

Glycyrrhetic Acid and Related Compounds Induce G1 Arrest and Apoptosis in Human Hepatocellular Carcinoma HepG2

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Abstract. Glycyrrhetic acid (GA) and its related compounds are known to have anti-inflammatory activity and also to inhibit liver carcinogenesis and tumor growth. GA and related compounds inhibited cell proliferation of the human hepatoma cell line, HepG2. Among five compounds tested, ursolic acid and 18 β -olean-12-ene-3 β , 23, 28-triol (18 β -erythrotriol) were comparatively effective, where the 50% inhibitory dose was 20 μ M and 25 μ M, respectively. Flow-cytometric analysis showed that GA and the related compounds arrested the cell cycle in the G1-phase; in addition, GA-related compounds induced apoptosis at high dose. Western blot analysis indicated that the induction of apoptosis by GA and ursolic acid was accompanied with an activation of caspase-8 and a reduction in the anti-apoptotic proteins, Bcl-2 and Bcl-xL, although the pro-apoptotic proteins, Bax and Bak, remained unaffected. These results suggest that GA and its related compounds may be potent agents in liver cancer treatment.

Glycyrrhetic acid (GA) is the aglycon of glycyrrhizin (GL), a naturally occurring triterpene saponin, found as a major constituent of licorice (the root of *Glycyrrhiza* spp.). GA and its related compounds (Figure 1) are known to possess anti-inflammatory activity, and GL has been clinically used in the treatment of hepatic inflammation. A Japanese clinical study demonstrated the preventive effect of GL in the development of hepatocellular carcinoma in chronic hepatitis C patients (1). Additionally, GL and GA inhibited liver carcinogenesis in a mouse model (2) and tumor cell

growth *in vitro* (3, 4). Although several biological activities have been reported for GA and its related compounds such as anti-viral (5), cytokine-inducing (6, 7) and anti-tumor promoter effects (8, 9), the precise mechanism by which these compounds act remains unknown.

In this study, the effect of GA and its related compounds on the growth of HepG2 cells, a human hepatoma cell line, were examined and found to induce G1 arrest and apoptosis. This was associated with a reduction in the expression levels of Bcl-2 and Bcl-xL and activation of caspase 8. Further, the effect of GA and its related compounds on the expression of connexin was assessed, since GA is known to inhibit the gap junction (10-12), and the relationship between their anti-tumor activity and gap junction inhibitory activity are discussed.

Materials and Methods

Chemicals. 18 β -Olean-12-ene-3 β , 23, 28-triol, newly named 18 β -erythrotriol (18 β ETol), was prepared from naturally occurring hederagenin by a previously described method (9) (Figure 1). All other chemicals were of biological grade.

Cell culture and cell proliferation assay. Human hepatocellular carcinoma HepG2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, at 37°C in a humidified 5% CO₂ atmosphere. The cells were seeded at a density of 2x10⁴ cells/2 ml of medium. After 1 day, the cells were treated with GA or its related compounds, which were dissolved in dimethyl sulfoxide. On day 3, cell viability was ascertained by the trypan blue dye exclusion method.

Flow-cytometric analysis. The cells were plated at a density of 1x10⁵ cells/10 ml of medium. GA or its related compounds were added to the medium 24 h after inoculation. The cells were harvested at specified times and sample cell suspensions (10⁶ cells) were centrifuged, washed with PBS (-) and resuspended in 1 ml of 0.1% Triton-X 100 solution. These were then filtered through 50- μ m nylon mesh, after which 10 μ l of 100 mg/ml RNase A and 20 μ l of 2.5 μ g/ml propidium iodide were added. The DNA content of the stained cells was measured using a FACSCalibur™ flow cytometer

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Key Words: Glycyrrhetic acid, glycyrrhizin, G1 arrest, apoptosis, Bcl-2 family, hepatocellular carcinoma.

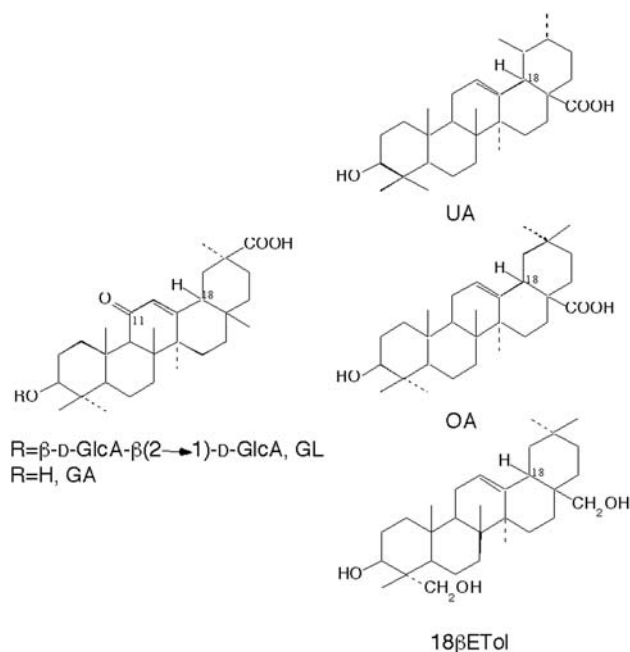


Figure 1. Chemical structure of GA and related compounds. GL, glycyrrhizin; GA, glycyrrhetic acid; UA, ursolic acid; OA, oleanolic acid; 18βETol, 18β-olean-12-ene-3β, 23, 28-triol (18β-erythrotriol).

(Becton-Dickinson, Franklin Lakes, NJ, USA) and analyzed by the ModFit LT™ program.

DNA ladder assay. The cells (2×10^6) were washed twice with PBS (-), centrifuged and then lysed in 100 μl of lysis buffer (1% NP-40, 20 mM EDTA, 50 mM Tris-HCl, pH 7.5) by mixing for 15 s. Following centrifugation (1600 xg for 5 min), the supernatant, containing fragmented DNA, was recovered, and to this was added 100 μl of 2% SDS. The samples were incubated with RNase (5 μg/μl) at 56°C for 2 h, and then treated with proteinase K (2.5 μg/μl) at 37°C for 4 h. DNA was precipitated in ethanol with 5 M of NH₄OAc and resuspended in loading buffer (0.25% bromophenol blue, 40% sucrose, 10 mM EDTA, pH 8.0). Following electrophoresis on a 2% agarose gel, the DNA was stained with ethidium bromide and visualized by UV light.

Northern blot analysis. Total RNA was extracted from cells using an RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany). Twenty μg of total RNA was electrophoresed and then transferred to a nylon membrane. Northern blots were hybridized with a ³²P-labelled probe. RT-PCR was performed to generate a cDNA probe for the *connexin 32* gene. The data was normalized to the level of 36B4 expression.

Western blot analysis. The cells were solubilized with cell lysis buffer containing 60 mM Tris-HCl (pH 6.8), 2% SDS, 12% glycerol, 10 mM NaF, 1 mM Na₃VO₄, 20 mM β-glycerophosphate, 1 mM PMSF and 0.5 μg/ml protease inhibitor mix (leupeptin, aprotinin and pepstatinA). The cell lysate was sonicated for 30 s on ice and then centrifuged at 12000 rpm for 30 min at 4°C. The protein content of

Table I. Growth inhibition of HepG2 cells by GA and related compounds.

| Compound | ID ₅₀ (μM) |
|-------------------|-----------------------|
| Glycyrrhizin | >1200 |
| Glycyrrhetic acid | 80 |
| Ursolic acid | 20 |
| Oleanolic acid | 70 |
| 18βETol | 25 |

Cells were treated with each reagent or vehicle alone and viable cells were counted on day 3. 18βETol: 18β-Olean-12-ene-3β, 23, 28-triol (18β-Erythrotriol).

the samples was determined using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Thirty to 100 μg of whole-cell extracts were separated by SDS-PAGE (12%) and then transferred to a PVDF membrane. The membranes were blocked overnight at 4°C with 5% non-fat dry milk in TBST buffer containing 20 mM Tris-HCl (pH 7.6), 137 mM NaCl and 0.1% Tween 20. Following washing with TBST, the membranes were incubated for 1 h at room temperature with primary antibody, washed and then incubated for 1 h with HRP-conjugated secondary antibody (1:1000, Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The proteins were visualized using the ECL detection system (Amersham). The antibodies used were rabbit anti-connexin 32 (1:500, Zymed Lab., Inc., South San Francisco, CA, USA), mouse anti-Bcl-2 (1:100, Santa Cruz Biotech. Inc., Santa Cruz, CA, USA), rabbit anti-Bcl-xL, -Bad, -Bax, -Bak and -caspase-9 (1:1000, Cell Signaling Tech., Inc., Beverly, MA, USA) and mouse anti-caspase-8 (1:1000, Cell Signaling).

Statistical evaluation. The data were analyzed using the Student's *t*-test. The significance was set at $p < 0.05$.

Results

Effects of GA and related compounds on the growth of HepG2 cells. GA and its related compounds inhibited the proliferation of HepG2 cells in a dose-dependent manner (data not shown), albeit with different effects. Table I summarizes the 50% inhibitory dose (ID₅₀) of the compounds tested. The inhibitory effect of GL was weak (ID₅₀ > 1200 μM), while ursolic acid and 18βETol were remarkably effective (ID₅₀ was 20 μM and 25 μM, respectively).

Induction of G1 arrest and apoptosis by GA and related compounds. GA and its related compounds induced G1 arrest in HepG2 cells (Figure 2). Treatment with GA or ursolic acid (ID₅₀) induced G1 arrest as early as 16 h; the effects continued for up to 2 days and were dose-dependent (not shown). Additionally, GA, ursolic acid and 18βETol induced apoptosis at high dose (ID₉₀, Figure 3). Oleanolic acid also induced apoptosis at high dose, although pre-apoptotic cells were not clearly detected by flow-cytometric analysis (data not shown). On the other hand, a high dose of GL induced G1 arrest, but did not induce apoptosis. The

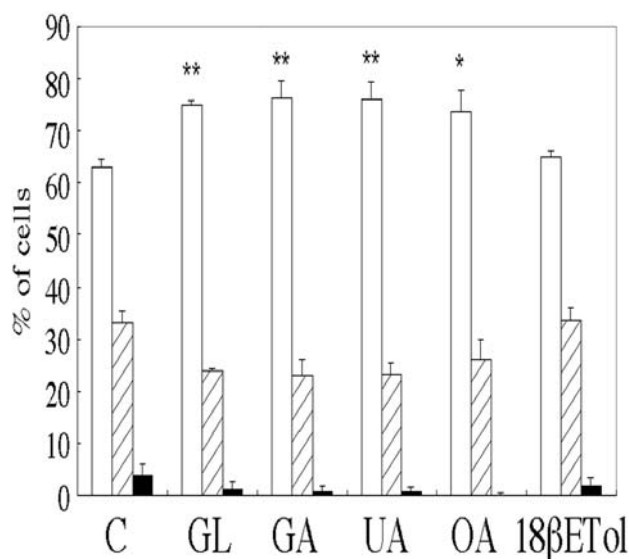


Figure 2. Induction of G1 arrest by GA and related compounds. Cells were treated with each reagent (ID_{50}) for 24 h and subjected to flow-cytometric assay. The data are expressed as average and SD of experiments performed in triplicate. C, control; GL, glycyrrhizin; GA, glycyrrhetic acid; UA, ursolic acid; OA, oleanolic acid; 18βETol, 18β-olean-12-ene-3β, 23, 28-triol (18β-erythrotriol). □, G1; ▨, S; ■, G2/M. **, $p < 0.01$; *, $p < 0.05$.

DNA ladder assay (Figure 4) and *in situ* staining of apoptotic cells (not shown) further confirmed the induction of apoptosis by ursolic acid and GA. Ursolic acid and GA yielded typical DNA fragmentation patterns in a dose-dependent manner at 24 h.

Reduction of anti-apoptotic proteins and activation of caspases 8 and 9 by ursolic acid and GA. The expressions of apoptosis-related proteins, the Bcl-2 family (13, 14) and caspases-8 and -9, initiator caspases in apoptosis execution (21), were assessed. Ursolic acid, the most effective compound tested in this study, and GA were selected for further analysis. As shown in Figure 5a, high-dose (ID_{90}) treatment with ursolic acid or GA decreased the expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL at 24 h, while the effect of a low-dose treatment was less pronounced. The expression of the pro-apoptotic proteins such as Bax (Figure 5a) and Bak (data not shown) remained unaffected following treatment with ursolic acid or GA. In addition, the activation and cleavage of caspases-8 and -9 was also observed by the same treatment (Figure 5b).

Reduction of connexin 32 gene and protein expression by ursolic acid and GA. Since GA is known to inhibit the gap junction, the effects of GA and its related compounds on

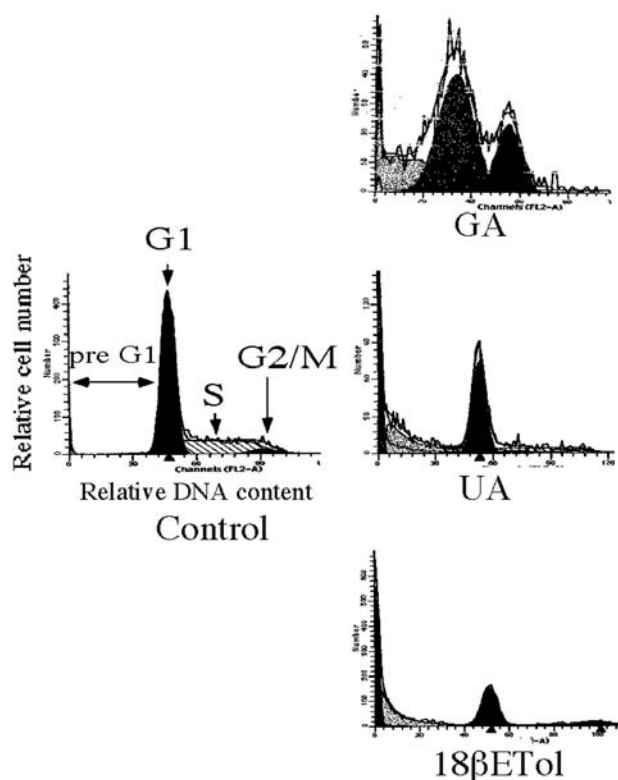


Figure 3. Induction of apoptosis by GA and related compounds. Cells were treated with a high dose (ID_{90}) of each reagent for 24 h and pre-G1 apoptotic cells were detected by flow-cytometric analysis. C, control; GA, glycyrrhetic acid; UA, ursolic acid; 18βETol, 18β-olean-12-ene-3β, 23, 28-triol (18β-erythrotriol).

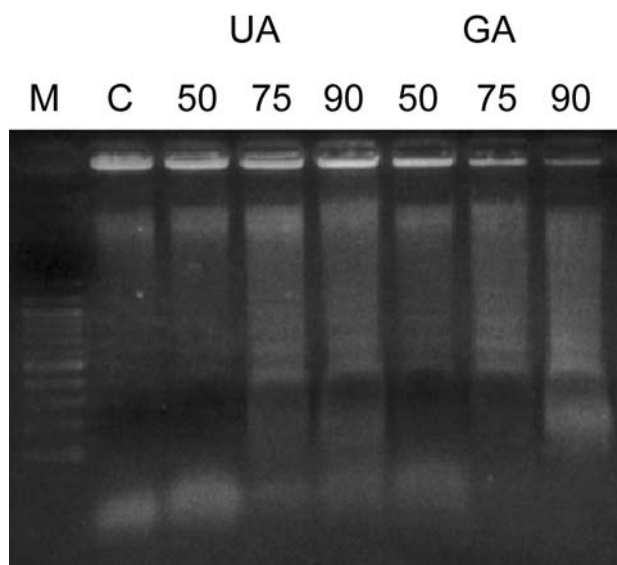


Figure 4. Induction of apoptosis by ursolic acid and GA. Cells were treated for 24 h and subjected to DNA ladder assay. DNA fragmentation was induced by treatment with ursolic acid or GA, in a dose-dependent manner. M, size marker; C, control; UA, ursolic acid; GA, glycyrrhetic acid; 50, 75 and 90, ID_{50} , ID_{75} and ID_{90} , respectively.

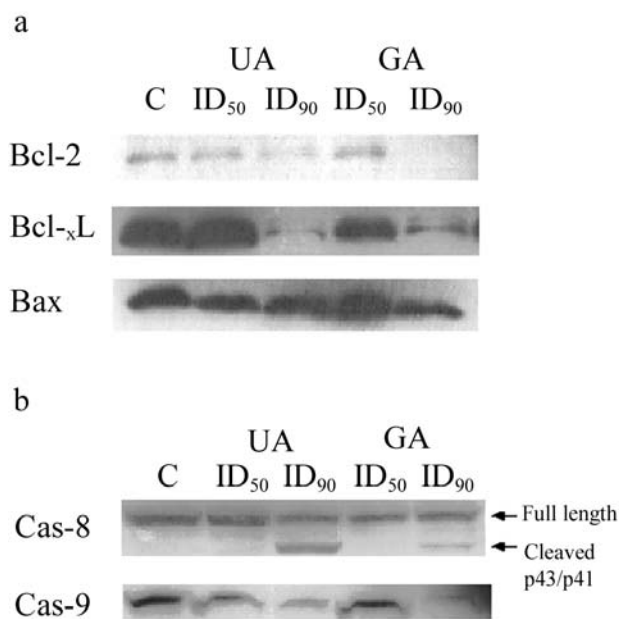


Figure 5. Reduction of Bcl-2 and Bcl-xL and activation of caspases-8 and -9 by ursolic acid and GA. Cells were treated for 24 h and then subjected to Western blot analysis. The same amount of proteins was applied. a, Both ursolic acid and GA decreased the expression of Bcl-2 and Bcl-xL, although Bax remained unaffected. b, Decrease of the full length of caspases-8 and -9, and increase of the cleaved form of caspase-8 were observed. C, control; UA, ursolic acid; GA, glycyrrhetic acid; Cas-8, caspase-8; Cas-9, caspase-9.

the expression of connexins, molecules of gap junctional channels, were examined. *Connexin 32* was detected in HepG2 cells. Treatment of the cells with GA, ursolic acid, oleanolic acid or 18βETol (ID₅₀) reduced the expression of the *connexin 32* gene at 24 h (Figure 6a). The reduction in connexin 32 protein levels was also observed following treatment with ursolic acid or GA (ID₉₀, but not ID₅₀) (Figure 6b). 18βETol at high dose also reduced the level of connexin 32 protein (not shown), although an unambiguous reduction was not observed following treatment with GL or oleanolic acid, even at high dose. Additionally, the decrease in actin observed was concomitant with the reduction in connexin 32.

Discussion

It was previously reported that GA induced G1 arrest (3) and apoptosis (4, 15), and enhanced mitochondrial permeability transition (16). Furthermore, a certain herbal medicine containing GL and its derivatives induced G0/G1 arrest and apoptosis (17, 18). The precise mechanism by which these compounds act remains largely unknown. Thus, we examined the anti-tumor effect of GA and its related compounds on human hepatoma cells. GA and the related

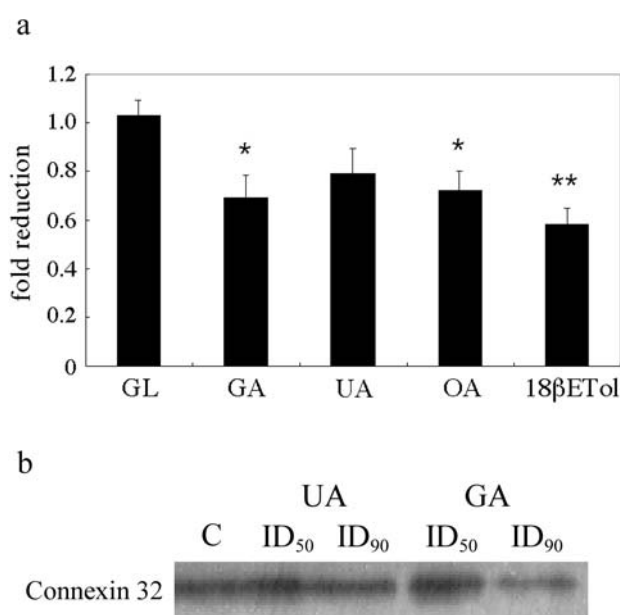


Figure 6. Effect of GA and related compounds on the expression of connexin 32. Cells were treated with each reagent for 24 h and then subjected to Northern and Western blot analyses. a, Results of Northern blot analysis. All compounds except GL reduced expression of the *connexin 32* gene. The expression level of connexin 32 was normalized to that of 36B4. The data are shown as average and SD of experiments performed in triplicate. b, Results of Western blot analysis. Cells were treated with ursolic acid or GA for 24 h. The same amount of proteins was applied. C, control; GL, glycyrrhizin; UA, ursolic acid; GA, glycyrrhetic acid; OA, oleanolic acid; 18βETol, 18β-olean-12-ene-3β, 23, 28-triol (18β-erythrotriol); ID₅₀ and ID₉₀, 50% and 90% inhibitory dose, respectively. **, $p < 0.01$; *, $p < 0.05$.

compounds inhibited the proliferation of HepG2 cells, a human hepatocellular carcinoma cell line, in a dose-dependent manner, arrested the cell cycle in the G1-phase at low dose, and induced apoptosis at high dose. GL induced G1 arrest but not apoptosis. Given that GL is known to be hydrolyzed to GA *in vivo*, it is likely that GL also has apoptosis-inducing activity, in addition to G1 arrest-inducing activity.

The effect of GA and its related compounds on the expression of apoptosis-related proteins was examined. The Bcl-2 family of proteins is known to play an intrinsic role in apoptosis, thus accelerating tumorigenesis (13, 14). GA and ursolic acid reduced the expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL, but had no effect on the pro-apoptotic proteins Bax and Bak. 18βETol also reduced the Bcl-2 protein levels. Therefore, it is suggested that GA and its related compounds induce apoptosis by reducing the expression of Bcl-2 and Bcl-xL. It seems that Bcl-xL is especially crucial in HepG2 cells, since the level of Bcl-xL expression was significantly greater than that of Bcl-2.

However, the activation of caspase-8 was also induced by treatment with GA and ursolic acid. It is known that caspase-8 mediates two downstream pathways of apoptosis, the mitochondrial pathway and direct caspase-3 activation (21). Thus, other pathways independent of the Bcl-2 family are not eliminated in the apoptosis induced by GA and ursolic acid.

GA is known to inhibit the gap junction, whereas GL, oleanolic acid and ursolic acid do not (11). In the present study, GA, ursolic acid, oleanolic acid and 18 β ETol were shown to decrease the expression of the *connexin 32* gene. A decrease in the level of connexin 32 protein resulted following treatment with GA or ursolic acid. Since the gap junction is thought to be important in maintaining cell homeostasis in normal cells, the inhibitory activity of GA on the gap junction seems to be inconsistent with its anti-tumor activity. However, it is also known that the gap junction decreases in cancer cells generally (19, 20) and does not work in HepG2 cells, thus the reduction of connexin 32 by GA may be independent of its inhibitory activity against the gap junction, at least in tumor cells. In this context, further investigations are required.

In summary, GA and its related compounds induced cell cycle arrest in the G1-phase and apoptosis in HepG2 cells. The reduction in the anti-apoptotic proteins Bcl-2 and Bcl-xL and the activation of caspases-8 and -9 seem to play an important role in the apoptosis effect induced by GA and its related compounds. Given the known inhibitory activity of GL and its derivatives on liver carcinogens, GA and its related compounds might prove useful in the control of liver cancer.

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