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# Antitumor activity of glycyrol via induction of cell cycle arrest, apoptosis and defective autophagy

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ABSTRACT

Glycyrol is a coumestan isolated from *Glycyrrhiza uralensis* and synthesized to use. In this study, the antitumor activity and the underlying mechanism of glycyrol were evaluated *in vitro* and *in vivo*. It was shown that glycyrol induced cell death associated with apoptosis and autophagy as evidenced by morphological changes in AGS and HCT 116 cells. The apoptosis-inducing effect was characterized by increase in ratio of sub-G1 phase, poly (ADP-ribose) polymerase-1 (PARP-1) cleavage and caspase-3 activation. Mechanistic studies showed that glycyrol induced G0/G1 phase cell cycle arrest as indicated by increase in p21. Furthermore, c-Jun *N*-terminal kinase (JNK)/p38 mitogen-activated protein kinases (MAPKs) activation induced caspase-dependent apoptosis accompanied by adenosine monophosphate-activated protein kinase (AMPK) activation. Defective autophagy was triggered, which stopped the autophagic flux by the slowing of lysosomal degradation. In addition, glycyrol suppressed tumor growth in a nude mouse tumor xenograft model bearing HCT 116 cells. Taken together, glycyrol is demonstrated to have antitumor activity, and might potentially serve as potential candidate for cancer therapy.



## 1. Introduction

Apoptosis, also known as type I programmed cell death, is characterized by cell shrinkage, DNA condensation and fragmentation, membrane blebbing, and apoptotic body formation. Apoptosis is induced mainly by the extrinsic (death receptor) pathway or the intrinsic (mitochondrial) pathway through caspase cascade activation (Fulda and Debatin, 2006). Macroautophagy (simply autophagy) involves the lysosome-mediated degradation and recycling of cytoplasmic components. It is known to play an important role in developmental processes, human disease, the cellular response to nutrient deprivation, and other stresses (Klionsky and Emr, 2000). Once autophagy is induced, microtubule-associated protein 1 light chain-3 I (LC3-I) is directly conjugated to the lipid phosphatidylethanolamine (PE) and inserted into autophagic membranes to produce LC3-II, a protein marker of autophagy (Baehrecke, 2005). Autophagic membrane sequesters cargo to form a double membrane vesicle called autophagosome, which docks with lysosome for degradation (Klionsky and Emr, 2000). The p62 protein (also called sequestosome 1, SQSTM1) binds directly to LC3-II to facilitate the degradation of ubiquitinated protein aggregates by autophagy (Pankiv et al., 2007).

Autophagy can serve as a cell survival pathway by removing scavenge-damaged organelles and protein aggregates to reduce cellular stresses that cause apoptosis, whereas excessive autophagy can cause cell death through over-degradation of cytoplasm, also known as type II programmed cell death (Baehrecke, 2005; Eisenberg-Lerner et al., 2009; Lockshin and Zakeri, 2002). Due to its pro-survival function, autophagy could make cancer cells resistant to chemotherapy, radiotherapy or anti-angiogenic therapy, so autophagy inhibition is being widely investigated to improve the efficacy of anti-cancer agents (Hu et al., 2012; Maycotte and Thorburn, 2011). Chloroquine, the anti-malaria drug, is the only U.S. Food and Drug Administration-approved agent able to inhibit autophagy by its accumulation in the lysosome and the resulting decrease in lysosomal function. However, the side effect of kidney injury limits its clinical use (Kimura et al., 2013).

Naturally occurring compounds, such as resveratrol, curcumin and (-)-epigallocatechin-3 gallate (EGCG), are reported to possess the capacities to induce oxidative stress, DNA damage, autophagy and apoptosis, and are under consideration as cancer chemoprevention agents due to their long standing efficacy and safety in many traditional usages (Mukhtar et al., 2012). Cancer cells have alterations and complexities in the communication between multiple signal transduction pathways, so targeting multiple signaling pathways might be more effective than targeting only one pathway (Sarkar et al., 2009).

*Glycyrrhiza uralensis* (Leguminosae) is a well-known traditional medicine to have anti-ulcer, anti-cancer, anti-oxidative, antiviral and hepatic-protective effects, due to the triterpene saponins,

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flavonoids and isoflavonoids that are considered to be its main bioactive compounds (Asl and Hosseinzadeh, 2008). Glycyrol is a coumestan compound isolated from *G. uralensis* and synthesized to use (Jin et al., 2008), and has been reported to have anti-inflammatory activity in RAW264.7 macrophages and apoptosis-inducing activity in human Jurkat T cell lymphocytes previously by our group (Jin et al., 2008; Shin et al., 2008, 2011a). In this study, the antitumor activity and the underlying mechanism of glycyrol were further evaluated *in vitro* and *in vivo*. We demonstrate that glycyrol induced cell cycle arrest, apoptosis and defective autophagy in AGS human gastric cancer cells and HCT 116 human colon cancer cells, and also suppressed tumor growth in the nude mouse tumor xenograft model bearing HCT 116 cells.

## 2. Materials and methods

### 2.1. Chemicals, reagents and antibodies

Glycyrol (purity 99%) was first isolated from the root of *G. uralensis*, and synthesized as previously described (Shin et al., 2011a). Glycyrol was dissolved in DMSO, and stored at −20 °C. The final DMSO concentrations in culture medium were less than 0.1% and did not interfere with any of the assays. Bafilomycin A1, SP600125, SB202190, compound C, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), JC-1, and acridine orange were purchased from Sigma-Aldrich (St. Louis, MO, USA). The annexin V/propidium iodide (PI) kit was acquired from BD Biosciences (San Diego, CA, USA). RNase A was purchased from Affymetrix, Inc (Santa Clara, CA, USA). The antibodies for PARP-1, FasL, p-ERK1/2 (Thr177), ERK1, p-p38α (Thr180/Tyr182), p38α/β, JNK, β-actin, and the secondary antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Cleaved caspase-8, pChk1 (Ser345), pChk2 (Thr68), p21, cyclin D1, cyclin E, cyclin B1, and E2F-1 were purchased from Cell Signaling Technology, Inc (Beverly, MA, USA). pJNK1/2/3 (Thr183/Tyr183/Tyr221), pAMPK alpha 1/2 (T183/T172), and AMPK alpha 1/2 were purchased from Abcam plc (Cambridge, UK). Caspase-3 (Active) and p62 were purchased from GeneTex, Inc (Irvine, CA, USA).

### 2.2. Cell culture

AGS human gastric cancer cell line and HCT 116 human colon cancer cell line were obtained from the Korea Cell Line Bank (Seoul, Korea). These cells were maintained at sub-confluence in a 95% air and 5% $CO_2$ humidified atmosphere at 37 °C. RPMI medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin was used for routine subculture and all experiments.

### 2.3. Cell viability assay

The cell viability was evaluated using an MTT assay. Cells were seeded at a density of $2 \times 10^4$ per well into 96-well plates and stabilized at 37 °C for 24 h. Glycyrol at different concentrations was added to each well, and then the cells were incubated for 24 h or 48 h. The MTT solution (0.5 mg/mL) was added to each well and the cells were incubated for another 2 h. The crystal of produced formazan was dissolved with DMSO and the optical density was measured at 595 nm using an Emax microplate reader (Molecular Devices, Sunnyvale, CA).

### 2.4. Flow cytometric analysis for measurement of sub-G1 phase

After treatment with various concentrations of Glycyrol, cells were collected and washed with PBS followed by fixation with 70% ethanol and were incubated at −20 °C overnight. Cells were then collected by centrifugation and washed. The pellet was re-suspended in PBS and incubated with RNase A (50 μg/mL) for 30 min at room temperature, then stained with PI for 10 min. Then, the samples were analyzed using a FACSCalibur flow cytometry (Becton Dickinson, San Jose, CA, USA).

### 2.5. Western blot analysis

After treatment with various concentrations of glycyrol, cells were collected 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). A 20 μg aliquot of total protein were separated by 10% SDS-PAGE gel and transferred to nitrocellulose membranes, blocked with 5% BSA and probed with a primary antibody (1:1000) followed by the corresponding secondary antibody (1:3000). The signal was detected with WEST-SAVE Up™ luminal-based ECL reagent (ABFrontier, Korea).

### 2.6. In vivo *tumor xenograft model*

Animal care and all the procedures were conducted in accordance with the approval and guidelines of the Seoul National University Institutional Animal Care and Use Committees (IACUC; permission number: SNU-130227-1). Male BALB/c nude mice at the age of 4 weeks were purchased from the Central Animal Laboratory Inc. (Seoul, Korea) and housed in the animal care facility at Seoul National University under pathogen-free conditions with a 12 h light–dark schedule. After the animals were acclimated for 1 week, HCT 116 cells ($2 \times 10^6$ cells/0.2 mL) were injected subcutaneously into the right flanks of the mice. When the tumor size reached 60 $mm^3$, the mice were randomly divided into the treatment and control groups (n = 6). The animals were treated intraperitoneally with glycyrol (10 mg/kg body weight dissolved in 0.2 mL of saline with 10% Tween 80) three times a week for 4 weeks. The control group was treated with an equal volume of vehicle. The body weight and tumor sizes were measured twice a week, and the tumor volume was determined by caliper measurements and calculated using the following formula: length × width$^2$ × π/6. The experiment was terminated when the average tumor volume of the control group reached 1500 $mm^3$. The mice were sacrificed, and the tumors were removed and weighted. The body weight of each mouse was monitored for toxicity.

### 2.7. Statistical analysis

The results are presented as the mean ± standard deviation (S.D.). An analysis of variance (ANOVA) with the Dunnett's *t*-test was used for the statistical analysis of multiple comparisons. *P*-values less than 0.05 were considered statistically significant.

## 3. Results

### 3.1. Glycyrol inhibits the growth of cancer cells

Previous work has shown that glycyrol (Fig. 1A) induces apoptosis through the extrinsic pathway in human Jurkat T cell lymphocytes. As part of our continuous study, to better understand the antitumor effect of glycyrol in different cancer cells, first, the MTT assay was conducted in several human cancer cell lines and non tumoral cell lines. Glycyrol showed a more potent cytotoxic effect against AGS human gastric cancer cells and HCT 116 human colon cancer cells than Chang human non tumoral hepatic cells, MRC5 human non tumoral lung fibroblast cells, and MDA-MB-231, MCF7 and T47D three human breast cancer cells (Appendix: Supplementary Fig. S1). So, the cytotoxicity effect of glycyrol was evaluated against AGS and HCT 116 cells in different dose for 24 h and 48 h treatment, and the $IC_{50}$ value is 34.5 μM for 24 h and 23.0 μM for 48 h in AGS cells, and 35.8 μM for 24 h and 21.0 μM for 48 h in HCT 116 cells, respectively (Fig. 1B). From the morphological observation, the high dose (40 μM and 50 μM) treatment of glycyrol induced massive cell rounding, shrinkage, membrane blebbing, apoptotic body formation and detachment from the culture plates (Fig. 1C).

### 3.2. Glycyrol induces caspase-mediated apoptosis

DNA fragmentation occurs in the late stage of apoptosis, and is a key characteristic of apoptosis. So, to evaluate whether glycyrol-induced cell death is triggered by apoptosis, the sub-G1 phase ratio was measured by flow cytometry. The 50 μM glycyrol treatment increased the sub-G1 phase to 10% in AGS cells, and 20% in HCT 116 cells (Fig. 2A). Western blot analysis was performed to detect mediators of apoptosis. Caspase-3 activation and PARP-1 cleavage were increased in a dose-dependent manner. In addition, the mediator of extrinsic pathway caspase-8 was activated, and the mediator of intrinsic pathway procaspase-9 was decreased in AGS cells (Fig. 2B). The same results were observed in HCT 116 cells (Fig. 2C). FasL was increased independent of Fas death receptor in AGS cells (Fig. 2B). Mitochondria membrane potential was checked by using JC-1 staining, and mitochondria membrane potential disruption was detected after glycyrol treatment (Appendix: Supplementary Fig. S2). These data indicate that glycyrol induced extrinsic and



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