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ORIGINAL ARTICLE

Piperine alkaloid induces anticancer and apoptotic effects in cisplatin resistant ovarian carcinoma by inducing G2/M phase cell cycle arrest, caspase activation and inhibition of cell migration and PI3K/Akt/GSK3β signalling pathway

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Summary

Purpose: *Ovarian cancer is one the prevalent cancers in women and is responsible for 5% of all the cancer-related mortality. Owing to late diagnosis, frequent relapses, side effects of chemotherapy, development of drug resistance, there is pressing need to screen out novel and effective treatment options. Herein, we examined the anticancer effects of a secoiridoid glycoside Piperine against ovarian cancer cells.*

Methods: *CCK8 assay was used to examine the anti-proliferative effects. DAPI and annexin V/propidium iodide (PI) staining assays were used to examine apoptotic cell death. Cell cycle analysis was performed by flow cytometry. The protein expressions were examined by western blotting.*

Results: *Piperine inhibited the growth of the ovarian cancer OVACAR-3 cell with IC_{50} of 28 μM. In contrast, Piperine had low cytotoxic effects on the normal astrocytes (SV40) cells with an IC_{50} of 200 μM. Also, Piperine exerted antiproliferative effects on the OVACAR-3 ovarian cancer cells by apoptotic cell death. This was concomitant with upregulation of apoptotic proteins such as caspase 3 and 9 and Bax expressions. Piperine also induced arrest of the OVACAR-3 cells at the G2/M phase of the cell cycle. Finally, Piperine also blocked the PI3K/Akt/GSK3β signal transduction pathway in OVACAR-3 ovarian cancer cells.*

Conclusions: *These results suggest that Piperine exerts potent anticancer effects on ovarian cancer cells and may prove beneficial in the management of ovarian cancer.*

Key words: *ovarian cancer, piperine, apoptosis, cell cycle arrest*

Introduction

Ovarian cancer is one of the severe female reproductive tract cancers accounting for 2.5% of all malignancies in women [1]. It is also responsible for 5% of all cancer related deaths in women throughout the world [2]. The frequency of ovarian cancer has been reported to vary among different ethnic groups and geographical areas [3]. Its treatment is highly dependent on the stage of disease. In case of localised ovarian cancers surgery is generally recommended. However, if the disease has spread beyond the ovaries, a combination of surgery and chemotherapy is recommended [3]. The currently used drugs exhibit severe side effects that impair the patient's quality of life [4]. Moreover, the development of drug resistance among cancer cells imposes a great challenge in the treatment of cancers [4]. Henceforth, the new treatment options that would overcome these issues may prove beneficial in the management of ovarian cancer. In order to combat the severe environmental stresses, plants

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have developed diverse ways to adapt themselves over the course of evolution. One of the imperative mechanisms is to synthesize a battery of chemicals, often referred as secondary metabolites [5]. Since, times immemorial, the plant extracts rich in secondary metabolites have been used for treating human abnormal conditions. Some of the plant extracts are used as medicines in different traditional medicine systems even today. However, the advent of natural product chemistry allowed the isolation of the bioactive molecules from plants and their subsequent use as drugs in the purest form [6]. Piperine is an important alkaloid that has been reported to exhibit enormous pharmacological potential [7]. It is isolated from several plant species such as *Piper longum* and *Piper nigrum* [8]. Several of the bioactivities of piperine have been reported which include insecticidal, anti-inflammatory, anti-bacterial and anticancer [9]. Herein, we examined the anticancer effects of Piperine, an important alkaloid of plant origin, against the cisplatin-resistant human OVACAR-3 ovarian cancer cells and attempted to explore the molecular mechanisms responsible for its anticancer effects.

Methods

Cell viability determination

The cell viability was monitored by the CCK8 assay. In brief, the transfected OVACAR-3 ovarian cancer cells and normal human astrocytes cells were seeded in 96-well plates and subjected to treatment with varied concentrations of Piperine at 37°C for 24 h. Thereafter, 10 µL of CCK-8 solution were added to the cell culture and incubated for 2 h at 37°C in a humidifier (5% CO_2, 95% O_2). Optical density $(OD)_{450}$ was taken with the help of a microplate reader to determine the cell viability.

Apoptosis detection

The OVACAR-3 cells were cultured at the density of 0.6×10^6 in 6-well plates. Following 12-h incubation, the OVACAR-3 cells were subjected to Piperine treatment for 24 h at 37°C. As the cells cast off, 25 µl cell cultures were put onto glass slides and stained with DAPI. The slides were cover-slipped and examined with a fluorescent microscope. Annexin V/PI staining was performed as described previously [10].

Cell cycle analysis

The OVACAR-3 cells were treated with varying concentrations of piperine and incubated for 24 at 37°C. The cells were washed with phosphate buffered saline (PBS). Afterwards, the Piperine-treated OVACAR-3 cells were stained with PI and the distribution of the cells in cell cycle phases was assessed by FACS flow cytometer.

Western blot analysis

Protein expression estimation was carried out by western blotting. The Piperine-treated OVACAR-3 cells were harvested with centrifugation and were then lysed in lysis buffer containing protease inhibitor. Around 50 µg of proteins from each sample were subjected to separation and transferred to polyvinylidene difluoride (PVDF) membrane. Next, fat-free milk was used to block the membrane at room temperature for 1 h. Afterwards, the membranes were treated with primary antibodies at 4°C overnight. Subsequently, the membranes were incubated with secondary antibodies. Finally the band signal was detected by Odyssey Infrared Imaging System. Actin was used as control for normalisation.

Results

Piperine inhibits OVACAR-3 ovarian cancer cell growth

To determine the anticancer effects of Piperine, the OVACAR-3 cells were treated with 0-100 µM concentrations of Piperine and then subjected to CCK8 assay. The results of the CCK8 cell viability assay showed that Piperine caused concentration-dependent decrease in the viability of the OVACAR-3 cells (Figure 1A). It was further found that at 24 h of incubation, Piperine showed an IC_{50} of 28 µM against the OVACAR-3 ovarian cancer cells. In

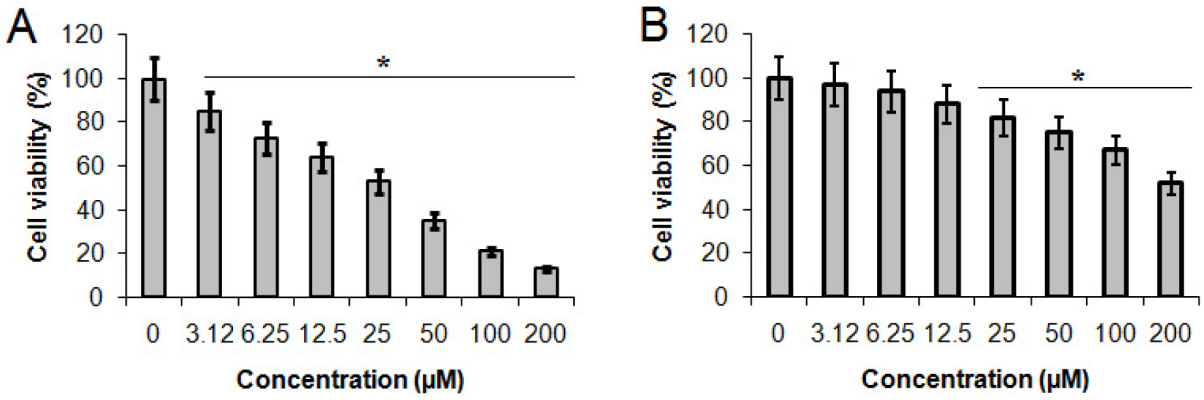

Figure 1. Effects of Piperine on the **(A)** human ovarian cancer OVACAR-3 and **(B)** normal human SV40 cells as determined by the cell viability assay. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

contrast, the Piperine did not exhibit significant toxic effects on the normal human SV40 astrocytes as evidenced from the IC_{50} of 200 μM (Figure 1B).

Piperine triggers apoptotic cell death of OVACAR-3 cells

The apoptosis in the Piperine-treated OVACAR-3 cells was determined by DAPI staining. The DAPI staining revealed that Piperine triggered apoptosis as evidenced from nuclear fragmentation of the Piperine-treated OVACAR-3 cells (Figure 2). Moreover, the results of the orange colored cells increased with increase in the concentration of Piperine, indicative of apoptotic cell death. Annexin V/PI staining showed that the apoptotic OVACAR-3 cell percentage increased to about 26.27% at 56 μM concentration of Piperine as compared to approximately 9% in the untreated OVACAR-3 cells (Figure 3). Further, the expression of Bax was significantly enhanced while that of Bcl-2 was decreased upon Piperine treatment (Figure 4).

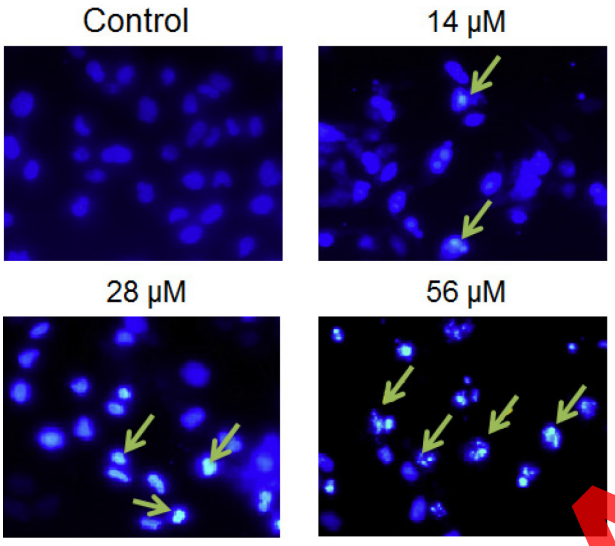

Figure 2. DAPI staining showing that Piperine triggers apoptotic cell death in the human OVACAR-3 ovarian cancer cells. The experiments were performed in triplicate. The arrows indicate apoptotic cells which increased with increasing dose of Piperine.

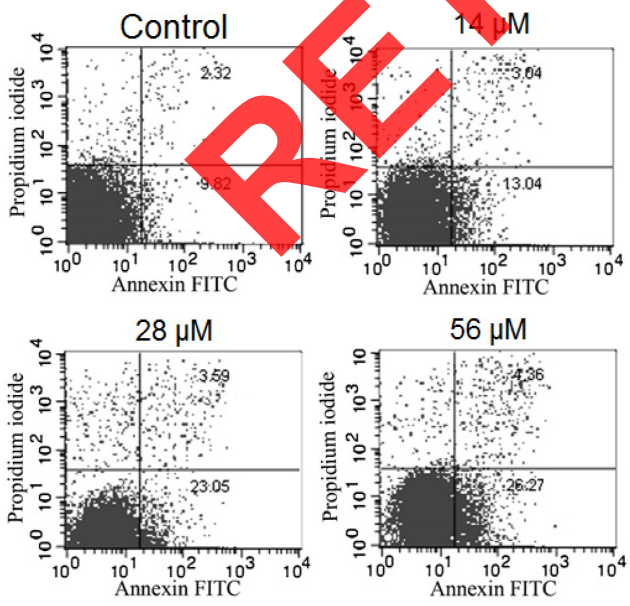

Figure 3. Annexin V/PI staining showing the effect of Piperine on the percentage of the apoptotic of OVACAR-3 cells. The experiments were performed in triplicate. The Figure indicates increase in the percentage of apoptotic cells (both early and late apoptotic cells) with increasing piperine dose.

Piperine activates caspases in OVACAR-3 cells

As Piperine induced apoptosis in the OVACAR-3 ovarian cancer cells, immunoblotting was carried out to assess the effects on the expression of Caspase-3 and Caspase-9. The results showed that Piperine caused a significant increase in the expression of Caspase-3 and 9. Piperine exerted these effects on the OVACAR-3 ovarian cancer cells in a concentration-dependent manner.

Piperine causes the G2/M arrest of OVACAR-3 cells

The effects of Piperine were also investigated on the cell cycle distribution of the OVACAR-3 cells

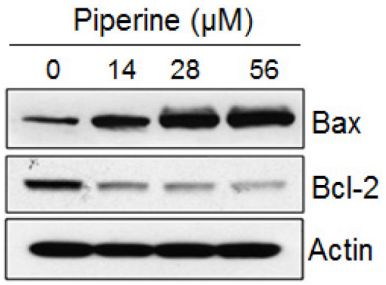

Figure 4. Piperine increased the expression of Bax and decreased the expression of Bcl-2 dose-dependently as determined by western blot analysis. The experiments were performed in triplicate.

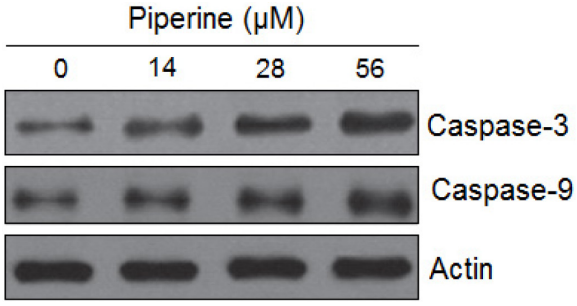

Figure 5. Activation of caspase-3 and caspase-9 by Piperine as determined by the western blot analysis. Piperine treatment led to increased expression of both caspase-3 and caspase-9 in a dose-dependent manner. The experiments were performed in triplicate.

by flow cytometry and showed that Piperine caused significant increase in the percentage of the G2/M phase OVACAR-3 cells. The percentage of the G2/M phase cells increased from 21.05% in the control to 65.1% at 56 μM (Figure 6).

Piperine suppresses the migration of the OVACAR-3 cells

The effects of piperine were examined on the migration of the OVACAR-3 cells by transwell assay. The results showed that piperine suppressed the migration of the OVACAR-3 cells (Figure 7). Moreover, these effects of piperine on the migration of the OVACAR-3 cells were concentration-dependent.

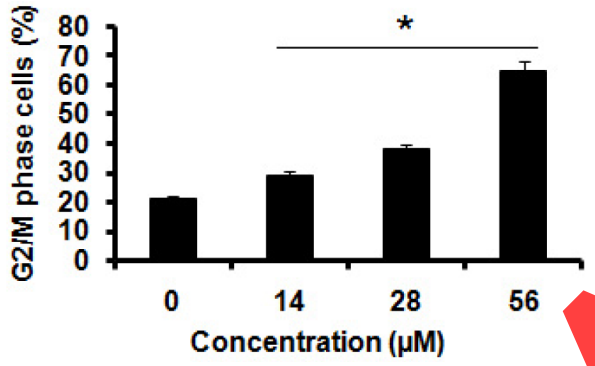

Figure 6. Effects of Piperine on the distribution of the OVACAR-3 cells in different cell cycle phases as determined by flow cytometry. Piperine treatment led to G2/M phase cell cycle arrest (increase in G2/M phase cells) dose-dependently. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

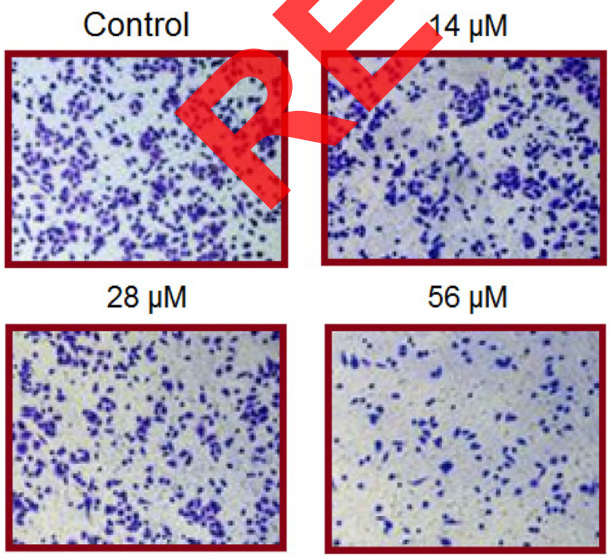

Figure 7. Effect of Piperine on the migration of the OVACAR-3 cells as determined by the transwell analysis. The experiments were performed in triplicate. Piperine treatment led to inhibition of cell migration in OVACAR-3 human ovarian cancer cells.

Piperine inhibits the JNK/MAPK signalling pathway in OVACAR-3 cells

We also wanted to study the effects of Piperine on the PI3K/Akt/GSK3β signalling pathway by western blot analysis at 0, 14, 28 and 56 μM concentrations. The results showed that Piperine caused decrease in the expression of p-PI3K, p-AKT and GSK3β in a concentration-dependent manner (Figure 8). However, no visible effects were observed on the total PI3K and AKT.

Discussion

Ovarian cancer is one the prevalent cancers in women and is responsible for 5% of all the cancer-related mortality. Owing to late diagnosis, frequent relapses, side effects of chemotherapy and the development of drug resistance, there is pressing need to screen out novel and effective treatment options [3,11]. Plant-derived molecules have shown exceptional potential to inhibit the growth and development of cancers [12]. They inhibit the growth of cancer cells via a diversity of mechanisms such as apoptosis, autophagy and cell cycle arrest [13]. Plant-derived secondary metabolites have also been shown to block signalling pathways that are generally overexpressed in cancer cells, while in other cases, they activate the signalling pathways that are generally blocked in cancer cells [14]. Moreover, plant-derived molecules are believed to be safer for human consumption owing to their minimal adverse effects [15]. Therefore, it is believed that anticancer drugs that are of plant-origin may show low or even no adverse effects on human

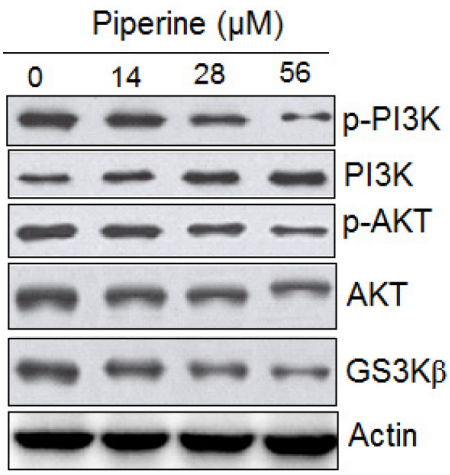

Figure 8. Effect of Piperine on PI3K/Akt/GSK3β signalling pathway in OVACAR-3 ovarian cancer cells as determined by western blot analysis. The results showed that Piperine caused decrease in the expression of p-PI3K, p-AKT and GSK3β in a concentration-dependent manner. The experiments were performed in triplicate.

body [15]. Herein, the anticancer effects of a plant-derived alkaloid Piperine was examined against the human OVACAR-3 cells as well as the normal SV40 astrocytes. The results showed that Piperine inhibited dose-dependently the growth of cancer cells an exhibited an IC_{50} 28 µM. However, it was interesting to see that Piperine exhibited minimal growth inhibitory effects on the normal human ovarian SV40 astrocytes, showing an IC_{50} of 200. These findings suggest that Piperine selectively targets the ovarian cancer cells. A number of investigations carried out on plant-derived molecules have shown that such molecules suppress the growth of cancer cells, for example, Isoalantolactone has been shown to halt the growth of the UM-SCC-10A cancer cells (head and neck squamous cell carcinoma) by triggering apoptosis [16]. In a yet another study, a plant-derived molecule Quinacrine has been reported to curb the growth of breast cancer cells by inhibiting of the topoisomerase activity [17].

The underlying mechanisms for the anticancer effects of Piperine were examined by carrying out DAPI and annexin V/PI staining. The results showed that Piperine induces apoptosis and the percentage of the apoptotic cells increases with increase in the concentration of Piperine. Bax and Bcl-2 are considered as vital biomarker proteins of apoptosis [18] and herein we observed that Piperine caused increase in the expression of Bax and decrease in the Bcl-2 expression, ultimately favoring apoptosis. The triggering of apoptotic cell death has also been reported to be associated with upregulation of different caspases [19] and herein we examined the impact of Piperine on the expression of caspase-3 and 9 and found that this molecule caused enhancement in the expression of both caspases 3 and 9 in a concentration-dependent manner.

Cell cycle arrest is another mechanism by which plant-derived anticancer agents have been reported to exert their anticancer effects [20]. Herein, we found that Piperine caused arrest of the OVACAR-3 cells in the G2/M checkpoint of the cell cycle. PI3K/Akt/GSK3β signalling cascade has been shown to be activated in the cancer cells and believed to be responsible for the development and progression of different mechanisms [21] and herein we found that Piperine blocks this pathway, suggestive of the potent anticancer effects of this molecule.

Conclusion

The findings of the present study suggest Piperine exerts significant anticancer effects on the human ovarian cancer cells. The anticancer effects of Piperine are mainly due to apoptosis induction and cell cycle arrest. Piperine may be utilised in the development of systemic therapy for ovarian cancer and deserves further studies.

Conflict of interests

The authors declare no conflict of interests.

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