes (B) and apoptosis rates (C) were measured.

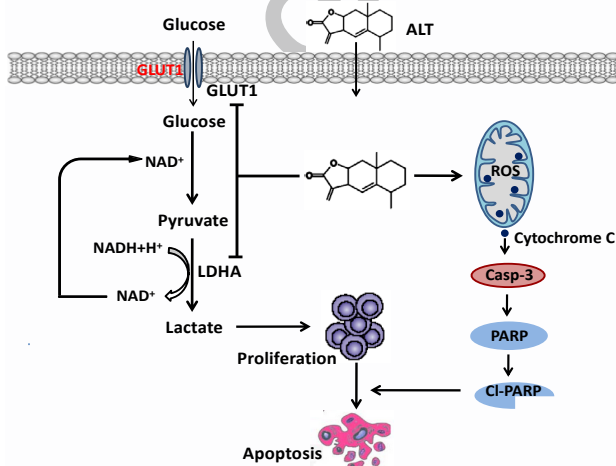


Fig. 7. A schematic diagram showing the molecular mechanism of ALT-mediated apoptosis in human ovarian cancer cells.

develop resistance against conventional mono-targeted therapeutics and targeted therapies. It is logical to argue that cancer heterogeneity is the key component of chemoresistance against mono-target chemotherapy and ovarian cancer is no exception. Targeting altered cancer metabolism has emerged as a new paradigm in cancer drug discovery since genetic mutations ultimately results in altered cancer metabolism which drive the cancer cells on the way to cancer progression (Vander-Heiden, 2011). Therefore, exploring the bioactive molecules which could not only exhibit potential anticancer activity but also interfere with altered tumor metabolism are highly desirable.

Here, we have probed the anticancer potential of ALT, a well-known anticancer compound of sesquiterpene lactone family in parental and cisplatin resistant ovarian cancer cells. ALT has been reported to inhibit the growth and trigger apoptosis in a wide range of human cancers (Cai *et al.*, 2021). As expected, ALT effectively inhibited the growth of parental and A2780/CR cancer cells as evident from CCK-8 assay, colonogenic assay, cell cycle analysis and Edu assay. Next we were anxious to know if ALT could modulate the altered tumor metabolism in ovarian cancer cells. The altered tumor metabolism was initially reported by Otto Warburg about a century ago, most commonly known as “Warburg Effect” which has been verified by a plethora of research reports (Liberti and Locasale, 2016). According to Warburg effect, cancer cells fulfill their energy demands by enhanced aerobic glycolysis which represents a striking metabolic difference between cancer cells and normal cells (Luengo *et al.*, 2017). It is well recognized now that cancer cells have up-regulated glucose transporters (GLUTs) and Lactate dehydrogenase (LDH) to achieve increased glucose up-take and regenerate NAD⁺ for sustainable glycolysis, respectively (Meng *et al.*, 2019; Urbanska and Orzechowski, 2019). To determine the effect of ALT on glycolysis, first we measured the level of glucose and lactate in culture media. Our findings demonstrated that glucose consumption as well as lactate contents in cells treated with ALT was significantly lower as compared to control cells. In line with above findings, a significant lower expression of GLUT1 and LDH1 was observed in cells treated with ALT compared to untreated cells. To validate the above findings, next we measured glycolysis by measuring ECAR using Seahorse an XF24 extracellular flux analyzer. Our data clearly demonstrated that ALT could effectively inhibit glycolysis in ovarian cancer cells. Our findings are also supported by various previous research reports indicating inhibitory effects of several sesquiterpene lactone compounds on glycolysis and glycolytic enzymes (Hartmann *et al.*, 1978; Pan *et al.*, 2020; Jing *et al.*, 2021).

Because ALT affectively inhibited the growth and

aerobic glycolysis, we sought to investigate if these effects of ALT will ultimately set the ovarian cancer cells on the way to apoptosis which is considered a highly planned mode of cell death in which a series of cellular events such as membrane blebbing, DNA fragmentation, caspases activation and PARP cleavage commence to decommission the cancerous cells (Khan *et al.*, 2015). In agreement with well-defined hallmarks of apoptotic cell death, ALT remarkably enhanced the expression of cleaved caspase-3 and cleaved PARP and induced apoptosis in both parental and cisplatin resistant ovarian cancer cells. Since sesquiterpene lactones have been well known for their potential to induce apoptosis in a variety of human cancer primarily via induction of ROS generation (Maryam *et al.*, 2017; Khan *et al.*, 2012, 2020), therefore, we ask if ALT also induces apoptosis in ovarian cancer cells through a mechanism that involves ROS generation. In agreement with previous findings, ALT promoted ROS generation and induces apoptosis which was effectively abrogated by pre-treatment with NAC, assuring a ROS-dependent apoptosis.

In conclusion, we have shown that ALT is a promising bioactive molecule which could conclusively suppress the growth of parental and cisplatin resistant ovarian cancer and trigger apoptosis at least partially by inhibition of glycolysis, and induction of ROS generation and activation of caspase-3 and PARP cleavage.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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