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ORIGINAL ARTICLE

Anticancer activity of cucurbitacin-A in ovarian cancer cell line SKOV3 involves cell cycle arrest, apoptosis and inhibition of mTOR/PI3K/Akt signaling pathway

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Summary

Purpose: *Ovarian cancer is one of the deadly causes of gynecological cancer related mortality worldwide. Despite initial responses to chemotherapy, the disease consistently relapses. Therefore there is an urgent need for identification of anticancer lead molecules for treatment and management of ovarian cancer. The present study evaluated the anticancer activity of cucurbitacin-A on ovarian SKVO3 cancer cells.*

Methods: *The cell viability of SKVO3 cells was evaluated by MTT assay, while clonogenic assay was used to evaluate the effects on cancer cell colony formation. DAPI staining using fluorescence microscopy was used to evaluate the effects of the compound on apoptosis. Flow cytometry was used to study the effects on cell cycle phase distribution, reactive oxygen species (ROS) generation and mitochondrial membrane potential (MMP) loss.*

Results: *Cucurbitacin-A exhibited an IC_{50} of 40 μM against ovarian SKVO3 cancer cell line. It also caused DNA damage in SKVO3 cells and also prompted ROS mediated alterations in MMP. On the other hand, it triggered cell cycle arrest of SKVO3 at G2/M checkpoint. The activation of the PI3K/AKT/mTOR pathway plays a vital role in ovarian cancer tumorigenesis, progression and chemotherapy resistance. The results showed that cucurbitacin-A considerably inhibited the expression levels of key proteins of PI3K/Akt/mTOR signaling pathway.*

Conclusion: *The study showed that cucurbitacin-A is a potent agent against ovarian cancer cells, can be considered for further in vivo research and can also be developed as a possible lead molecule.*

Key words: *apoptosis, cell cycle, cucurbitacin-A, ovarian cancer, reactive oxygen species*

Introduction

Ovarian cancer is one of the most deadly causes of cancer related deaths across the world and chemotherapy still remains the keystone for its management [1,2]. Nonetheless, despite frequent initial responses to chemotherapy, the disease often relapses. Moreover, only limited chemotherapeutic agents are available for the management of ovarian cancer [3,4]. So far bevacizumab is the only approved therapy in ovarian cancer for which consistent analytical markers are yet to be established. Furthermore, except for p53 signaling pathway, the PI3K/Akt/mTOR pathway is probably the most repeatedly altered signaling pathway in cancer [1,5]. Consistent with this, first generation mTOR inhibitors exhibit substantial anticancer properties and several have even been approved for controlling several types of cancers which include pancreatic, renal and breast cancers [1,5]. Furthermore, PI3K, Akt together with second generation inhibitors of mTOR are undergoing clinical

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trials. Cucurbitacins are plant secondary metabolites chemically categorized as steroids. These are synthesized by a few species of plants, especially the members of the family cucurbitaceae. Normally cucurbitacins are found as glycosides and help plants to deter predators [6] and have been reported to exhibit anticancer activities against a diversity of cancer cell types [7-9]. However, antitumor activity of cucurbitacin-A against ovarian cancer cells has not been evaluated as yet. Therefore, the present study was aimed to evaluate the anticancer activity of cucurbitacin-A against human ovarian cancer cell line SKVO3.

Methods

Chemicals, reagents, cell lines and culture conditions

The following drugs and chemical reagents were used in the current study. Cucurbitacin-A and propidium iodide (PI) were procured from Sigma Aldrich, St. Louis, MO, USA. MTT kit was purchased from Roche (USA). DMEM was obtained from Gibco BRL, Carlsbad, CA, USA. All primary and secondary antibodies were purchased from Cell Signaling Technology, USA. Ovarian cancer SKOV31 cell line was procured by the Institute of Cell kept in DMEM and was supplemented with 10% FBS and antibiotics (100 µg/ml streptomycin and 100 U/ml penicillin G) in an incubator at 37°C (5% CO_2 and 95% air).

Cytotoxicity by MTT and colony formation assays

The cancer growth inhibitory effect of cucurbitacin-A on ovarian cancer SKOV3 cells was demonstrated by MTT assay. SKVO3 cells were grown at 1×10^6 cells per well in 96-well plates for 12 hrs and then exposed to 0, 10, 20, 40, 80, and 160 µM cucurbitacin-A dose for 48 hrs. To each well, MTT solution (20 µl) was added. Before the supplementation with 500µl of dimethylsulfoxide (DMSO), the medium was entirely removed. Afterwards, 500 µl DMSO was added to solubilize MTT formazan crystals. Finally, for determination of the optical density (OD), ELISA plate reader was used. To determine the effect of cucurbitacin-A on colony formation potential of ovarian cancer cell line SKOV3, exponentially growing cells were collected and counted with a hemocytometer. The cells were seeded at a density of 200 cells per well, followed by incubation for 48 hrs to allow the cells to attach. Later on, different doses (0, 20 40 and 80 µM) of cucurbitacin-A were added to the cell culture. The cells were again kept for incubation for 6 days following treatment with cucurbitacin-A. Afterwards, washing was done with PBS and methanol was used to fix colonies. Before being counted under light microscope, cells were stained with crystal violet for about 30 min.

Apoptosis and cell cycle phase distribution

For DAPI staining, SKOV3 cells were seeded at the density of 2×10^5 cells/well (2 ml) in 6 well plates and supplemented with with 10 to 40 mM cucurbitacin-A for 48 hrs. Thereafter, cells were subjected to DAPI staining. Finally, the cell samples were examined and photographed under fluorescence microscopy as previously described [10]. For cell cycle phase distribution the cells were seeded in 6-well plates (2×10^5 cells/well) and cucurbitacin-A was administered to the cells at doses of 0, 20 40 and 80 µM followed by 24-h incubation. Further procedure for cell cycle distribution was carried out as previously described [11].

Determination of ROS and MMP

For determination of ROS and MMP, SKOV3 cells were seeded at a density of 2×10^5 cells/well in a 6-well plate, kept for 24 hrs and then administered 0 to 80 µM cucurbitacin-A and incubated for 72 hrs at 37°C in 5% CO_2 and 95% air. Afterwards, the treated cells from all samples were harvested and washed twice with PBS. The cells were then re-suspended in 500 µl of DCFH-DA (10 µM) for ROS and $DiOC_6$ (1 µmol/l) for evaluation of MMP at 37°C in the dark for 30 min. The samples were then evaluated immediately using flow cytometer as described previously [12].

Protein expression by western blot analysis

Cucurbitacin-A-administered cells were washed twice in ice-cold PBS and then extracted with a RIPA buffer comprising of a mixture of 5% each of phosphate inhibitor and protease inhibitor. The cell lysates were centrifuged at 15,000g for 15 min. The protein concentrations were thereafter determined using Bio-Rad protein assay. The proteins' samples were then subjected to SDS-PAGE and then shifted to nitrocellulose membranes. The membranes were then probed with specific antibodies at 4°C overnight, followed by washing with buffered saline and incubation with the appropriate secondary antibody for 1 hr. The bands were then pictured using an ECL chemiluminescent detection kit (Perkin Elmer Cetus, Foster City, CA, USA).

Statistics

Data were expressed as mean±SD. Statistical significance was analysed by GraphPad Prism Demo, version 5 (GraphPad Software, San Diego, California, USA). $p<0.05$, $p<0.01$ and $p<0.001$ were considered as statistically significant.

Results

Cucurbitacin-A exerts growth inhibitory effects on SKOV3 cells

The results of MTT assay revealed that cucurbitacin-A exerted a potent antiproliferative effect on SKOV3 cells and reduced the cell viability concentration-dependently (Figure 1A). The IC_{50} of cucurbitacin-A was found to be 40 µM. In colony formation assay, it was seen that cucurbitacin-A-administered cells had decreased capacity to form colonies (Figure 1B).

Cucurbitacin-A induces apoptosis and G2/M cell cycle arrest

After separating the cells from cucurbitacin-A, apoptotic cells were identified by DAPI staining. Our results revealed that cucurbitacin-A induced apoptosis in a dose-dependent manner. The apoptotic cells were characterized by chromatin condensation, apoptotic body formation and deformed cell morphology (Figure 2). Analysis of cell cycle phase distribution by flow cytometry indicated that cucurbitacin-A caused cell arrest at G2/M checkpoint. After 48 hrs of treatment, cells in the G2/M population increased from 5.24% in control to 63.66% at 80 μM concentration (Figure 3). Furthermore, cucurbitacin-A prompted G2/M phase increase of SKOV3 cancer cells in a dose-dependent manner.

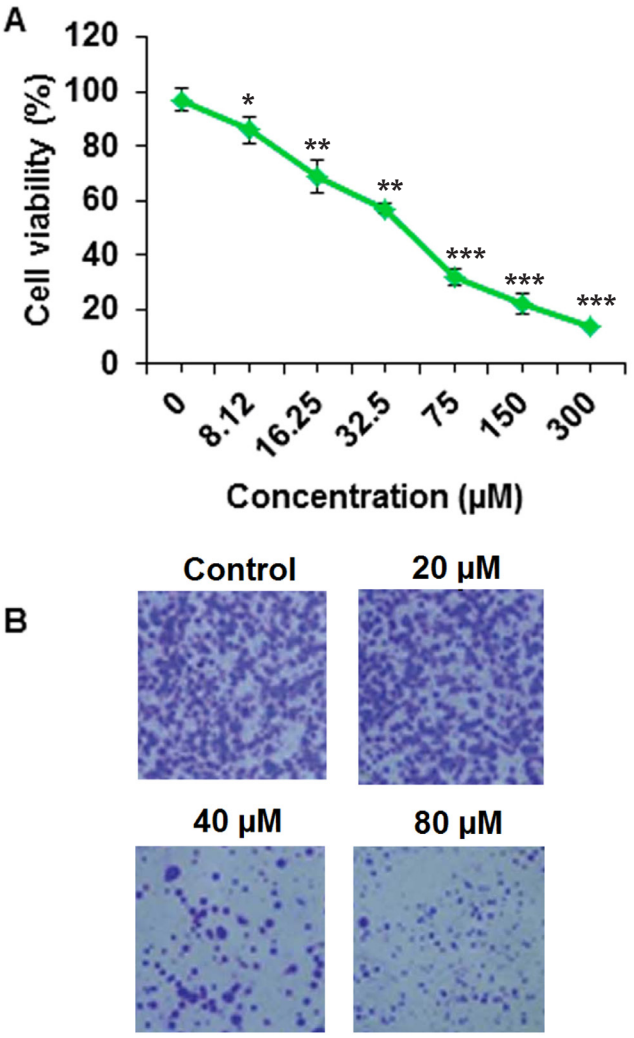

Figure 1. Cucurbitacin-A exerts a potent anti-proliferative effect on SKOV3 cells and reduces the cell viability in a concentration-dependent manner. (**A)** Colony formation assay, showing that cucurbitacin-A-administered cells had decreased capacity to form colonies (**B**). All the experiments were carried out in triplicate. *p<0.05, **p<0.01, ***p<0.001

Cucurbitacin-A triggers the ROS production and MMP reduction

From apoptosis inducing properties of cucurbitacin-A, we speculated that it might lead to ROS production and hence we calculated the ROS levels at different concentrations of cucurbitacin-A for 48 hrs. The results revealed that intracellular ROS levels of treated cells increased up to 197% as compared to untreated cells (Figure 4A). Therefore, cucurbitacin-A is an effective molecule for activating ROS in SKOV3 cells, since ROS produc-

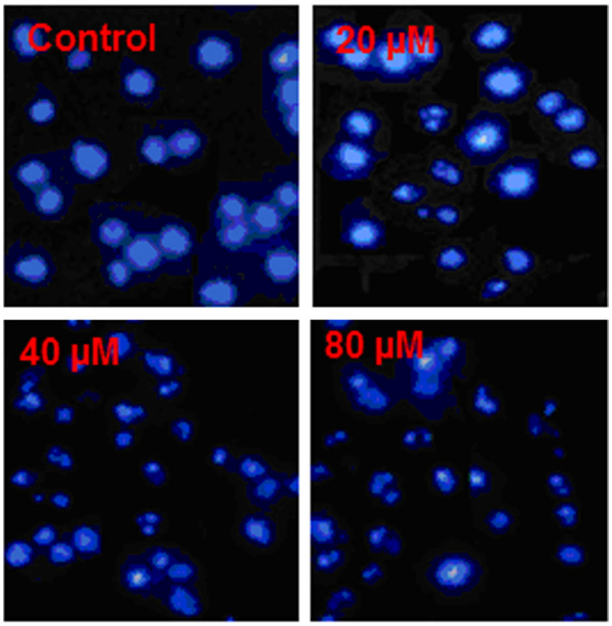

Figure 2. Cucurbitacin-A induced apoptosis in a dose-dependent manner. The cells were stained using DAPI and then analyzed by flow cytometry.

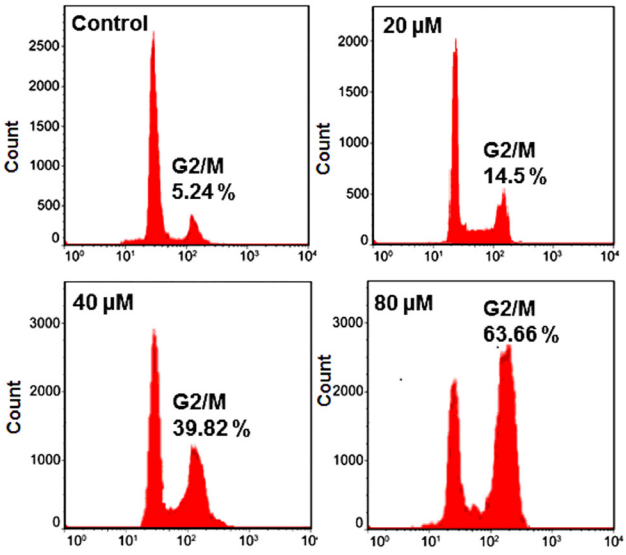

Figure 3. Analysis of cell cycle phase distribution by flow cytometry after 48 hrs of cucurbitacin-A treatment of SKOV3 cells. Cucurbitacin-A triggered cell cycle arrest of SKV03 cells at G2/M checkpoint in a dose-dependent manner.

tion is associated with mitochondrial malfunction as it interrupts the outer mitochondrial potential, and it induces release of the death-promoting proteins [13]. Therefore, we examined whether cucurbitacin-A decreased the MMP in SKOV3 cells and observed that cucurbitacin-A-treated SKOV3 cells displayed a considerable reduction in MMP in a dose-dependent manner. The MMP reduced up to 65% at 80 μM of cucurbitacin-A as compared to untreated control (Figure 4B).

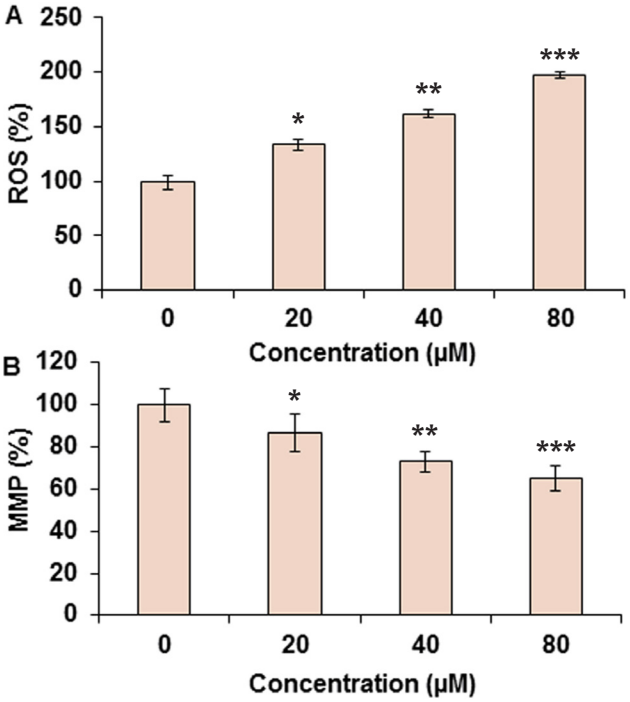

Figure 4. A: Effect of cucurbitacin-A on ROS levels at different concentrations in SKOV3 cells for 48 hrs, and **B:** on MMP in SKOV3 cells. All the experiments were carried out in triplicate. *p<0.05, **p<0.01, ***p<0.01

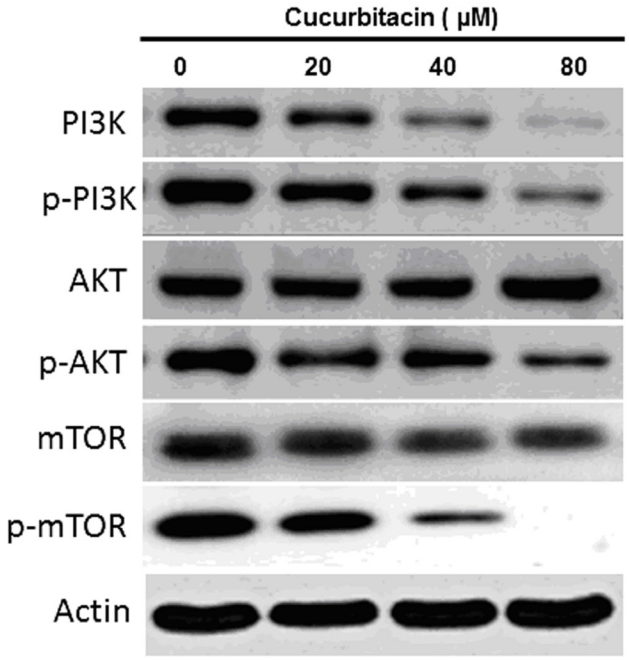

Figure 5. Western blotting revealed that cucurbitacin-A-administered cells showed a concentration-dependent downregulation of m-TOR and pm-TOR proteins.

Cucurbitacin-A targets m-TOR/PI3K/Akt signalling pathway

The m-TOR/PI3K/Akt signalling pathway is one of the main signaling cascades activated in cancer cells. To confirm whether cucurbitacin-A could modulate the protein expressions of m-TOR/PI3K/Akt signalling pathway, western blotting was carried out. The findings shown in Figure 5 indicate an interesting outcome. Compared to the untreated control cells, cucurbitacin-A administered cells showed a concentration-dependent downregulation of m-TOR and pm-TOR proteins. It also led to downregulation of PI3K/Akt protein expressions. Henceforth, it may be concluded that cucurbitacin-A prompted anticancer effects via modulation of m-TOR/PI3K/Akt signalling pathway.

Discussion

Ovarian cancer ranks fifth among the most lethal cancers and one of the leading causes of death among women in USA. The mortality due to ovarian cancer is 68% and 75% of ovarian cancers are diagnosed at stage III. With lower efficacy of presently available therapies, there is a tremendous demand for development of novel chemotherapeutic agents for this deadly disease [14,15]. Plant secondary metabolites have often been found useful as anticancer agents and with lower related toxicities, while they have gained much fame in recent times [16]. Cucurbitacin-A is also a plant-derived secondary metabolite and has been shown to possess important pharmacological potential [7-9]. In the present study we evaluated its anticancer activity against ovarian cancer cells. Our results indicated that cucurbitacin-A exhibited a low IC_{50} of 40 μM. It was observed that cucurbitacin-A significantly decreases the cancer cell viability in a dose-dependent manner and also lessens their colony forming potential. Moreover, it also induced apoptosis in SKOV3 cells and caused G2/M cell cycle arrest in a dose-dependent manner. Our results are in agreement with a previous study where several anticancer drugs, such as cisplatin, have been reported to induce apoptosis in cancer cells [17]. From the antiproliferative and apoptosis inducing potential of cucurbitacin-A, we speculated that cucurbitacin-A administration may lead to generation of ROS. Interestingly, cucurbitacin-A did induce production of ROS which was also associated with reduction in the MMP.

Our results are supported by several studies already reported in the literature. For instance, capsaicin causes ROS-mediated alterations in MMP which ultimately lead to apoptosis in pancreatic cancer cells [18]. These findings are promising since it is well established that ovarian cancer is one of the most lethal cancers and cucurbitacin-A could convert this undesirable outcome. Lastly, we also evaluated the effects of cucurbitacin-A on the protein expression levels of several proteins such as m-TOR, pm-TOR, PI3K, p-PI3K and Akt using western blot assay. Our results revealed that cucurbitacin-A-treated cells exhibited a concentration-dependent downregulation of m-TOR and pm-TOR proteins. Furthermore, it also led to suppression of PI3K/Akt protein expressions. It has been reported that activation of the PI3K/AKT/mTOR pathway plays a vital role in ovarian cancer tumorigenesis, progression and chemotherapy resistance [1]. Therefore, the inhibitory effect of cucurbitacin-A on these pathways may prove crucial in the treatment of ovarian cancer.

Conclusion

Taken together, we conclude that cucurbitacin-A may prove a potential candidate for the treatment of ovarian cancer by controlling m-TOR/PI3K/Akt signalling pathway. With limited drug options available for ovarian cancer and limited toxicity associated with this natural product, cucurbitacin-A represents a strong option and deserves further research.

Acknowledgements

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Conflict of interests

The authors declare no conflict of interests.

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