Acta Biochim Biophys Sin 2013, 45: 946–952 | © The Author 2013. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmt097.
Advance Access Publication 15 September 2013

Original Article

Glaucocalyxin A, a negative Akt regulator, specifically induces apoptosis in human brain glioblastoma U87MG cells

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Akt is becoming an attractive target in the development of anti-tumor agents. In the present study, we aimed to discover novel negative Akt regulators against malignant glioma. An Akt regulator screening platform performed in an Akt-GFP overexpression cell line was developed, and natural product library was screened and evaluated using this platform. In addition, the cytotoxic effect of the regulator was detected by MTT assay. Cell apoptosis was assayed by Hoechst 33342 staining and flow cytometry analysis. Afterwards, the apoptotic signaling pathway was investigated by western blot analysis. Glaucocalyxin A, isolated from *Rabdosia japonica*, was identified as a potent negative regulator of Akt. In human-derived malignant glioma U87MG cells, glaucocalyxin A inhibited Akt phosphorylation, suppressed proliferation, and promoted apoptosis in a dose-dependent manner, but not in normal glial cells. Furthermore, glaucocalyxin A activated caspase-3, decreased BAD phosphorylation, and reduced the expression of X-linked inhibitor of apoptosis protein. Taken together, these results indicated that glaucocalyxin A may become a promising candidate in the treatment of malignant glioma.

Keywords apoptosis; glaucocalyxin A; glioblastoma; Akt regulator

Received: April 5, 2013 Accepted: June 20, 2013

Introduction

The annual incidence of malignant glioma is close to 1/20,000, of which glioblastoma accounts for about 60%–70% [1]. In addition, the malignant gliomas detected in clinics account for 15%–25% of all intracranial tumors [2]. Nearly 70% of low-grade glioma patients inevitably incur secondary malignant glioma because of malignant progression and recurrence risk during their limited lifetime [3]. In the past 30 years, the incidence of these tumors was still increasing, especially in young people. They have been the second largest cause of death in young cancer patients [4]. Even after surgery, radiotherapy and chemotherapy, the median survival of glioblastoma patients is only 12–15 months [1]. In addition, glioblastoma patients are prone to early recurrence, of which the median survival time is about 7 months from the first recurrence to death [5]. Therefore, developing new drugs to treat malignant glioma is urgently needed.

Serine/threonine kinase Akt (also known as protein kinase B) plays an important role in cell signaling pathways, which regulates cell survival and metabolism [6,7]. As a proto-oncogene, *Akt* is named as *v-Akt* in mice [8]. There are three subtypes in Akt families: Akt1 (PKBα), Akt2 (PKBβ), and Akt3 (PKBγ). They are all associated with cancers, manifesting regular and unique features in cells. Once properly located in the plasma membrane, Akt is phosphorylated and then activated. It is well known that Akt has anti-apoptotic activity and plays a vital role in cell viability and proliferation [7]. The dysregulation of Akt is closely related to a variety of human diseases, including cancer, diabetes, and schizophrenia. In cancers, the activation of Akt is not only associated with the tumor origin, but also has a close relationship with tumor grade and transfer rates [9]. Nowadays, Akt has become an attractive drug target to treat diseases with abnormal Akt signaling [10]. Many studies have suggested that Akt over-activation has been detected in various types of human malignancy including malignant glioma, pancreatic carcinomas, ovarian cancer, and breast cancer [11–15]. Moreover, knockdown of Akt by antisense or siRNA could significantly induce apoptosis of Akt overexpressing tumor cells as well as reduce tumor growth and invasiveness [16–20]. Meanwhile, some results indicated that knockdown of Akt could induce cell cycle arrest in U87MG cells and inhibit the growth of glioma xenograft [21]. Since the excessive activation of Akt in a vast majority of human malignant tumor cells, many compounds acting on PI3K/Akt pathway have been reported to treat cancers in the literature [22].

Based on the available evidence, Akt has been proved to be an effective anti-glioma target.

The natural products have always been an important resource library for drug discovery because of their structural diversity. There are a lot of natural products in anti-cancer treatment drugs, such as paclitaxel. Medical herbs are widely used in anti-tumor treatment for cancer patients in some Asian countries. Many of them have satisfactory efficacy. *Rabdosia japonica* has been mainly used to prevent and treat esophageal cancer, while its effect against malignant glioma has not been reported. In the present study, we reported that a natural product glaucocalyxin A [23], which was isolated from *R. japonica* and identified as a potent negative Akt regulator, showed specifically pro-apoptotic effects in human brain glioblastoma U87MG cells. Therefore, glaucocalyxin A might be a promising candidate in the treatment of malignant glioma.

Materials and Methods

Cell culture and specimens

Human glioblastoma U87MG cells (purchased from ATCC, Manassas, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin. CHO-Akt-GFP cells (obtained from Amersham, Buckinghamshire, UK) were cultured in F12 medium to select stable cell strains containing 10% FBS, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 400 μg/ml of G418. All of the cells were cultured at 37°C in an incubator with 5% CO_2. Primary glial cells were isolated from the gray matter of normal brain tissue samples from patients with traumatic brain injury. Tissue aperture was washed with phosphate-buffered saline (PBS; 0.01 M, pH 7.4), and sieved with 200 μm mesh. Glial cells were collected by centrifuge at 1000 *g* for 5 min. The cell pellets were then washed and resuspended with PBS and plated into T75 cell culture flasks (Greiner Bio-One GmbH, Frickenhausen, Germany). Cells were cultured in DMEM containing 30% FBS in an incubator with 5% CO_2 at 37°C. Samples were collected with the approval of the Medical Laboratory Ethics Committee, Xijing Hospital, Fourth Military Medical University (Xi' an, China). All of the patients gave written informed consent.

Akt regulator screening

Compounds that can inhibit Akt phosphorylation were screened from natural product library with the Akt-GFP fusion protein in the stable cell line (CHO-Akt-GFP) by IN CELL 1000 instrument (Amersham). Cells were plated into 96-well plate (Costar, New York, USA) and grown to 50% convergence. Cells were then incubated for 1 h in the presence of different compounds and stimulated by insulin-like growth factor-1 (IGF-1) for another 5 min. Wortmannin (Sigma, St Louis, USA) was used as a positive control in the test. Each assay was performed in triplicate wells. Pictures were taken by the IN CELL equipment, and the distribution of the Akt-GFP protein on the plasma membrane was analyzed by the in cell analyzer software to reflect the level of phosphorylation.

Akt kinase activity assay

The effect of glaucocalyxin A on the kinase activity of Akt was determined using the commercially available kit (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Briefly, the Akt activity was, respectively, accessed in 25 μl of a buffer containing 50 mM HEPES (PH 7.5), 0.0015% Brij-35, 10 mM $MgCl_2$, 2 mM dithiothreitol (DTT), and 30 nM enzyme in the presence of the indicated concentrations of glaucocalyxin A or the inhibitor staurosporine (Invitrogen). The mixture was incubated at room temperature (RT) for 10 min. Then, 3 μM carboxyfluorescein-labeled peptide and 2 μM ATP were added and the mixtures were incubated for another 5 h at 28°C. The reaction was terminated by adding 25 μl buffer containing 50 mM HEPES (PH 7.5), 0.0015% Brij-35, 10 mM $MgCl_2$, and 2 mM DTT. Data were collected on Caliper.

MTT assay

U87MG cells were seeded at a density of 5×10^3 per well in 96-well plates. After reaching the logarithmic growth phase, cells were treated with 0, 0.1, 1, 5, and 10 μM glaucocalyxin A for 24, 48, and 72 h, respectively. The assay was performed in six wells at each experimental condition. To test the cell viability, 20 μl of MTT (5 g/l) was added to each well and incubated for 4 h. The purple crystal was dissolved by adding 150 μl dimethyl sulfoxide (DMSO). The optical density was measured at a wavelength of 490 nm with a Bio-Rad 680 microplate reader (California, USA). Cell viability was calculated as the percentage of the control (considering as 100%). The primary glial cells treated with glaucocalyxin A in different concentrations (0, 0.1, 1, 5, and 10 μM) for 72 h were used as the control in the MTT assay.

Morphological analysis

Logarithmic growths of U87MG cells were plated into 96-well plates at a density of 5×10^4 per ml, 200 μl per well, and cultured for 24 h. The cells were then treated with different concentrations (0, 5, and 10 μM) of glaucocalyxin A for 24 h. The final concentration of DMSO was 0.1% (v/v) in the medium. Cell morphology was observed under light microscope and photographed using imaging-pro (Olympus, Tokyo, Japan). Furthermore, the cell morphology was also observed by Hoechst 33342 nuclear staining. After the treatment, cells were stained by 2 μg/ml of Hoechst 33342 dyes for 5 min. The cells were then washed twice with PBS. Nuclei were detected with an excitation wavelength of 350 nm and emission wavelength at 460 nm by a fluorescence microscopy (Leica Microsystems, Wetzlar, Germany).

Flow cytometry experiments

Logarithmic growths of U87MG cells at a density of 5×10^5/ml were cultured in T25 flasks (Greiner Bio-One GmbH). The cells were treated with 1, 5, and 10 μM of glaucocalyxin A, while control cells were treated with 0.1% (v/v) DMSO. After being incubated for 24 h at 37°C with 5% CO_2, cells from each flask were collected, respectively. After being centrifuged at 50 *g* for 5 min, the cells were then washed and resuspended with PBS. A total of 100 μl cell suspension from each sample was added with 1 μl Annexin V-fluorescein isothiocyanate (200 μg/ml) and 10 μl propidium iodide (PI; 30 μg/ml). After 5 min of incubation, each sample was analyzed by flow cytometry (Beckman Coulter, Brea, USA) (excitation wavelength = 488 nm, emission wavelength = 530 nm). The percentage of apoptotic cells is analyzed by Cell Quest software (Becton Dickinson, San Jose, USA).

Western blot analysis

Cells were lysed in RIPA buffer (Thermo Scientific, Waltham, USA) and proteins were quantified by BCA protein

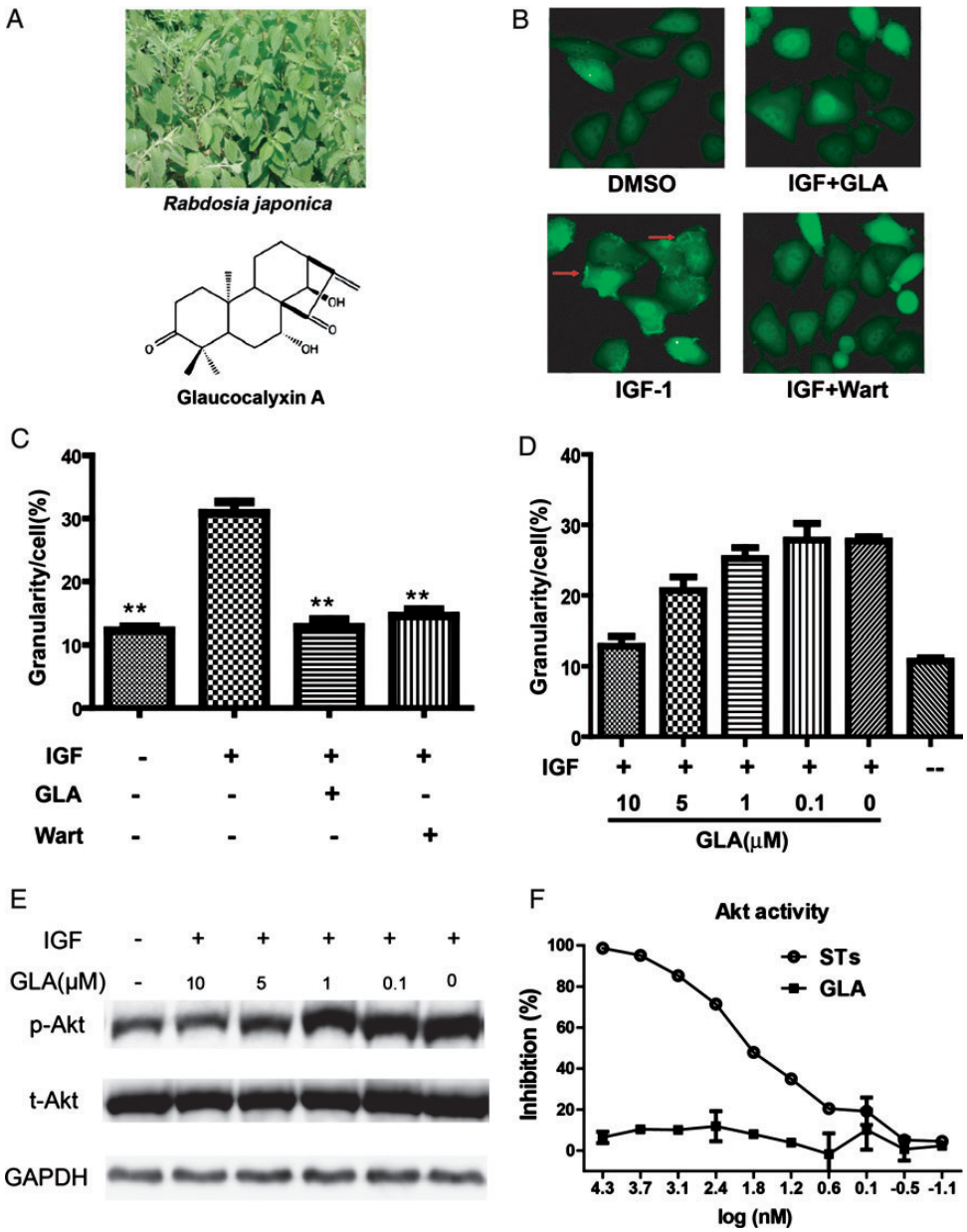

Figure 1 Glaucocalyxin A was identified as a potent Akt regulator (A) The structure of glaucocalyxin. (B) The distribution of Akt-GFP in CHO-Akt-GFP cells after treating with DMSO, IGF-1, IGF-1+glaucocalyxin A, IGF+Wartmanin for 1 h (magnification, ×200). (C) Quantifying analysis (granularity per cell, %) of three assays. (D) Glaucocalyxin A inhibited IGF-1-stimulated Akt translocation in a dose-dependent manner. (E) Glaucocalyxin A inhibited IGF-1-stimulated Akt phosphorylation in a dose-dependent manner. (F) Glaucocalyxin A had no effects on Akt kinase activity. Data were presented as the mean ± SD ($n = 3$). $^{**}P < 0.001$, compared with vehicle (DMSO). These data are the representatives of three independent experiments. GLA, glaucocalyxin A.

assay kit (Thermo Scientific). Equal amounts of samples were resolved by 10% polyacrylamide gels and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, USA). Antibodies against Akt, p-Akt, BAD, p-BAD, XIAP, caspase 3, cleaved caspase 3 (1 : 1000 dilution; Cell signaling Technology, Beverly, USA) were used to incubate the membrane at 4°C overnight. GAPDH was used as an internal control. Anti-rabbit secondary antibodies (1 : 3000 dilution) were then incubated at RT for 1 h. The bands were visualized with Dura (Thermo Scientific). Images were taken by Image Quant LAS 4000 Mini (GE, New York, USA) and the value of the gray densities of the bands were analyzed by Image Quant TL (GE).

Statistical analysis

Data were expressed as the mean ± SD. One-way analysis of variance followed by Dunnett's *post hoc* test was applied for multiple comparisons between treatments and control. Statistical analysis was performed using the SPSS software (version 15.0). Differences were considered significant at $P < 0.05$.

Results

Glaucocalyxin A was identified as a potent Akt regulator

A natural product library was screened by using IN Cell Analyzer 1000 with CHO-Akt-GFP cells, and glaucocalyxin A, a natural product isolated from *R. japonica*, was identified as a potent negative Akt regulator [**Fig. 1(A)**]. IGF-1 acted as a positive regulator of Akt, markedly increased the distribution of protein granularity in the plasma membrane, indicating that the phosphorylation and translocation of Akt were promoted. However, the effect of IGF-1 was almost completely inhibited by adding glaucocalyxin A or Wortmannin ($P < 0.001$, respectively) [**Fig. 1(B,C)**]. Moreover, glaucocalyxin A could inhibit translocation in the presence of IGF-1 in a dose-dependent manner [**Fig. 1(D)**].

To verify the regulation of glaucocalyxin A on Akt, we detected the phosphorylation of Akt in CHO-Akt-GFP cells. Glaucocalyxin A could inhibit IGF-stimulated Akt phosphorylation in a dose-dependent manner [**Fig. 1(E)**]. To identify the possible target of glaucocalyxin A, we investigated its direct effect on Akt kinase activity. As shown in **Fig. 1(F)**, glaucocalyxin A exerted no effects on Akt kinase activity indicating that glaucocalyxin A-induced Akt inhibition in an indirect manner.

Taken together, we identified a natural product glaucocalyxin A, which could indirectly inhibit Akt phosphorylation and translocation and might function as a negative Akt regulator.

Glaucocalyxin A promoted apoptosis in malignant glioma U87MG cells

To test the specific anti-tumor role of glaucocalyxin A, its effects on cell viability were accessed in U87MG cells and normal glial cells. Results showed that glaucocalyxin A significantly inhibited the viability of U87MG cells in a time- and dose-dependent manner [**Fig. 2(A)**]. After being treated with 10 μM glaucocalyxin A for 24, 48, and 72 h, the cell viability of U87MG cells was maximally decreased to 38.5%, 25.7%, and 5.1% of the control, respectively. In contrast, glaucocalyxin A had little effect on the cell viability of human-derived normal glial cells, which indicated the good selectivity of glaucocalyxin A in tumor cells.

To study the effects of glaucocalyxin A on cell apoptosis, we observed the morphological changes of U87MG cells treated with 10 μM glaucocalyxin A for 48 h. As shown in **Fig. 2(B,C)**, the cytoskeletal structures of a large number of U87MG cells were damaged, and the cells became round, shrinking, and aggregative. The dendritic structure of the rest

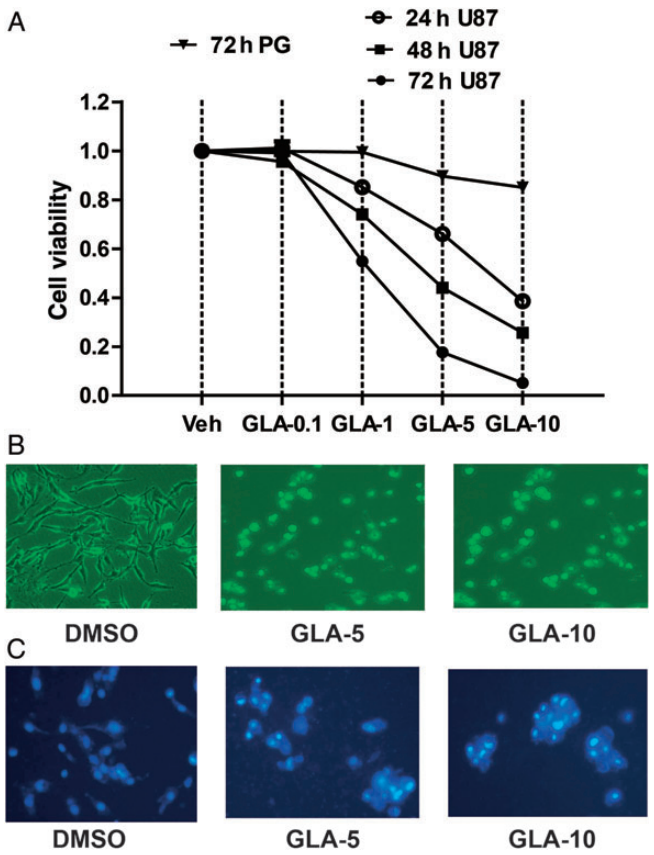

Figure 2 Glaucocalyxin A promoted apoptosis in malignant glioma U87MG cells (A) Glaucocalyxin A decreased cell viability in a time- and dose-dependent manner. U87, U87MG cells; PG, primary glia cells; GLA, glaucocalyxin A. (B,C) The morphological changes and Hoechst 33342 staining of U87MG cells, which were incubated with 0 (control), 5, and 10 μM glaucocalyxin A for 48 h (magnification, ×100). Data were presented as the mean ± SD ($n = 3$). These data are the representatives of three independent experiments.

cells became shorter and narrower compared with normal tumor cells. Hoechst 33342 staining more clearly showed these changes induced by glaucocalyxin A. Compared with the control cells, cells treated with glaucocalyxin A showed significant apoptotic changes such as characteristic condensation of the chromatins and nuclear fragmentation. Furthermore, results from flow cytometry confirmed that glaucocalyxin A promoted the apoptosis of glioma cells [**Fig. 3(A)**]. There were 14.6%, 60.9%, and 40.6% of early-apoptotic cells and 2.5%, 9.9%, and 32.8% of late-apoptotic cells in U87MG cells after being treated with 1, 5, and 10 μM glaucocalyxin A for 48 h, respectively [**Fig. 3(B,C)**]. These results suggested that glaucocalyxin A could not only specifically suppress the cell growth, but also promote apoptosis in human brain glioblastoma cells.

Glaucocalyxin A-induced apoptotic signaling in malignant glioma U87MG cells

In U87MG cells, glaucocalyxin A could rapidly and dose-dependently inhibit Akt phosphorylation after 3 h [**Fig. 4(A,C)**] or 6 h [**Fig. 4(B,D)**] of treatment. Meanwhile, the apoptotic pathway including caspase 3, X-linked inhibitor of apoptosis protein (XIAP), and BAD were observed [**Fig. 4(E)**]. As shown in **Fig. 4(F,G)**, glaucocalyxin A significantly increased cleaved caspase 3, and decreased the XIAP expression. Moreover, the phosphorylation of BAD was markedly blunted by glaucocalyxin A [**Fig. 4(H)**]. These results indicated that glaucocalyxin A promoted apoptosis probably via the mitochondrial pathway.

Discussion

Considering the important role of Akt in cell metabolism and the fact that the dysfunction of Akt always happens in human malignancy; to develop new Akt regulators is an attractive way for treating tumors. In the present study, we developed the Akt regulator screening platform, and after screening the natural product library, we finally discovered that glaucocalyxin A could inhibit Akt phosphorylation and translocation. As a potent negative regulator of Akt, glaucocalyxin A could specifically suppress proliferation and promote apoptosis in U87MG cells. In order to at the most, reduce the damage caused by chemotherapy in the treatment of cancer, the selectivity of the drug is of great importance. In this study, glaucocalyxin A showed strong specificity in the malignant glioma U87MG cells, and exerted few effects on the normal glial cells. Therefore, these results largely supported the efficiency of this platform, and showed that

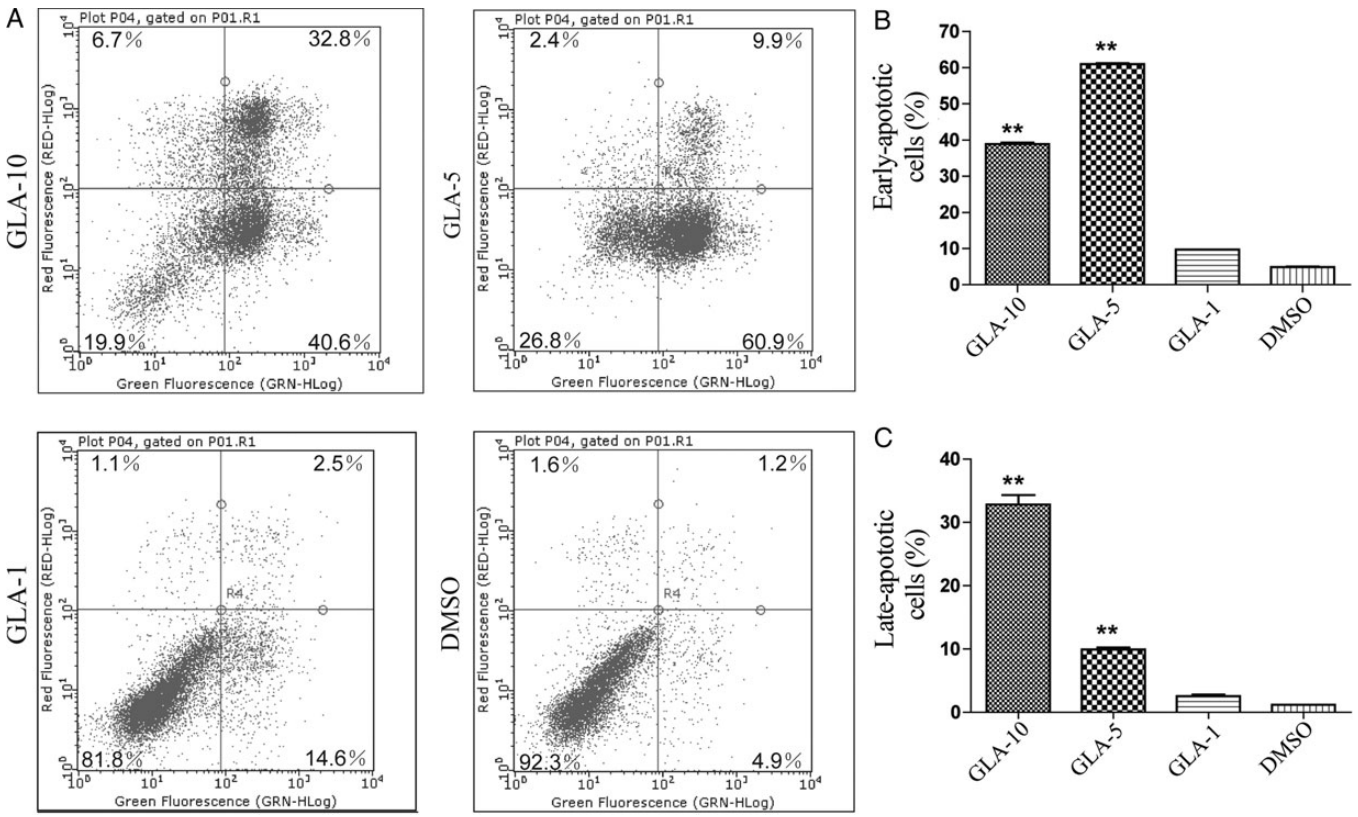

Figure 3 Flow cytometry analyses of glaucocalyxin A-induced apoptosis in malignant glioma U87MG cells Cells were treated with 0 (control), 1, 5, and 10 μM glaucocalyxin A for 48 h, respectively. (A) Reprehensive figures of flow cytometry by PI staining and Annexin V staining. The lower-right phase was the early-apoptotic cells. Quantification of three independent assays for early apoptosis (B) and late apoptosis (C). Data were presented as the mean ± SD. $^{**}P < 0.001$. These data are the representatives of three independent experiments. GLA, glaucocalyxin A.

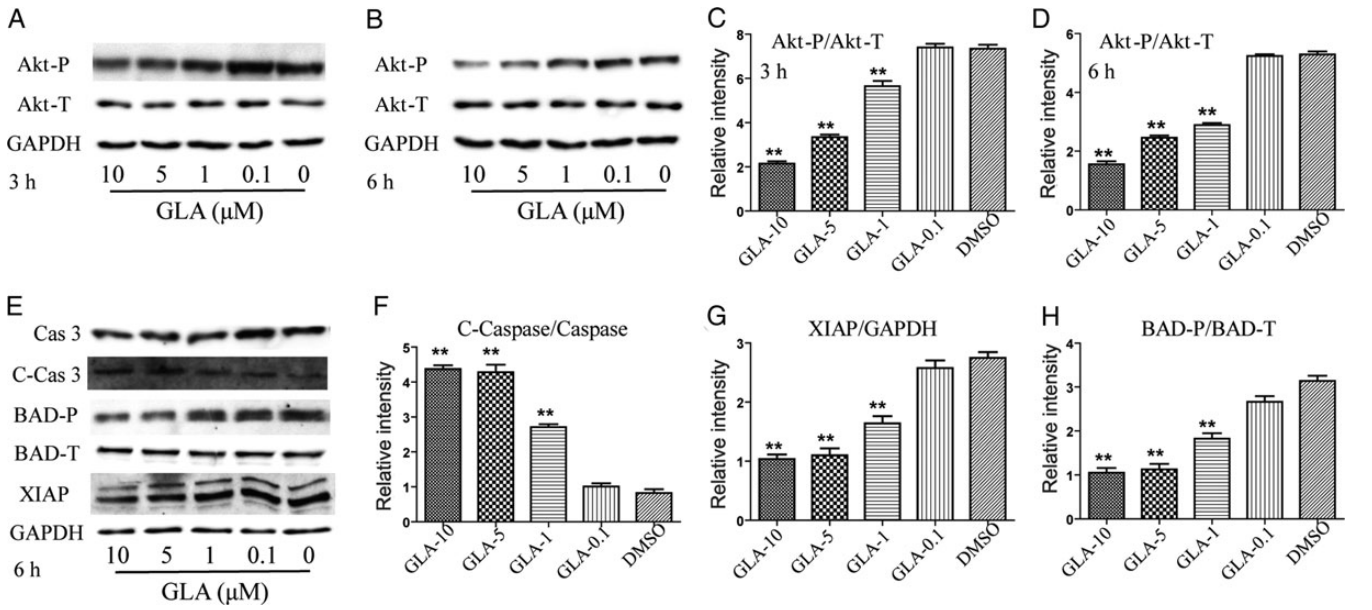

Figure 4 Western blot analysis of malignant glioma U87MG cells Cells were treated with 0 (control), 0.1, 1, 5, and 10 μM glaucocalyxin A for 3 or 6 h, respectively. The level of Akt and p-Akt after 3 (A) and 6 h (B) of incubation. Quantification of p-Akt/Akt after 3 (C) and 6 h (D). The levels of caspase 3, cleaved caspase 3 (C-Cas 3), p-BAD, BAD, and XIAP (E). GAPDH was used as an inner reference. Quantification of C-Cas 3 (F), XIAP (G), and p-BAD (H). Data were presented as the mean ± SD ($n = 3$). $^{**}P < 0.001$. These data are the representatives of three independent experiments. GLA, glaucocalyxin A.

glaucocalyxin A could become a promising compound against malignant glioma.

Signal transduction pathway is closely related to cancer development, and molecular-targeted therapy is of great interest in recent drug development [24–26]. Akt is an attractive target of cancers because of its important role in cell proliferation and cell cycle regulation [27]. In the present study, we showed that glaucocalyxin A could inhibit the phosphorylation of Akt, and significantly suppress cell growth and promote apoptosis in glioma cells. Akt was activated through phosphorylation under the action of PI3K or mTORC2, and then activated BAD, caspase 9, and other related downstream molecules, and further promoted or inhibited cell survival. Our results showed that glaucocalyxin A could activate caspase 3 and BAD, which are probably involved in Akt phosphorylation inhibition, and thus promote apoptosis in U87MG cells.

XIAP is often overexpressed in malignant tissue [28], which is the most potent inhibitor of caspase and reduces the release of cytochrome C [29]. In this study, we confirmed that glaucocalyxin A can significantly reduce the expression of XIAP. XIAP that has multiple domains can selectively inhibit caspase 9 (through its BIR3 domain) and caspase 3 and 7 (through its BIR2 domain) [30]. Asselin *et al*. [31] showed that XIAP could affect the sensitivity of tumor cells to chemotherapeutic drug by PI3K/Akt pathway. Anandharaj *et al*. [7] further suggested that the inhibition of the PI3K/Akt pathway could increase apoptotic signaling pathway. Therefore, glaucocalyxin A may decrease XIAP expression by down-regulating Akt signaling, which leads to cell apoptosis.

Tamm *et al.* [32] found that XIAP and C-IAP1 could increase expression in most human tumor cell lines and a high expression of XIAP was related to chemotherapy resistance and poor prognosis. The previous study reported that XIAP antisense oligonucleotides significantly enhanced the sensitivity of tumor cell to chemotherapeutic drugs [33]. Therefore, glaucocalyxin A may enhance the sensitivity of chemotherapy in the treatment of tumors. However, more studies are still needed to clarify this notion.

In summary, our study confirmed the strong anti-glioma activity of glaucocalyxin A *in vitro*, which specifically promoted apoptosis of malignant glioma U87MG probably via Akt signaling pathway. Glaucocalyxin A showed a good prospect in clinical anti-glioma therapy. Further studies of related molecular mechanisms remain to be clarified in future.

Funding

This work was supported by a grant from the National Natural Science Foundation of China (81072083).

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