ORIGINAL ARTICLE ────────────────────────────

Shionone suppresses the growth, migration and invasion of human breast cancer cells via induction of apoptosis and inhibition of MEK/ERK and STAT3 signalling pathways

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Summary

Purpose: _Breast cancer is responsible for high morbidity and mortality across the globe. Studies are focusing to develop novel systemic therapies for the treatment of this disease. The present study was designed to examine the anticancer effects of Shionone against human breast cancer cells along with the underlying mechanism of its action._

Methods: _The breast cancer SK-BR-3 and normal breast MB-157 cell lines were used in the study. CCK8 assay was used for cell viability assessment. DAPI was used for the assessment of nuclear morphology. Acridine orange (AO)/ethidium bromide (EB) and annexin V/propidium iodide (PI) assays were used for detection of apoptosis. Cell cycle analysis was done by flow cytometry. Protein expression was examined by western blot analysis._

Results: _The results showed that in vitro administration_

of Shionone led to decline of proliferation of breast cancer cells. The reduction of proliferative rates was attributed to the induction of apoptosis of breast cancer cells. Shionone caused cleavage of caspase-3 and 9. The expression of Bax was increased and that of Bcl-2 was decreased upon Shionone treatment. The transwell assays showed that Shionone suppressed the migration and invasion of breast cancer cells in a dose-dependent manner. Finally, western blot analysis showed that Shionone blocked the Ras/Raf/MEK/ERK and STAT3 signaling pathways in breast cancer cells.

Conclusion: _Taken all together, the study established the anticancer role of triterpenoid Shionone in restricting the growth and proliferation of human breast cancer cells._

Key words: _shionone, breast cancer, anticancer, apoptosis, cell cycle arrest, flow cytometry_

Introduction

With recent advancements in the field of cancer research, a vast number of natural compounds have been screened for their anticancer properties. The natural compounds possess health beneficial effects on human body and they act as vital source for the development of effective drug molecules [1]. These compounds possess antioxidant and anticancer properties [2]. Against this background, this study was undertaken to investigate the anticancer effects of a triterpenoid compound, Shionone, against human breast cancer cells. Over the years, breast cancer incidence has increased substantially and has become one of serious health issues in women world over [3]. More than 1.3 billion breast cancer cases and approximately half million deaths are attributed globally to breast cancer each year. In China alone, more than 0.45 million new breast cancer cases and 0.13 million deaths were reported to be due to breast cancer [4]. The heterogeneous nature, diagnosis at advanced stage, unreliable molecular markers and

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inefficient treatment regimes makes it very difficult to manage this disease [5]. MEK/ERK and STAT3 signalling pathways have been shown to be activated in cancer cells and have been reported to be involved in several of the cancer-related processes such as apoptosis, proliferation and metastasis [6,7]. Herein, we investigated the effects of the MEK/ERK and STAT3 signalling pathways and found that Shionone suppresses the growth of the human breast cancer cells via induction of apoptosis. Additionally, Shionone suppresses the migration and invasion of the human breast cancer cells in a dose-dependent manner. Taken all together, Shionone may be an essential lead molecule for the development of systemic therapy for breast cancer.

Methods

Cell lines and culture

The human breast cancer cell line SK-BR-3 and the normal breast cell line MB-157 were acquired from the ATCC collection center, USA. The cell lines were cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640) (Thermo Scientific, Waltham, Mass, USA). The cell lines were maintained in a humidified incubator at 37°C with 5% CO_2/95% air.

Analysis of cell proliferation

The proliferation of SR-BR-3 cells was determined by cell counting kit-8 (CCK8, Thermo Scientific) following the manufacturer's protocol. The breast cell lines (both normal and cancer) were cultured in 96-well plates at 37°C for 48h. The cell cultures were treated with 0 to 200 μM of Shionone for 24 h. Afterwards, 10 μl CCK8 reagent were added to each well and incubation at 37°C was continued for 4 h. Finally, a microplate reader was used to record the optical density (OD) at 450nm which was used to calculate the percent cell proliferation without Shionone treatment as 100%.

Analysis of nuclear morphology

The SK-BR-3 breast cancer cells were cultured for 24h at 37°C at the cell density of 10^6 cells/well in 6-well plates. The cell cultures were then administered with 0, 7, 14 or 28 μM Shionone. After 24-h incubation at 37°C, the cells were harvested by centrifugation, phosphate buffered saline (PBS)-washed, fixed with 70% ethanol and subsequently stained with 4',6-diamidino-2-phenylindole (DAPI) solution. Then the cells were examined under fluorescent microscope.

AO/EB dual staining

The SK-BR-3 cancer cells treated with 0, 7, 14 or 28 μM Shionone for 24h were centrifuged and the collected cell pellets were washed with PBS and fixed using 70% ethanol. The cells were then stained with acridine orange and ethidium bromide (AO/EB) dual staining and analyzed for the morphological changes under fluorescent microscope.

Annexin V- FITC/PI assay

The annexin V-FITC/PI staining assay was performed to assess the level of cell apoptosis under 0, 7, 14 or 28 μM Shionone administration for 24h. The SK-BR-3 cancer cells were fixed using methanol and stained with dual annexin V-FITC/PI staining solution. Then, the cells were examined for nuclear morphology under fluorescent microscope and the percentage of apoptotic cells was determined.

### Flow cytometric examination of the cell cycle

For studying the mitotic cell cycle phase distribution, the SK-BR-3 breast cancer cells were treated with 0, 7, 14 or 28 μM Shionone for 24h. Centrifugation was used to harvest the treated cells which were then washed with PBS three times, fixed using 4% formaldehyde and stained with PI solution. The cells were then examined using flow cytometer for the analysis of mitosis.

Western blot analysis

To extract the total proteins the SK-BR-3 cells were lysed with RIPA lysis buffer (Thermo Scientific). The Bradford method was used to determine the total protein concentrations of the cell lysates. Equal protein concentrations were loaded on 8% SDS-PAGE gel, which was then blotted to transfer the contents to nylon membranes which were exposed to primary and secondary antibodies designed for the respective proteins of interest which were then visualized and their expression levels assessed by the chemiluminescence method.

Statistics

The experiments were performed in triplicate. The final values were presented as average±standard deviation. The t-test was performed using the GraphPad Prism 7.0 software and p values ≤0.05 were considered statistically significant.
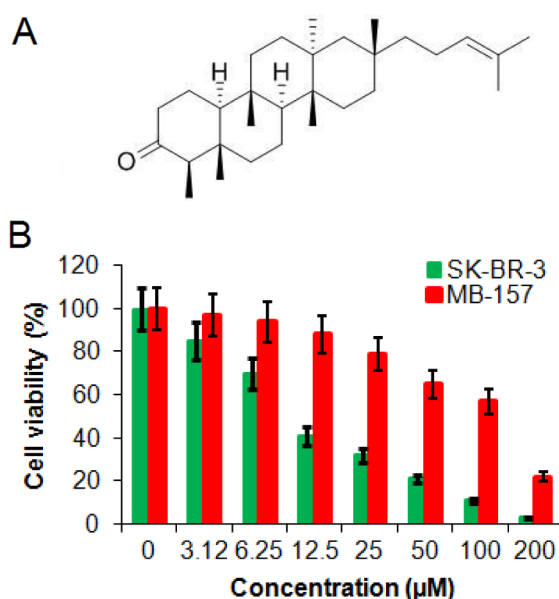
Figure 1. Shionone suppresses the growth of breast cancer. **A:** Chemical structure of Shionone. **B:** CCK8 assay showing the viability of SK-BR-3 and MB 157 cells. The experiments were performed in triplicate and expressed as mean ± SD (p<0.05).

Results

Shionone inhibited selectively the breast cancer cell proliferation

Shionone is a triterpenoid compound (Figure 1A). To examine the effects of Shionone on the cell proliferation, the SK-BR-3 cancer and MB-157 normal breast cell lines were treated for 24 h with 0 to 100 µM Shionone. CCK-8 assay was performed to determine the proliferation rates. It was observed that Shionone decreased the proliferation of both SK-BR-3 and MB-157 breast cells (Figure 1B). However, the antiproliferative effects were significantly more severe on the SK-BR-3 cancer cells. The IC_{50} of Shionone for SK-BR-3 cancer cells was 14 µM but it was 105 µM for MB-157 breast cells which suggests that Shionone selectively targets the breast cancer cells and has limited effect against the normal breast cells.

Shionone triggers apoptosis in breast cancer cells

To know, whether the antiproliferative effects of Shionone against the breast cancer cells was because of the induction of cell apoptosis, the SK-BR-3 cancer cells were treated with 0, 7, 14 and 28 µM Shionone. The cells were assessed for nuclear morphology by DAPI staining. The observations made indicated that Shionone treatment led to nuclear deformation of breast cancer cells and the effects were more prominent at higher concentrations (Figure 2). Similar observations were noted in the fluorescent microscopic examination of the AO/EB stained cells (Figure 3). The results were further confirmed by the annexin V-FITC/PI dual staining procedure. The percentage of apoptotic cells was seen to increase from 0.89% to 12.5% under 0 and 28 µM Shionone administration, respectively
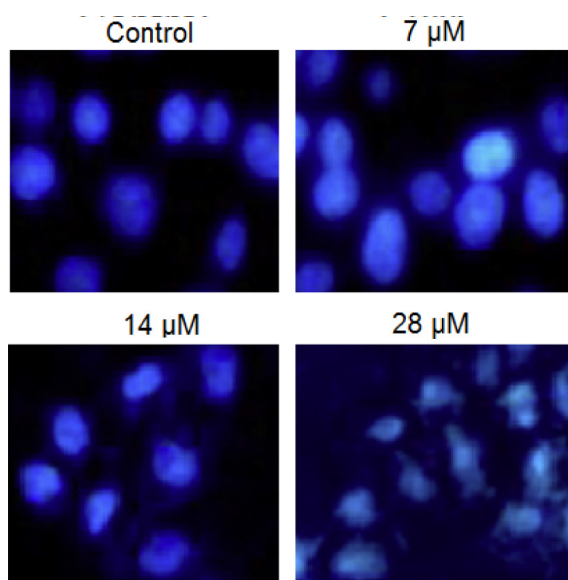
Figure 2. Shionone triggers apoptosis in breast cancer cells. DAPI staining of SK-BR-3 cells at indicated concentrations of Shionone showing induction of apoptosis in a dose-dependent manner. The experiments were performed in triplicate.
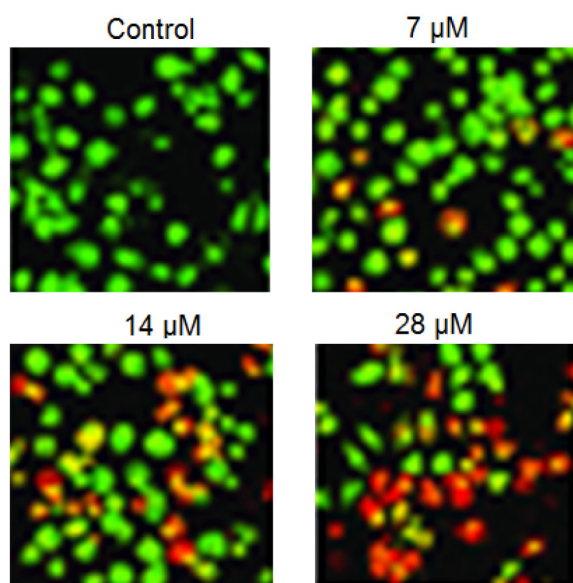
Figure 3. Shionone induces apoptotic cell death of breast cancer cells. AO/EB staining of SK-BR-3 cells at indicated concentrations of Shionone showing induction of apoptosis in a dose-dependent manner. Green color depicts early apoptotic cells and red color depicts late apoptotic cells. The experiments were performed in triplicate.
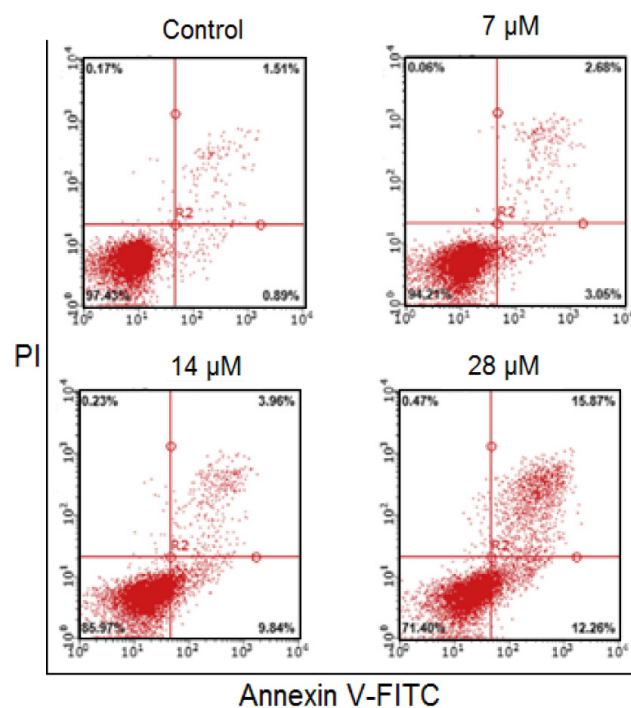
Figure 4. Annexin V/PI staining of SK-BR-3 cells at indicated concentrations of Shionone showing that the percentage of apoptotic SK-BR-3 cells increase with a dose-dependent manner. The experiments were performed in triplicate.

(Figure 4). These results clarified that Shionone induces apoptosis in breast cancer cells. The western blot analysis showed that Shionone triggered the cleavage of caspase-3 and 9 in a dose-dependent manner. The expression of Bax increased and that of Bcl-2 decreased with increase in the dosage of Shionone (Figure 5).

Shionone suppressed the migration and invasion of breast cancer cells

The effects of Shionone were also examined on the migration and invasion of the human breast cancer cells which showed that this molecule in-

hibited the migration and invasion of the human breast cancer cells. The percent migration was 100, 74, 42 and 25% at 0, 7, 14 and 24 µM concentration, respectively (Figure 6). The percent invasion was 100, 78, 55 and 37% at 0, 7, 14 and 24 µM concentration, respectively (Figure 7).

*Shionone modulated MEK/ERK and STAT3 signaling pathway*

The western blotting studies carried out for the expression analysis of MEK and ERK proteins revealed that the expression of phosphorylated MEK and ERK (p-Raf, p-MEK and p-ERK) decreased dose-dependently (Figure 8). The concentrations of MEK and ERK remained unaltered. Similarly, the expression of phosphorylated STAT3 decreased dose-dependently while that of normal STAT3 remained constant. The results suggest that the growth inhibitory effects of Shionone against the breast cancer cells were exerted through inhibition of Ras/Raf/MEK/ERK and STAT3 pathways.
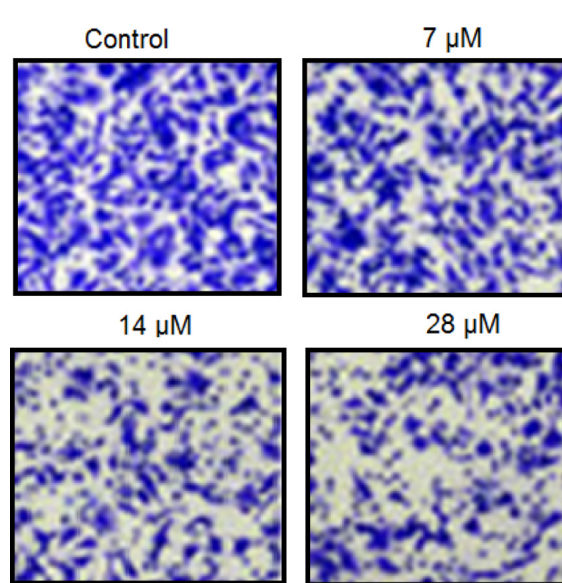
Discussion

Breast cancer imposes significant health and economic burden on human populations across the globe. Although surgery followed by chemotherapy is utilized for the breast cancer treatment, unfortunately the frequent relapses and advanced stages form a bottleneck in its treatment [8]. The mortality associated with the human breast cancer has been seen to result from the metastasis of this
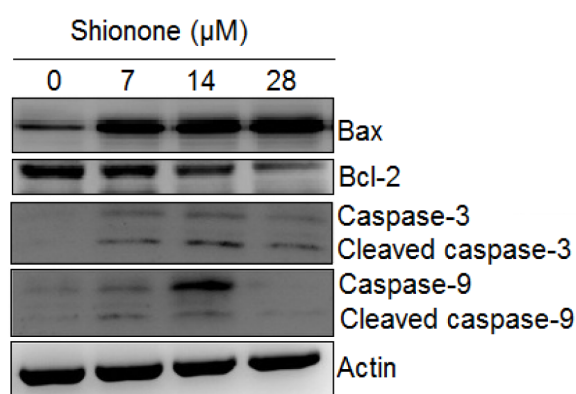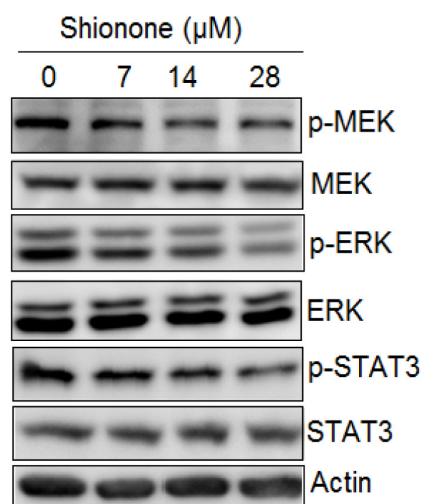

Figure 5. Western blot analysis showing the effect of Shionone on the expression of apoptosis-related proteins. The Figure shows that the expression of Bax cleaved caspase-3 and cleaved caspase-9 increase while Bcl-2 decreases in a dose-dependent manner. The experiments were performed in triplicate.
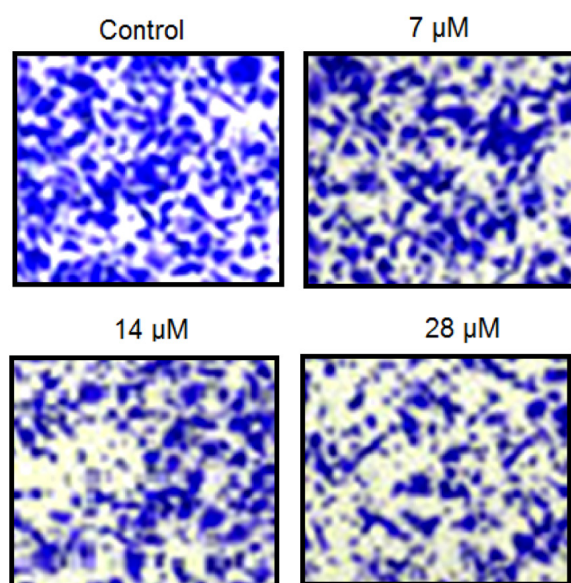
Figure 6. Effect of Shionone on the migration of the SK-BR-3 cells as depicted by transwell assay. The Figure shows that the migration of the SK-BR-3 cells decreeses with increase in the concentration of Shionone. The experiments were performed in triplicate.

Figure 7. Effect of Shionone on the invasion of the SK-BR-3 cells as depicted by transwell assay. The Figure shows that the invasion of the SK-BR-3 cells decreases with increase in the concentration of Shionone. The experiments were performed in triplicate. The experiments were performed in triplicate.

Figure 8. Western blot analysis showing the effect of Shionone on the MEK/ERK and STAT3 signalling pathway in SK-BR-3 cells. The Figure shows that the expression of p-ERK, p-MEK and p-STAT-3 decreases in a dose-dependent manner upon Shionone treatment. The experiments were performed in triplicate. The experiments were performed in triplicate.

cancer to the neighboring tissues and distal organs [9]. The currently employed anticancer approaches against breast cancer are rather unsatisfactory and are associated with many undesirable effects [10]. Against this background, the researchers are trying searching for the more effective strategies to combat this malignancy in a sustainable manner. For this, the natural products are being evaluated for their anticancer role against the growth and proliferation of human breast cancer [11,12]. In a similar type of study, we herein deduced the anticancer role of a bio-active substance, Shionone, which is a plant-derived triterpenoid compound [13]. The anticancer effects of Shionone have not been reported but the anticancer effects of triterpenoids are well reported. For instance, a natural triterpenoid plectranthoic acid has been reported to inhibit the growth of the prostate cancer cells [14]. Similarly, Taraxastane-type triterpenoid derivatives have been reported to induce apoptotic cell death in several cancer cell types [15]. In the

current study, Shionone exhibited selective proliferative inhibition against the human breast cancer cells and its effects were limited against the normal breast cells. Such selective inhibitory potential has been noticed for other natural compounds also [16,17]. The inhibitory effects of Shionone on the proliferation of breast cancer cells were due to the induction of cell apoptosis. This finding is in conformity with a previous study wherein triterpenoids, such as lupane triterpenoid derivatives, have been shown to induce apoptosis in cancer cells [18]. The migration and invasion of cancer cells are the initial steps in the metastasis of cancer cells to neighboring tissues and distal organs [19]. Herein, we found that Shionone suppresses both migration and invasion of the breast cancer cells suggestive of its anti-metastatic potential. The MEK/ERK and STAT3 pathways have been shown to act as a vital target in cancer management and it was stated that the molecules may be designed to target this pathway to effectively inhibit the cancer progression [6,7]. In our study, we have shown that Shionone exerted its anticancer effects on breast cancer cells by inducing apoptosis and blocking the MEK/ERK and STAT3 signaling pathways which highlights its potential to serve as a vital lead molecule in anticancer drug discovery.

Conclusion

Taken all together, the results of the present research work indicate that Shionone is effective in inhibiting the growth and proliferation of breast cancer cells by inducing apoptosis and blocking STAT3 and MEK/ERK pathways. Additionally, Shionone also inhibits the migration and invasion of the breast cancer cells indicating Shionone could prove an essential lead molecule in the development of systemic therapy for breast cancer.

Conflict of interests

The authors declare no conflict of interests.

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