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Original Article

Globularifolin exhibits potent anticancer activity on A549 human lung cancer cell line via induction of mitochondrial apoptosis, cell cycle arrest and NF-kB inhibition

Yingou Zeng, Yongming Zhang

Department of Thoracic Surgery, Shanghai University of Medicine & Health Sciences, Shanghai 201318, China

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Abstract: Globularifolin is an iridoid glucoside and has only been isolated from some plant species of *plantaginaceae*. Iridoids glucosides have been reported to exhibit tremendous pharmacological potential. However, the anticancer activity of the rare acylated iridoid glucoside globularifolin has not been reported so far. In the present study we determined its anticancer potential against human lung cancer A549 cancer cell line. The results showed that globularifolin has an IC_{50} value of 12.5 μM human lung cancer cells and inhibited the colony formation potential in a dose-dependent manner. The molecule exerted its anticancer activity through induction of apoptosis by regulating Bcl-2/Bax signaling pathway. Additionally, it caused cell cycle arrest of human lung cancer A549 cancer cells at G2/M phase. Globularifolin was also found to cause reduction in the mitochondrial membrane potential in a dose dependent manner. Additionally, globularifolin effectively inhibited NF-kB almost by 50% at 25 μM concentration at 24 h incubation. We conclude that globularifolin exerts its anticancer activity through induction of mitochondrial apoptosis, G2/M cycle arrest and NF-kB inhibition indicating it may prove as a lead molecule treatment of lung cancer.

Keywords: Lung cancer, globularifolin, apoptosis, cell cycle arrest, NF-kB

Introduction

Iridoids are monoterpenes and consist of a basic scaffold consisting of cyclopenta[c]-pyran ring system. A diversity of medicinal plants showing the presence of iridoids have been used in several traditional systems of medicine for the treatment of several diseases and disorders. The medicinal properties of these plants are mainly due to the presence iridoids as active constituents. The plant derived iridoids and their glucosides have been reported to exhibit tremendous pharmacological potential including neuroprotective, anticancer and others [1, 2]. Globularifolin (**Figure 1**) is a rare acylated iridoid glucoside isolated from a few genera of family *Plantaginaceae* of plants. Mainly, globularifolin has been reported to be isolated from *Globularia cordifolia* [3, 4]. Despite the pharmacological potential of iridoids, the pharmacological activities of globularifolin have not been studied so far. The present study was therefore designed to evaluate the anticancer potential of globularifolin against A549 human lung cancer cell line and to decipher the underlying mechanism. Lung cancer is as one of the main causes of mortality and the major cause of cancer related deaths in China [5, 6]. The severe increase in the frequency of cancers, the dearth of suitable cure and the severe side effects accompanied with the synthetic drugs has made it compulsory to explore new and more effective molecules. With upsurge in the incidence of drug-resistance, the treatment and management of cancers has become even difficult [5, 6]. In the current study, globularifolin was evaluated against human lung cancer cells and it was found to exhibit an IC_{50} at 12.5 μM concentration. Of note, the results of the present study indicated that globularifolin exhibits a significant anticancer activity by inducing apoptosis in human lung cancer A549

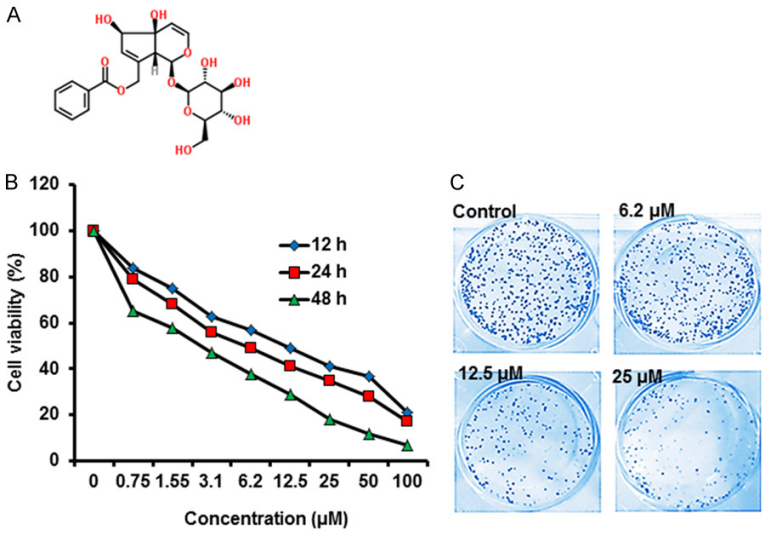

Figure 1. A. Chemical structure of Globularifolin; B. Effect of globularifolin on human A549 Lung cancer cells at 12, 24 and 48 h of incubation; C. Colony forming potential of lung cancer A549 cells at varied doses of 6.2, 12.5 and 25 μM of globularifolin. All experiments are representatives of three biological replicates ± SD. Results were considered significant at $P^*<0.05$.

cancer cell, altering mitochondrial membrane potential (MMP) and by causing cell cycle arrest via downregulation of the expression of Bcl-2 and upregulation of Bax expression. In conclusion, we propose that globularifolin may prove a potential candidate towards the development of anticancer chemotherapy for lung cancer.

Materials and methods

Cell lines and culture conditions

Human lung cancer cell line (A549) was purchased from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin and maintained in a humidified atmosphere containing 5% CO_2. All of the reagents were procured from Hyclone (Logan, UT, USA).

Anti-proliferative and colony formation assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] assay [7] was used to determine the anti-proliferative activity of globularifolin against the human lung cancer A549 cells. The A549 cells in 100 μL culture medium were seed in a 96-well plate at a density of 3×10^3 cells/mL and kept at 37°C in 5% CO_2 for a time period of 24 h. After 24 h, an additional 100 μL of complete medium with either no additions or different concentrations (0-100 μM) of globularifolin were added. Thereafter the cells were incubated for 12, 24, and 48 h. This was followed by the addition of 20 μL of MTT solution (5 mg/mL) and an incubated of 4 h. Afterwards the medium was removed and 150 μL DMSO was add. The absorbance (OD) of each well was measured at 490 nm using a Tunable Mi-185 croplate Reader (EL-x 800, BioTek Instruments, USA). To evaluate its effect on the colony formation, human lung cancer A549 cells at the exponential growth phase were harvested and counted with a hemocytometer. Platting of the cells was done at 200 cells per well. This was followed by incubation for a time period of 48 h to allow the cells to adhere and then different doses (0, 6.2, 12.5 and 25) of globularifolin were added. After administration of globularifolin acid, the cells were again kept for incubation for 6 days, washing was done with PBS and methanol was used to fix colonies and then stained with crystal violet for about 30 min before being counted under light microscope.

Apoptosis and comet assays: Human A549 cells at a density of 2×10^5 cells/well were seeded in 6-well plates, which were administrated with 0, 6.2, 12.5 and 25 μM globularifolin for 48 h. The cells were then subjected to DAPI staining for detection of apoptosis by fluorescence microscopy [8]. For estimation of apoptotic cell populations, an FITC-Annexin V/PI Apoptosis detection kit was used following the manufacturer's instructions (Beijing Biosea Biotechnology, China). Alkaline comet assay was carried out to evaluate DNA damage efficacy of globularifolin as described previously [9]. Briefly, 1×10^5 A549 cells were seeded in 24-well plates and treated with various concentrations of globularifolin for 48 h. The slides were processed and stained with SYBR green and the migration of DNA was observed using a fluorescence microscope (Nikon, Japan) at 10 × magnification. The comet tail lengths were ana-

lyzed by TriTek CometScore™ software (Tritek Corporation, VA, USA).

Determination of cell cycle phase distribution of A549 cells

For cell cycle analysis, the A549 cells were treated with 0, 6.2, 12.5 and 25 μM globularifolin concentration of KAM and the percentage of cells in each of the cell cycle phases were estimated by using Muse™ Cell Analyzer and Muse™ Cell Cycle Kit according to the manufacturer's protocol (Merck Millipore).

Flow cytometric determination of mitochondrial membrane potential (MMP)

Human Lung cancer A549 cells were seeded at a density of 2×10^5 cells/well in a 6-well plate and kept for 24 h and treated with 25 μM Hesperidin for 6-72 h at 37°C in 5% CO_2 and 95% air. Thereafter cells from all treatment were collected, washed 2 times by PBS and resuspended in 500 μl of DOC_6 (1 μmol/l) for MMP at 37°C in dark room for 30 min. The samples were then analyzed instantly using flow cytometry.

Luciferase reporter assay

A549 cells were transfected with NF-kB-Luc reporter plasmid in combination with pRL-TK Renilla luciferase vector (Promega). After 24 hours post transfection, the cells were lysed and the luciferase activity was determined using Promega Dual Reporter Assay following manufacturer's protocol. Relative luciferase activity was determined as the ratio of reporter to transfection control.

Western blotting analysis

After administration with various concentrations of globularifolin, cells were harvested and lysed in lysis buffer (20 mM HEPES, 350 mM NaCl, 20% glycerol, 1% Nonidet P 40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, protease inhibitor cocktail, and phosphatase inhibitor cocktail). Out of the total protein samples, 20 μg aliquot was separated on 10% SDS-PAGE gel. The gel was then transferred to nitrocellulose membranes, blocked with 5% BSA and probed with a primary antibody (This was followed by probing with the required secondary antibody). Finally, the signal was perceived with WEST-SAVE Up™ luminal-based ECL reagent (ABFrontier, Korea).

Statistical analysis

All experiments were carried out in triplicates and the results were expressed as mean ± standard deviation (SD). Statistical analysis was carried by GraphPad prism 7 using one-way analysis of the variance (ANOVA) and Tukey test. The values were considered significant at $P^*<0.05$ and $P^{**}<0.01$.

Results

Anti-proliferative activity of globularifolin on A549 human lung cancer cells

Anti-proliferative activity of globularifolin was evaluated against human cancer cell A549. The assay of MTT assay at 0-100 μM concentration showed that Globularifolin exhibited a concentration dependent activity. The IC_{50} of Globularifolin against humman A549 cells was found to be IC_{50} 12.5 μM (**Figure 1B**). In the colony formation assay carried out at 0, 6.2, 12.5 and 25 μM concentration of globularifolin revealed that globularifolin administration reduced the number of colonies in a dose-dependent manner (**Figure 1C**). The colony formation was reduced by upto 78% at 25 μM concentration.

Globularifolin induced apoptosis and DNA damage in human A549 lung cancer cells

DAPI staining indicated that globularifolin induced apoptosis in A549 human cancer cells in a dose dependent manner (**Figure 2A**). At the concentrations of 12.5 and 25 μM, apoptotic corps were clearly visible. In order to confirm apoptotic cell death induced by globularifolin, annexin V/PI staining was carried out at the concentrations of 0, 6.2, 12.5 and 25 μM. Flow cytometric results showed that the percentage of apoptotic cell increased to 16%, 28% and 48% in human A549 cancer cells after 48 h at the concentrations of 6.2, 12.5 and 25 μM, respectively, as compared to untreated control (**Figure 2B**). Thus the results indicate that globularifolin caused apoptosis of human A549 cancer in a concentration dependent manner. Moreover, the results of comet assay showed that globularifolin caused DNA damage in A549 human lung cancer cells in a dose dependent way (**Figure 3A**, **3B**).

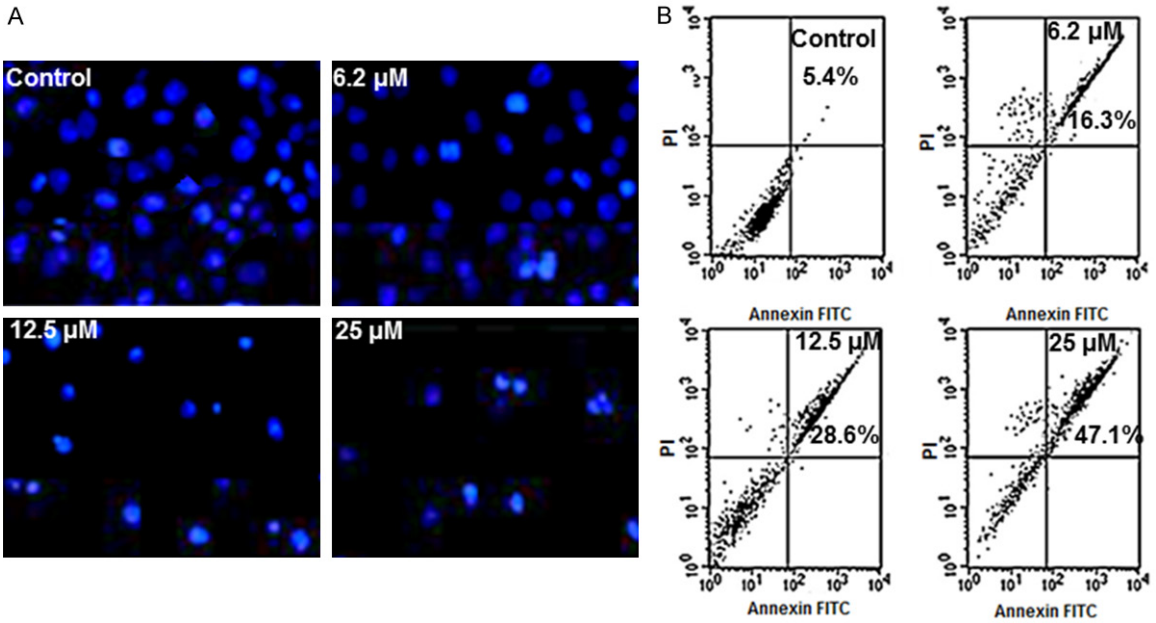

Figure 2. Induction of apoptosis by globularifolin at the doses of 6.2, 12.5 and 25 μM as depicted by (A) DAPI staining observed by fluorescence microscopy (B). Annexin V/IP staining observed by flow cytometry. All experiments are representatives of three biological replicates.

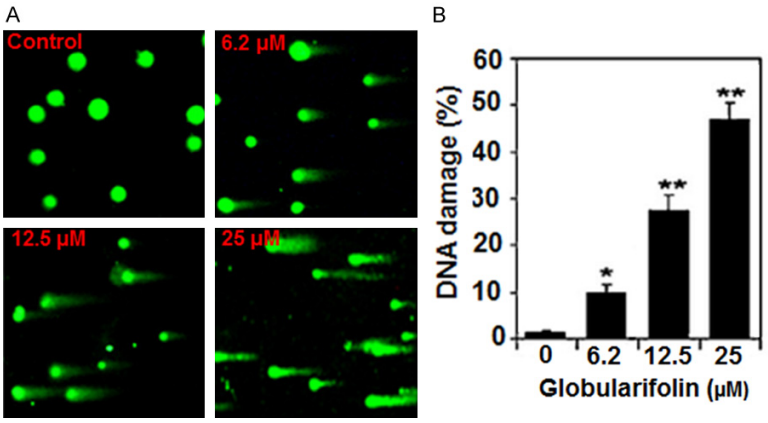

Figure 3. Effect of indicated doses of globularifolin on DNA damage (A). Comet assay (B) quantification of DNA damage. The experiments were carried out in triplicates and expressed as mean ± SD. The values were considered significant at $P^*<0.05$ and $P^{**}<0.01$.

Globularifolin caused stimulated G2/M phase arrest of A549 cancer cells

Our results indicated that globularifolin caused G2/M cell cycle arrest in a dose-dependent manner. It was observed that the percentage of cells was considerably increased at G2/M phase by the treatment of Globularifolin (**Figure 4**). Additionally, the populations of A549 cells at G2/M phase were only slightly elevated at a dose of 6.2 μM, however, the apoptotic cell populations significantly increased at G2/M phase at the concentration of 25 μM. The globularifolin induced cell cycle arrest of A549 cancer cells showed a dose-dependent pattern.

Globularifolin triggered the MMP loss in human A549 lung cancer cells

Cells were administrated with 0, 6.2, 12.5 and 25 μM globularifolin for various time periods and the levels MMP were evaluated. A significant reduction of MMP level (**Figure 5A**) was experienced in the treated A549 cells as compared to the control. At concentrations of 0, 6.2, 12.5 and 25 μM, the MMP was found to be 84, 75 and 57% as compared to the untreated human A549 cancer cells.

Effect of globularifolin on Bcl-2/Bax signalling pathway

The fact that globularifolin could induce apoptosis; we determined the expressions of proto-

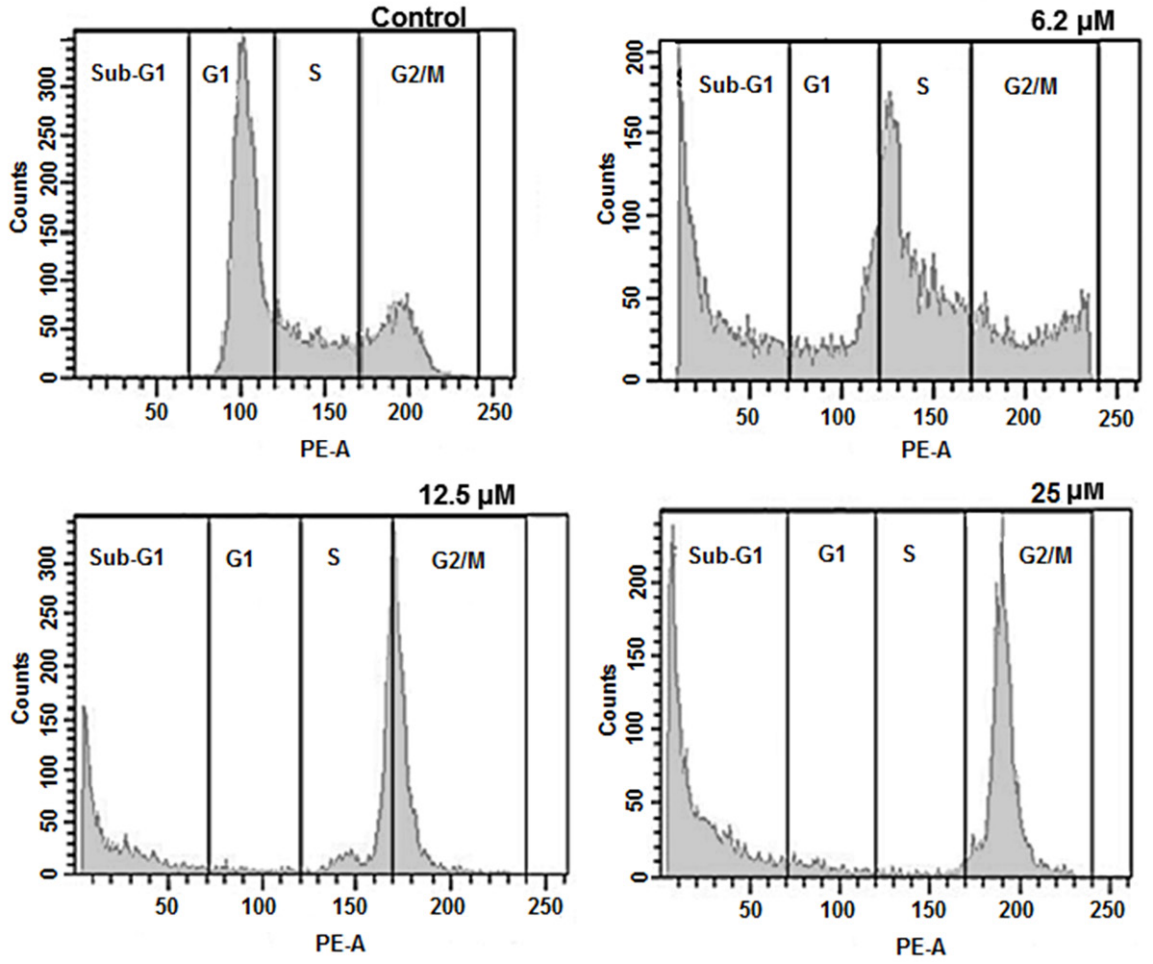

Figure 4. Effect of varied doses 6.2, 12.5 and 25 μM of globularifolin on cell cycle phase distribution of A549 cancer cells. All experiments are representatives of three biological replicates.

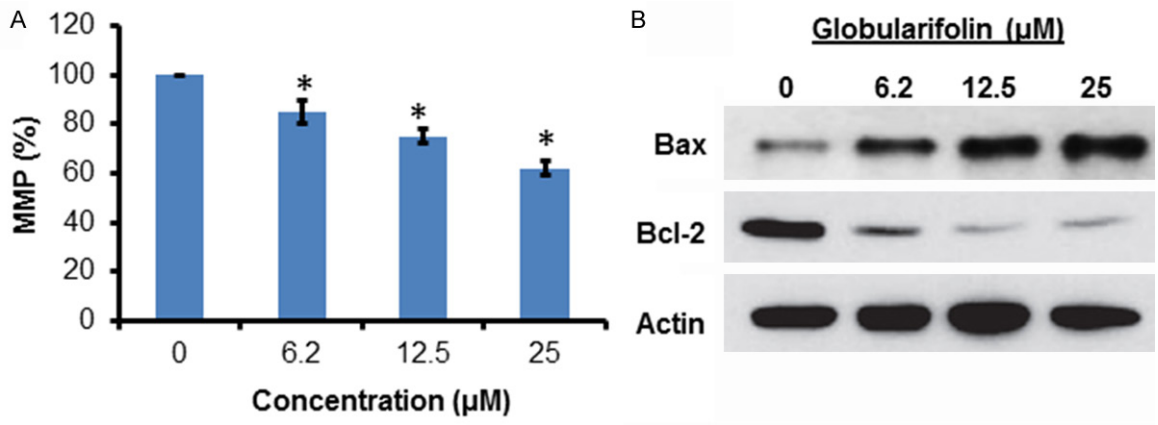

Figure 5. Effect of indicated doses globularifolin on (A) mitochondrial membrane potential of A549 cancer cells (B). Expression of Bax/Bcl-2 by western blotting. All experiments are replicates of three biological replicates. All experiments are representatives of three biological replicates ± SD. Results were considered significant at $P^*<0.005$.

pototic proteins Bcl-2/Bax by western blotting. The findings are shown in **Figure 5B** and indicate an interesting outcome. The increased Bax/Bcl-2 ratio causes activation of caspase 3

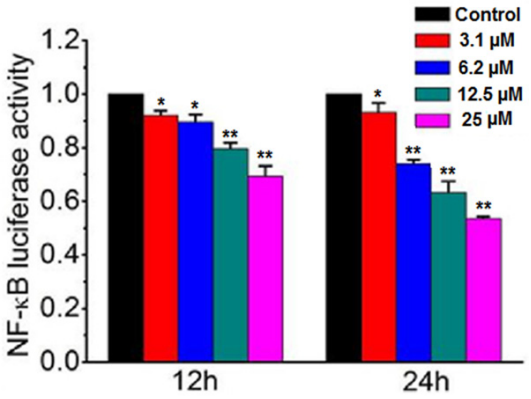

Figure 6. Luciferase reporter assays monitoring NF-kB activity in A549 cancer cells. All experiments are representatives of three biological replicates ± SD. Results were considered significant at $P^*<0.05$ and $^{**}P<0.01$.

and hence apoptosis. In our results, Compared to the untreated control cells, globularifolin treated cells showed a concentration-dependent downregulation of Bcl-2 and upregulation of Bax proteins. Hence increasing the Bax/Bcl-2 ration and ultimately inducing apoptosis.

Globularifolin inhibits NF-kB

The NF-kB-Luc luciferase reporter assay in human A549 lung cancer cells, revealed that globularifolin apparently inhibited the NF-kB-Luc reporter at 12 and 24 h in a concentration-dependent manner, (**Figure 6**) and 25 μM globularifolin decreased NF-kB activation by almost 50% at 24 h.

Discussion

Lung cancer is one of the deadly cancers detected around the globe. Lakhs of patients are diagnosed for this disease annually [5]. Nevertheless, existing treatment options exhibit decent clinical results, still lakhs of cancer related deaths are accredited to lung cancer. Moreover, existing treatment options have severe side effects which badly influence the quality of life [6]. Furthermore, development of drug resistance has made cancer very difficult to treat. Consistently, natural products are known to have multiple targets and able to suppress or activate complicated signaling pathways to inhibit cancer cell growth [7]. In the current study, globularifolin showed potential growth inhibiting activity against human lung A549 cancer cells as evident from the proliferation assay. As reported previously, many drugs exhibit antiproliferative effects via induction of apoptosis. Several existing drugs have been reported to alter explicit apoptotic signalling pathways [9-14]. Additionally, resistance to drug is partially enlightened by the potential of cancer cells to escape apoptosis [15]. To determine whether globularifolin induces apoptosis in A549 cells, we carried out the DAPI staining of the globularifolin treated cells. It was observed that globularifolin induces apoptosis in a concentration dependent manner. Mitochondrial outer membrane permeability (MOMP) is an important process involved in the apoptotic pathway. In the present study it was observed that globularifolin treated showed a significant reduction in the MMP and exhibited a concentration dependent pattern. Our results are consistent with studies carried out earlier [16]. Therefore the results suggest that globularifolin may induce apoptosis by cumulative intracellular reduction in MMP. It has been reported that many anti-cancer drugs target cancer cells partially by generation reduction of MMP [17]. Analysis of apoptotic cell populations revealed that globularifolin induce apoptosis and DNA damage in human lung cancer cells as evident from DAPI staining and comet assay. These are in agreement with previous studies wherein natural products such as isoliquiritigenin, to inhibit cancer cell growth and induce apoptosis [18]. Flow cytometry using propidium iodide as a probe was used to study effects of Globularifolin on cell cycle progression. Globularifolin induced G2/M cell cycle arrest and led to a significant increase of G2/M cells dose dependently. Our results are consistent with previous studies wherein natural products such as genestien have been reported to inhibit cell proliferation and induce cycle arrest in a dose-dependent manner [19]. Additionally, effects of globularifolin on Bcl-2/Bax signalling were studied using western blot assay. Results showed globularifolin-treated cells showed a concentration-dependent downregulation of Bcl-2 and upregulation of Bax proteins ultimately inducing apoptosis. Members of the Bcl-2 family proteins, such as Bcl-2, Bax and Bak, are believed to play key controlling roles in the execution of cell apoptosis [19]. However, several studies have shown that Bcl-2 family proteins also aim at the apoptotic pathway [20]. We also evaluated the effect of globularifolin on NF-kB and it was observed that it apparently inhibited the NF-kB-Luc reporter at 12 and 24 h in a concentration-dependent

manner, and 25 μM globularifolin decreased NF-kB activation by almost 50% at 24 h. NF-kB plays essential part in carcinogenesis by controlling the expression of a number of proteins[16] and thus its inhibition is considered as an important aspect of anticancer drug development.

Conclusion

In conclusion, in the present study, our data provides a framework for the construction of cell death pathways in A549 cells in response to globularifolin by inducing apoptosis, regulating Bcl-2/Bax signaling pathway and mainly by inhibition of NF-kB. All of these contribute to the growth inhibition of cancer cells and globularifolin might potentially serve as a potential candidate for cancer therapy.

Disclosure of conflict of interest

None.

Address correspondence to: Yongming Zhang, Department of Thoracic Surgery, Shanghai University of Medicine & Health Sciences, Shanghai 201318, China. Tel: 0086-21-68135590; Fax: 0086-21-68135590; E-mail: yongmingzhang135@hotmail.com

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