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ORIGINAL ARTICLE

Gammacerane suppresses the growth of human endometrial carcinoma cells via induction of apoptosis, cell cycle arrest, inhibition of cell migration, invasion and STAT3 signaling pathway

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Summary

Purpose: *This study was conducted to assess the anticancer role of gammacerane against human endometrial cancer.*

Methods: *The human RL-95 cell line (endometrial cancer) and SV40 (normal endometrial cells) were used in this study. The MTT-based estimation of cell proliferation assay along with the colony formation assay were used for assessing the cell viability. Acridine orange (AO)/Ethidium bromide (EB) staining followed by fluorescent microscopy was performed for estimation of cell apoptosis. Flow cytometry was used to assess the cell cycle phase distribution of cancer cells. Cell migration and invasion were estimated using wound healing and transwell assay, respectively. Western blotting was used for protein expression studies.*

Results: *The cell proliferation assay revealed that gammacerane treatment led to loss of viability of RL-95 cancer cells in a concentration-dependent manner. However, the antiproliferative effects were comparatively less prominent when gammacerane was used against the SV40 normal endometrial cells. AO/EB staining of cancer cells showed that gammacerane is active in inducing apoptosis in RL-95 cells and apoptotic induction effects were more evident at higher concentrations of the molecule. Flow cytometric analysis with Annexin V-FITC/Propidium iodide (PI) fixed cells showed that the percentage of apoptotic cells increased with increase in gammacerane concentration. Apoptotic signal was mediated via the modulation of Bax/Bcl-2 protein ratio. Western blot analysis of STAT3 protein showed that gammacerane treatment reduced the protein levels of STAT3 and the effects were more prominent at higher treatment concentrations.*

Conclusion: *Gammacerane, by its ability to take control over the transcription of STAT3 transcription factor, inhibits the proliferation of human endometrial cancer cells. The effects revealed loss of viability, arrest of mitosis and cellular apoptosis.*

Key words: *anticancer, gammacerane, migration, invasion, endometrial cancer, apoptosis*

Introduction

Isoprenoids are highly abundant and a structurally most diverse class of natural products [1]. Terpenoids, belonging to isoprenoid class of naturally occurring compounds, constitute about 60% of known natural products [2]. Terpenoids are mainly isolated from microbes or plants. These molecules possess impressive medicinal potentials and are mainly seen to exert antimicrobial properties like artemisinin which is used as antimalarial drug [3-5]. The anticancer potential of terpenoids is no longer an obscure area and there is a good number of reports on anticancer activities of these molecules [6,7]. Gammacerane (Figure 1A) is a pentacyclic triterpene type of terpenoid. Gammacerane

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is commonly found as sediment deposition under the abnormal salinities [8]. Tetrahymanol, which occurs abundantly in marine sediments, is most likely considered as the precursor of gammacerane [9]. Prokaryotic source of gammacerane has also been discovered way back in 1990 [10]. From the plant world, the stem bark of the species of *Abies* like *A. veitchii* and *A. mariesii* has been reported as source of gammacerane terpenes [11,12]. There is accumulated evidence regarding the pharmacological activities of tritepenoids among which betulinic acid, ursolic acid, oleanolenic acid and cucuribacin B have been studied most and are shown to possess strong anticarcinogentic properties [13]. Here we investigated the anticancer effects of gammacerane against the human endometrial cancer. Endometrial cancer is considered as the most common malignancy in females which affects about 2-3% of women in developed countries [14]. The prominent effect associated with the development of this endometrial disorder is believed to be the unstopped stimulation of estrogen [15]. However, there is a large proportion of such cases where hormonal basis cannot be established. The advancement in cancer research has shown that the molecular changes are important for development of human cancers. Here in this study, the anticancer effects of gammacerane were examined against the endometrial cancer cells and the underline mechanism was explored.

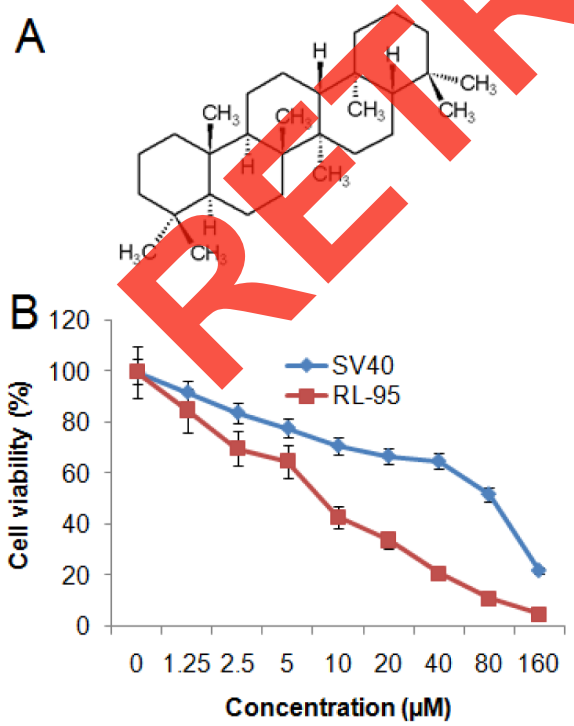

Figure 1. A: Chemical structure of Gammacerane. **B:** Effect of Gammacerane on the viability of the RL-95 endometrial carcinoma and SV40 normal cells. The experiments were performed in triplicate and expressed as mean ± SD ($p<0.05$).

Methods

Cell culture

The human cell line RL-95 (endometrial cancer) and SV40 (normal endometrial cells) were purchased from Bioresource Collection and Research Center (BCRC), Taiwan. The cells were cultured using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), streptomycin/penicillin (1%) and $NaHCO_3$ (0.02%). The cell lines were maintained at 37°C with 5% CO_2.

Cell viabilty assay

RL-95 cancer cells and SV40 normal endometrial cells were treated with 0, 1.25, 2.5, 5, 10, 20, 40, 80 and 160 µM gammacerane for 24 h at 37°C in 96-well plates with cellular density of 10^4 cells/well. The 3,4-[4,5-dimethylthiazolyl-2]-2, 5-diphenyltetrazolium bromide (MTT) reagent was used for the determination of cancer cells. Then, MTT was added to each well with a final concentration of 0.5% and plates were incubated for 4 h at 37°C. To each well, 150 µl of DMSO were added for solubilizing the product of MTT-formazan. Optical density (OD) at 570nm was taken using spectrophotometer for each well to determine the cell viability which was finally presented in the form of percentages.

Clone formation assay

Cancer cells treated with 7, 14 and 28µM gammacerane for 24 h were plated in 6-well plates at 10^3 cells/100µl of DMEM. The plates were incubated at 37°C and 5% CO_2 for 48 h. The cell mass was collected by centrifugation and washed with PBS. This was followed by incubation of cancer cells for 30 min after being resuspended in 0.1% crytal violet dye (1Ml). Washing of cell pellet with PBS after centrifugation was done till no dye remained in PBS. Finally, pictures of clones were taken and colonies were counted.

Assessment of cell apoptosis

AO/EB staining was used to determine the apoptosis of cancer cells treated with 7, 14 and 28 µM gammacerane for 24h. The treated cells were plated in 96-well plates (2×10^5 cells/well). Twenty five µl of cell culture were taken from each well and mixed with 1 µl of dye solution (100 µg of each AO/EB in PBS). Ten µl aliquot of this mix were put on glass slides. The slides were covered with a cover slip and the nuclear morphology was examined using fluorescent microscope to assess the cell apoptosis. Further, the treated cells were fixed using Annexin V-FITC/PI and the percentage of apoptotic cells was determined through flow cytometry.

Analysis of cell cycle phase distribution

Flow cytometry was used to analyze the cell cycle of cancer cells after 24 h of gammacerane treatment of 7, 14 and 28 µM. The treated cancer cells were fixed using 70% ethanol. This was followed by centrifugation and resuspension of cancer cells in PBS containing 0.1% Triton X-100, 200µg/ml RNase A and 50µg/ml PI. Then,

incubation in the dark for 30 min was followed and finally the flow cytometric determination of the cell cycle phase distribution was carried out.

Migration and invasion assays

The ability of cancer cells to migrate was determined using wound healing assay. In brief, untreated and 7 µM gammacerane-treated cancer cells were cultured in 6-well plates. This was followed by making a scratch line perpendicular to cell surface using 200 µl sterile pipette tip. The scratched area was analyzed after 0 and 24 h of 37oC and 5% CO_2 incubation.

Transwell assay-based estimation of invasion of cancer cells was carried out in transwell chambers. Cancer cells treated with 0 and 7 µM gammacerane were cultured in transwell chambers. The chambers were put into the 24-well plates containing growth medium with 20% FBS and incubated at 37°C and 5% CO_2 for 2 days. The cells that were not able to invade to the lower chamber were cleared with cotton swabs. Invaded cells were fixed using 0.5% crystal violet for 35 min. High magnification (200×) light microscopy was used to examine at least 10 random fields for cell invasion.

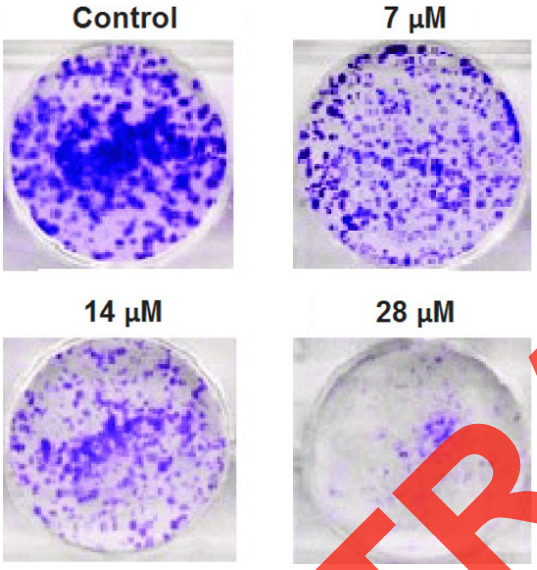

Figure 2. Effect of Gammacerane on the colony formation potential of the RL-95 cells as depicted by the colony formation assay. The Figure shows that Gammacerane inhibits the colony formation of cancer cells in a dose-dependent manner. The experiments were performed in triplicate.

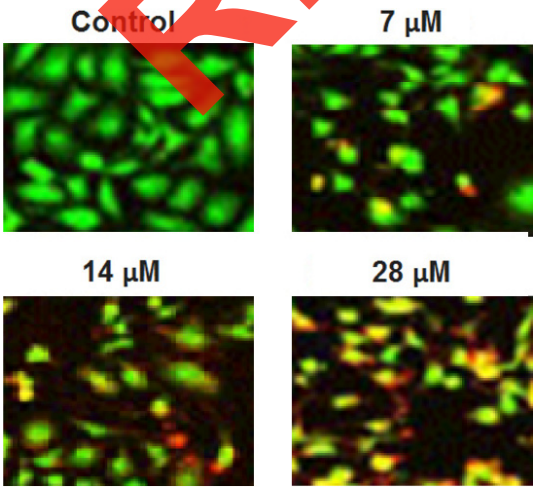

Figure 3. AO/EB staining showing induction of apoptosis in endometrial cancer cells by Gammacerane at indicated concentrations. The experiments were performed in triplicate.

Assessment of protein concentrations

The cells were washed with ice-cold PBS and then suspended in RIPA lysis buffer at 4°C. Subsequently, Bradford assay was used to evaluate the protein content of each cell extract. About 50 µg of protein were loaded from each sample and separated by SDS-PAGE before being shifted to polyvinylidene fluoride membrane. The membranes were then subjected to tris-buffered saline (TBS) treatment and then exposed to primary antibodies at 4°C. Subsequently, the cells were treated with appropriate secondary antibodies and the proteins of interest were visualised by enhanced chemiluminescence reagent. Finally, the protein signal was detected by Odyssey infrared imaging system. Actin and GAPDH were used as controls for normalization.

Statistics

Graphpad7 prism software was used for data analysis. Values obtained were given as mean±standard deviation. Student's *t*-test was performed to calculte the p value. A p value <0.05 was indicative of statistically significant difference of a particular parameter between two samples.

Results

Gammacerane inhibits the cancer cell growth

RL-95 cancer cells treated with of 0, 1.25, 2.5, 5, 10, 20, 40, 80, 160 µM of gammacerane were analyzed for proliferation using MTT assay. Of note, the proliferation was inhibited in a dose-dependent fashion. The same was true for normal SV40 endometrial cells but the inhibitory effects were significantly less prominent (Figure 1B). This was evident from the gammacerane IC_{50} values of 14 µM and 80 µM, repectively for cancer and normal cells. The antiproliferative effects of gammacerane against RL-95 cells were also evident from the reduction in potential of RL-95 cells to form clones when they were treated with varrying concentrations of the molecule and assessed for the same (Figure 2).

Gammacerane induces apoptosis in cancer cells through Bax and Bcl-2 apoptotic proteins

The RL-95 cells treated with 0, 7, 14 and 28µM of gammacerane were analyzed for nuclear morphology with fluorescent microscope employing AO/EB staining. It was clearly visible from the microscopic results that the chromatin of cancer cells had undergone denatunation which suggested that

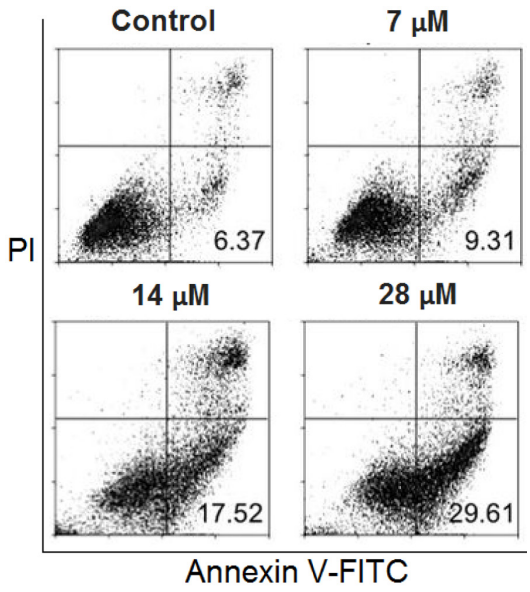

Figure 4. Annexin V/PI staining showing the extent of apoptosis in RL-95 cells at indicated concentrations of Gammacerane. The experiments were performed in triplicate.

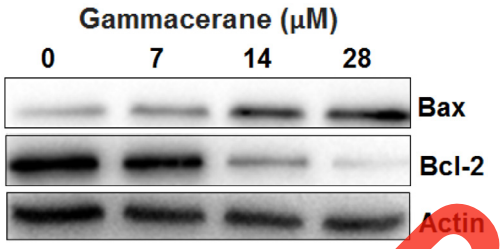

Figure 5. Western blot analysis showing the effect of Gammacerane on the expression of Bax and Bcl-2 in RL-95 cells. The Figure shows that Bax expression increased and Bcl-2 decreased dose-dependently upon Gammacerance treatment. The experiments were performed in triplicate.

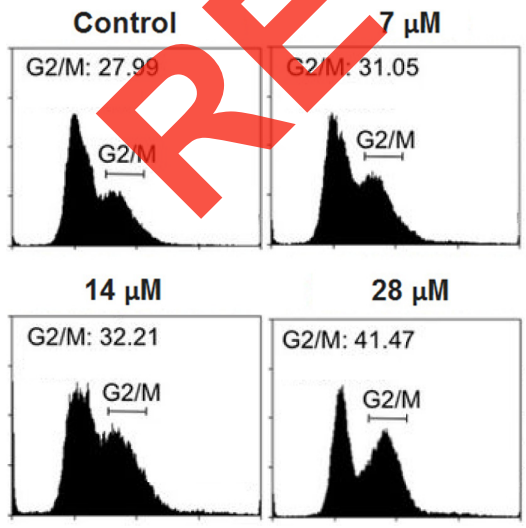

Figure 6. Effect of Gammacerane on the distribution of RL-95 cells in different phases of the cell cycle. The Figure shows that Gammacerance induces G2/M cell cycle arrest of the endometrial cancer cells. The experiments were performed in triplicate.

gammacerane has a potential to induce apoptosis of endometrial cancer cells and it did so in a concentration-dependent manner (Figure 3). The percentage of apoptotic cells treated with 0, 7, 14 and 28 μM gammacerane was estimated using flow cytometry which showed 6.37, 9.31, 17.52 and 29.61, repectively for 0, 7, 14 and 28 μM gammacerane concentrations (Figure 4). The flow cytometric results corellated with the ones obtained through fluorescent microscopy. To infer whether there was any involvement of apoptosis-related proteins Bax and Bcl-2 in the induction of cell apoptosis, the concentration of Bax and Bcl-2 was determined using western blotting. Bax increased with increase in the concentration of gammacerane while Bcl-2 decreased when gammacerane concentration was increased (Figure 5). This suggests that the apoptotic signal is mediated via Bax and Bcl-2 proteins.

Gammacerane mediated cell cycle arrest in RL-95 cancer cells

The anticancer effects of gammacerane against the human endometrial cancer cells were also evident from its potential to arrest the cell cycle of cancer cells. The flow cytometry was used to analyze the cell cycle with different concentrations of gammacerane and it was observed that the treatment of gammacerane caused cell cycle arrest at G2/M phase of the cell cycle (Figure 6).

Gammacerane inhibits the migration and invasion of endometrial carcinoma cells

Gammacerane-treated cells were assessed for their ability to migrate and invade the surroundings. It was observed that both cell migration and invasion were remarkably affected in a negative way and the effects were more prominent at higher concentrations of gammacerane (Figures 7 and 8).

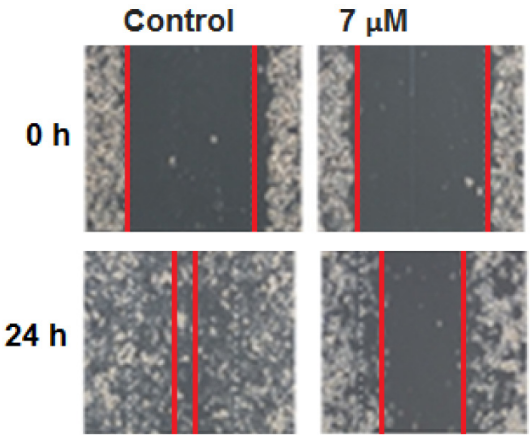

Figure 7. Effect of Gammacerane on the migration of RL-95 cells as depicted by the wound healing assay. The experiments were performed in triplicate.

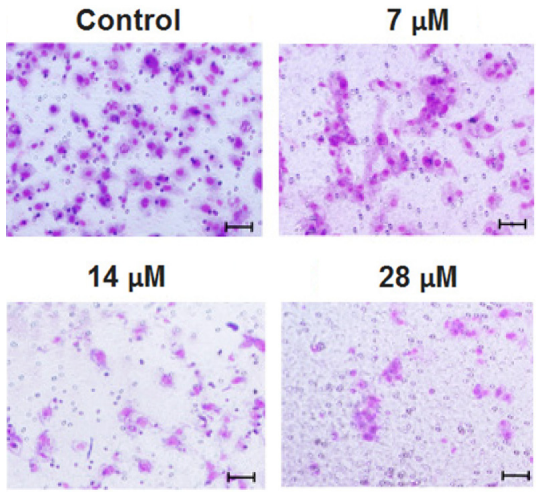

Figure 8. Transwell assay showing the effect of different concentrations of Gammacerane on the invasion of RL-95 cells. The experiments were performed in triplicate.

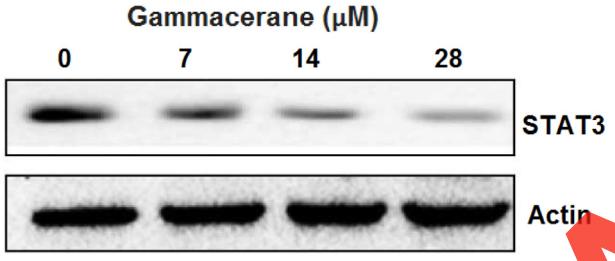

Figure 9. Western blot analysis showing the effect of Gammacerane on the expression of STAT3 in RL-95 cells. The Figure shows that Gammacerane inhibits the expression of STAT3 dose-dependently. The experiments were performed in triplicate.

Gammacerane inactivates the STAT3 cancer cell signalling

To check whether the growth inhibitory effects of gammacerane against RL-95 cancer cells were propagated through STAT3 signalling pathway, cancer cells were treated with 0, 7, 14 and 28 μM of this molecule and analyzed for STAT3 protein concentration using actin as internal control. The results suggested that STAT3 protein was expressed in lower quantities at higher gammacerane concentration (Figure 9). This indicates that the anticancer effects of the molecule was mainly modulated through decline in STAT3 protein concentration, which otherwise is constitutively expressed in cancer cells.

Discussion

Despite a lot of research going on cancer, cancer is still the second leading cause of death in developed countries and estimations made have predicted that in few decades it will be the major cause of mortality in all the countries [16]. Due to extensive cancer research, since 1991 a decline of about 27% has been observed in the cancer cases. Lot of research studies aim to investigate the anticancer potential of natural products. Terpenoids, the most dominant natural products, have been shown to possess a variety of health beneficial effects and thus a growing interest among the scientific community is being observed to assess the anticancer effects of natural and synthetic terpenoid compounds. The present study was also conducted for the same purpose and provides the effects of gammacerane, a pentacyclic triterpene, against human endometrial carcinoma. The results gathered revealed that gammacerane is effective in inhibiting the growth and proliferation of endometrial carcinoma cells. The peculiarity of this inhibitory effect was that the inhibition of cancer cell proliferation occurred in a dose-dependent manner. However, when the normal endometrial cells were treated with gammacerane, the antiproliferative effects were very less prominent. Such results have also been previously reported by some other investigators. To assess if the antiproliferative effects of gammacerane were mediated as induction of cell apoptosis, DAPI staining based analysis of cell apoptosis was performed. What was observed was that gammacerane treatment induced apoptosis of cancer cells in a dose-dependent manner. This is in concordance with the anticancer effects of some previously studied natural products [17,18]. At molecular level, the induction of apoptosis was shown to be prompted via the modulation of STAT3 signalling pathway. Such results were also obtained by other researchers for cycloastragenol against gastric cancer cells [19]. Compelling evidence suggests that signal transducer and activator of transcription 3 (STAT3), a cytoplasmic transcription factor, is constitutively expressed in cancer cells and is important for cancer cell proliferation and metastasis [20]. The western blotting experiments revealed that STAT3 protein expression in cancer cells decreased under gammacerane treatment and the trend observed showed that decline in protein concentration progressed in a dose-dependent manner. The migration and invasion of cancer cells are prime activities to propagation of cancer to neighbouring tissues. Such propagation results in metastasis of cancer. To check whether gammacerane is active in the prevention of endometrial cancer cells to metastatize, cancer cells were treated with gammacerane and assessed for migration and invasion abilities. The results showed that the migration and invasion of gammacerane-treated cancer cells were remarkably inhibited and the analysis of cell cycle showed that this molecule was able

to arrest the cancer cell cycle. In summary, gammacerane treatment resulted in loss of cell viabilty in endometrial cancer cells. The anticancer effects of this molecule were seen as inhibition of cell growth via promotion of cell apoptosis mediated through STAT3 pathway. The cancer cell migration and invasion were also seen to be prevented to a great deal under gammacerane treatment.

Conclusion

From this study, the conclusion drawn is that the natural products like gammacerane can be seen as a promise to act against human health diseases like cancer and there is a vast field to look for measures to enhance their health beneficial effects. The current study may act as a base for future research for exploration of natural products to be used against human health disorders like cancer.

Conflict of interests

The authors declare no conflict of interests.

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