ORIGINAL ARTICLE

Anticancer activity of globularifolin against human adenoid cystic carcinoma cells is due to ROS-mediated apoptotic cell death and modulation of the JAK/STAT signalling pathway

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Summary

Purpose: Adenoid cystic carcinoma is a rare and underresearched disease. There is hardly any chemotherapy available for it, hence the urgent need to develop novel and efficient chemotherapy. Therefore, we examined the anticancer effects of globularifolin, an acylated iridoid glucoside, against salivary adenoid cystic carcinoma (SACC-83) cell line and normal human salivary gland (HSG) cell line.

Methods: Cell counting and colony formation assays were used to determine cell viability. Acridin orange (AO)/ethidium bromide (EB) staining and comet assay were used for the detection of apoptosis. Reactive oxygen species (ROS) determination and cell cycle analysis were performed by flow cytometry. Transwell assay was used to monitor cell migration and Western blot analysis was used to determine protein expression.

Results: Globularifolin inhibited the growth of SACC-83 cell line and exhibited an IC_{50} of 10 μ M. Nonetheless, the cytotoxic effects of globularifolin were comparatively negligible against normal HGS cells with an IC_{50} of 80 μ M. The investigation of the mechanism of action revealed that the anticancer effects of globularifolin against the SACC-83 cells was due to the induction of apoptotic cell death as indicated by AO/EB staining. Globularifolin treatment also resulted in enhancement of the Bax, Caspase 3 and 9 expression and decline of the Bcl-2 expression. Globularifolin also blocked the SACC-83 cells at the GO/G1 phase of the cell cycle. Moreover, cell invasion assay revealed that globularifolin inhibited the migration of the SACC-83 cells concentration-dependently, which was also coupled with the downregulation of metalloproteinase (MMP) 2 and 9. JAK/STAT is an important pathway involved in the proliferation and tumorigenesis of cancer cells and this research found that globularifolin could inhibit this pathway.

Conclusion: We conclude that globularifolin may prove essential in the development of systemic therapy for adenoid cystic carcinoma.

Key words: adenoid cystic carcinoma, globularifolin, apoptosis, cell cycle arrest, invasion

Introduction

cer mainly originating from the salivary glands as such, there are only a few treatment options

Adenoid cystic carcinoma is a rare type of can- been carried out on adenoid cystic carcinoma and [1]. Owing to its slow growth, little research has available [2]. It has been reported that adenoid

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cystic carcinoma constitutes about 1% of all malignancies and 10% of all neoplasms of the salivary glands [3]. Studies have shown that there is hardly any chemotherapeutic agent available for adenoid cystic carcinoma and patients barely benefit from the currently available chemotherapy [4]. Hence, the identification and development of new and efficient chemotherapy for adenoid cystic carcinoma is urgently required. Nature has bestowed mankind with an inexhaustible array of chemical scaffolds. These amazing chemical entities represent an exceptional source of drugs for the management of human disorders [5]. Although herbal extracts have been used since times immemorial, the use of pure isolated compounds started only a few decades ago [6]. Since then, a wide array of molecules have been isolated, evaluated and used for the treatment of several diseases and disorders [7]. Globularifolin is a pharmacologically important acylated iridoid glucoside mainly isolated from plant species belonging to the Plantaginaceae family [8]. A recent study reported that it inhibited the growth of glioma cells both in vivo and in vitro [9]. This study was designed to investigate the anticancer effects of globularifolin against human adenoid cystic carcinoma cells.

Methods

Cell viability and colony formation assays

SACC-83 cell viability was examined by cell counting assay. In brief, 5×10^4 cells/well were seeded in 12-

well plates and subjected to incubation for 24 h with different concentrations of globularifolin. The aliquots of cells were then removed and counted in triplicate following Trypan blue staining. The effect of globularifolin on the formation of SACC-83 colonies was investigated as previously described [11].

Apoptosis assays and estimation of ROS

For AO/EB staining, SACC-83 cells (0.6×10^6) were grown in 6-well plates. Following an incubation period of around 12 h, SACC-83 cells were subjected to globularifolin treatment for 24 h at 37°C. As the cells sloughed off, 25 µl cell cultures were put onto glass slides and subjected to staining with a solution (1 µl) of AO and EB. The slides were cover-slipped and examined under a fluorescent microscope. Comet assay was performed as described previously [12]. ROS levels were also estimated at different globularifolin concentrations and time intervals as previously described [13].

Cell cycle analysis

After incubating the SACC-83 cells with varied concentrations of globularifolin (0, 7.5, 15 and 30 μ M) for 24 h, the cells were subjected to washing with phosphate buffered saline (PBS). Afterwards, the SACC-83 cells were stained with propidium Iodide (PI) and the distribution of the cells in cell cycle phases was assessed by FACS flow cytometer.

Cell invasion assay

For cell invasion analysis, the SACC-83 cells were subjected to treatment with 0, 5, 10 and 20 μ M concentrations of globularifolin. The cell invasion assay was then performed as described previously [14].



Figure 1. A: Chemical structure of globularifolin. **B:** Effect of globularifolin on the viability of SACC-83 cells and **C:** normal HGS ovarian cells. The values represent the mean of three experiments ± SD (*p<0.05).

Western blotting

The SACC-83 cells were first subjected to washing with ice-cold PBS, suspended in a lysis buffer at 4°C and then at 95°C. After that, the protein content of each cell extract was checked by Bradford assay. About, 40 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 treatment with tris buffered saline (TBS) and exposed to pri-

mary antibody at 4°C. Next, the cells were treated with appropriate secondary antibodies and the proteins of interest were visualized by enhanced chemiluminescence reagent.

Statistics

Data are shown as mean \pm standard error of the mean (SEM). Statistical analysis was done using Student's *t*-test with GraphPad prism 7 software. Values of p<0.05 were regarded as statistically significant.



Figure 2. Effect of globularifolin on the colony formation of SACC-83 cells showing that globularifolin inhibits the colony formation of SACC-83 cells in a concentration-dependent manner. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).



Figure 3. Globularifolin triggered apoptosis in SACC-83 cells as indicated by AO/EB staining (arrows show apoptotic cells). The experiments were performed in triplicate.

Figure 4. Alkaline comet assay showing DNA damage in SACC-83 cells at various concentrations. The experiments were performed in triplicate.

Results

Globularifolin suppressed the growth of adenoid cystic carcinoma cells

The anti-proliferative effects of globularifolin on SAAC-83 cell line and one non-cancer HGS cell line were assessed (Figure 1A). Globularifolin treatment caused dose-dependent inhibition of the growth of the SACC-83 cells. The IC₅₀ of globularifolin against the SACC-83 cell line was found to be 10 μ M (Figure 1B). However, the cytotoxicity of globularifolin on non-cancer HGS cells was comparatively negligible (IC₅₀; 80 μ M) (Figure 1C). The effects of globularifolin treatment on the colonyformation potential of SACC-83 cells were also assessed and it was found that globularifolin exerted dose-dependent inhibitory effects (Figure 2).



Figure 5. Effect of globularifolin on the apoptosis-related protein expression in SACC-83 cells as indicated by Western blotting, which showed that globularifolin enhanced the cleavage of caspase-3, 8, 9 and PARP. The experiments were performed in triplicate.

Globularifolin activated ROS-mediated apoptosis of adenoid cystic carcinoma cells

To investigate the mechanism of the anticancer effects of globularifolin, AO/EB staining was performed and remarkable changes in the nuclear morphology and membrane blebbing of SACC-83 cells were observed (Figure 3). Furthermore, comet assay also showed that globularifolin induced DNA damage in the SACC-83 cells (Figure 4). Globularifolin also caused considerable increase in the expression of Caspase 3, 9, PARP and Bax and decreased the expression of the Bcl-2 in SW480 cells (Figure 5). To find out if globularifolin induces the generation of ROS in SACC-83 cells, ROS levels were estimated by flow cytometry. It was found that ROS levels increased from 100% at control concentrations to around 210% at 20 µM of globularifolin (Figure 6A). ROS levels also increased from 100% at 12 h to 175% at 48 h (Figure 6B).

Globularifolin caused the G0/G1 arrest of adenoid cystic carcinoma cells

In order to asses if globularifolin affects the distribution of SACC-83 cells in various cell cycle phases, flow cytometry was performed. It was found that globularifolin caused remarkable increase in the percentage of SACC-83 cells in the G0 phase of the cell cycle. The percentage of SACC-83 cells in the G0 phase increased from 56.27% to 90.39% upon treatment with globularifolin (Figure 7). These results clearly indicate that globularifolin induced G0/G1 cell cycle arrest of adenoid cystic carcinoma cells. Additionally, globularifolin also caused considerable downregulation in the expression of Cyclin B1 and Cdc2 proteins (Figure 8).

Globularifolin inhibited the invasion of adenoid cystic carcinoma cells

The effects of globularifolin were also examined on the SACC-83 cell invasion by Boyden cham-



Figure 6. Effect of globularifolin on ROS in SACC-83 cells at **A:** different concentrations and **B:** time intervals. The Figure shows that ROS levels increased upon globularifolin treatment in a concentration-dependent manner. The values represent the mean of three experiments ± SD (*p<0.05).



Channels (FL2-A)

Figure 7. Globularifolin triggered G0/G1 cell cycle arrest in SACC-83 cells as indicated by flow cytometry. The G0/G1 phase cells increased in a concentration-dependent manner. The experiments were performed in triplicate.



Figure 8. Globularifolin affected the cell cycle-related protein expression in SACC-3 cells as indicated by Western blotting which showed inhibition of expression of cyclin B1 and Cdc2. The experiments were performed in triplicate.

ber assay. It was found that globularifolin treatment could significantly inhibit the invasion of the cancer cells in a dose-dependent manner (Figure 9).

Globularifolin inhibited the JAK/STAT signalling pathway

Next, we sought to know the effects of globularifolin on the JAK/STAT3 signalling pathway of SACC-83 cancer cells. It was revealed that globularifolin caused concentration-dependent decline in the phosphorylation of p-JAK2 and p-STAT3, while no apparent effect was observed on the expression of total JAK2 and STAT3 (Figure 10).

Discussion

Adenoid cystic carcinoma is considered one of the rarest types of cancer with few treatment options available [15]. Moreover, the limited chemotherapeutic agents available today are less efficacious and patients show a very poor response to these drugs [16]. Hence, the development of novel and efficient chemotherapy for adenoid cystic carcinoma is urgently required. Therefore, we examined the anticancer potential of globularifolin against the SACC-83 cells. It was found that globularifolin exerted growth inhibitory effects on SACC-83 cells. However, the cytotoxic effects of globularifolin were comparatively negligible on HGS normal cells, indicative of the specific activity of globularifolin on adenoid cystic carcinoma cells. The antiproliferative effects of globularifolin were also confirmed by colony formation assay. Previous studies have also shown that globularifolin inhibits the growth of glioma and lung cancer cells [9,17]. In order to understand the mechanism of the antican-



Figure 9. Effect of globularifolin on the cell invasion of SACC-3 cells. The transwell assay showed that globularifolin inhibited the invasion of SACC-3 cells in a concentration-dependent manner. The values represent the mean of three experiments \pm SD (*p<0.05).



Figure 10. Globularifolin inhibited JAK/STAT3 signalling pathways as indicated by Western blot analysis. The Figure shows that globularifolin blocked the phosphorylation of JAK and STAT3 in a concentration-dependent manner. The experiments were performed in triplicate.

cer activity of globularifolin, we performed AO/EB staining, which clearly showed membrane blebbing and induction of apoptotic cell death. The apoptotic death of the SACC-83 cells was also confirmed by examining the expression of marker proteins of apoptosis. It was found that globularifolin treatment prompted an increase in the expression of Bax and cleavage of caspase 3, 8 and 9. Moreover, the expression of Bcl-2 was considerably down-regulated. In addition, comet assay also showed that globularifolin treatment caused DNA damage in SACC-83 cells.

Apoptosis is a vital process that removes defective cells from the body and maintains tissue homeostasis. It also prevents the development of chemoresistance in cancer cells [18]. In addition, ROS has been implicated in the induction of

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apoptosis [19]. Many natural products cause ROSmediated apoptosis of cancer cells [20]. In this study, we observed that globularifolin also caused the generation of significant amounts of ROS both time- and dose-dependently.

Globularifolin was also found to block SACC-83 cells at the GO/G1 checkpoint. Previously, several plant-derived molecules have been shown to cause cycle arrest of the cancer cells. For example, genipin causes cycle arrest of gastric cancer cells [21].

Next, the anti-metastatic potential of globularifolin was examined by cell invasion assay which showed that this molecule inhibited the migration of SACC-83 cells concentration-dependently, thus indicating that globularifolin may prove beneficial against metastatic cancers.

The JAK/STAT signal transduction pathway is considered an important pathway that regulates the proliferation and tumorigenesis of several types of cancers [22]. In this study we found that globularifolin blocked this pathway by inhibiting the phosphorylation of JAK2 and STAT3 proteins.

Conclusion

Globularifolin is an important iridoid glucoside with anticancer potential. Globularifolin inhibits the growth of adenoid cystic carcinoma cells by induction of ROS-mediated apoptosis and cell cycle arrest. Hence, it may prove beneficial in treating adenoid cystic carcinoma and therefore warrants *in vivo* evaluation.

Conflict of interests

The authors declare no conflict of interests.

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