

Glycyrrhizic Acid Inhibits Proliferation of Gastric Cancer Cells by Inducing Cell Cycle Arrest and Apoptosis

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Purpose: Glycyrrhizic acid (GA) is the main active ingredient extracted from Chinese herb licorice root, and it shows anti-tumor effects in many cancer types, while its role in gastric cancer (GC) is still unknown. In this study, we evaluated the effects of GA on GC cells and explored the underlying mechanisms.

Methods: The anti-proliferation effect of GA on GC cells was assessed by CCK-8, colony formation, and EdU assay. The effects of GA on cell cycle and apoptosis were detected by flow cytometer. Western blotting was performed to explore the underlying mechanisms.

Results: Our results showed that GA had a time- and dose-dependent inhibitory effect on proliferation of GC cells. Flow cytometer analysis demonstrated that GA would lead to G1/S-phase arrest and apoptosis. GA treatment down-regulated the levels of G1 phase-related proteins, including cyclin D1, D2, D3, E1, and E2. In terms of apoptosis, GA treatment up-regulated the levels of Bax, cleaved PARP, and pro-caspase-3, -8, -9, but did not influence their cleavage patterns. The expression of Bcl-2, survivin and p65 was attenuated after treatment. Besides, GA would down-regulate the phosphorylation of PI3K/AKT pathway.

Conclusion: This study focused on inhibitory effect of GA on GC cells by inducing cell cycle arrest and apoptosis. Several important cyclins- and apoptosis-related proteins were involved in the regulation of GA to GC cells, and phosphorylated PI3K and AKT were attenuated. The results of this study indicated that GA is a potential and promising anti-cancer drug for GC.

Keywords: glycyrrhizic acid, gastric cancer, cell cycle, apoptosis, PI3K/AKT pathway

Introduction

Gastric cancer (GC) is the sixth most common malignancy following breast, prostate, lung, colorectal, and cervical cancers. It is the fifth leading cause of cancer-associated deaths following lung, breast, colorectal and liver cancers. Approximately half of the GC cases encountered occur in developing countries.¹⁻⁴ Despite the improvement in diagnostic and treatment techniques, GC remains a major health issue.⁵ Current treatments for GC include surgery and chemotherapy, despite more and more drugs have been investigated for GC treatment,⁶⁻⁸ they still exhibit certain disadvantages. Therefore, it is urgent to develop molecular-targeted agents for the improvement in the treatment of this disease.

Glycyrrhizic acid (GA) is the main active ingredient of Chinese herb licorice root (Figure 1). Previous studies have shown that GA and its derivatives exhibit a variety of pharmacological effects, such as detoxification, anti-inflammatory,

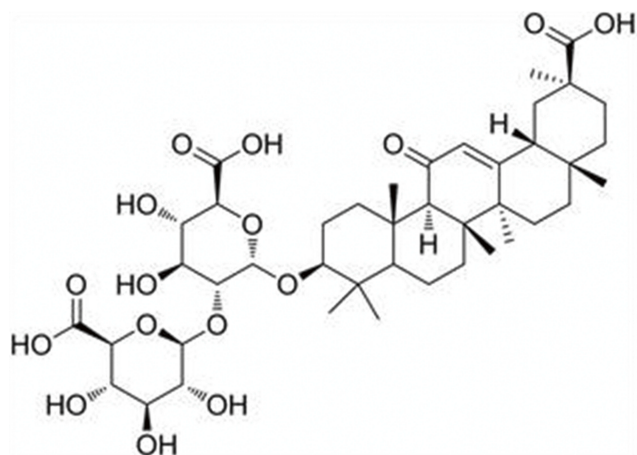


Figure 1 Chemical structure of glycyrrhizic acid.

bronchodilatory, anti-tumor, anti-ulcer, and anti-viral functions.⁹ The anti-tumor effect of GA has been reported in various types of tumors, such as those of the lung, liver, breast and cervix as well as in hematological malignancies, such as leukemia.^{10–14} GA demonstrated low toxicity and its LD50 value was estimated to 2000 mg/kg in mice following a single oral dose.¹⁵ In the clinic, GA compounds are widely used in the treatment of viral hepatitis and hepatocellular carcinomas.^{16–18} Previous studies have shown that GA can regulate several important signaling proteins, including those that belong to cysteine-dependent aspartate-specific protease (caspase) and the Bcl-2 families, the nuclear factor-kappaB (NF-κB) protein, the high mobility group box-1 (HMGB1) protein, the extracellular regulated protein kinases (ERK), the phosphatidylinositol 3-kinase (PI3K)/AKT kinases and the c-Jun N-terminal kinase (JNK).^{19–22} However, a limited number of reports have investigated mechanism by which GA affects GC.

The present study investigated whether GA influenced the biological behavior of GC cells *in vitro*. Furthermore, the potential mechanism of this process was explored in order to provide evidence for the application of GA as an effective treatment regimen for GC.

Materials and Methods

Reagents

GA (purity $\geq 98\%$; MW, 822.93), penicillin-streptomycin, Phenylmethanesulfonyl fluoride (PMSF) and phosphatase inhibitor were obtained from Solarbio Technology Co., Ltd; RPMI 1640 culture medium was purchased from Hyclone. Fetal bovine serum (FBS) was obtained from

Gibco. The Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Chemical Technology Co., Ltd. The 5-ethyl-2'-deoxyuridine (EdU) proliferation kit was purchased from Guangzhou RiboBio Co., Ltd. Annexin V FITC Apoptosis Detection Kit and PI/RNase Staining Buffer were purchased from BD Biosciences Company. The primary and secondary antibodies for the investigation of apoptosis, cell cycle and the PI3K/AKT pathway were all acquired from Cell Signaling Technology.

Cell Culture

The human GC cell lines (MGC-803, BGC-823, SGC-7901) were purchased from the Cell Bank of the Chinese Academy of Sciences and stored in the translational medical center and central laboratory of Wuxi No.2 People's Hospital. All cells were cultured in RPMI-1640 medium containing 10% FBS and 1% penicillin-streptomycin and incubated with 5% CO₂ at 37°C. GA was dissolved in culture medium at the desired concentrations.

Cell Viability Assay and Colony Formation Assay

Cell viability was assessed by the CCK-8 assay. Cells were seeded in 96-well culture plates (3×10^3 cells/well). Following 24 h of incubation, cells were treated with different concentrations of GA for 48 h and cell viability was assessed by the CCK-8 solution. Subsequently, a concentration close to IC₅₀ (1 mg/mL) of GA was selected in order to examine cell viability at different time periods. Cell viability was subsequently detected by the CCK-8 assay from 12 to 72 h time points of incubation. All experiments were repeated three times.

To examine the effects of GA on clonogenic ability, MGC-803 cells were seeded in the 6-well culture plate at a density of 200 cells per well. Following 24 h of incubation, cells were treated with GA (1 mg/mL) for 2–3 weeks. When the clones were visible to the naked eye, cells were fixed with methanol and stained with 0.1% crystal violet. All colonies were counted manually and each single colony was observed under the microscope.

EdU Assay

MGC-803 cells were seeded in a 96-well culture plate (4×10^3 – 1×10^4 cells/well) and treated with GA (1 mg/mL) for 48 h. The culture medium was replaced with EdU solution and cells were incubated for 2 h. Cells were subsequently fixed and permeabilized. The cells

were stained successively by Apollo[®] and Hoechst 33342 staining. Ultimately, detection of EdU staining was performed using a fluorescence microscope (OLYMPUS). Cells were counted by the Image J software (National Institutes of Health) and the experiment was repeated three times.

Apoptosis Assay

MGC-803 cells were transplanted to 6-well plates and treated with GA (1 mg/mL) for 48 h. Subsequently, they were collected and washed twice with cold PBS. The cells were resuspended in 100 μ L binding buffer containing 5 μ L FITC Annexin V and 5 μ L PI. Following 15 min of incubation at 25°C in the dark, 400 μ L binding buffer was added to the cell suspension and the samples were analyzed by flow cytometry (Beckman Coulter) within 1 h.

Cell Cycle Assay

MGC-803 cells were transplanted to 6-well plates and treated with GA (1 mg/mL) for 48 hours. Following treatment, cells were collected and washed twice with cold PBS and fixed with 70% ethanol at -20°C for at least 2 h. The cells were treated with 0.5 mL staining buffer and incubated at room temperature for 15 min. The samples were analyzed by flow cytometry (Beckman Coulter) within 1 h.

Western Blot Analysis

The proteins were extracted from cells using RIPA buffer with 1 mM PMSF and phosphatase inhibitor. Concentration of each protein was subsequently detected by the bicinchoninic acid assay kit (Beyotime). Then, samples were denatured and separated in 12% SDS gels. Following electrophoresis, proteins were transferred on the PVDF membranes. All membranes were blocked with 5% skimmed milk for 2 h and primary antibody incubation was performed at 4°C overnight. The membranes were subsequently placed in the secondary antibody solution for 2 h at room temperature and protein bands were visualized with the ECL detection kit (Millipore).

Statistical Analysis

Statistical analysis was carried out by GraphPad Prism 6 (GraphPad Software, Inc.). All results were expressed as mean \pm SD. A two-tailed Student's *t*-test was used for comparison between GA-treated and control groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

GA Inhibits the Proliferation of GC Cells

The inhibition of GC proliferation by GA was assessed by CCK-8, clonogenic and EdU assays. As shown in Figure 2, GA significantly suppressed the proliferation of GC cells in a dose- and time-dependent manner.

Furthermore, the clonogenic assay indicated that GA treatment decreased the colony formation rate of MGC-803 cells (Figure 3A and B) and the number and size of colonies in the GA-treated group were considerably lower than those of control group (Figure 3C), indicating that GA exerted a long-time inhibitory effect on the proliferation of MGC-803 cells. The EdU assay detects the cells present in mitosis that are by this dye. These cells appear red under the fluorescence microscope (Figure 3D and E). Following treatment, the rate of proliferated cells decreased from 51.86 ± 4.44 to $32.25 \pm 8.52\%$ ($P < 0.001$), which demonstrated directly that GA treatment inhibited proliferation of MGC-803 cells. The aforementioned results revealed that GA could inhibit the proliferation of GC cells.

GA Inhibits Proliferation of MGC-803 Cells by Inducing G1/S-Phase Arrest

To further explore the underlying mechanisms of GA-mediated inhibition of GC proliferation, the changes in cell cycle following GA treatment were investigated by flow cytometry. The cell cycle distribution is shown in Figure 4A–C. Following GA treatment, percentage of S-phase cells was dramatically decreased from 42.86 ± 5.22 to $32.54 \pm 2.47\%$ ($P < 0.05$), while the percentage of G0/G1-phase cells was significantly increased from 24.49 ± 2.43 to $33.13 \pm 3.53\%$ ($P < 0.05$). The percentage of GC cells at the G2/M phase exhibited no significant changes between the treated and the control groups. These results suggested that GA treatment induced G1/S-phase arrest, which contributed to the GA-mediated inhibition of cell proliferation.

The expression levels of several cell cycle-associated proteins, including cyclins A2, D1, D2, D3, E1, E2, B1 and H were examined in order to explore the effects of GA on the GC cell cycle. GA treatment downregulated the levels of G1 phase-associated proteins, including cyclins D1, D2, D3, E1 and E2, while the expression of other cyclin proteins remained unchanged (Figure 4D). These findings confirmed that GA induced G1/S-phase arrest in GC cells.

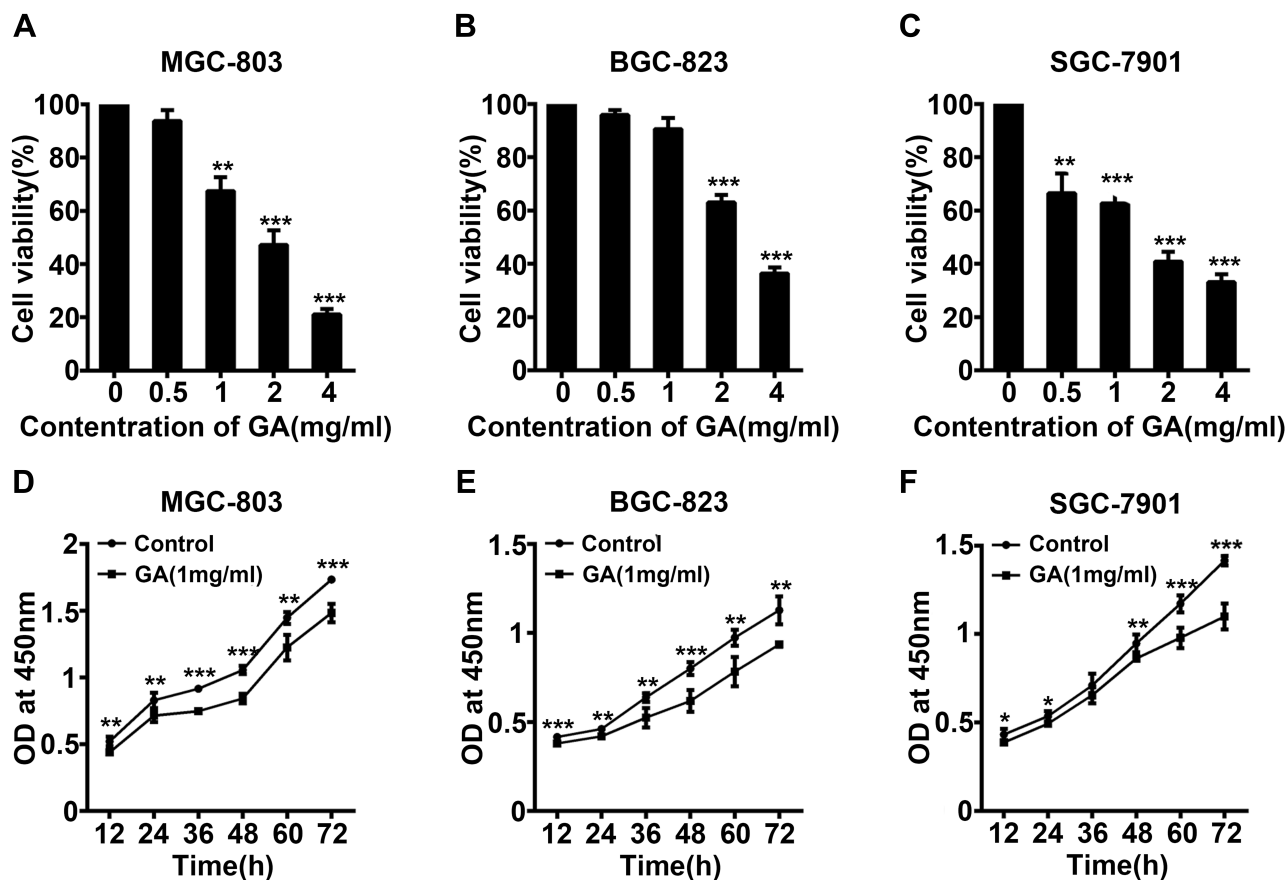


Figure 2 GA inhibits proliferation of GC cells in vitro. GC cell lines were treated with different concentrations of GA or an appropriate concentration for different time periods, cell viability was examined by CCK-8 assay. (A–C) GA inhibited proliferation of GC cells in a dose-dependent manner. Different cell lines showed different sensitivities to GA, while high concentration of GA (4 mg/mL) exerts certainly inhibitory effects on all the three cell lines. (D–F) GA inhibited proliferation of GC cells in a time-dependent manner. The longer of treatment time period, the more significant of the inhibitory effects of GA on GC cell lines. The results were expressed as mean \pm SD (n=5) and all data were from three independent experiments. *P<0.05, **P<0.01, ***P<0.001.

Abbreviations: GA, glycyrrhizic acid; GC, gastric cancer.

GA Inhibits Proliferation of MGC-803 Cells by Inducing Caspase-Independent Apoptosis

To confirm whether the inhibitory effect of GA on GC cells was associated with induction of apoptosis, this process was detected by flow cytometry. As shown in Figure 5A–C, the early apoptotic rate of cells was increased significantly following GA treatment (from 3.37 ± 1.10 to $12.79 \pm 2.23\%$, $P < 0.01$) and the late apoptotic rate was also increased (from 3.74 ± 0.34 to $9.54 \pm 2.34\%$, $P < 0.05$).

In addition to caspases, the Bcl-2, Bcl-2 antagonist X (Bax), PARP, survivin and p65 proteins play important roles in apoptotic process. Therefore, expression levels of these apoptotic proteins were assessed by Western blotting (Figure 5D and E). The results indicated that GA treatment increased levels of Bax and cleaved PARP. In addition, the

expression levels of pro-caspase-3, -8 and -9 were increased following treatment, while their cleavage patterns did not increase. The expression levels of caspase-7 and cleaved caspase-7 remained unchanged. The expression levels of Bcl-2, survivin and p65 were attenuated following GA treatment. The aforementioned results indicated that GA could inhibit proliferation of MGC-803 cells by inducing caspase-independent apoptosis.

GA Downregulates the Phosphorylation Levels of the PI3K and AKT Signaling Pathway Proteins

The PI3K/AKT pathway is an intracellular signaling pathway that is closely associated with development of tumors. Previous studies have demonstrated that PI3K/AKT pathway plays a key role in transition of cells from the G1 to the

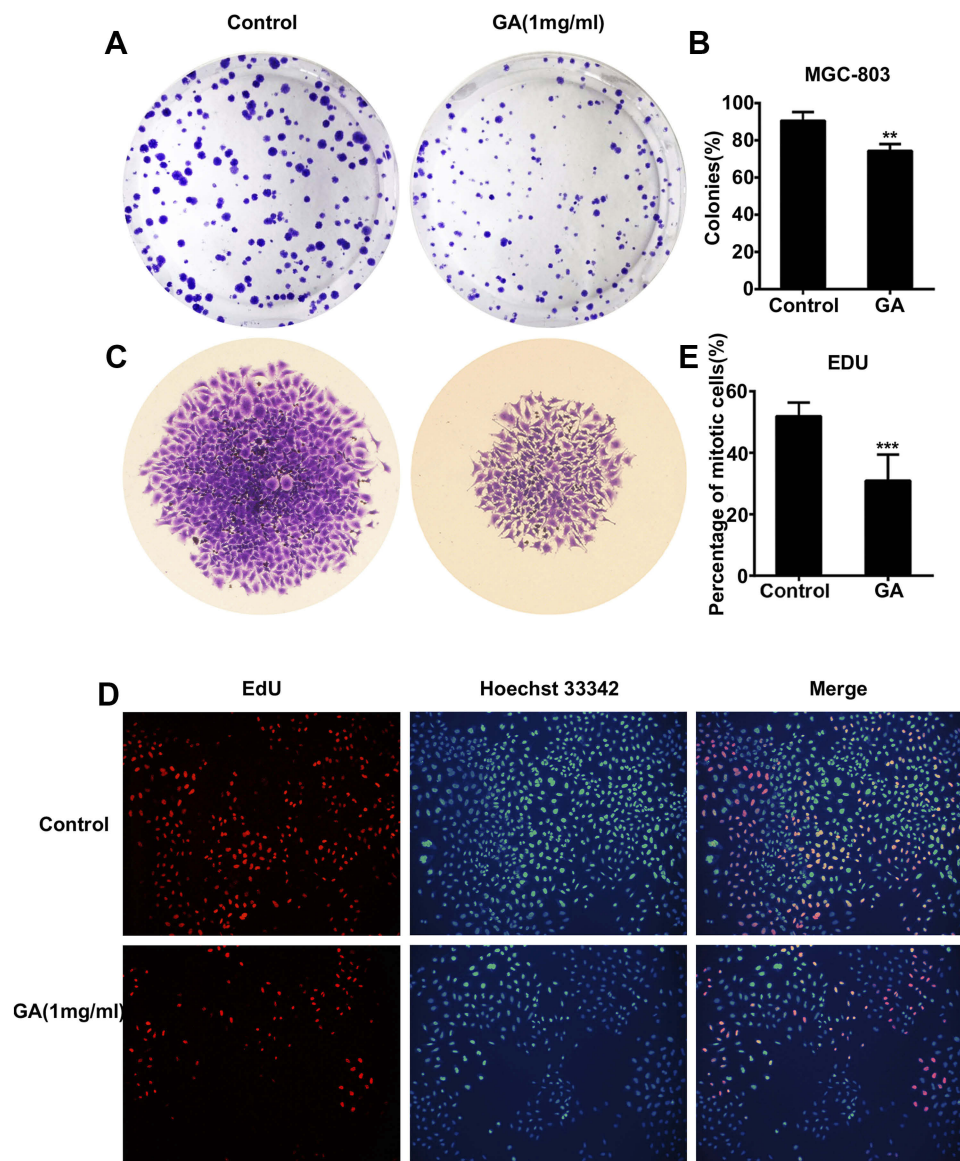


Figure 3 GA suppresses the colony formation ability and proliferation of MGC-803 cells. **(A and B)** GA suppressed colony formation rate of MGC-803 cells. **(C)** Single colonies of MGC-803 pretreated with or without GA were observed under the microscopy, and GA treatment inhibited the size of single colony of MGC-803 cells. **(D and E)** The inhibition of cellular proliferation was observed by Apollo and Hoechst 33342 staining and visualized by fluorescence microscopy. GA treatment significantly reduced the percentage of mitotic MGC-803 cells. The results were expressed as mean \pm SD (n=3). All data were from three independent experiments. **P<0.01, ***P<0.001. **Abbreviation:** GA, glycyrrhizic acid.

S phase of cell cycle. Moreover, this pathway can regulate the inhibition of apoptosis. Based on these findings, phosphorylation levels of PI3K and AKT were assessed by Western blotting. The phosphorylation levels of these two proteins were significantly decreased, whereas the levels of their total forms remained unchanged (Figure 6), suggesting the inhibition of PI3K/AKT signaling pathway.

Discussion

The use of Chinese medicinal herbs has been widely applied in the clinic in China.²³ Previous studies have

shown that GA possesses anti-cancer activity against several types of cancers. In the present study, GA decreased proliferation of GC cells by inducing G1/S-phase arrest and apoptosis.

The inhibition of cellular proliferation can be caused by cell cycle arrest. Cyclins and cyclin-dependent kinases (CDKs) are critical proteins that regulate cell cycle progression. The results of the present study demonstrated that GA treatment attenuated the expression levels of G1 phase-related proteins. Activated D-type cyclins (cyclin D1, cyclin D2 and cyclin D3) bind to CDK4 or CDK6 to

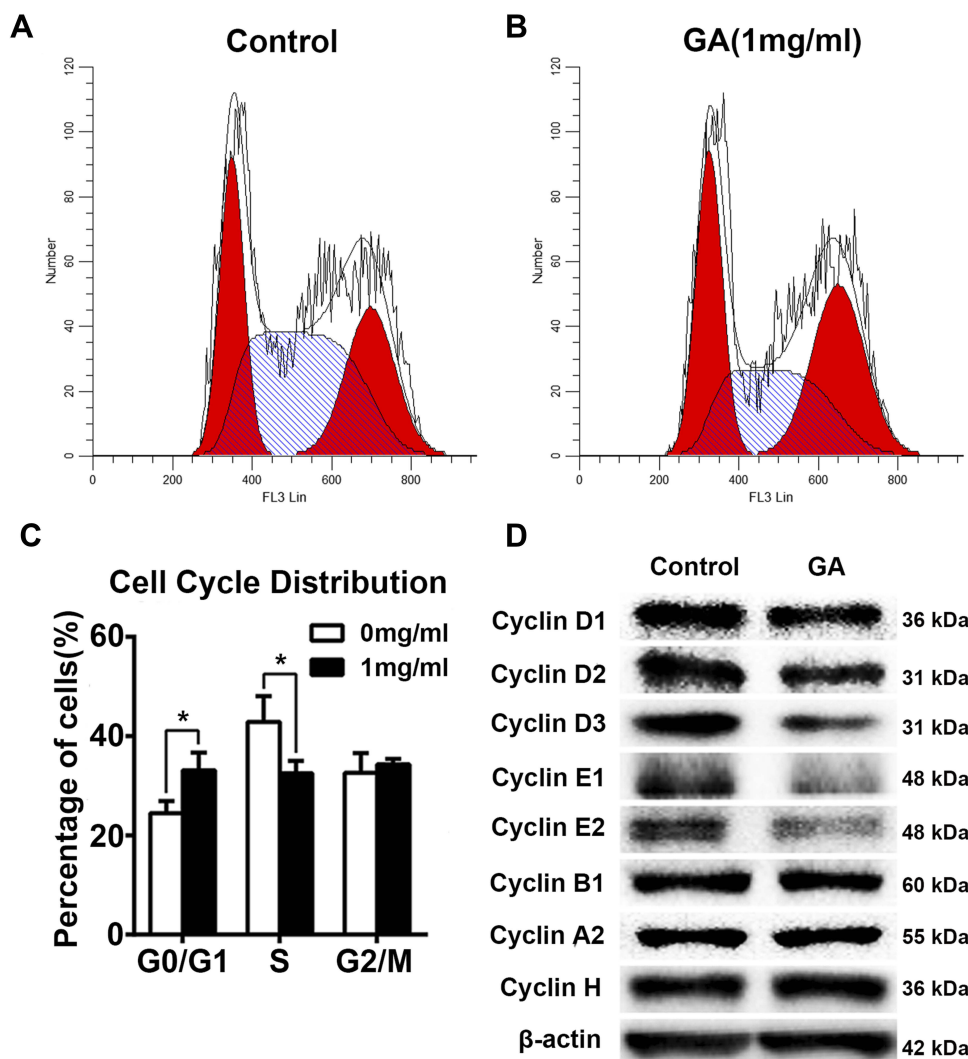


Figure 4 Effects of GA on GC cell cycle distribution. (A–C) GA induces cell cycle arrest at G1/S-phase in MGC-803 cells. Percentage of S-phase cells was significantly decreased following GA treatment, while the amount of G0/G1-phase cells was increased and G2/M-phase cells remained unchanged. The results were expressed as mean \pm SD (n=3). (D) The levels of cyclin proteins were inconsistent with the result of cell cycle assay, the proteins related to G1/S check point were attenuated following GA treatment. All experiments were repeated three times. *P<0.05.

Abbreviations: GA, glycyrrhizic acid; GC, gastric cancer.

form the cyclin D-CDK4/CDK6 complexes, which result in phosphorylation of the retinoblastoma protein and subsequent dissociation of the E2F transcription factor. Once released, the E2F transcription factor promotes expression of downstream target genes and facilitates the S-phase entry. Deregulation of D-type cyclins, notably cyclin D1, can directly lead to proliferation and cell cycle progression from G1 to S phase.²⁴ E-type cyclins (cyclin E1 and cyclin E2) are synthesized initially in the mid G1 phase. Their expression reaches its maximum levels at G1/S transition followed by their degradation at the end of S phase. They act as key regulators of the G1 to S phase transition. A similar action has been noted for the D-type cyclins. E-type cyclins bind to CDK2 and activate it, resulting in

the S phase entry of the cells.²⁵ The changes in the expression levels of cyclin proteins caused by GA treatment of GC cells were accompanied by G1/S-phase arrest of cell cycle.

The results of the present study demonstrated that both early and late apoptotic rates of MGC-803 cells were increased following GA treatment. Current studies have shown that GA exhibits different effects on the induction of apoptosis or necrosis under different conditions. In tumors, GA promotes apoptosis, while during liver, brain or kidney injury, it impedes apoptosis.^{26–28} It is well known that caspase family of proteins plays an important role in the progression of apoptosis. Caspase-dependent apoptosis is a “waterfall pattern” cascade reaction

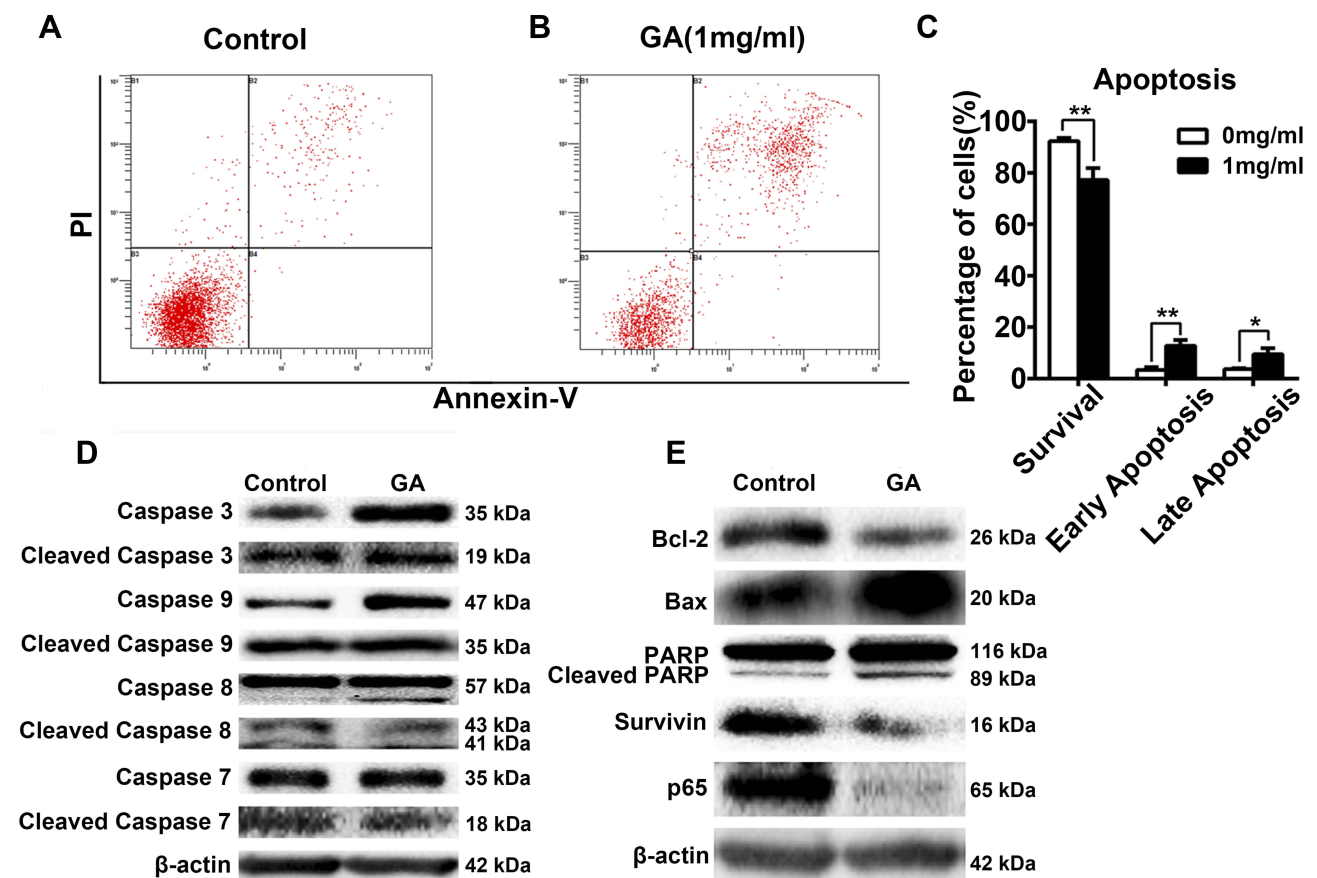


Figure 5 Effects of GA on the induction of cell apoptosis. (A–C) GA induces apoptosis in MGC-803 cells. Both the early and late apoptosis rates were significantly increased by GA treatment. The data were expressed as mean \pm SD ($n=3$). (D and E) The levels of apoptosis-related proteins were measured by Western blotting and β -actin was used as a loading control. GA treatment induced the apoptosis without activating the caspase pathway. All experiments were repeated at least three times. * $P<0.05$, ** $P<0.01$.

Abbreviation: GA, glycyrrhizic acid.

beginning with the activation of initiator-like caspase-2, -8, -9, and -10 enzymes and is continued with the increase in their expression levels. The self-activation activates endogenous and exogenous apoptotic pathways. Once activated, the initiators induce the expression of a series of downstream caspase proteins and ultimately activate caspase-3, -6, -7 and -14, which are the effectors of apoptotic process.²⁹ However, the present study demonstrated that GA treatment did not change the levels of cleaved caspase proteins in MGC-803 cells, indicating GA mediated caspase-independent apoptosis in GC cells. Similar results were noted in breast cancer.¹⁰ Moreover, the expression levels of other key apoptosis-associated proteins changed, including Bcl-2, Bax, cleaved PARP, survivin, and p65. Bcl-2 is an anti-apoptotic protein that prevents the release of apoptotic factors into cytoplasm, while Bax permeabilizes the outer mitochondrial membrane to promote the release of apoptotic factors and

accelerate the apoptotic process.³⁰ The ratio of expression levels of Bcl-2 and Bax is used to reflect the apoptotic cascade.³¹ PARP is regarded as a DNA repair enzyme and a substrate of caspase family of enzymes. During induction of apoptosis, the full-length PARP protein is cleaved into a catalytic domain (89 kDa) and a binding domain (24 kDa), which is considered to be the hallmark of apoptosis.³² The survivin protein exhibits anti-apoptotic effects by inducing inhibition of caspase family proteins.³³ Previous studies demonstrated that GA treatment attenuated survivin by blocking AKT/mTOR/STAT3 signaling pathway.¹¹ P65 is an active subunit of NF- κ B protein, which is responsible for regulation of cellular proliferation and apoptosis.³⁴ It was reported that GA inhibited cell growth and induced apoptosis possibly by downregulating p65.³⁵ The results presented in the current study revealed that GA acted via a caspase-independent apoptotic pathway.

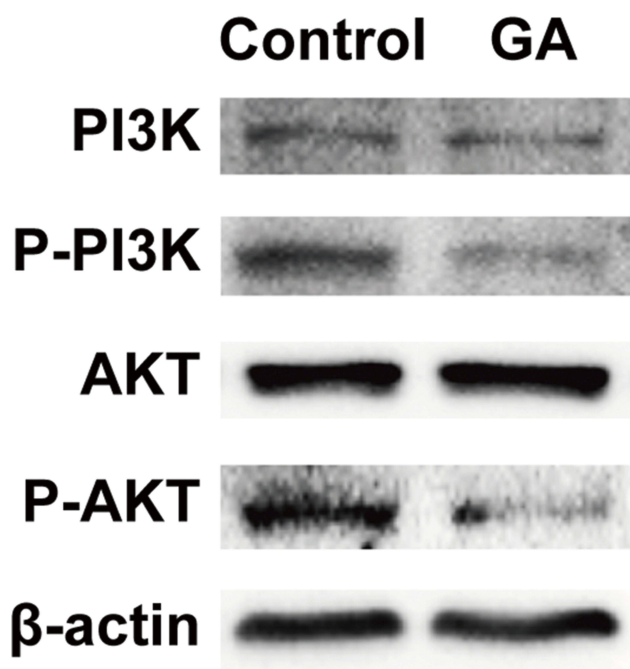


Figure 6 GA downregulates levels of the phosphorylated PI3K and AKT proteins. β -actin was used as a loading control. All experiments were repeated three times. **Abbreviation:** GA, glycyrrhizic acid.

The PI3K/AKT signaling pathway is involved in the regulation of several biological processes, including inhibition of apoptosis and cell proliferation. PI3K can be activated by tyrosine kinase receptors, G-protein-coupled receptors or Ras protein. This activation leads to the phosphorylation and subsequent activation of AKT at Ser473 and Thr308 residues.³⁶ Phosphorylated AKT targets more than 100 proteins and leads to oncogenesis. It can protect cardiomyocytes from H_2O_2 -induced injury by abating apoptosis.³⁷ Low levels of phosphorylated AKT upregulated p21^{cip1} and p27^{kip1} leading to the induction of G1/S-phase arrest in thyroid carcinoma cells.³⁸ Furthermore, downregulation of phosphorylated AKT inhibited proliferation and induced G1/S-phase arrest and apoptosis in glioma cells resulting to a significant reduction in the levels of Bcl-2 and cyclin D1.³⁹ In the present study, GA attenuated phosphorylation of PI3K and AKT, which was probably responsible for cell cycle arrest and apoptosis in GC cells.

In conclusion, GA exhibited anti-tumor property in GC cells partly by inducing apoptosis and cell cycle arrest. These effects were associated with corresponding protein changes. In addition, the phosphorylation of PI3K/AKT pathway proteins was inhibited. The aforementioned data indicated that GA was a potential anti-tumor drug for GC treatment.

Data Sharing Statement

All data generated or analyzed during this study are included in this published article.

Author Contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests.

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