

Original Research Article

Globularifolin inhibits CAMA-1 human breast cancer cell line via cell cycle arrest, apoptosis and inhibition of PI3K/AKT signalling pathway

Yu Chen¹, Zhenwu Wang², Mei Liu¹, Xiaodong Wang^{1*}, Linghan Zhang³, Can Gong¹

¹Department of Breast Surgery, ²Department of the Graduate's Training, ³Department of Surgery, West China Hospital, Sichuan University, Chengdu, 610041, China

*For correspondence: **Email:** WGargilak@yahoo.com

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Abstract

Purpose: To investigate the anticancer activity of globularifolin against CAMA-1 breast cancer cells.

Methods: The viability of CAMA-1 cells was assessed by MTT assay. DAPI and annexin V/PI staining were used for the determination of apoptotic cell death. Flow cytometry was employed for cell cycle analysis, while wound healing and immunoblotting assays were used to measure cell migration and protein expression, respectively.

Results: Globularifolin decreased the viability of CAMA-1 breast cancer cells dose-dependently, with half-maximal inhibitory concentration (IC_{50}) of 10 μ M, relative to its IC_{50} of 65 μ M against non-cancerous CMMT breast cells. It also initiated apoptotic cell death and cell cycle arrest. Moreover, it inhibited the migration of CAMA-1 breast cancer cells, and PI3K/AKT signalling cascade.

Conclusion: These results suggest that globularifolin has promising potential for use in the treatment of breast cancer.

Keywords: Breast cancer, Globularifolin, Apoptosis, Cell cycle, Cell migration

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INTRODUCTION

Breast cancer is one of prevalent cancers in women the world over. In the United States alone, it has been reported that one out of eight women is diagnosed with breast cancer [1]. About 400,000 breast cancer-related deaths are reported annually [2]. The heterogeneity of breast cancer poses a major impediment to its early detection [3]. Currently, breast cancer treatment involves surgery, chemotherapy and/or

radiotherapy. However, despite recent progress in cancer research, the clinical outcomes are very poor [4].

Plant-derived natural products have been considered as prospective anticancer agents, and are therefore regularly screened for their anticancer properties. Globularifolin is one of the rare acylated iridoid glucosides isolated from some species of *Plantaginaceae* family [5,6]. Studies have shown that it exhibits an array of

pharmacological properties. Recently, it was reported that it exhibited significant anticancer effects against glioma U87 cell line [7]. In the present study, the anticancer effects of globularifolin against CSAMA-1 breast cancer cell line was investigated.

EXPERIMENTAL

Chemicals, reagents and culture conditions

All chemicals and reagents were obtained from Sigma-Aldrich Company. Human breast cancer CAMA-1 and non-cancerous CMMT cell lines were purchased from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. The cells were grown in RPMI-1640 with the composition 10 % fetal bovine serum (FBS), and appropriate antibiotics and atmosphere of 5% CO₂.

Effect of globularifolin on proliferation of CAMA-1 cancer cells

The proliferation rate of CAMA-1 cancer cells and CMMT cells was determined with MTT assay as described previously [8]. The cells were cultured in 96-well plates at 5×10^3 cells per well. They were then incubated overnight, and the culture medium was changed and a fresh media with different concentrations of globularifolin (0-100 μ M) was added.

After 24 h, addition of MTT (0.5 mg/mL) was followed, and the wells were subjected to incubation 3-4 h. The absorbance was then taken at 570 nm.

Effect of globularifolin on apoptosis of CAMA-1 breast cancer cells

The assessment of the nuclear morphology of CAMA-1 cells was done by fluorescence microscopy following DAPI treatment as described previously [9]. Ten fields with 100 cells/ field were selected randomly for estimation of cells with condensed nuclei. Annexin V/PI double staining was used for the determination of apoptotic CAMA-1 cancer cells by flow cytometry.

Cell cycle analysis

The distribution of the CAMA-1 cells in different cycle phases was performed by flow cytometry after PI stained by following the method reported in literature [9]. In brief, the CAMA-1 cells were grown in 6-well plates and treated with globularifolin for 24 h. The cells then collected and PBS washed followed by fixation in ethanol

(70%). After overnight incubation at 4 °C, the cells were subjected to PI staining and subjected to flow cytometry.

Wound healing assay

The migration of CAMA-1 cells was determined with wound healing assay. The cells were grown to confluence, and a scratch was made with a scratching device. The cells were then incubated again for 48 h, and the wound healing capacity was evaluated by comparing the widths of the wounds.

Determination of protein expressions by western blotting

After lysis of the CAMA-1 cells in RIPA lysis buffer, the protein content of the each lysates was estimated by BCA assay as described previously [10]. The samples were then loaded on the SDS-PAGE. The gels were then transferred to nitrocellulose membranes and subjected to treatment with primary antibody at 4 °C for a period of 24 h.

After this the membranes were incubated with HRP-conjugated secondary antibody (1:1000) for 50 min at 25 °C. Enhanced chemi-luminescence reagent was used to visualise the protein bands.

Statistical analysis

The experiments were done in triplicate and the data is presented as mean \pm SD. GraphPad prism was employed to carry out the statistical analysis by student's t test and the values at $p < 0.05$ were considered statistically significant.

RESULTS

Globularifolin decreased the viability of CAMA-1 breast cancer cells

The impact of globularifolin on cell proliferation was evaluated with MTT assay. The CAMA-1 cells were subjected to globularifolin treatment at varied concentrations (0, 5, 10 and 20 μ M). The results of MTT assay revealed that globularifolin exhibited considerable and dose-dependent anti-proliferative effects on CAMA-1 cells (Figure 1).

It was observed that the IC₅₀ for the inhibition of CAMA-1 cancer cells by globularifolin was 10 μ M, while its IC₅₀ for the inhibition of the non-cancerous CMMT cells was 65 μ M. These results clearly show that globularifolin selectively targets breast cancer cells.

Globularifolin induced apoptosis in CAMA-1 breast cancer cells

Previously, it was reported that globularifolin could induce apoptosis in glioma cells [7]. Therefore, herein the impact of globularifolin on CAMA-1 breast cancer cell apoptosis was investigated. The CAMA-1 cells were first exposed to globularifolin at different concentrations, then subjected to DAPI staining, and finally observed under fluorescence microscope. The results showed that globularifolin induced apoptosis in CAMA-1 breast cancer cells, as was apparent from the increased number of cells with colourless nuclei (Figure 2). Annexin V/PI further revealed that the apoptotic cells were increased from 2.6% in control to 61.3% at 20 μM of globularifolin (Figure 3). To further confirm apoptosis at the molecular level, the expressions of Bax and Bcl-2 proteins were assessed. It was found that globularifolin treatment enhanced Bax expression, but downregulated the expression of Bcl-2 expression in a dose-dependent manner (Figure 4).

Globularifolin caused G2/M cell cycle arrest in CAMA-1 breast cancer cells

The distribution of CAMA-1 cells in the different cell cycle phases after treatment with various concentrations of globularifolin was determined by flow cytometry. The results revealed that globularifolin caused accumulation of CAMA-1 in G2 phase, which ultimately led to G2/M cell cycle arrest (Figure 5).

Globularifolin inhibited migration of CAMA-1 cells

The inhibitory effect of globularifolin at IC_{50} on the migration of CAMA-1 breast cancer cells was determined by wound healing assay. The results showed that globularifolin considerably inhibited the migration of the CAMA-1 breast cancer cells (Figure 6).

Globularifolin inhibited PI3K/AKT signalling pathway

The PI3K/AKT signalling pathway is involved in the tumorigenesis of different cancer types [11]. Herein, the effect of globularifolin on the PI3K/AKT pathway was determined. It was found that globularifolin dose-dependently decreased expressions of p-PI3K and p-AK. However, the expressions of PI3K and AKT remained almost unaltered (Figure 7).

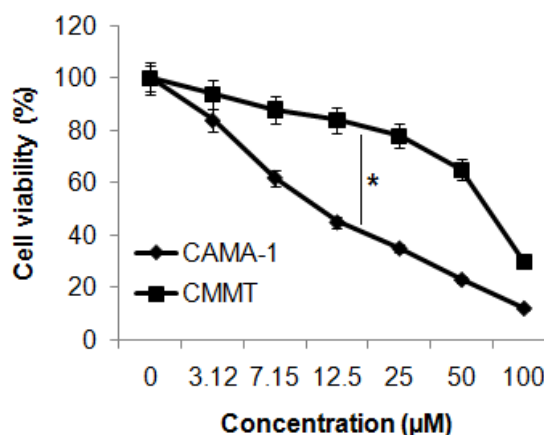


Figure 1: Effect of globularifolin on cell viability of CAMA-1 and CMMT cells. The values are mean \pm SD ($n = 3$, $*p < 0.05$)

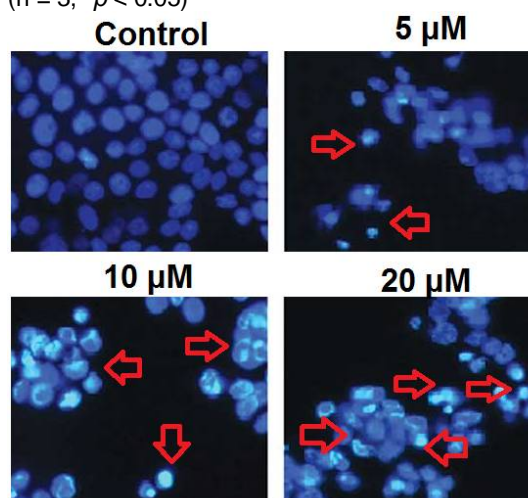


Figure 2: Effect of globularifolin on apoptosis of CAMA-1 breast cancer cells. The experiments were carried in triplicate

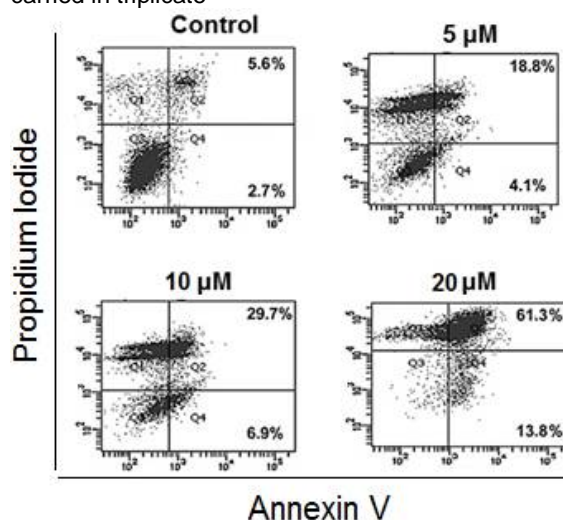


Figure 3: Percentage of apoptotic CAMA-1 cells, as depicted by Annexin V/PI staining. Experiments were carried in triplicate

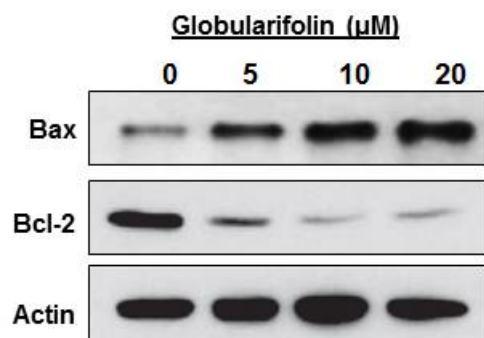


Figure 4: Effect of globularifolin on Bax and Bcl-2 expressions, as determined with western blotting. The experiments were carried in triplicate

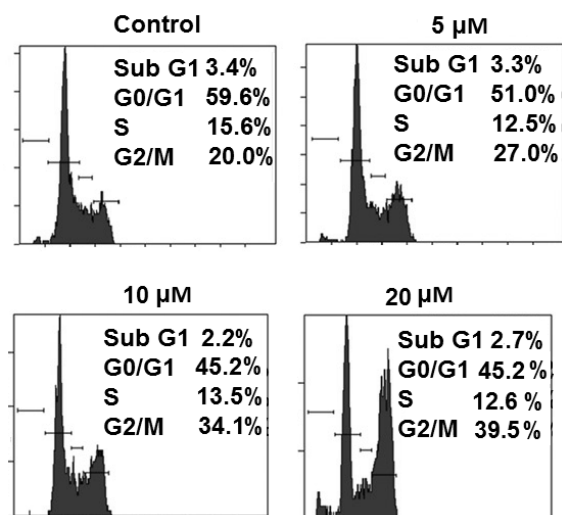


Figure 5: Effect of globularifolin acid on cell cycle distribution of CAMA-1 breast cancer cells. The experiments were carried in triplicate

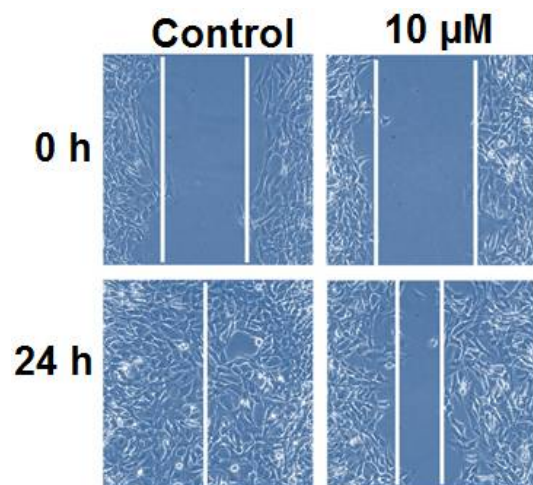


Figure 6: Effect of globularifolin on migration of CAMA-1 breast cancer cells, as assessed using wound healing. The experiments were carried in triplicate

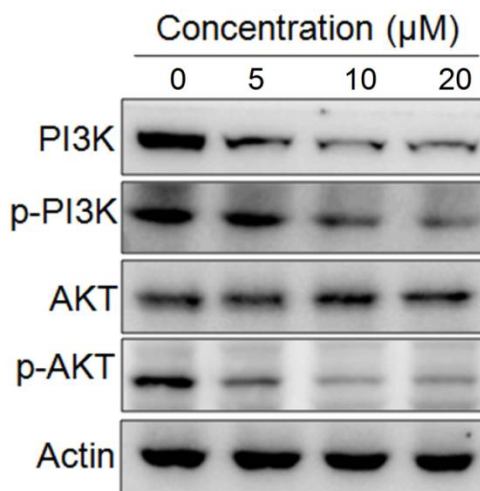


Figure 7: Effect of globularifolin on protein expressions of PI3K/AKT pathway in CAMA-1 cells, as determined using western blotting. The experiments were carried in triplicate

DISCUSSION

Breast cancer is one of the devastating cancers causing considerable mortality in women across the globe [12]. It is one of the frequently detected cancers in women, and is mostly diagnosed at the advanced stages due to the unavailability of reliable biomarkers [13]. Currently, the disease is managed with chemotherapy, but the available drugs are associated with a number of adverse side effects [14]. Over the years, plant-derived secondary metabolites have gained considerable attention as bioactive molecules. They have been shown to possess anticancer effects on different types of cancers [15].

Against this backdrop, this study was carried out to unravel the growth-inhibitory effects of globularifolin against CAMA-1 cells. The results showed that globularifolin exhibited considerable anticancer activity with an IC_{50} of 10 μ M against CAMA-1 cells, as against its IC_{50} of 65 μ M against normal CMMT breast cells. To further determine the mechanism of the anticancer effects of globularifolin, DAPI staining was performed and it was found that globularifolin exerted anticancer effects via induction of apoptosis. Moreover, the apoptotic effects of globularifolin were concentration-dependent, as was apparent from the annexin V/PI staining. Apoptosis is an important mechanism by which several chemotherapeutic drugs exert their anti-proliferative actions [16]. These results are supported by reports from a previous investigation in which globularifolin was shown to trigger apoptosis in glioma cells [7]. Herein, the expressions of Bax and Bcl-2 were determined to see if the globularifolin-induced apoptosis

followed the mitochondrial pathway. Western blotting results revealed Bax expression was enhanced, while the Bcl-2 expression was suppressed in response to globularifolin treatment. Another important mechanism that has been reported to contribute to the anticancer effects of many well-known drugs is cell cycle arrest [17]. Some anticancer drugs halt the progression of cells from one phase of the cell cycle to another by targeting specific proteins, leading to the accumulation of cancer cells at a particular stage. Arrest of the cell cycle prevents the cancer cell from developing into tumours, and from spreading to other parts of the body [18]. Consistent with this, it was observed that globularifolin caused G2/M cell cycle arrest of CAMA-1 breast cancer cells in a concentration-dependent fashion. Anticancer agents that suppress the migration of cancer cells have been reported to be important, since they can efficiently inhibit the metastasis of cancer cells [19].

It was also found that globularifolin efficiently inhibited the migration of CAMA-1 cancer cells. Earlier on, it was reported that many anticancer molecules target PI3K/AKT signalling pathway in cancer cells [9]. Therefore, the effect of globularifolin on the expressions of p-AKT, AKT, p-p-PI3K and PI3K was investigated. It was found that globularifolin decreased the expression of p-PI3K and p-AKT, indicating that the anticancer effects of globularifolin may in part, be due to blockage of the PI3K/AKT signalling pathway.

CONCLUSION

The findings of this study indicate that globularifolin possesses significant anticancer activity against CAMA-1 breast cancer through a mechanism that involves apoptosis, cell cycle arrest and inhibition of the PI3K/AKT signalling pathway. These results also suggest that globularifolin may be a promising lead molecule for development of an effective chemotherapy for breast cancer.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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