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ORIGINAL ARTICLE

Inhibition of *in vitro* and *in vivo* ovarian cancer cell growth by pinoresinol occurs by way of inducing autophagy, inhibition of cell invasion, loss of mitochondrial membrane potential and inhibition Ras/MEK/ERK signalling pathway

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Summary

Purpose: *Ovarian cancer causes considerable mortality in women across the globe. The limited availability of the efficient chemotherapeutic agents and associated side effects of the existing drugs forms a bottle neck in the treatment of ovarian cancer. In this study, the in vitro and in vivo anticancer activity of a plant derived lignan pinoresinol was investigated along with deciphering its mode of action.*

Methods: *The anticancer activity was evaluated by MTT cell viability assay and its effects on mitochondrial membrane potential loss was checked by flow cytometry. Effects on cell invasion were measured by Matrigel invasion assay, whereas effects on autophagy were evaluated by electron microscopy and Western blotting assay. Protein expressions of the phosphor (p)-MEK and p-ERK were measured by Western blot.*

Results: *Results revealed that Pinoresinol inhibits the growth of the ovarian SKOV-3 cancer cells and exhibits an IC_{50} of 20 μM. The anticancer effects were found to be due to the induction of autophagy which was associated with increase in the expression of LC3 II and Beclin and decrease in the expression of p62. Furthermore, pinoresinol also caused reduction in the mitochondrial membrane potential (MMP) of the SKOV-3 cells and inhibited their invasion capacity. The effects of pinoresinol were also investigated on the Raf/MEK/ERK signalling pathway and it was observed that pinoresinol inhibited the expression of phosphore (p)-MEK and p-ERK in a concentration-dependent manner. Finally, in vivo evaluation revealed that pinoresinol significantly inhibited the growth of xenografted tumors in mice, indicating the potential of pinoresinol in the treatment of ovarian cancer.*

Conclusions: *Pinoresinol, as per the current study, has the potential to inhibit in vitro and in vivo cancer cell growth of SKOV-3 human ovarian cancer cells and as such could be a possible drug candidate for future research.*

Key words: *autophagy, invasion, ovarian cancer, pinoresinol, western blot*

Introduction

Ovarian cancer causes considerable mortality in women throughout the world. In United States alone, more than twenty thousand women are diagnosed annually with ovarian cancer [1]. Owing to its late diagnosis the treatment of ovarian cancer is often difficult. Moreover, the chemotherapeutic agents used in the treatment of ovarian cancer are associated a number of side effects [2]. Hence, there is an urgent need to develop new treatment strategies for the treatment of ovarian

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cancer. Plants are natural chemical factories producing a wide diversity of chemical scaffolds. The metabolites produced by plants are either primary metabolites without which plants can't survive or secondary metabolites which are produced as a defense against the biotic and abiotic stresses [3]. Since, plants are exposed to extreme environmental conditions, they have evolved to produce different types of secondary metabolites [4]. These metabolites have been shown to prove beneficial in the treatment of human diseases. They have been used as antimicrobial and anticancer agents among others [5]. Moreover, these plant secondary metabolites have now gained so much attention that they are screened for their bioactivities especially anticancer activities every now and then [6]. Pinoresinol is an important lignin isolated from different species of plant and has been reported to have immense pharmacological potential. It has been found to inhibit the growth of cancer cells [7]. However, the anticancer effects of pinoresinol have not been evaluated against the ovarian cancer.

The purpose of this study was to investigate the anticancer effects of pinoresinol against human ovarian cancer cells and to explore the underlying molecular mechanisms.

Methods

Cell lines and culture conditions

Ovarian cancer SKOV-3 cell line was obtained from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China and was cultured in RPMI 1640 complete medium containing 10% FBS and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin), at 37°C in a humidified incubator.

MTT assay

For examination of the proliferation rate the SKOV-3 ovarian cancer cells were cultured in ninety-six well plates at 5×10^3 cells per well. The cells were then incubated for one night followed by replacement of the media with a new media containing linderalactone at different concentrations (0-100 μM) for one day. Addition of MTT (0.5 mg/mL) was followed and then the cells were incubated for 3-4 hrs and finally the absorbance at 570 nm was measured by spectrophotometer.

Electron microscopy

The induction of autophagy in Pinoresinol-treated ovarian cancer cells was assessed by electron microscopy. In brief the ovarian cancer cells were treated with 0, 10, 20 and 40 μM pinoresinol for 24 hrs. The cells were collected by trypsinization and were subjected to washing which was followed by fixation in glutaraldehyde (2%) in phosphate buffer (0.1 M). The cells were then post-fixed in osmium tetroxide (1%). This was followed by the treatment of the cells with ethanol and embedding in resin. Thin sections were then cut with the help of an ultramicrotome and subjected to electron microscopy.

Invasion assay

Invasion was evaluated with the help of Matrigel®-coated invasion chambers. The Pinoresinol treated and untreated SKOV-3 cells that reached the lower surface of the membrane were subjected to staining with crystal violet (CV), and images of CV-stained cells were taken. The CV complexes formed were dissolved in 10 % acetic acid and the cell invasion was determined by measuring the absorbances of the resultant solutions at 600 nm with a spectrophotometer.

Western blotting

After lysis of the ovarian cancer cells in lysis buffer, the protein content of each lysate was estimated by bicinchoninic acid (BCA) assay. The samples were then loaded on the SDS-PAGE. The gels were then transferred to nitrocellulose membranes and subjected to treatment with primary antibody at 4°C for 24 hrs. Following this, the membranes were incubated with HRP-conjugated secondary antibody (1:1000) for 50 min at 25°C. Enhanced chemi-luminescence reagent was used to visualise the protein bands.

In vivo study

National Institutes of Health standards for the care and use of laboratory animals were adopted and approved by The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei. The mice (4 weeks old) were injected with 5×10^6 SKOV-3 cells subcutaneously at the left flank. As the tumors became apparent, the mice (n=5) for each group were injected intraperitoneally with DMSO (0.1%) dissolved pinoresinol and diluted with 100 μL normal saline at 40 mg/kg per body weight and taken as the day one of the experiment. Pinoresinol was given to the mice thrice a week and the control mice were given DMSO (0.1%) in normal saline only. At the end of 6 weeks, the mice were euthanized and tumors were harvested for assessment of tumor growth and other investigations

Statistics

The experiments were performed in triplicate and values were presented as mean ± SD. Student's t-test was used for statistical analyses and $p<0.05$ showed statistical significance.

Results

Pinoresinol inhibits the growth of ovarian cancer cells

The growth inhibitory effects of pinoresinol (Figure 1) were determined on the SKOV-3 ovarian cancer cells by MTT assay. The SKOV-3 cells were treated with 0-100 μM of pinoresinol and the proliferation rate was measured. The results revealed that pinoresinol inhibited the growth of the SKOV-3

cancer cells in a concentration-dependent manner. The IC_{50} of pinoresinol against the SKO-V3 was 20 μM (Figure 2).

Pinoresinol triggers autophagy

Many of the plant-derived lignans have been reported to trigger autophagy in cancer cells. Hence, we investigated whether pinoresinol could induce autophagy in the SKOV-3 cancer cells. The results of electron microscopy revealed that pinoresinol triggers the formation of the autophagic vesicles in the SKOV-3 cancer cells which is indicative of autophagy (Figure 3). The autophagy was further confirmed by determining the expression of the autophagy-related proteins. It was found that pinoresinol enhanced the expression of LC3 II and Beclin 3, whereas the expression of p62 was considerably decreased. Furthermore, no effect was observed on the expression of LC3 I and Vps34 (Figure 4).

Pinoresinol causes reduction in MMP levels of SKOV-3 cells

The effect of pinoresinol was also investigated on the MMP levels of the SKOV-3 cancer cells. It was found that upon treatment with pinoresinol the MMP levels decreased up to 65% at 40 μM concentration (Figure 5). These results indicate that decrease in MMP levels might play a role in the induction of autophagy of the SKOV-3 cells.

Pinoresinol inhibits the invasion of the SKOV-3 cells

The effects of pinoresinol were also examined on the invasion of the SKOV-3 cancer cells by transwell assay and it was found that this molecule

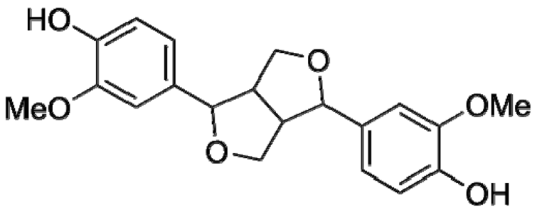

Figure 1. Chemical structure of pinoresinol.

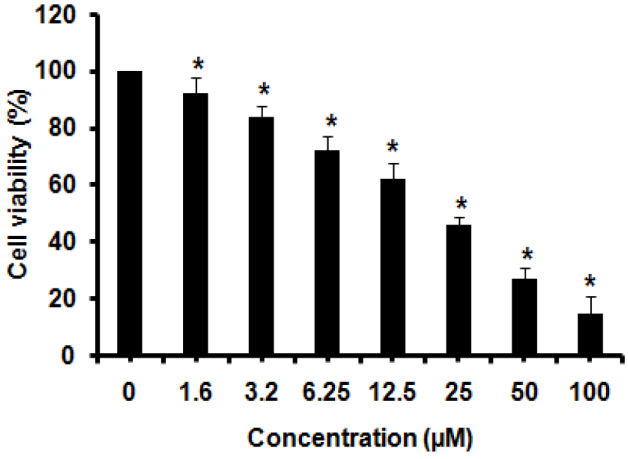

Figure 2. Antiproliferative effects of pinoresinol in SKOV-3 human ovarian cancer cells determined by MTT assay. The experiments were repeated three times and expressed as mean ± SD (*p<0.05).

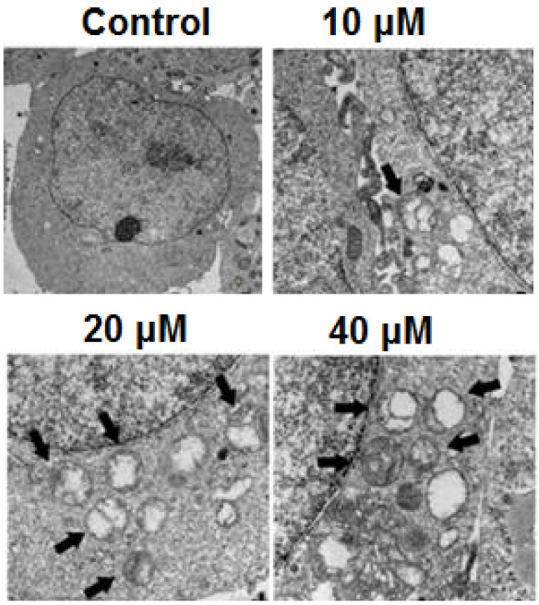

Figure 3. Induction of autophagy in SKOV-3 human ovarian cancer as indicated by Transmission electron microscopy (TEM) at 0 μM, 10 μM, 20 μM and 40 μM concentrations of pinoresinol. The experiments were repeated three times (Arrows show autophagosomes, which are a hallmark of autophagy).

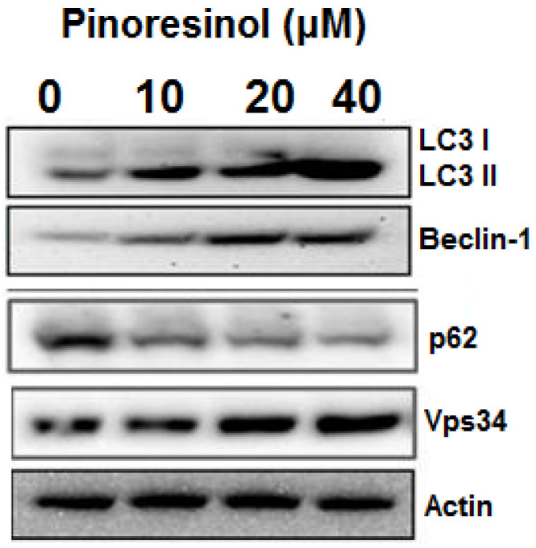

Figure 4. Effect of varying concentrations of pinoresinol on the expression of autophagy-related proteins as determined by western blotting. The Figure shows that pinoresinol enhances the expression of LC3II and Beclin and decreases the expression of p62 in ovarian cancer cells. The experiments were performed in triplicate.

inhibits the migration and invasion of the SKOV-3 cells in a concentration-dependent manner (Figure 6).

Pinoresinol inhibits the Raf/MEK/ERK signalling pathway

Raf/MEK/ERK signalling pathway is an important cascade that has been shown to play a vital role in the proliferation of the cancer cells, and it was found that pinoresinol inhibits the expression of p-MEK and p-ERK. However, no apparent expression of MEK and ERK proteins was observed (Figure 7).

Pinoresinol inhibits the ovarian tumor growth in vivo

The effects of pinoresinol were also investigated on the xenografted mice *in vivo* and it was found that this molecule inhibits the growth of the xenografted tumors (Figure 8A). Further administration of pinoresinol also decreased the xenografted tumor volume (Figure 8B) and weight (Figure 9).

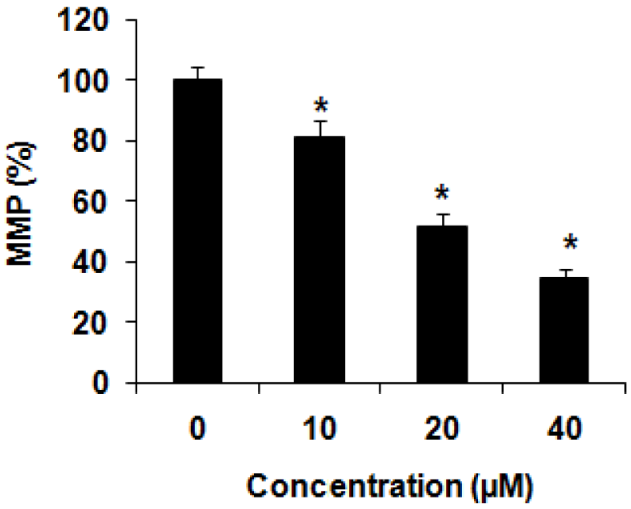

Figure 5. Effect on mitochondrial membrane potential (MMP) of pinoresinol in SKOV-3 human ovarian cancer cells. The Figure shows that pinoresinol decreases the MMP in ovarian cancer cells concentration-dependently (*p<0.05). The experiments were performed in triplicate.

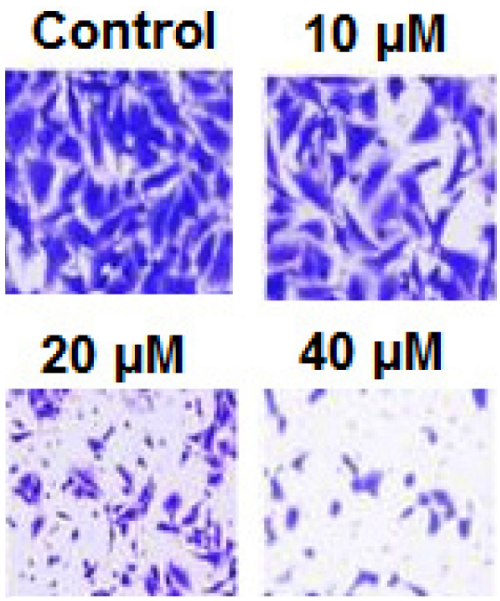

Figure 6. Inhibition of invasion of SKOV-3 human ovarian cancer cells treated with pinoresinol at increasing doses. The Figure depicts that pinoresinol inhibits the invasion of ovarian cancer cells concentration-dependently. The experiments were performed in triplicate.

Discussion

Ovarian cancer is one of the most lethal malignant diseases and is commonly known as silent killer owing to lack of specific symptoms that leads to late diagnosis [8]. It causes considerable mortality and more efficient treatment strategies need to be developed for the treatment of ovarian cancer [9]. In this study the anticancer activity of a natural lignan was examined against the SKOV-3 cancer cells and it was found that pinoresinol reduces the viability of the SKOV-3 cells in a concentration-dependent manner. These results are in agreement with previous investigations of plant-derived lignans that have been reported to inhibit the growth of cancer cells [10]. For instance, pinoresinol has been reported to inhibit the growth of the HepG2 cells by triggering apoptosis [11]. Pinoresinol derivatives have also been reported to inhibit the growth of breast cancer cells [12]. The underlying mechanism for the antiproliferative effects of

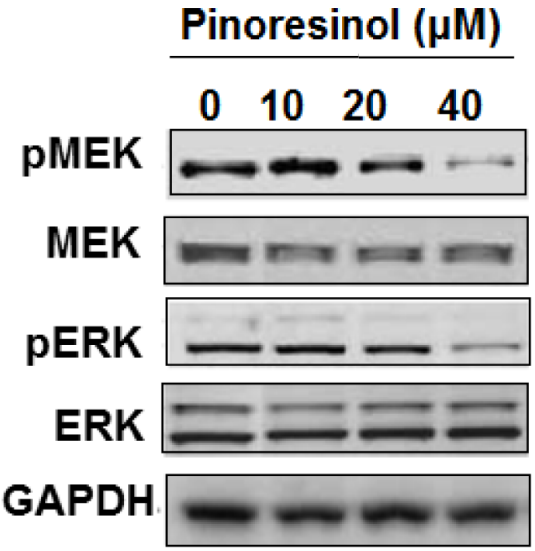

Figure 7. Effect of pinoresinol on the Ras/MEK/ERK signalling pathway as measured by western blotting analysis. The Figure depicts that pinoresinol blocks the Ras/MEK/ERK signalling pahway in ovarian cancer cells concentration-dependently. The experiments were repeated three times.

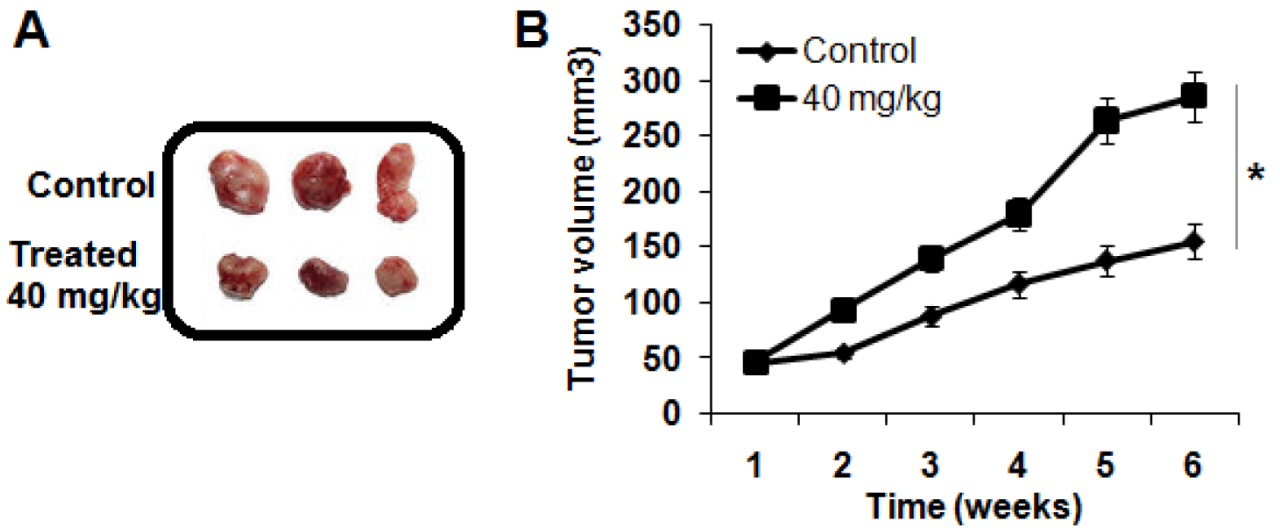

Figure 8. Pinoresinol inhibits *in vivo* ovarian cancer tumor growth **(A)** and tumor volume **(B)**. There was a significant reduction in tumor volume at indicated dose of pinoresinol. Results are representatives of three biological experiments and expressed as mean±SD. The values were considered significant at p*<0.01.

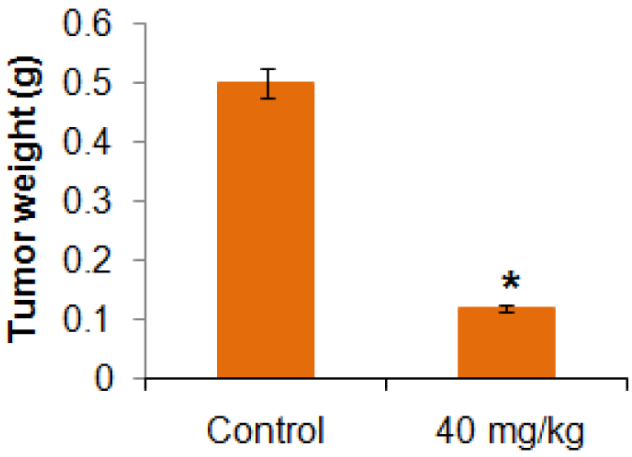

Figure 8. Pinoresinol inhibits *in vivo* ovarian cancer tumor growth. There was a significant reduction in tumor weight at indicated dose of pinoresinol. Results are representatives of three biological experiments and expressed as mean±SD. The values were considered significant at p*<0.01.

pinoresinol were also investigated and it was found that this molecule could trigger autophagic cell death of the ovarian SKOV-3 cells. Autophagy is an important process which involves the removal or death of harmful cells under stress conditions or in response to chemotherapeutic agents [13]. The pinoresinol¬-induced autophagic cell death was also associated with alteration in the expression of the autophagy-related proteins. For instance, the expression of LC3 II and Beclin 1 was increased and that of p62 was decreased, hence favouring autophagy. Furthermore, MMP is an important factor that determines the survival of the cells [14]. It was found that the pinoresinol caused reduction in MMP, thereby favouring autophagy. Invasion of cancer cells to the neighbouring tissues causes spread of cancer to other parts of the body and hence the molecules that can inhibit the invasion of cancer cells may also inhibit the invasion and metastasis of cancer cells *in vivo* [15]. In this study we found that pinoresinol inhibits the invasion of cancer cells concentration-dependently, indicating the potential of pinoresinol as anticancer agent. Raf/MEK/ERK signalling pathway has been reported to be involved in the proliferation, progression and tumorigenesis of several types of cancers and in this study it was observed that pinoresinol could efficiently inhibit this pathway [16]. In addition, pinoresinol also inhibited the xenografted tumor growth *in vivo*, indicating the potential of this molecule in the treatment of ovarian cancer.

Conclusion

Taken together, we conclude that pinoresinol inhibits the growth of human ovarian tumor cells by triggering autophagy and also inhibiting their invasion capability. In addition, pinoresinol inhibited the xenografted tumor growth *in vivo*, indicating the potential of this molecule in the treatment of ovarian cancer and as such warrants further investigation.

Conflict of interests

The authors declare no conflict of interests.

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