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ORIGINAL ARTICLE

Tanshinone l exhibits anticancer effects in human endometrial carcinoma HEC-1-A cells via mitochondrial mediated apoptosis, cell cycle arrest and inhibition of JAK/STAT signalling pathway

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Summary

Purpose: *Tanshinone I is an important plant-derived natural product that has been reported to exert impressive bioactivities, including antiproliferative effects against different types of cancer cells. In this study the anticancer effects of tanshinone I were examined on human endometrial cancer cells along with its mechanism of anticancer action.*

Methods: *Antiproliferative activity and apoptosis were investigated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and DAPI (4',6-diamidino-2-phenylindole) staining, respectively. Effects on reactive oxygen species (ROS) and mitochondrial membrane potential (MMP) were estimated by flow cytometry and western blotting was performed to examine the effect of tanshinone I on JAK/STAT signalling pathway proteins.*

Results: *The results of this study revealed that tanshinone I inhibited the proliferation of the human endometrial carcinoma HEC-1-A cells in a dose-dependent manner. The IC_{50} of tanshinone I was 20 μM. The antiproliferative effects were due to induction of apoptosis in human endometrial carcinoma HEC-1-A cells. Tanshinone I also caused increase the ROS levels in these cells which was linked with the reduction the MMP levels. Tanshinone I also modulated the expression of JAK/STAT signalling pathway proteins.*

Conclusion: *In conclusion, tanshinone I may prove beneficial in the development of systemic therapy for endometrial carcinoma and deserves further research including its in vivo anticancer effects which shall be our future research work in this project.*

Key words: *apoptosis, human endometrial carcinoma, reactive oxygen species, tanshinone I, HEC-1-A*

Introduction

Plants are natural chemical factories producing a wide diversity of chemical scaffolds. The metabolites produced by plants are either primary metabolites without which plant can't survive and secondary metabolites which are produced as a defence against the biotic and abiotic stresses [1]. Since, plants are always exposed to extreme environmental conditions, they have evolved to produce different types of secondary metabolites [2]. These metabolites have been shown beneficial in the treatment of human diseases. They have been used as antimicrobial and anticancer agents besides others [3,4]. 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 [5]. *Salvia miltiorrhiza* is an important medicinal herb and has been used in the treatment of several diseases and disorders. It has been reported to be a rich source of tanshinones [6,7]. Tanshinone I is a diterpenoid

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isolated from this plant and has been reported to exert anticancer effects on breast cancer cells [8]. In this study, the anticancer effects of tanshinone I were evaluated on the human endometrial carcinoma HEC-1-A cells. It has been reported that endometrial carcinoma causes significant number of deaths in women worldwide [9]. The therapeutic options for endometrial carcinoma include radiation or hormonal therapy as postoperative adjuvant treatment in most of the patients [10]. In this study it was observed that tanishone 1 caused significant reduction in the viability of these cells in a dose-dependent manner. The antiproliferative effects were found to be due to induction of apoptosis as evidenced from the results of DAPI and annexin V/propidium iodide (PI) staining. The induction of apoptosis was further confirmed by the Bax and Bcl-2 ratio. It was further observed that tanshinone I increased the ROS levels and decreased the MMP. Tanshinone I also inhibited the JAK/STAT pathway in HEC-1-A cancer cells. Taken together these results suggest tanshinone I could prove beneficial in the treatment of cancers in general and endometrial carcinoma in particular.

Methods

Cell lines and culture conditions

The human endometrial carcinoma HEC-1-A cells were procured from American Type Culture Collection. The cell line was then cultured and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBV), antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin), and 2 mM glutamine. The cells were cultured in CO_2 incubator (at 37°C with 98% humidity and 5% CO_2 for 24 hrs.

MTT assay

Human endometrial carcinoma HEC-1-A cell viability was determined by assay. In brief, the cultured endometrial cancer cells were seeded at a density of 1.5×10^4 in 96-well plates. This was followed by the addition of MTT solution in all the wells and then the absorbance at 570 nm was assessed using an ELISA plate reader.

Apoptosis assay

The human endometrial carcinoma HEC-1-A cells were seeded in 6-well plates (2×10^5 cells per well) and cultured for 24 hrs. The cells were then DAPI-stained to detect apoptosis by fluorescence microscopy as previously reported [11]. For the percentage of the apoptotic cells FITC-Annexin V/propidium iodide (PI) apoptosis detection kit was employed as per the instructions of the manufacturer.

Determination of ROS and MMP

Human endometrial carcinoma HEC-1-A cells were cultured in 6-well plates with a density of 2×10^5 cells per well and incubated for 24 hrs . The cells were then subjected to treatment with 0, 10, 20 and 40µM tanshinone 1 for 24 hrs at 37°C in 5% CO_2 and 95% air. Afterwards, the cells were harvested, subjected to PBS (phosphate buffered saline) washing and resuspended in 500 µL of DCFH-DA (10 µM) for determination of ROS and $DiOC_6$ (1 µmol/l) for assessment of MMP levels at 37°C in the dark for 30 min. The samples were then analyzed by flow cytometer.

Western blotting

The HEC-1-A endometrial carcinoma cells treated with tanshinone I were collected and treated with lysis buffer [Tris-HCl, sodium-dodecyl sulfate (SDS)], mercaptoethanol and glycerol. The extracts were boiled for 10 min in the presence of loading buffer followed by separation of cell extracts using 15% SDS-PAGE gel. The samples were then put onto polyvinylidene fluoride membranes and blocked using 5% skimmed milk powder. Membrane incubation with primary antibodies was performed overnight at 4°C with horseradish peroxidase-linked biotinylated secondary antibodies at 1:1,000 dilution for 2 hrs. Washing of the membranes with PBS was followed by visualization of the immunoreactive bands using the ECL-PLUS Kit according to the manufacturer's instructions. The immune complexes development was carried out using an ECL detection kit according to the manual protocol. The bands were analyzed using GelG-Doc2000 imaging system (Bio-Rad Laboratories GmbH, Munich, Germany).

Statistics

Data is shown as mean ± SEM and was statistically analyzed using Students-Newman-Keul's test or t-test. A p value<0.05 was considered to be statistically significant.

Results

Tanshinone I exerts antiproliferative effects on human endometrial carcinoma HEC-1-A cells

Tanshinone I (Figure 1) treatment of human endometrial carcinoma HEC-1-A cells caused significant reduction in the cell viability. With increase in the concentration of the natural compound tanshinone I, the cell viability decreased significantly, indicative of dose-dependent antiproliferative effects (Figure 2). The IC_{50} of Tanshinone I was also

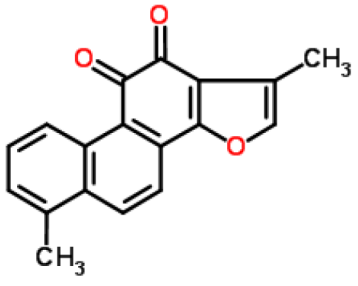

Figure 1. Chemical structure of Tanshinone I.

determined against the HEC-1-A cells and was found to be 20 μM at 24-h incubation.

Tanshinone I causes apoptosis of human endometrial carcinoma HEC-1-A cells

Tanshinones have previously been reported to exert antiproliferative effects on cancer cells by prompting apoptosis. Therefore, the apoptosis inducing potential of tanshinone was examined on the HEC-1-A cells by DAPI staining. The results showed that tanshinone I caused shrinkage and blebbing of HEC-1-A cells and with increase in the concentration of the tanshinone I, the number of cells with white colour nuclei increased (Figure 3). These charactertirestics clearly demonstrate that tanshinone I induced apoptosis in HEC-1-A carcinoma cells. Thereafter, the percentage of the apoptotic cell populations were also estimated by annexin V/PI staining and it was observed that tanshinone I caused a significant increase in the percentage of the apoptotic cell population (Figure 4). The apoptotic cells increased from 0.8% in the control to 45.62% at 40 μM tanshinone I concentration. The induction of apoptosis by tanshinone I on HEC-1-A cells was further confirmed by examining the expression of apoptosis-related proteins Bax and Bcl-2 where it was observed that the expression of Bax increased in a concentration-dependent manner while the expression of Bcl-2 was significantly decreased (Figure 5).

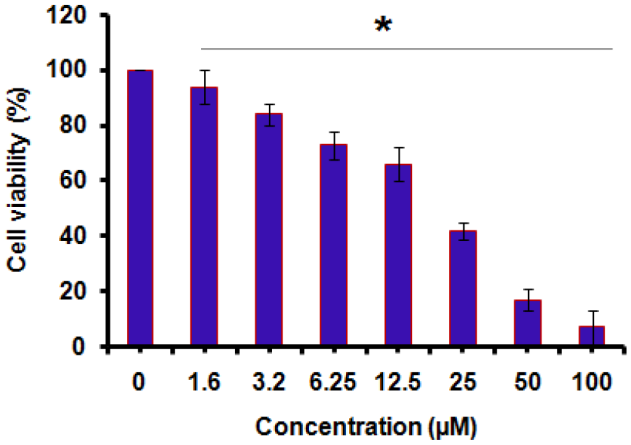

Figure 2. Tanshinone I effected the viability of human endometrial carcinoma HEC-1-A cells in a concentration-dependent manner. The values represent mean of three biological experiments (*p <0.05).

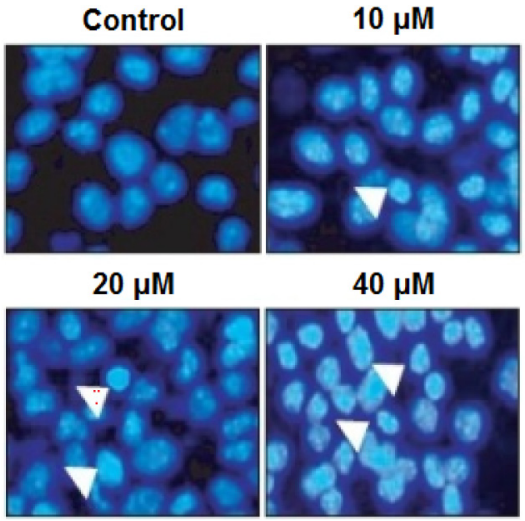

Figure 3. Tanshinone I triggered apoptosis in human endometrial carcinoma HEC-1-A cells as indicated by DAPI staining. The experiments were performed in triplicate. The Figure shows that Tanshinone I induces apoptosis in HEC-1A cells in a concentration-dependent manner (arrows).

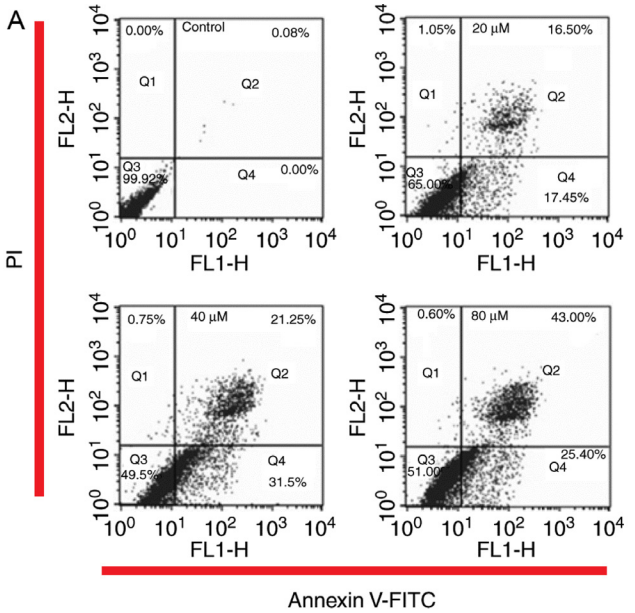

Figure 4. Annexin V/PI staining showing the percentage of apoptotic endometrial cells. The experiments were performed in triplicate. The Figure shows that apoptotic cell populations increase in a concentration-dependent manner upon Tanshinone I treatment.

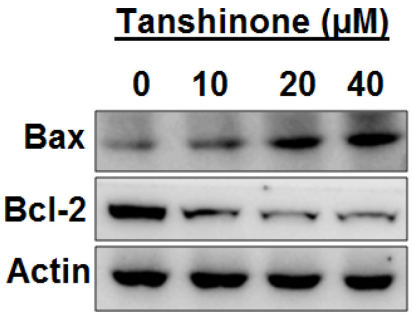

Figure 5. Western blotting analysis showing the effect of Tanshinone I on Bax and Bcl2 expression. The experiments were performed in triplicates. The Figure depicts that Tanshinone I triggers increase in the expression of Bax and decrease in the expression of Bcl-2.

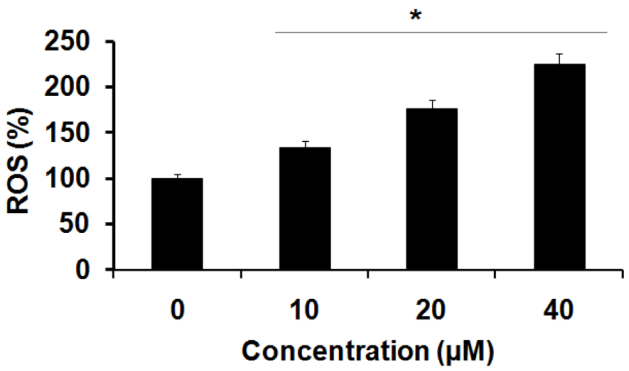

Figure 6. Effect of Tanshinone I on ROS levels in human endometrial carcinoma HEC-1-A cells. The values represent the mean of three biological experiments (*p <0.05). The Figure depicts that Tanshinone I causes upsurge of ROS in HEC-1A cells.

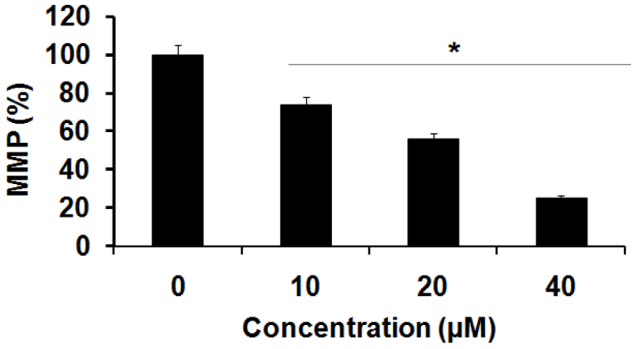

Figure 7. Effect of Tanshinone I on MMP levels in human endometrial carcinoma HEC-1-A cells. The values are the mean of three biological experiments (*p <0.05). The Figure shows Tanshinone I causes decline of MMP in HEC-1A cells.

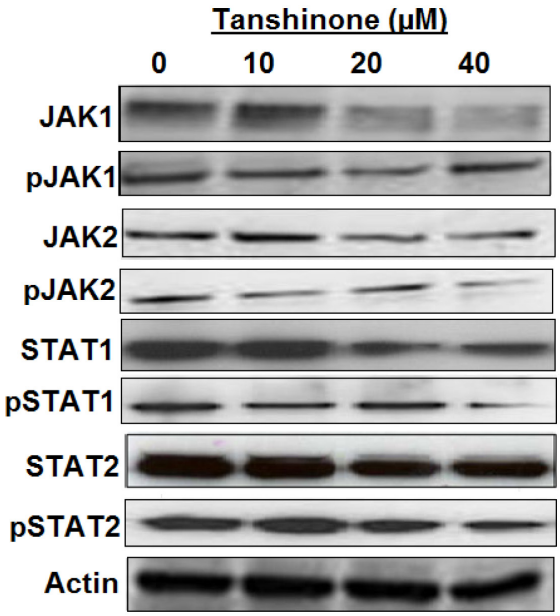

Figure 8. Western blotting analysis showing the effect of Tanshinone I on JAK/STAT pathway. The experiments were performed in triplicate. The Figure depicts that Tanshinone I causes inhibition of JAK/STAT pathway in HEC-1A cells.

Effect of tanshinone on ROS and MMP levels in human endometrial carcinoma HEC-1-A cells

The results showed that treatment of HEC-1-A cells with tanshinone I caused significant increase in the ROS levels (Figure 6). The ROS levels increased up to 225% at 40 μM concentration. Increase in the levels of ROS was directly linked to reduction in the MMP levels in a concentration-dependent manner (Figure 7).

Tanshinone 1 causes upregulation of JAK/STAT pathway in human endometrial carcinoma HEC-1-A cells

It was observed that tanshinone I could inhibit the expression of STAT1, JAK1 and JAK2. Moreover, tanshinone I could also inhibit the phosphorylation of pSTAT1, pSTAT-2, pJAK1 and pJAk2. These results clearly indicate that Tanshinone I inhibits the JAK/STAT pathway in human endometrial carcinoma HEC-1-A cells (Figure 8).

Discussion

Endometrial carcinoma arises in the female genital tract and causes significant mortality in women worldwide. The treatments modalities for endometrial carcinoma are limited and also create adverse effects, disturbing the patient quality of life [12]. Therefore, new drug options are being identified for the treatment of this disease. In this study we examined the effects of tanshinone I on human endometrial carcinoma HEC-1-A cells. The results showed that tanshinone I could inhibit the growth of the HEC-1-A cells in a dose-dependent manner. The IC_{50} of tanshinone I against HEC-1-A cells was found to be 20 μM. These results are in concordance with studies carried out previously. For example, tanishones have been reported to inhibit the proliferation of several types of cancers which included lung and breast cancers [13,14]. It has been reported that many of the currently used anticancer agents induce apoptosis in cancer cells [15]. Apoptosis is considered an important mechanism by which malignant cells are eliminated from the body. Moreover, apoptosis of cancer cells prevents the development of chemoresistance by cancer cells [16]. In this study we also examined whether tanshinone I exerts its growth inhibitory effects via apoptosis and the results confirmed that tanshinone I triggers apoptosis in HEC-1-A cells. The apoptotic potential of tanshinones has also been reported previously by other authors, e.g. tanshinones have been reported to cause apoptosis of A459 lung cancer cells [17]. Furthermore, generation of ROS in cancer cells has significant implications in the induction of apoptosis. Increase in

the levels of intracellular ROS levels causes reduction in the MMP, thus favouring apoptosis [18]. It was observed that tanshinone I caused increase in the ROS levels which was further associated with decrease in MMP, ultimately leading to apoptosis [18]. In this study, apoptosis was further confirmed by increased expression of Bax and decrease in the expression of Bcl-2. JAK/STAT signalling pathway is highly activated in cancer cells and plays a pivotal role in the progression of tumors [19]. Interestingly, in the present study we observed that tanshinone I caused inhibition of JAK/STAT signalling pathway, indicative of the potential of this compounf as treatment for endometrial carcinoma.

Conclusion

In conclusion, the results of the present study show that tanshinone I inhibits the proliferation of human carcinoma HEC-1-A cells. The antiproliferative effects are due to the induction of mitochondrial apoptosis and inhibition of JAK/STAT signalling pathway. Taken together, the results of this study indicate that tanshinone I may prove a potential lead molecule for the treatment of endometrial carcinoma and deserves *in vivo* evaluation.

Acknowledgement

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Conflict of interests

The authors declare no conflict of interests.

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