Anticancer effects of Pimaric acid is mediated via endoplasmic reticulum stress, caspase-dependent apoptosis, cell cycle arrest, and inhibition of cell migration in human ovarian cancer cells

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Pimaric acid is a naturally occurring resin and has been found to perform many pharmacological activities including, anticancer activity. However, the role of Pimaric acid in ovarian cancer is still not known. This investigation aimed to evaluate the anticancer effects of Pimaric acid and its molecular mechanism in human ovarian cancer cells. MTT assay was used to examine cell viability. Cell morphology was determined through phase contrast microscopy. DAPI staining and TUNEL assay were performed for apoptotic study. Examination of cell cycle phase distribution was carried out through flow cytometry. In vitro wound healing assay was used for cell migration determination. Pimaric acid induced cytotoxicity in human ovarian cancer cells (PA-1) in a dose-dependent manner without causing too much cytotoxicity in human ovarian epithelial cells (T1074). Cell morphology in treated cancer cells showed significant changes compared to untreated controls. Furthermore, it was observed that the cytotoxic effects of Pimaric acid were apoptosis-mediated and caspase-dependent cascade. Western blotting analysis showed that the expression of apoptosis-associated proteins like BAX, p-53 and caspase-3 was enhanced and BCL-2 expression was diminished. The induction of cytotoxicity was mediated via endoplasmic reticulum stress through expressions of related proteins which showed a tremendous increase in p-PERK, PERK, AT-4, CHOP and IRE-1 levels after treatment. Cell cycle analysis through cytometry showed significant results as it revealed G2/M phase cell cycle arrest. Furthermore, the in vitro wound healing assay showed specific anti-migratory effects of Pimaric acid on PA-1 cells. In conclusion it can be assumed that Pimaric acid may act as a potential anticancer agent against ovarian carcinoma, however further investigations are required to validate this initial claim.

Keywords: Pimaric acid, ovarian cancer, apoptosis, cell cycle, caspase

INTRODUCTION

Ovarian cancer (OC) is a lethal malignancy affecting large numbers of women across the globe (Miree et al., 2021). Due to its asymptomatic behaviour, the maximum number of patients is diagnosed at later disease stages. Only 2.5% of women are diagnosed of OC worldwide, but the death rate is 5%. Poor prognosis results show decreased 5-year overall survival (OC) rate, which stands at 25% to 30% (Fleming et al., 2011). In 2018 alone, around 22,000 new patients and 14,000 deaths due to OC were expected in United States.

Ovarian cancer is divided into three main subtypes: epithelial OC, germ cell OC and sex cord-stromal OC (Miree et al., 2021). Various factors contribute to the etiology of this disease including biological, physical, chemical, genetic, immune factors, carcinogenic factors, and poor lifestyle choices. Intraperitoneal chemotherapy has revealed significant improvements in later stages of epithelial OC. Still, in 2012, only less than 50% of the patients were treated with intraperitoneal chemotherapy due to a higher number of side effects (Reid et al., 2017; Kurnit et al., 2021).

OC has a very high relapse rate and develops high chemotherapy resistance, resulting in high mortality and poor prognosis (Feng et al., 2021). An increased overall survival rate was reported after secondary surgery in case of relapsing. Thus it may be considered for the patients who have a disease-free time interval of half a year (Fleming et al., 2011; Al Rawahi et al., 2013). Lethality of OC gets amplified mainly of poor prognosis, diagnosis at later stages, drug resistance and side effects of chemotherapy. Therefore there is a pressing need to overcome the shortcomings of conventional chemotherapeutic agents and move to novel and effective ones.

Apoptosis plays a crucial role in maintaining the balance between cell division and death, mandatory for the normal physiology of cells. Hence, downregulation of this process results in leading disorders, including cancer. Moreover, minimal inflammation is witnessed during apoptotic cell death, which has attracted strong attention in developing chemo-preventives targeting apoptosis. Apoptosis is modulated by intra and extra-cellular signalling transduction known as intrinsic and extrinsic apoptosis, respectively (Putcha et al., 2002). Death receptor proteins stimulates extrinsic pathway, while intrinsic pathway is mostly mitochondrial mediated. Intrinsic apoptosis begins with the insertion of Bax/Bak proteins into the mitochondrial membrane, which stimulates the release of cytochrome-c into cytosol (Pena-Blanco and Garcia-Saez, 2018). Cytochrome-c initiates a number of proapoptotic interactions within cytosol which ultimately leads to apoptotic cell death. One of the main functions performed by cytochrome-c is activation of different caspases (Jiang & Wang, 2004). So, anticancer molecules are always tested in their capacity to activate apoptotic pathways in cancer kills.
Due to their amazing structural diversity and potential bioactivity profiles, natural products continuously influence the drug discovery researchers (Tang et al., 2010). The carboxylic acid group shows a key role in bio-systems and drug design. Over 450 drugs containing carboxylic acid functional group are being used for humans (Kuo et al., 2010). Pimaric acid—a naturally occurring resin carboxylic acid—has revealed several pharmacological activities, including cancer (Zi & Simoneau, 2005). Treatment of brain tumor cells with Pimaric acid has been reported to significantly kill the tumour cells (Hoa et al., 2009). Herein, the current study was performed to examine the anticancer effects of Pimaric acid in human ovarian cancer cells.

METHODS

Cell culture

Cell lines T1074 (human ovarian normal epithelial cells) and PA-1 (human ovarian cancer cells) were purchased from ATCC. Cells were grown in culture for 24 h in RPMI-1640 medium mixed with 10% FBS and supplemented with antibiotic solution (100 U/ml). Cells were grown under normal conditions (5% CO₂ with 95% humidified air). Cells were either left untreated or treated with different concentrations of Pimaric acid. Pimaric acid (CAS 127-27-5) was obtained from Santa Cruz.

Reagents

Antibodies against p-PERK (Cat no. 3179S), PERK (Cat no. 3192S), AT-4 (Cat no. 11815S), CHOP (Cat no. 2895S), IRE-1 (Cat no. 3294S), Cyclin-B1 (Cat no. 4138S) and β-actin (Cat no. 49678) were purchased from Cell Signalling Technology, MA, United States. All other chemicals used were acquired from Sigma-Aldrich (St. Louis, MO, USA).

MTT assay. The cytotoxicity of Pimaric acid was determined through MTT assay. Both the cell lines T1074 and PA-1 (1×10⁴ cells) were plated using 96-well plates and subjected to incubation for 12 h in a 5% CO₂ incubator. Incubation was followed by treatment with different doses of Pimaric acid (0, 2.5, 5, 10, 20, 40, 80, 160 and 320 μM). After treatment, cells were further incubated for 48 h in a 5% CO₂ incubator at 37°C. At the end of Pimaric acid treatment, MTT (Sigma) stock solution of 5 mg/mL concentration and volume 100 μL was supplemented to cells with 4 h of incubation. The formazan crystals then produced are dissolved using DMDO, and thereafter, absorbance was measured at 580 nm using OPTImax microplate reader (Molecular Devices, San Jose, California, USA).

Cell morphology determination by phase-contrast microscopy. Cell morphological changes of Pimaric acid-treated cells were determined by phase contrast microscopy. Briefly, 4×10⁴ PA-1 human ovarian cancer cells were incubated for 48 h with varying concentrations of Pimaric acid (0, 5, 10 and 20 μM) in 6-well plates. Afterward, RPMI-1640 medium was discarded and cells were washed using phosphate buffered saline (PBS). The morphological differences between cells were visible. Phase contrast inverted microscope (Leica DMI 3000B, Germany) was used (magnification x200) to detect the morphological changes in apoptotic cells (Syed et al., 2015).

Cell apoptosis determination. PA-1 human ovarian cancer cells were seeded at a concentration of 3×10⁴ cells per well in 96-well plates. Seeding was followed by treatment with Pimaric acid at varying concentrations (0, 5, 10 and 20 μM). Cells were then incubated at 37°C for 24 h. After incubation of treated cells, 4’,6-diamidino-2-phenyindole staining (DAPI) was performed. The treated cancerous cells were then washed in PBS and fixed using 10% formaldehyde. Finally, DAPI-stained cells were examined under fluorescence microscope.

To determine the extent of apoptotic cells TUNEL assay was used. PA-1 human ovarian cancer cells were seeded with different concentrations of Pimaric acid using 6-well plates with coverslips to protect from any contamination for 12 h. Seeded cells were washed and fixed with PBS with acetone-methanol (1:1), respectively, for 15 min. DNA damage by apoptosis induction was evaluated through terminal deoxynucleotidyl transferase-UTP mediated nick end labelling using in Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s protocol.

Cell cycle analysis. For cell phase distribution of PA-1 human ovarian cancer cells, cells were harvested and PBS-washed. Washing was followed by treatment with varying doses of Pimaric acid (control, 5, 10 and 20 μM). These treated cells were then subjected to further washing and fixation (70% ethanol). After that, cells were suspended in 250 μg/mL of RNase1 and 50 μL/mL of propidium iodide (PI) solution and incubated for one hour at room temperature. Finally, grouping was performed such that 10000 cells were arranged in each group and examination was done through fluorescence-activated cell sorting using FACSCalibur flow cytometer.

In vitro wound healing assay. PA-1 human ovarian cancer cells at a density of 3×10⁴ were placed in 6-well plates using RPMI-1640 medium. Using a 10-μL pipette tip, scratch or wound was produced in each well, and then cells were left untouched till they reached 90% confluence of growth. RPMI-1640 medium was decanted and cells were washed with PBS. Cultures cells were treated with Pimaric acid at the varying concentrations (control, 5, 10 and 20 μM). Six randomly selected fields were captured (magnification ×200) using microscopy before and after treatment (24 h).

Western blotting analysis. For western blotting analysis, cells were lysed using RIPA buffer. Estimation of protein lysate was performed through bicinchoninic acid (BCA) assay. Protein samples were loaded on SDS-PAGE and were transferred to PVDF membrane. Next, these membranes were subjected to primary antibody treatment for 15 min. DNA damage by apoptosis induction was evaluated through terminal deoxynucleotidyl transferase-UTP mediated nick end labelling using in Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s protocol.

Statistics. All the data were presented as mean ± standard deviation (S.D.) from at least three independent experiments. Statistically, significant difference was taken as P<0.05. Differences among the groups were investigated through one-way ANOVA with Graphpad Prism 7 software.

RESULTS

Effects of Pimaric acid on cell viability

Cell viability effects on T1074 and PA-1 by Pimaric acid (Fig. 1A) were analysed through MTT assay. Cell viability was determined after treatment with different dos-
Anticancer effects of Pimaric acid. The results depicted that the viability of PA-1 cells decreased significantly with increasing drug concentrations (Fig. 1B). The cell cytotoxicity induced in normal T1074 ovarian epithelial cells was much lower than the cancer cells indicating its selective cytotoxicity.

Pimaric acid induced significant morphological alterations in PA-1 ovarian cancer cells

Morphological changes were determined by using phase contrast microscopy after exposure to varying doses of Pimaric acid (control, 5, 10 and 20 μM). Control cells showed no significant changes in the cell morphology and maintained their originality. But the morphology of treated PA-1 cells showed significant changes including cell shrinkage, membrane blebbing, cell rounding, and dropping contact with nearby cells (Fig. 2).

Induction of caspase-dependent apoptosis by Pimaric acid in PA-1 cells

Estimation of apoptosis in PA-1 cells was performed via DAPI staining and TUNEL assay. DAPI staining revealed significant changes in the morphology of the cells after Pimaric acid exposure (control, 5, 10 and 20 μM). Fluorescence microscopy showed membrane blebbing and the onset of formation of apoptotic crops after treatment. No such effect was observed

Figure 1. (A) Chemical structure of Pimaric acid. (B) Effect of Pimaric acid on the proliferation of T1074 and PA-1 cells were evaluated through MTT assay.

The experiments were performed three times and in triplicates, *p<0.05.

Figure 2. The effects of Pimaric acid on morphology of PA-1 human ovarian cancer cells.

Pimaric acid-treated cells showed significant morphological changes like cell shrinkage, membrane blebbing cell rounding, and dropping contact with nearby cells. The experiment was performed three times and in triplicates.

Figure 3. Title for the whole figure

(A) DAPI staining (blue) and TUNEL assay. Apoptosis determination at varying concentrations of Pimaric acid (control, 5, 10 and 20 μM). As indicated treated cells showed apoptotic crop formation and membrane blebbing. TUNEL-positive apoptotic cells were detected by localized FITC-fluorescence (green). (B) Effect of Pimaric acid assessed by western blotting on the expression of apoptosis related proteins including BCL-2, BAX, p-53 and caspase-3. The results depicted that expression of BAX, p-53 and caspase-3 increased and that of BCL-2 decreased post Pimaric acid treatment. Actin was taken as loading control. (C) Shows densitometry analysis of western blots. The experiments were performed three times and in triplicates.
Apoptotic studies were not carried out in case of normal cells (T1074 ovarian epithelial cells). Furthermore, it was revealed that the number of apoptotic cells increased with increasing dose of Pimaric acid. Next, TUNEL assay revealed that the apoptosis induction by Pimaric acid was mediated via DNA damage as the frequency of stained cells increased with increasing molecule concentration (Fig. 3A). Furthermore, western blotting analysis was performed to evaluate the effects of Pimaric acid on the expressions of apoptosis-related proteins. Results showed that the expression of BAX, p-53 and caspase-3 increased post Pimaric acid exposure, however expression level of BCL-2 decreased significantly (Fig. 3B), revealing induction of caspase-dependent apoptosis.

Antiproliferative effects of Pimaric acid via endoplasmic reticulum stress and cell cycle arrest

The expression of endoplasmic reticulum (ER) stress related proteins in treated PA-1 human ovarian cancer cells were evaluated via western blotting analysis, depicting that the levels of ER stress related proteins (p-PERK, PERK, AT-4, CHOP and IRE-1) increased significantly on increasing the molecule concentration (control, 5, 10 and 20 μM), suggesting that the antiproliferative effects of Pimaric acid on PA-1 cells are clearly induced via induction of ER stress (Fig. 3A).

After exposure of PA-1 human ovarian cancer cells with the test molecule at different concentrations (control, 5, 10 and 20 μM), cell cycle analysis was performed via fluorescence-activated cell sorting using flow cytometer. The results showed that the number of S phase and G0/G1 phase cells decreased with increased dose concentrations of Pimaric acid. There was a tremendous increase in the number of G2/M phase cells, suggestive of cell cycle arrest at this phase of the cell cycle distribution (Fig. 4B). It was further validated via western blotting analysis which revealed significant decrease in the expression of Cyclin-B1 protein (Fig. 4C).

Inhibition of cell migration by Pimaric acid on PA-1 human ovarian cancer cells

The impact on PA-1 cell migration by Pimaric acid was studied through *in vitro* wound healing assay. Cells were treated with different doses of Pimaric acid after an *in vitro* wound was scratched in each well. Photographs were captured after 0 h and 24 h of exposure at 0 μM (untreated control) and 10 μM of Pimaric acid concentration. The pictures suggested that the number of migrated cells was reduced compared to untreated controls (0 μM) after 24h of exposure (Fig. 5).

DISCUSSION

There is an alarming increase in the incidence rate of ovarian cancer resulting in high mortality worldwide. Poor prognosis, diagnosis at later-stage, high relapse, chemotherapy resistance and chemo-side effects are the major causes of mortality of this malignancy. To overcome these drawbacks of the conventional chemotherapy, we need to switch to novel and efficient methods, chemotherapeutic agents and explore different target pathways to curb OC (Rikova et al., 2007; Bergethon et al., 2012; Siegel et al., 2020). Endoplasmic reticulum response (ERR) plays a key role in regulating cell apoptosis, cell proliferation and cell autophagy in different human cancers. Its association is perhaps found in large numbers of biological systems. When there is an increase...
in the accumulation of unfolded proteins within a cell, ER releases large quantity of Bip proteins, enhancing and amplifying protein folding, reducing the amount of unfolded proteins and restoring ER function (Shaw et al., 2014). Herein, we testified the activity of Pimaric acid on cell proliferation of ovarian cancer cells via ER stress, caspase-dependent apoptosis, cell cycle arrest and inhibition of cell migration. Cell viability was determined by MTT assay and the results revealed that the cytotoxicic effects were significant on PA-1 cells as compared to T1074 cells. It also revealed that the cytotoxic effect of the test molecule were dose-dependent. Similar type of inhibitory function of Pimaric acid has been reported in brain tumor cells (Hoa et al., 2009). Furthermore, cell morphological changes were determined by phase-contrast microscopy, which showed that control cells retained their original identity in contrast with treated cells which showed significant morphological changes like cell shrinkage, membrane blebbing, cell rounding, and dropping contact with nearby cells. Next, DAPI staining and TUNEL assays were performed to assess whether the cytotoxic effects were due to apoptosis induction. Both the assays showed an increase in the number of apoptotic cells after treatment with Pimaric acid, showing membrane blebbing with formation of apoptotic crops. It was also unveiled that the number of apoptotic cells increases with increasing dose concentrations. Furthermore, the expressions of apoptosis-related proteins were altered by the exposure of target cells to the test molecule, and the expression of BAX, p-53 and caspase-3 was increased as compared to the BCL-2 expression which was suppressed. It was also observed through western blotting analysis that whether the apoptosis was caspase and ER stress mediated the results showed tremendous increase in the expressions of p-PERK, PERK, AT-4, CHOP and IRE-1, indicating ER stress. Activation of IRE1-JNK-CHOP signalling also been reported by levopimaric acid in gastric cancer cells (Kim et al., 2018). Afterwards, cell cycle analysis was performed after treatment with Pimaric acid. The results suggested a tremendous increase in the number of G2/M phase cells compared to the S phase and G0/G1, clearly indicating that Pimaric acid induced G2/M phase cell cycle arrest. Cell cycle arrest has been reported by levopimaric acid in human hepatoma cells (Qi et al., 2018). Western blotting analysis also showed that the expression of CyclinB1 diminished after increasing the molecule concentrations. Finally, cell migration results depicted that the cell migration of PA-1 human ovarian cancer cells was significantly suppressed with increasing doses of the test molecule. The above results indicate that Pimaric acid is a potential anticancer agent against human ovarian cancer cells and merits further investigations.

CONCLUSIONS

In conclusion, through all the above assays and results it is evident that Pimaric acid is a potential anticancer agent against PA-1 human ovarian cancer cells. The results revealed that Pimaric acid induces antiproliferative effects in human ovarian cancer cells via ER stress, caspase-dependent apoptosis, cell cycle arrest, and inhibition of cell migration.

Conflict of interest

The authors declare that there is no conflict of interest to indicate.

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