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Research Article

Anti-Hepatoma Activity of a Novel Compound Glaucocalyxin H *In Vivo* and *In Vitro*

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Abstract. Glaucocalyxin H (GLH) is a new compound isolated from a traditional Chinese medical herb *Isodon japonica* var. *glaucocalyx* which has been used for folk medicine. This study was carried out for the first time to investigate the potential role of GLH in anti-hepatoma activity and underlying mechanisms in it. GLH could inhibit the growth of tumor in mice and induce $HepG_2$ cells to death as assessed by the tumor reduction assay, toxic assay, morphological change, and survival rate assay. Many antitumor drugs originated from plants could inhibit the growth of tumor by inducing cells to apoptosis. The morphological changes of $HepG_2$ cells treated with different concentrations of GLH under fluorescence and electron microscope and apoptotic rates were detected to verify its effect on apoptosis. As shown in the study, GLH could induce $HepG_2$ cells to apoptosis in a dose-dependent manner. Bcl_2 and Bax proteins played important roles in apoptosis and the disequilibrium between Bcl_2 and Bax might result in apoptosis. The expression of Bax protein was upregulated and Bcl_2 protein was downregulated in $HepG_2$ cells treated with GLH assessed by Western blotting, and they were in a dose-dependent manner. Taken together, GLH can inhibit the growth of hepatoma cells *in vivo* and *in vitro* by inducing cell apoptosis due to the decreased Bcl_2 and increased Bax proteins suggesting that GLH could be a potential candidate as an anti-hepatoma agent for the therapeutic treatment of hepatoma.

KEY WORDS: apoptosis; Glaucocalyxin H; hepatoma; $HepG_2$ cell.

INTRODUCTION

Isodon japonica (Burm. f.) Hara var. *glaucocalyx* (Maxim.) Hara is widely distributed throughout northern China, and it has been used as an antibacterial, anti-inflammatory, stomachic, and anthelmintic agent in Chinese folk medicine (1). Its anticancer activities were proven in the early 1960s (2). Phytochemical analysis has shown that the main chemical compositions of *I. japonica* var. *glaucocalyx* are diterpenoids (3,4). Glaucocalyxin A and glaucocalyxin B are the two important diterpenoids from *I. japonica* var. *glaucocalyx* (5). Glaucocalyxin A is capable of inducing human leukemia HL-60 cells to apoptosis (6), inhibiting platelet-activating factor (PAF)-induced platelet aggregation (7), and suppressing immune activity (8). It has also been reported that glaucocalyxin A has cytotoxic activity (9). Glaucocalyxin B possesses cytotoxicity of AGZY-83A cells by blocking entry to the G_2/M phase (10) and may induce Rb cells to apoptosis (11). As of yet, a total of more than 30 *ent*-kaurane diterpenoids have been extracted from *I. japonica* var. *glaucocalyx* found in different areas of China, including glaucocalyxin C, glaucocalyxin D, glaucocalyxin E, glaucocalyxin F, etc. (12–15). However, there are seldom studies relating to the pharmacological effects and mechanisms of these diterpenoids.

Glaucocalyxin H (GLH), a new natural compound, was isolated from the ethyl acetate (EtOAc) extract of the dried leaves of *I. japonica* var. *glaucocalyx* in our laboratory (16). The structural elucidation demonstrated that GLH is an acetal derivative of the two hydroxyl groups of glaucocalyxin A with the aldehyde group of protocatechuic aldehyde (Fig. 1). Glaucocalyxin A, glaucocalyxin B, and glaucocalyxin H are the *ent*-kaurane diterpenoids, containing the same pharmacophore α,β-unsaturated cyclopentanone in their structure (17). In our early studies, it was found that GLH had potent cytotoxicity on EC-1, U87, A549, MCF-7, Hela, K562, and $HepG_2$ cells. Its antitumor activity on $HepG_2$ cells was stronger than both glaucocalyxin A and glaucocalyxin B. Therefore, further studies were carried out regarding the effects of GLH on liver tumor both *in vivo* and *in vitro* to determine its potential clinical value.

Guangfan Hai and Chong Zhang are contributed equally to this work.

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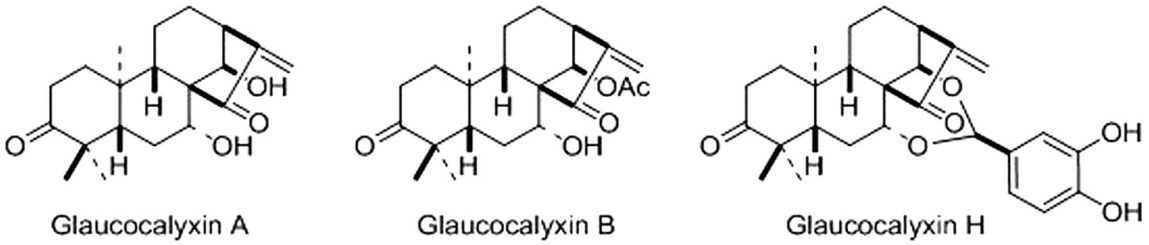

Fig. 1. The structures of glaucocalyxin A, glaucocalyxin B, and glaucocalyxin H

MATERIALS AND METHODS

Reagents

Compound GLH (purity 95.5%) was provided by our laboratory (School of Pharmacy, Xinxiang Medical University). Cyclophosphamide injection (no. 09123121) was purchased from Jiangsu Hengrui Pharmaceutical Co., Ltd., China. Medium RPMI-1640 was produced by Gibco, USA. Mouse monoclonal antibodies (Abs) to human Bcl-2, Bax, and rabbit-antimouse immunoglobulin G (IgG) Ab were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Fetal bovine serum was provided by Hangzhou Sijiqing Biological Engineering Materials Co., Ltd, China. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were provided by Sigma, USA. Propidium iodide (PI) and Hochest 33258 were purchased from Guangzhou Weiga Technology Co., Ltd., China. 5-Fluorouracil (5-Fu) (no. 100301) was purchased from Hualian Shanghai Pharmaceuticals, China.

The Scheme of Synthesis of Glaucocalyxin H

In a 50-mL round-bottom flask, two drops of concentrated sulfuric acid was added to the solution of glaucocalyxin A (332 mg, 1.0 mmol) and protocatechuic aldehyde (207.2 mg, 1.5 mmol) in CH2Cl2 (8 mL) and THF (0.5 mL). The solution was stirred for 1 h at room temperature and then 20 mL of CH2Cl2 (20 mL) was added. The mixture was washed with water (2×30 mL) and saturated sodium carbonate solution (30 mL), dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography eluting with chloroform/methanol (100/1) to afford glaucocalyxin H as white solid (271.5 mg, 60% yield). Mp. 282-284oC; [α]32.4 D −74.4° (c 0.23, MeOH); 1H-NMR (C5D5N, 400 MHz, ppm): δ 6.30 and 5.36 (each 1H, s, 2H-17), 4.82 (1H, s, H-14), 4.74, 4.70 (1H, dd, J=12.88, 5.08Hz, H-7), 3.22 (1H, s, H-13), 1.13, 1.11 and 1.06 (each 3H, s, 3 × Me), 11.32 (2H, brs, ×2 Ar-OH), 6.27 (1H, s, H-1′), 7.68 (1H, s, H-7′), 7.25 (1H, s, H-3′, 6′). 13C-NMR (C5D5N, 100 MHz, ppm): δ 215.3 (s, C-3), 206.2 (s,C-15), 147.4 (s, C-16), 116.8 (t, C-17), 78.0 (d, C-14), 73.2 (d, C-7), 56.0 (s, C-8), 52.2 (d,C-9), 51.4 (d, C-5), 47.7 (s, C-4), 44.1 (d, C-13), 38.8 (s, C-10), 38.3 (t, C-1), 34.6 (t, C-2), 31.4 (t, C-12), 23.9 (t, C-6), 26.6 (q, C-18), 21.8 (q, C-19), 17.1 (q, C-20), 18.7 (t, C-11), 94.6 (d, C-1′), 131.8 (s, C-2′), 115.5 (d, C-3′), 148.5 (s, C-4′), 148.2 (s, C-5′), 116.3 (d, C-6′), 119.0 (d, C-7′). The scheme of synthesis of glaucocalyxin H was shown in Fig. 2.

Animals

Adult Kunming mice weighed 20±2 g, 50% female and 50% male, were purchased from the Henan Province Center of Laboratory Animals (Zhengzhou, China). All animals received humane care. The Animal Care Committee of Xinxiang Medical University of China approved the experimental protocol. These mice were maintained in a controlled environment at 24±2°C with a 12-h light/dark cycle and received food and water *ad libitum*. They were acclimatized for at least 3 days prior to the experiments.

Cell Lines

H_{22} cells and $HepG_2$ cells were obtained from Nanjing Kaiji Biological Engineering Co., Ltd. (Nanjing, China). $HepG_2$ cell lines were originally obtained from the Cell Center of the Chinese Academy of Sciences Library. The cells were cultivated in an incubator at 37°C in air containing 5% CO_2 with a relative humidity of 98%. The culture medium RPMI-1640 (Gibco, USA) contained 100 g/L of heat-inactivated fetal calf serum, 10 U/mL of penicillin, and 100 mg/mL of streptomycin. The cells were transferred to a new culture every 2–3 days. The hepatocytes in the exponential growth phase were used in the experiment.

Mouse Preparation of Experiment *In Vivo*

One milliliter (1×10^7cells) H_{22} cell solution was subcutaneously injected at the right oxter of the mice for 8 days to create the focal tumor models. Fifty mice of focal tumor models were randomly divided into five groups, namely, the model control group, 10 mg/kg cyclophosphamide control group, and 20, 40, and 80 mg/kg GLH groups. Each mouse in the GLH groups was injected with GLH by means of intraperitoneal injection every day at 8:00 am for 12 days. Physiological saline and cyclophosphamide were also injected for the model control group and 10 mg/kg cyclophosphamide control group at 8:00 am for 12 days, respectively.

Assay of Tumor Weight, Tumor Inhibitory Rate, and Morphological Changes of Tumor Tissue

Twelve days later, the mice were euthanized by dislocation after being weighed in balance. Then, they were sterilized in 75% alcohol and the skin of right axil was cut to retrieve the tumor with the membrane decoherence. The wet tumor was weighed in balance and the tumor inhibitory rate was calculated according to the following formula: tumor inhibitory

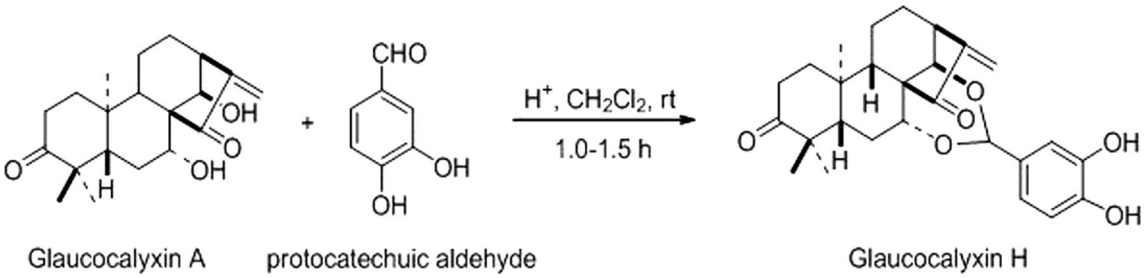

Fig. 2. The scheme of synthesis of glaucocalyxin H

rate=(mean tumor weight in model control group−mean tumor weight in GLH group)/mean tumor weight in model control group×100%. At the same time, the extent of tumor diffusion and necrosis as well as adherence with muscles and skin was observed.

Assay of Thymus Gland and Spleen

The thoracic cavity and the belly cavity of mice were cut open to extract the thymus gland and spleen out after the mice were euthanized and sterilized. The wet thymus gland and spleen were weighed in balance. The thymus gland and spleen indexes were calculated, respectively, according to the formula: thymus index=weight of thymus/body weight of mice×100%, spleen index=weight of spleen/body weight of mice×100%.

Assay of $HepG_2$ Survival Rates

The effect of GLH on cell viability was assessed by a colorimetric MTT assay. Briefly, 5×10^3 $HepG_2$ cells were seeded in each well of a 96-well culture plate and allowed to adhere overnight at 37°C in a humidified 5% CO_2 atmosphere. Twenty-four hours later, $HepG_2$ cells were incubated with GLH at the concentrations of 0.625, 1.25, 2.5, 5.0, and 10 μmol/L, respectively. The negative control group received drug-free medium with 0.05% *v/v* DMSO. The positive control group was treated with 5-Fu with the same concentrations of GLH. Subsequently, 5 mg/mL MTT was added to each well. The medium was discarded and 150 μL DMSO was added into each well after incubation at 37°C for 4 h. The optical density was measured by a microplate reader at a reference wavelength of 630 nm and a test wavelength of 570 nm. The percentage of cell growth was calculated as follows: cell survival rates (%) = $A570_{sample}/A570_{negative\ control}\times 100\%$

Assay of Morphology Under Fluorescence Microscope

$HepG_2$ cells were seeded into 6-well culture plates at a density of 1×10^5cells per well and incubated with GLH at concentrations of 0.625, 1.25, 2.5, 5.0, and 10 μmol/L. The negative control group received drug-free medium with 0.05% *v/v* DMSO. The cells were washed with 4°C phosphate buffer solution (PBS) three times, and 500 μL 4% paraformaldehyde (PFA) at 4°C was dropped in every well to fix the cells for 10 min 24 h later. Being washed with PBS three times, 10 μg/L Hoechst33258 was placed into each well for 10 min at room temperature away from light. Then, the cells were observed under reverse fluorescence microscopy after being washed with PBS three times.

Assay of Morphology Under Electron Microscope

$HepG_2$ cells (3×10^6) were seeded into each culture bottle and incubated with GLH at concentrations of 1.25 and 5.0 μmol/L for 24 h. The negative control group received drug-free medium with 0.05% *v/v* DMSO. Twenty-four hours later, the cells were collected and washed three times with 4°C regular saline and were detected with electron microscope.

Assay of Apoptotic Rates

$HepG_2$ cells (1×10^6) were seeded into 6-well plates and exposed to different concentrations (0.625, 1.25, 2.5, 5.0, and 10 μmol/L) of GLH for 24 h. The negative control group exposed to 0.05% *v/v* DMSO for 24 h. The positive control group was treated with 5-Fu with the same concentrations of GLH. The cell solution was centrifugated at 1200 rpm for 5 min to harvest the cells, and the cells were washed three times with cold PBS. Then, the cells were gently suspended in 500 μL binding buffer. Five microliters PI were added and incubated with cells in the dark for 10 min. At the end of incubation, analysis was carried out to discriminate between live and apoptotic cells by FACScan flow cytometry.

Detection of Bcl_2 and Bax Proteins

Protein expression of Bcl_2 and Bax was tested by Western blot. In brief, $HepG_2$ cells were washed with PBS (mM: NaCl 130, KCl 2.5, Na_2HPO_4 10, KH_2PO_4 1.5, pH 7.4) and lysed with solubilization buffer (mM: Tris-Cl 50, NaCl 150, 0.02%NaN_3, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, $NaVO_3$, 5 mg/mL leupeptin and 1 mg/mL aprotinin). After centrifugation, the supernatants were collected and equivalent protein concentrations were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The separated proteins were electrotransferred to polyvinylidene fluoride (PVDF) membranes. After blocking with PBST (PBS containing 0.05% Tween 20) containing 5% non-fat milk for 1 h, each membrane was incubated with primary antibodies 1 h at room temperature or overnight at 4°C, and then the membranes were probed with the appropriate secondary peroxidase-conjugated antibodies (HRP-linked anti-rabbit secondary antibody and HRP-linked anti-biotin antibody, 1 h at room temperature). The immunoblots were visualized by enhanced chemiluminescence.

STATISTICAL ANALYSIS

The results are expressed in terms of mean±standard deviation (SD). Statistical analysis was performed using the SPSS software package (version 16.0, SPSS Company, USA). Paired *t* tests, Student's *t* test, and one-way ANOVA, followed by the Student–Newman–Keuls test, were used to determine statistical significance. The difference between means was considered to be statistically significant when $P<0.05$.

RESULTS

Effects of GLH on Tumor Weight in Mice

The tumor weight showed a clear decrease in the GLH groups and cyclophosphamide group compared to the model control group. The tumor weights in the 20 and 40 mg/kg GLH groups increased compared to the cyclophosphamide group, while the weight in the 80 mg/kg GLH group was similar to that in the cyclophosphamide group. The data were shown in Fig. 3. Tumor tissues intruded extensively into the muscle of the chest wall tightly and colliquation, cellular necrosis in the center of tumors, and skin diabrosis at the right axil were obvious in some mice of the model group. There were fewer invasions and cellular necrosis in the center of the tumor in the 20 and 40 mg/kg GLH groups. Adherence and necrosis were seldom seen in the 80 mg/kg GLH group except fewer invasions and cellular necrosis compared with the model control group. The tumor inhibitory rate in the 80 mg/kg GLH group was similar to that in the cyclophosphamide group.

Effects of GLH on Thymus and Spleen Indexes in Mice

The effects of GLH on the thymus and spleen are shown in Fig. 4. As shown in the figure, 80 mg/kg GLH significantly decreases the thymus and spleen indexes compared to the model control group. However, there were little changes of GLH in the 20 and 40 mg/kg dosages on the thymus and spleen indexes. There was no clear difference for thymus and spleen indexes between GLH 80 mg/kg group and cyclophosphamide group. However, the thymus index was higher in the 20 and 40 mg/kg GLH groups than that in cyclophosphamide group.

Effects of GLH on $HepG_2$ Survival Rates

As shown in Fig. 5, GLH could significantly inhibit $HepG_2$ cells growth. Meanwhile, GLH inhibited $HepG_2$ cell growth in a concentration-dependent manner. A significant decrease in survival rates was shown in GLH-treated cells compared with 5-Fu-treated cells under the same experimental conditions. The survival rates were 93.17, 74.56, 59.7, 44.08, and 19.25% in GLH groups, respectively, while the survival rates were 98.66, 93.27, 82.19, 68.21, and 53.30% in 5-Fu groups, respectively.

Effects of GLH on Morphological Changes of $HepG_2$ Cells

It was shown under reverse fluorescence microscopy that $HepG_2$ cells treated with GLH presented obvious morphological changes. In Fig. 6, there were many apoptotic cells

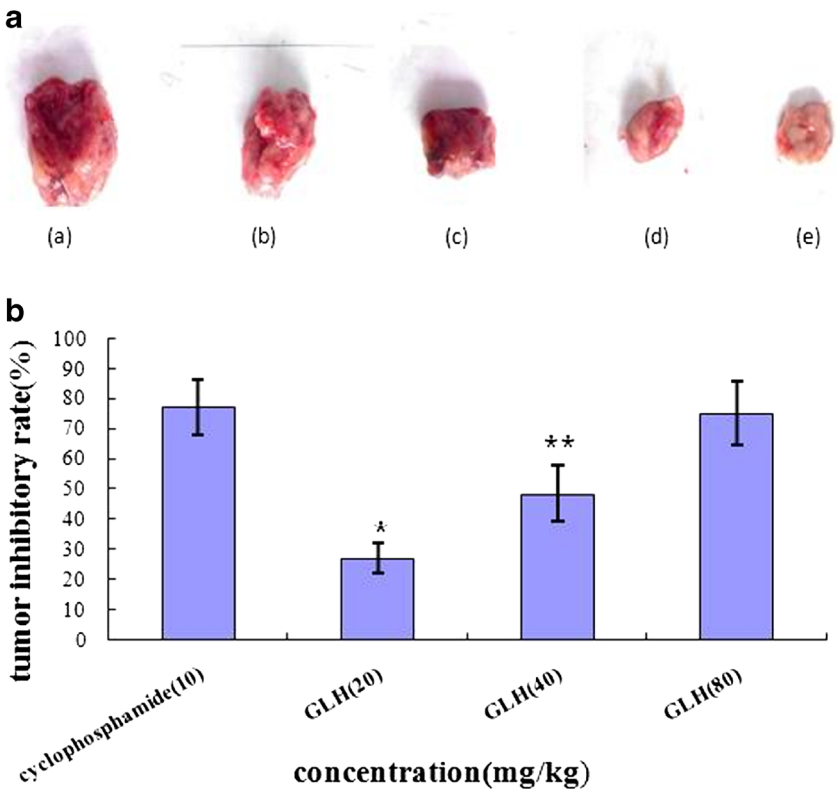

Fig. 3. **a** Effect of GLH on the size of tumor in mice. (*a*) The model control group, (*b*) the cyclophosphamide group, (*c*) the 20 mg/kg GLH group, (*d*) the 40 mg/kg GLH group, (*e*) and the 80 mg/kg GLH group. **b** Effect of different concentrations of GLH on the tumor inhibitory rate in mice

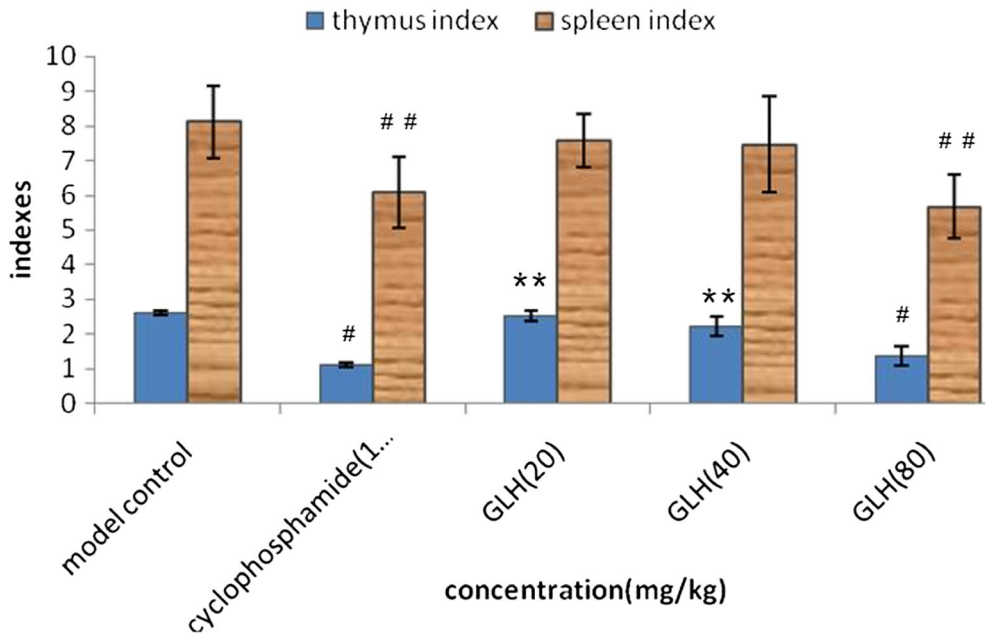

Fig. 4. Effect of glaucocalyxin H (GLH) on thymus and spleen indexes in tumor-bearing mice. Each data represents mean±SD of ten determinations (n=10). *P<0.05; **P<0.01 compared with the cyclophosphamide group. #P<0.05; ##P<0.01 compared with the model group

including shrinkage, fragmentation, and irregularity of nuclei in the GLH-treated cells, especially in the 2.5, 5.0, and 10.0 μmol/L GLH groups. Meanwhile, the number of apoptotic cells increased with the increasing of GLH concentration. There was little apoptotic cells in the non-GLH-treated group.

Effects of GLH on Microstructure of $HepG_2$ Cells

As shown in Fig. 7, $HepG_2$ cells were condensed with lunate and drifted nuclei in the 1.25 μmol/L GLH-treated cells compared to the no-GLH-treated $HepG_2$ cells with round nuclei and intercalated nuclei. There were no clear nuclei and many apoptotic bodies could be found in 5.0 μmol/L GLH-treated $HepG_2$ cells.

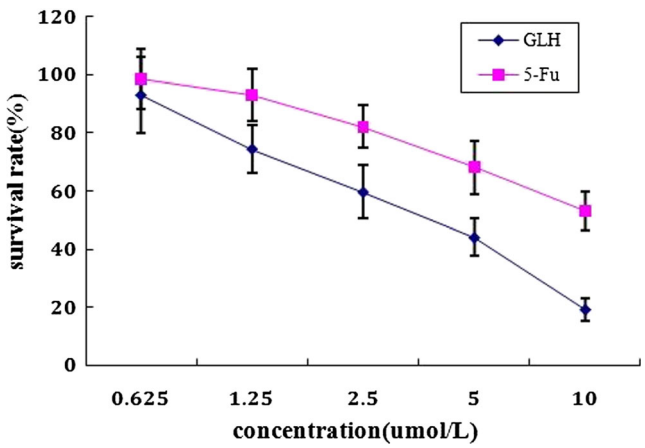

Fig. 5. Survival rates of $HepG_2$ cells. Each data represents mean±SD of six determinations (n=6)

Effects of GLH on the Apoptotic Rates of $HepG_2$ Cells

Different GLH concentrations could induce $HepG_2$ cells to apoptosis and the apoptotic rates were shown in Fig. 8. GLH induced $HepG_2$ cell to apoptosis in a concentration-dependent manner, and the apoptotic rates were 7.8, 27.6, 29.9, 33.7, and 55.4% in 0.625, 1.25, 2.5, 5.0, and 10.0 μmol/L GLH-treated cells, respectively, which showed significant difference between GLH-treated cells and no-GLH-treated cells with 0.4% apoptotic rate.

Effects of GLH on Protein Expressions of Bcl_2 and Bax of $HepG_2$ Cells

GLH was able to significantly upregulate the protein expression of Bax and downregulate that of Bcl_2 with increasing dose. The results are shown in Fig. 9. The changes of Bcl_2 and Bax proteins are in a concentration-dependent manner. Bcl_2/Bax ratio decreased with GLH dosage increasing.

DISCUSSION

Malignant tumors have been a serious threat to human health. However, currently, there are no effective drugs which only kill tumor cells and have little damages to normal cells. Anticancer drugs originating from natural plants provide some basis for such drugs. In China, *I. japonica* var. *glaucocalyx* is a type of folk medicine which is mainly used to cure inflammation, stomach pain, and cancer as well as heat-clearing and detoxification (18). Glaucocalyxin A and glaucocalyxin B, the main components of *I. japonica* var. *glaucocalyx*, play significant roles in disease treatment and prevention. Glaucocalyxin A is able to protect the heart and liver from injuries induced by ischemia-reperfusion and

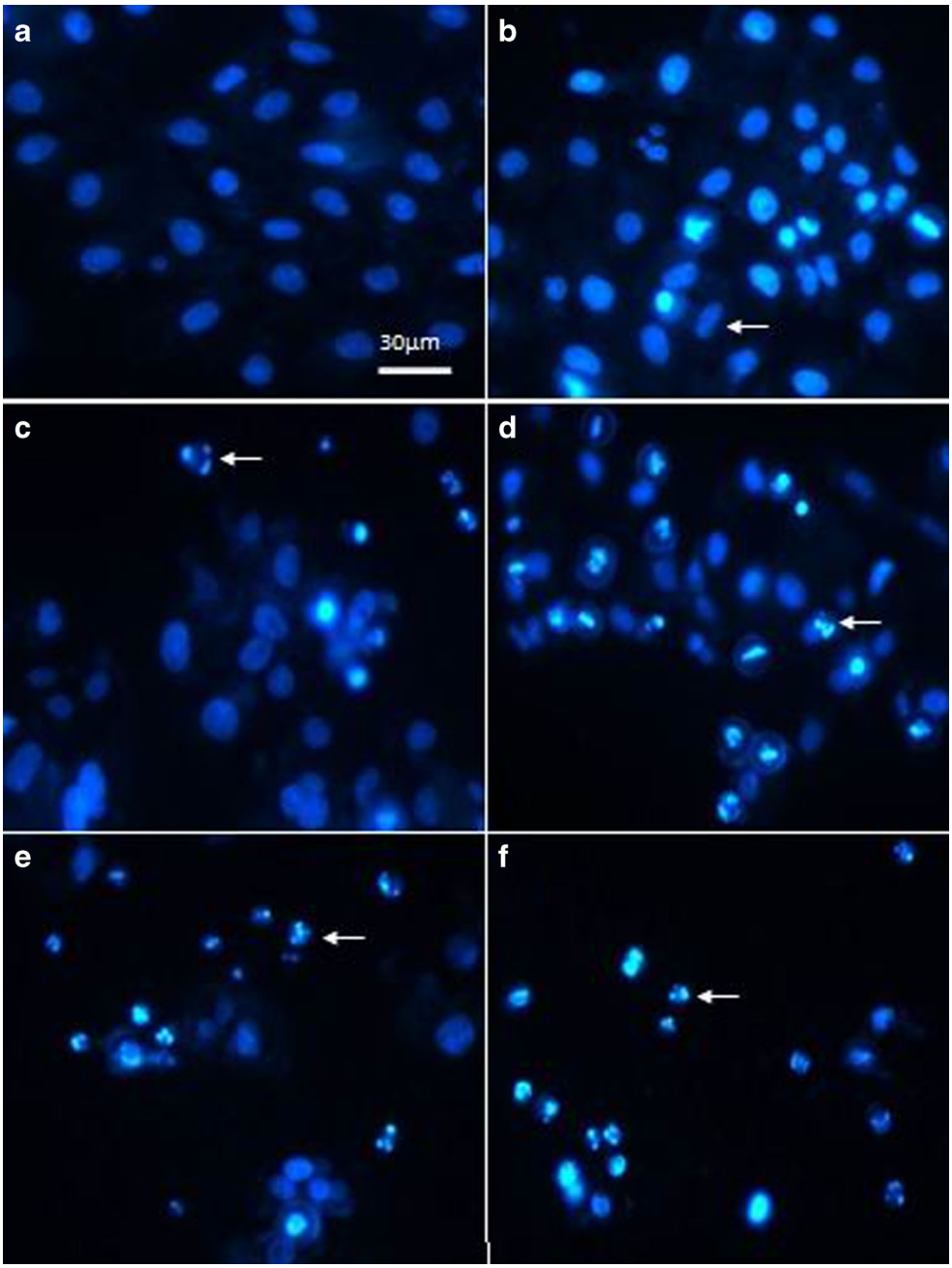

Fig. 6. Apoptosis of $HepG_2$ cells induced by GLH. (**a** $HepG_2$ cells un-treated with GLH, **b** $HepG_2$ cells incubated with 0.625 μmol/L GLH, **c** $HepG_2$ cells incubated with 1.25 μmol/L GLH, **d** $HepG_2$ cells incubated with 2.5 μmol/L GLH, **e** $HepG_2$ cells incubated with 5.0 μmol/L GLH, **f** $HepG_2$ cells incubated with 10.0 μmol/L GLH)

carbon tetrachloride (CCl_4) by decreasing the expression of c-fos genes (19,20). It also has antitumor activity for cells such as K562, $HepG_2$, HL-60, Hela, etc. *N*-Acetylcysteine (NAC), a type of antioxidant, is capable of inhibiting the cytotoxicity of glaucocalyxin A on K562 cells, which is related to reactive oxygen species (ROS) (21). Further studies regarding NAC's structure–function relationship with antitumor activity revealed that almost all diterpene components, including α-methylene cyclopentanone building block, may cause the tumor cells to die. Based on these observations, it may be concluded that the α-methylene cyclopentanone building block of diterpenes components is the pharmacophore (22). GLH, a newly discovered compound, isolated from *I. japonica* var. *glaucocalyx*, is an acetal derivative of glaucocalyxin A. It is derived by means of condensation reaction of two hydroxyl groups of glaucocalyxin A with the aldehyde group of protocatechuic aldehyde. GLH contains the same antitumor pharmacophore of cyclopentanone, conjugated with an exomethylene group in the structure as glaucocalyxin A. However, GLH exhibits higher inhibitory proliferation activities than its parent compound glaucocalyxin A, with 50% inhibitory concentration (IC_{50}) value of 1.97 μM against

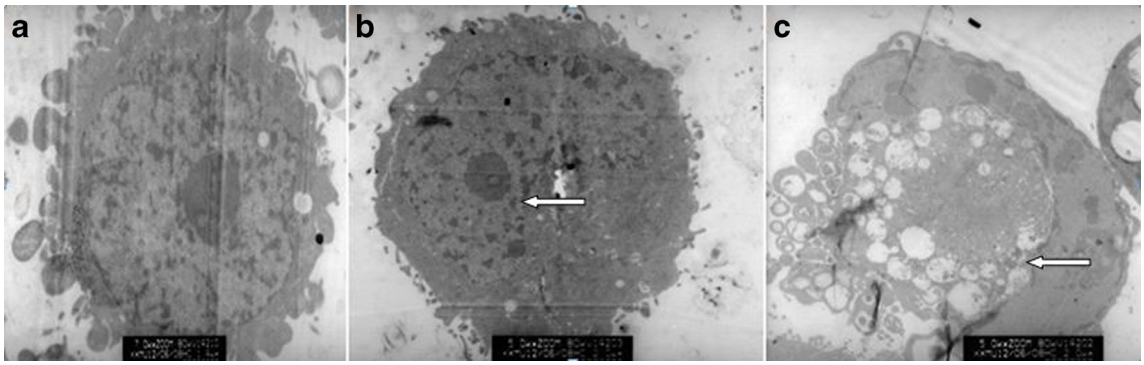

Fig. 7. Effects of GLH on the microstructure of $HepG_2$ cells. (**a** $HepG_2$ cells without being treated with GLH, **b** 1.25 μmol/L GLH, **c** 5.0 μmol/L)

$HepG_2$ cell line in our early studies (16). This suggests that the hydroxyl groups of protocatechuic aldehyde enhance the antitumor activities of GLH. In order to evaluate the antitumor therapeutic efficacy and mechanism of glaucocalyxin H, GLH has been synthesized artificially from glaucocalyxin A and protocatechuic aldehyde.

Cyclophosphamide, an antitumor drug, may be used to treat carcinomas of the lung, breast, ovary, etc. (23–25). It is activated by cytochrome P_{450}-mediated microsomal oxidation in the liver to produce aldophosphamide, which spontaneously decomposes to produce reactive phosphoramide mustard, which in turn may inhibit cell growth and multiplication by promoting DNA alkylation (26). In the present study, it was shown that 10 mg/kg cyclophosphamide may resist tumor growth in mice. The results showed that GLH decreased the tumor weight of mice in GLH groups (i.e., 20, 40, and 80 mg/kg) compared with the model group, and infiltration, colliquation, and celluar necrosis in the center of the tumors were improved especially in the 80 mg/kg group compared to the cyclophosphamide group and the model group which indicated that GLH played similar roles in inhibiting tumor growth to cyclophosphamide and may be related to its anti-inflammation effect (27). As shown in Fig. 4, thymus and spleen indexes decreased in the immune systems of the mice in GLH group, which was similar to cyclophosphamide which indicated that GLH had little toxicity to immune system of mice with the treatment of tumor.

The results in the experiments *in vitro* showed that GLH could decrease the survival rates of $HepG_2$ cells which made clues that GLH could inhibit the growth of $HepG_2$ cells. How did GLH inhibit its proliferation? As known to us, most of the present anticancer drugs mediate their effect *via* apoptosis induction in cancer cells, and apoptosis is suggested as one of the major mechanisms for the targeted therapy of various cancers including prostate cancer. To determine whether the growth inhibition induced by GLH in human liver cells was caused by apoptosis, $HepG_2$ cell was treated with different concentrations of GLH for 24 h and the morphological changes were examined. As shown in Fig. 6 and Fig. 7, typical characteristics (shrinkage, fragmentation, irregularity, and shift of nuclei) of apoptosis could be observed, which suggested that GLH suppress the growth of $HepG_2$ cells by inducing apoptosis. To further confirm the apoptotic effects of GLH in the cell, we also analyzed the apoptotic rates in different concentrations of GLH. The results suggested that

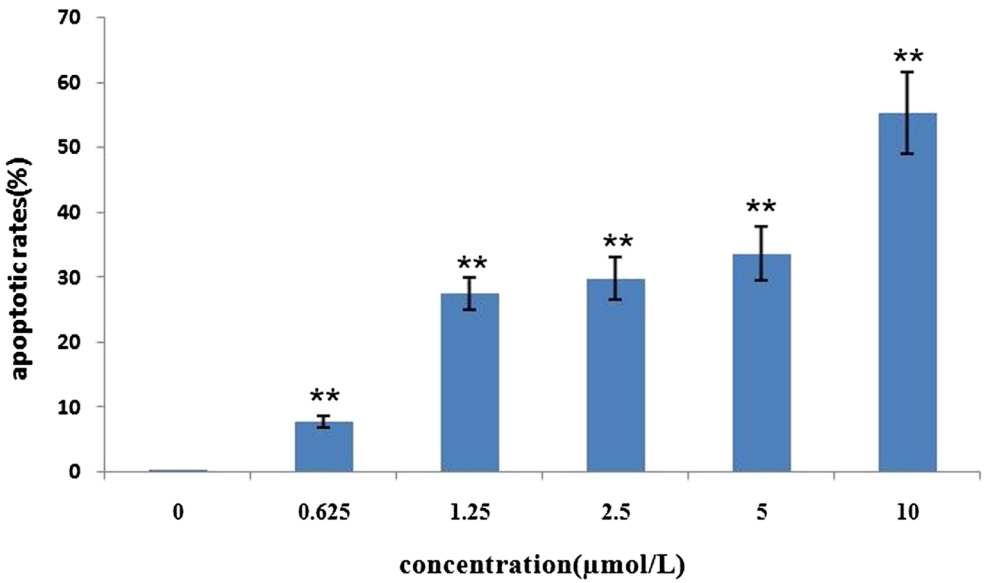

Fig. 8. The apoptotic rates of $HepG_2$ cells. Each data represents mean±SD of six determinations (n=6). $^{**}P<0.01$ compared with the control group

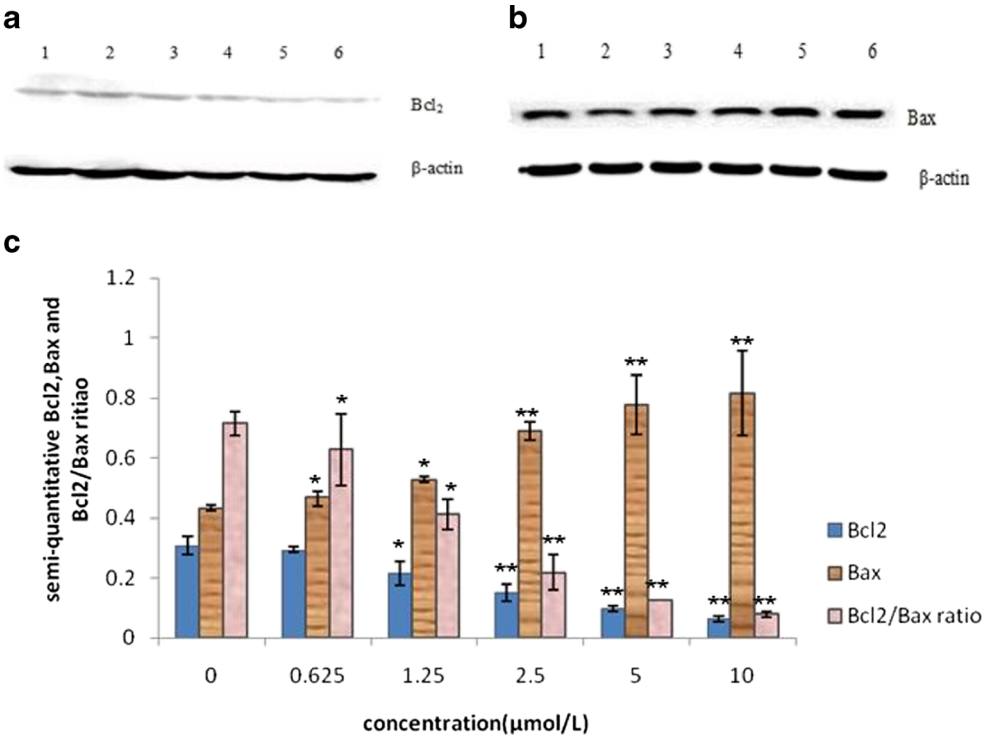

Fig. 9. Effect of GLH on Bcl_2, Bax proteins, and Bcl_2/Bax ratio of $HepG_2$ cells. **a** Effect of GLH on Bcl_2 protein of $HepG_2$ cells (*lane 1*: 0 μmol/L; *lane 2*: 0.625 μmol/L; *lane 3*:1.25 μmol/L; *lane 4*:2.5 μmol/L; *lane 5*: 5 μmol/L; *lane 6*:10 μmol/L). **b** Effect of GLH on Bax protein of $HepG_2$ cells (*lane 1*: 0 μmol/L; *lane 2*: 0.625 μmol/L; *lane 3*:1.25 μmol/L; *lane 4*:2.5 μmol/L; *lane 5*: 5 μmol/L; *lane 6*:10 μmol/L). **c** Effect of GLH on Bcl_2/Bax ratio of $HepG_2$ cells. Each data represents mean±SD of three determinations (n=3). *P<0.05; **P<0.01, compared with the control group

GLH induced $HepG_2$ cell to apoptosis in a concentration-dependent manner (Fig. 8). These results discovered that GLH suppress the growth of $HepG_2$ cells by inducing apoptosis.

Apoptosis is known as programmed cell death (PCD) or cell suicide and is a form of cell death in which a controlled sequence of events (or program) leads to the elimination of cells, without releasing harmful substances into the surrounding area (28). The disequilibrium between Bcl_2 and Bax may cause mitochondrial permeability transition and aid cytochrome c in releasing from mitochondria to cytosol, resulting in the activation of caspase cascades (29,30). It may also be concluded that the increased Bax may induce cells to apoptosis and the decreased Bcl_2 protein may reduce its anti-apoptosis effect, which could cause the cells to die. Did the changes of Bcl_2 and Bax protein induce $HepG_2$ cells to apoptosis? So, the expressions of the two kinds of proteins were detected by western blot. As shown in Fig. 9, the expression of Bax increased and Bcl_2 decreased with the increasing dose which showed that the apoptotic effect of GLH on $HepG_2$ cells was related to the regulations of Bcl_2 and Bax proteins. However, the reasons inducing cells to apoptosis were complex and many factors such as cytochrome C, tumor necrosis factor (TNF), etc. could induce cells to apoptosis. So, the exact mechanism of inducing $HepG_2$ cells to apoptosis requires further study, due to the fact that in this study, other apoptotic pathways were not covered.

CONCLUSION

In summary, there is a clear inhibitory effect of GLH on hepatoma *in vivo* and *in vitro*. The mechanism of the potent inhibitory effect of GLH on the tumor is a result of the decreased Bcl_2 and increased Bax, which can induce cells to apoptosis. GLH is a promising potential antitumor agent for further drug development.

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