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Original Article

11, 13-Dehydro Lactone Moiety in Gynecologic Cancer Cells

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Abstract

Background: To study the anti-cancer effect of isoalantolactone, a sesquiterpene lactoneisolated from the roots of *Inula helenium*on human gynecologic cancer cells.

Methods: A structure-activity relationship experiment was designed to identify the functional moiety of isoalantolactone for its significant anti-cancer activity. Five gynecologic cancer cell lines were treated with isoalantolactone. Cell proliferation was determined by MTT assay in vitro and cell apoptosis by flow cytometry.

Results: We found isoalantolactone strongly inhibited the cell proliferationofHEC-1, HAC-2, HOC-21, and HeLa cells. Its inhibitory effect was comparable to that of well-known chemotherapeutic agents, cisplatin and taxol. Furthermore, isoalantolactone induced apoptosis in HeLa cells via caspase. On the contrary, its 11, 13-dihydro derivatives had much weaker anti-proliferative activities than the parent compound.

Conclusion: Isoalantolactone exhibited strong anti-proliferative activities and apoptosis-inducing effects on gynecologic cancer cells. The 11, 13-dehydro lactone moiety was critical for its anti-proliferative activity.

Keywords: Apoptosis; Gynecologic cancer; Inulahelenium; Isoalantolactone; Proliferation

Introduction

Gynecologic cancers including cervical, ovarian, uterine, vaginal and vulvar cancer, are among the most common cancers in the world. Using ovarian cancer as an example, there will be approximately 22,240 new cases and 14,070 deaths in the US in 2017 (1). Surgery and radiotherapy are effective for treatment of primary gynecologic cancer, but not for the advanced or the recurrent cases. Chemotherapy, in particular combination chemotherapy, are important treatment choice for gynecologic cancers. Since neo-adjuvant chemotherapy became widely used in clinical treatment of gynecologic cancers, the 5-yr survival rate has improved in recent years (1). However, side effects of the chemotherapeutic drugs and drug resistance are constant threats to long-term survival and the quality of life of gynecologic cancer patients. Therefore, it is significant to develop novel anti-cancer agents for the treatment of gynecologic cancers.

Medicinal herbs are rich sources of potential anti-cancer lead compounds (2). Further structural modifications of these lead compounds would allow us develop better chemotherapeutic agents

with higher potency and better safety profiles (3). *Inula helenimu*, a perennial herb of *Compositae inula*, is widely distributed in Xinjiang, China and cultivated in many regions. Its root has been widely used as a traditional Chinese herbal medicine to relieve gastrointestinal, respiratory and genitourinary symptoms. Sesquiterpene lactones are the main chemical ingredients extracted from *I. helenimu* with anti-mycobacterial and anti-proliferative activities (4). Many efforts have been invested to develop plant-derived sesquiterpene lactones as anti-cancer agents (5). Among these, several compounds, e.g., artesunate, have been evaluated in clinical or preclinical trials. A number of structural analogues have been isolated or partially synthesized, and extensively investigated (6). For example, one compound, isoalantolactone, has been tested for its anti-proliferative effects on various cancer cells (7).

However, only a few studies investigated the effects of sesquiterpepe lactones on gynecologic cancer in the literature. Isoalantolacone induced autophagy in ascites ovarian carcinoma cells HRA, (SKOV3) ovarian cancer cells through up-regulation of PEA-15 (8). Atractylenolide I was found to change the conformational ensemble of human MD-2 on EOC cells and modulate the TLR4/MD-2 complex-mediated MyD88/NF-κB signaling (9). Eremophila dien - 1 (10) - 11-12, 8 – olide (EPD) was found to exert a synergistic effect with cisplatin on a drug-resistant ovarian cancer cell line, as well as a synergistic effect with paclitaxel on two other ovarian cell lines (10).We isolated three sesquiterpene lactones from the root of *I. helenimu,* isoalantolactone, 2-hydroxy-11,13-dihydroisoalantolactone and 11,13-dihydroisoalantolactone. We investigated their effects on cell proliferation and apoptosis of multiple gynecologic cancer cells. We were interested in identifying the functional moiety of isoanalolactone for its anti-cancer activity.

Materials and Methods

Reagents

Isoalantolactone, 2-hydroxy-11,13-dihydroisoalantolactone and 11,13-dihydroisoalantolactone were isolated from the root of *I. helenimu* at the Department of Medicinal Chemistry of Natural Products, School of Pharmaceutical Sciences, Hebei Medical University, as described in the literature (7, 11).Purity of the lactones was more than 99%. Structural formula of these compounds were shown in Fig. 1.

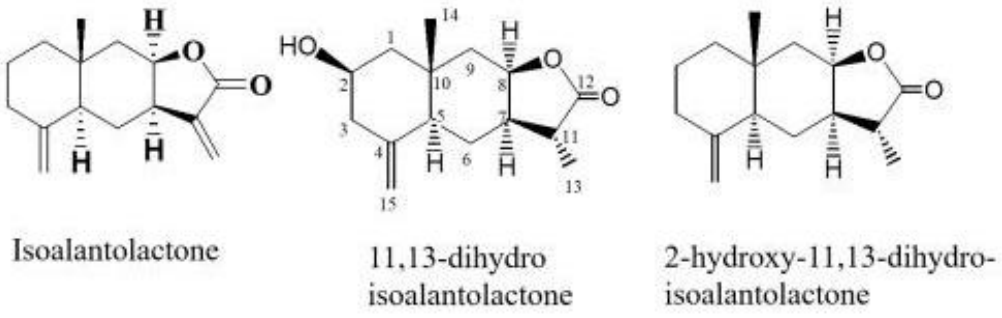

Fig. 1: Chemical structures of three sesquiterpenoid lactones: isoalantolactone,2-hydroxy-11,13-dihydroisoalantolactone, and 11,13-dihydroisoalantolactone

Taxol, cisplatin, MTT, and propidium iodidewere purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 medium and FBS were purchased from Invitrogen (Carlsbad, CA, USA).

Apoptotic rate was analyzed by flow cytometry using the FITC Annexin V Apoptosis Detection Kit (BD Biosciences; Frank Lakes, NJ, USA) according to manufacturer's instruction. Z-VAD-FMK, a cell permeable general caspase inhibitor, was purchased from Promega (Madison, WI, USA).

Cell culture

Cervical cancer cell line HeLa, endometrial adenocarcinoma cell line HEC-1, ovarian serous cystadenocarcinoma cell line SHIN-3, ovarian adenocarcinoma cell line HOC-21 and ovarian clear cell adenocarcinoma cell line HAC-2 were obtained from the Japanese Collection of Research Bioresources Cell Bank (Tokyo, Japan). Chinese hamster lung fibroblast cell line (CHL) was obtained from the Department of Cell Biology, School of Medicine, Hebei Medical University. Cells were cultured in RPMI1640 medium containing 10% (v/v) FBS at 37 °C in a humidified atmosphere containing 5% CO_2, and subcultured upon reaching confluence.

Cell proliferation assay

Cell proliferation was determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazoiium bromide, (MTT) assay. Logarithmically proliferating cells were collected after tripsinization and plated at 1.0×10^4 cells per well in 96-well plates with 100 µl of the medium containing a compound at the indicated concentrations. Two days after treatment, cells were incubated with 100 µl of the medium containing 0.5 mg/mL MTT for 4 h. Then 150 µl of stop solution (2-propanol containing 80 mM HCl) was added into each well and the plates were shaken for 10 min to dissolve formazan crystals. Finally, the absorbance was measured at a wavelength of 570 nm with a reference at 630 nm. The inhibition ratio was calculated with the following formula: Growth inhibition rate (%) = $[1 - A_{570(experimental)}/A_{570(control)}] \times 100\%$. When the effect of a caspase inhibitor (Z-VAD-FMK) was determined, cell survival ratio was calculated as follows: Cell survival rate (%) = $A_{570(experimental)}/A_{570(control)} \times 100\%$. The concentration effect curve of experimental compounds on the tumor cells was fit by Hill mathematical model to calculate the IC_{50} of medicine.

Analysis of apoptosis by flow cytometry

HeLa cells were treated by isoalantolactone (1 µM) for 12 h or 24 h. Then 5×10^5 cells were harvested, washed twice with ice-cold PBS, and resuspended in 100 ml of binding buffer. Following the manufacturer's instruction, 5 ml of Annexin V and 5 mL of PI were added to each sample and incubated for 15 min. Finally, each sample was evaluated for apoptosis in binding buffer by using a FACS Calibur flow cytometer (BD Biosciences).

Statistical analysis

Data were presented as mean ± SD. The statistical significance of difference was analyzed using Student's *t* test and $P<0.05$ was regarded as statistically significant. SAS6.12 software (SAS; Cary, NC, USA) was used for statistical analysis.

Results

Inhibition of cell proliferation by *isoalantolactone*

We first measured the anti-proliferative effect of isoalantolactone on five gynecologic cancer cells in comparison with cisplatin and taxol (Fig. 2A, B, C, D). Isoalantolactone showed strong anti-proliferative activity to HEC-1, HAC-2, and HOC-21 cells with an IC_{50} of 32.54, 19.65 and 11.53 µM, respectively. Their anti-proliferative activities were significantly stronger than those of paclitaxel and cisplatin. SHIN-3 cells were relatively more resistant to all these compounds.

On the other hand, the anti-proliferative effect of isoalantolactone on non-cancerous CHL cells was much weaker than that of cisplatin. At the concentration of 100 µM, the inhibition rates by

cisplatin and isoalantolactone were 80.00% and 46.33% in average (Fig. 2E).These data indicated that isoalantolactone had a strong anti-proliferative activity against gynecologic cancer cells in vitro and was lesstoxic to non-cancerous cells than cisplatin.

Induction of cell apoptosis by isoalantolactone

To figure out whether the anti-cancer activity of isoalantolactone was associated with apoptosis, HeLa cells were staining with annexin V-FITC/PI for flow cytometry analysis. As shown in Fig. 3A, the number of apoptotic cells was increased by isoalantolactone (1μM) after 12 and 24 h of treatment. Furthermore, a general caspase inhibitor, Z-VAD-FMK, was examined for its protection of cell death induced by isoalantolactone.

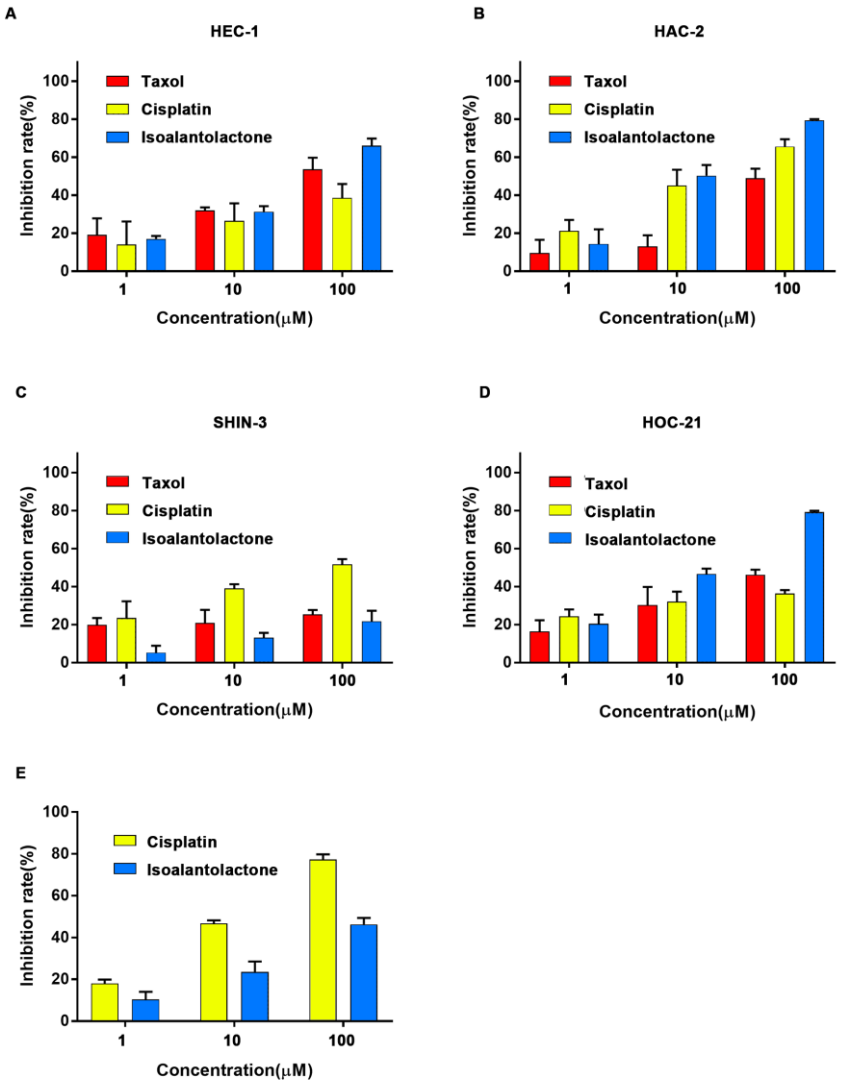

Fig. 2: Anti-proliferative effect of isoalantolactone on gynecologic cancer cells, HEC-1 **(A)**, HAC-2 **(B)**, SHIN-3 **(C)** and HOC-21 **(D)**, and non-cancerous fibroblast cells, CHL **(E)**. MTT assay was performed after treatment for 2 days. Data were expressed as mean ± SD of three independent experiments. * $P<0.05$; ** $P<0.01$, as compared with control (mock-treated) cells

Treating HeLa cells with isoalantolactone in the presence of Z-VAD-FMK (10 μM) cell survival was significantly, yet partially, restored. These data suggested that induction of caspase-dependent apoptosis was involved in the anti-proliferative activity of isoalantolactone (Fig. 3B).

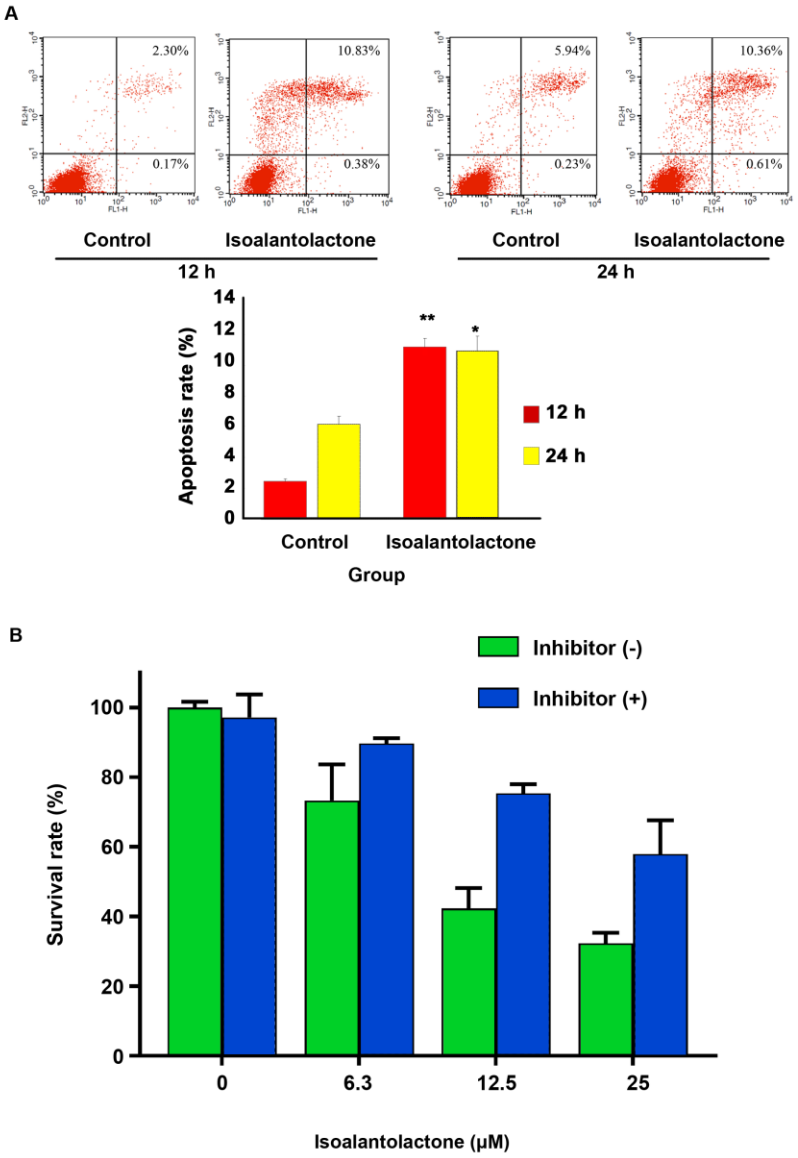

Fig. 3: Induction of apoptosis in HeLa cells by isoalantolactone

A: Annexin V-FITC/PI double staining followed by flow cytometry detected cell apoptosis. **B:** Cell survival after isoalantolactone treatment was compared with that after combination treatment with isoalantolactone and Z-VAD-FMK. Values were expressed as mean ± SD of three independent experiments. * $P<0.05$; ** $P<0.01$, as compared with control (mock-treated) cells

Structure-activity relationship of isoalantolactoneand its 11,13-dihydro derivatives

To further understand what chemical moiety of isoalantolactone was critical for the above effects, inhibitory effects of isoalantolactone and its

11,13-dihydro derivatives were compared with the parent compound for their anti-proliferative effects on HeLa cells. Taxol and cisplatin were used as positive control compounds. Isoalantolactone was significantly more effective in inhibiting cell survival after 2 days of treatment, in particular, at the concentrations of 10 and 100μM (Fig. 4). These data indicated that the 11,13-dehydro lactone moiety was critical for the strong anti-proliferative activity of isoalantolactone.

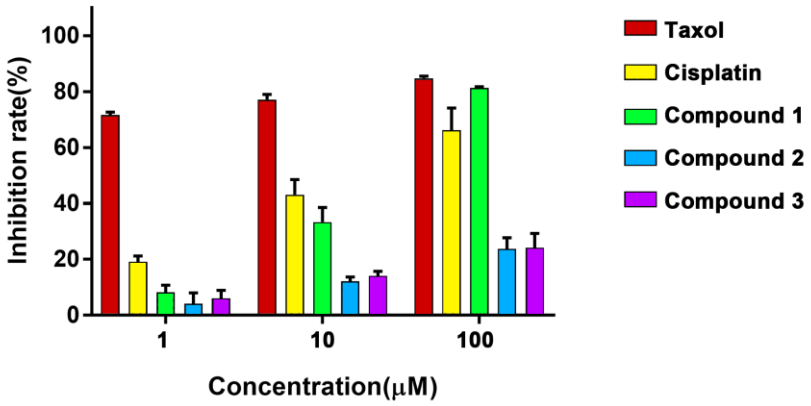

Fig. 4: Structure-activity relationship of isoalantolactone and its 11,13-dihydro derivatives. Isoalantolactone and its 11,13-dihydro derivatives were compared for their anti-proliferative effects on HeLa cells. Data were expressed as mean ± SD of three independent experiments. * $P<0.05$; ** $P<0.01$, as compared with control (mock-treated) cells

Discussion

We found strong anti-proliferative and apoptosis-inducing activities after treating with isoalantolactone on human gynecologic cancer cells, HEC-1, HAC-2, and HOC-21. These strong activities dramatically diminished due to the 11, 13-dihydro derivatives alternation (2-hydroxy-11,13-dihydroisoalantolactone and 11,13-dihydroisoalantolactone, each of them having an α,β-saturated lactone ring), which suggested that the 11,13-dehydro lactone moiety, i.e. an α,β-unsaturated lactone ring of eudesmane sesquiterpenoids, playeda critical role for the anti-cancer activities in the process of isoalantolactone treated on gynecologic cancer cells.

Sustaining proliferative signaling and resisting cell death have long been recognized as two important hallmarks of cancer cells (12). These mechanisms have been targeted as a rational approach in developing novel cancer chemotherapy (13).Isoalantolactone had anti-proliferative and apoptosis-inducing effects on multiple gynecologic cancer cells, but not the non-cancerous fibroblast cells. More importantly, following by using isoalantolactone and two 11, 13-dihydro derivatives, we further demonstrated a critical role by the function of the 11,13-dehydro lactone moiety. The anti-cancer activity of two derivatives (2-hydroxy-11,13-dihydroisoalantolactone and 11,13- dihydroisoalantolactone) were significantly different, so our results suggested that an α,β-unsaturated lactone ring of eudesmane sesquiterpenoids of the isoalantolactone is one of the important functional structure to affect the antitumor activity. These data provide an opportunity through further research on the structural modifications of isoalantolactone to develop novel chemotherapeutic agents for the treatment of gynecologic cancers.

Numerous evidence indicates that the sesquiterpene lactone compounds has the widespread application value and prospect, yet molecular mechanisms of isoalantolactone and other ses-

quiterpene lactones against cancer cells remain to be largely unknown. It was proved that isoalantolactone could induced reactive oxygen species and activated caspases and PARP cleavage to bring about apoptosis (14-16). As a result, it activated Nrf2-mediated induction of detoxifying enzymes (17). In addition, isoalantolactone acted on the mitochondrial and PI3K/Akt signaling pathway, inhibited via NF-κB pathway, and eventually inhibited Erk1/2 phosphorylation (16, 18, 19). Atractylenolide I, a naturally occurring sesquiterpene lactone, was found to change the conformational ensemble of human MD-2 on EOC cells and regulate the TLR4/MD-2 complex-mediated MyD88/NF-κB signaling pathways (9).Our results confirmed that the Z-VAD-FMK (caspase inhibitor) application was not completely blocked isoalantolactone induced apoptosis (Fig. 3B).

Isoalantolactone could induce prostate cancer cells apoptosis through ROS-mediated ER stress and inhibition of STAT3 (20). So we speculate that isoalantolactone probably also induces apoptosis through ROS-mediated ER stress pathway in gynecologic cancer cells.

Conclusion

Further preclinical studies in vivo on isoalantolactone and better derivatives will be detected in the near future to develop new chemotherapeutic agents for gynecologic cancers treatment. Meanwhile, we believe that these compounds are able to bring into valuable function when used in combination with other chemotherapeutic agents.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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