Original Research

Cold-water extracts of *Grifola frondosa* and its purified active fraction inhibit hepatocellular carcinoma *in vitro* and *in vivo*

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Abstract

Mushrooms are used in traditional Chinese medicine to treat a variety of diseases. *Grifola frondosa* (GF) is an edible mushroom indigenous to many Asian countries with a large fruiting body characterized by overlapping caps. In particular, GF is known for its anti-tumor activity, which has been targeted by scientific and clinical research. This study aimed to investigate the effects of the cold-water extract of GF (GFW) and its active fraction (GFW-GF) on autophagy and apoptosis, and the underlying mechanisms *in vitro* and *in vivo*. Our results revealed that GFW and GFW-GF inhibited phosphatidylinositol 3-kinase (PI3K) and stimulated c-Jun N-terminal kinase (JNK) pathways, thereby inducing autophagy. We also demonstrated that GFW and GFW-GF inhibited proliferation, induced cell cycle arrest, and apoptosis in Hep3B hepatoma cells. GFW and GFW-GF markedly arrested cells in S phase and promoted cleavage of caspase-3 and -9. In addition, GFW and GFW-GF decreased the expression levels of the anti-apoptotic proteins protein kinase B and extracellular signal-regulated kinase. We also found that GFW significantly inhibited tumor growth in nude mice implanted with Hep3B cells. Our work demonstrates that GF and its active fraction inhibit hepatoma growth by inducing autophagy and apoptosis.

Keywords: *Grifola frondosa*, bioactive, apoptosis, autophagy, cell cycle, Hep3B

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Introduction

Hepatocellular carcinoma (HCC) is a significant type of primary liver malignancy with a low worldwide survival rate. Unfortunately, patients with HCC are often diagnosed at a late stage, and prognosis is generally very poor. HCC is commonly associated with alcohol use, aflatoxin B1 exposure, viral infection (hepatitis virus B and C), contraceptives, obesity, diabetes, smoking, and metabolic liver diseases.[1–4] Globally, HCC accounts for 85–90% of primary liver cancers and is one of the leading causes of cancer-related death.[5] In patients who cannot receive a transplant, HCC is particularly lethal, with a 5-year survival of less than 5%.[6] Unlike many cancers that have decreased over the years, HCC incidence is still increasing.[7] When HCC is identified at an advanced and inoperable stage, chemotherapy is the only remaining therapeutic option. In recent years, many new drugs such as paclitaxel, gemcitabine, and capecitabine have been used to treat various types of cancer, and their efficacies have been tested for liver cancer patients.[8–10] In general, new drugs for liver cancer patients have not afforded any dramatic progress because the therapeutic efficacies of the drugs are low.

Grifola frondosa (GF) is a mushroom that grows in Japan and the temperate forests of Asia. It has been known for a long time among Chinese and Japanese people who consumed or utilized GF for dietary or medicinal purposes. GF extracts contain polysaccharides, proteins, nucleic and amino acids, minerals, organic selenium, vitamins C, E, B1, and B2, phenols, and flavonoids.[11] Compounds extracted from GF are claimed to have various health benefits for cancer, hyperlipidemia, hypertension,[12] diabetes,[13] obesity treatment, and prevention of viral infection.[14] GF anticancer medications contain various compounds with diverse biological and therapeutic effects.[15] The content and bioactivity of these compounds depend on how GF is prepared and consumed. Most of the anticancer effects of GF

polysaccharides (D-fraction) have been attributed to modulation of the immune system through the activation of macrophages, dendritic cells, natural killer cells, and cytotoxic T cells.[16] In addition, a recent study reported that polysaccharides suppress HCC growth *in vitro* and *in vivo*.[17] Although preclinical studies suggest that GF extract may have anti-tumor activity, scientific evidence from rigorously designed prospective trials is not available.

Apoptosis (type I cell death) is a form of programmed cell death and the major cell death pathway that occurs upon DNA damage and abnormal cell proliferation.[18] Cancer cells do not undergo apoptosis but bypass it through a series of complex mechanisms involving dynamic interplays between oncogenes and mutated tumor suppressor genes.[19] Therefore, induction of apoptosis is a major objective of anticancer therapies. Autophagy (type II cell death) is a protective mechanism that removes superfluous or damaged cellular constituents to maintain the quality of the cellular components; however, hyperactivation of autophagy can lead to cell death. Autophagy is an evolutionarily conserved catabolic process that involves the entrapment of cytoplasmic components within characteristic double-membrane vesicles, termed autophagosomes, which translocate to the lysosome for degradation.[20] When cells are exposed to stress conditions, such as oxidative stress, starvation, infection, or accumulation of protein aggregates, autophagy begins with membrane isolation and expansion to form autophagosomes that sequester unwanted cytoplasmic materials.[21] Various proteins, including Beclin-1, autophagy-related gene (Atg) proteins, and microtubule-associated protein 1 light chain 3 (LC3), mediate autophagosome formation to engulf cytoplasmic material and fuse with the lysosome for degradation.[20] Numerous studies have reported that autophagy is activated in various anticancer therapies.[22] Autophagy is regulated by several cell signaling pathways such as the class I phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) and c-Jun N-terminal kinase (JNK) pathways.[23,24] PI3K promotes cell growth and proliferation when overexpressed under stress conditions, and its aberrant activation has been implicated in the development of malignancies and anticancer therapy resistance.[25] Studies indicate that autophagy plays a significant role in cancer initiation and progression.[26] Clinical therapeutic approaches currently used in cancer therapy, including cytotoxic chemotherapy, radiation, and hormone therapy, can induce autophagy in cancer cells.[26,27] Multiple studies have demonstrated that these stimuli can simultaneously induce both apoptosis and autophagy.[26,28]

In this study, we found that cold-water but not hot-water extracts of GF polysaccharides exert higher anti-HCC effects *in vitro* and *in vivo*. The active fractions of cold-water extracts of GF (GFW-GF) have molecular weights around 91–198 kDa. Furthermore, GF and GFW-GF significantly induced autophagy and apoptosis in HCC cells. To our knowledge, this is the first demonstration that GF extracts induce autophagy and apoptosis in Hep3B cancer cells.

Materials and methods

Bioactive extract from GF

GF was purchased from a local supermarket (Taichung, Taiwan), and 100 g fresh fruiting bodies were cleaned with wet tissue paper, dried, and ground to a fine powder. Freeze-dried GF powder (4 g) was extracted with water (30 mL) at 4°C, 25°C, 50°C, and 100°C for 24 h. The extract was centrifuged at 3000 rpm at 4°C to give a clear supernatant and filtered through Whatman Number 4 filter paper. Crude extract filtrates (GFW) were successively filtered using 0.45 and 0.2-μm Acrodisc syringe filters (Pall Life Sciences, USA), then concentrated by ultrafiltration using 100-kDa cut-off Macrosep centrifugal devices (Pall Life Sciences) at 3000 rpm for 1 h at 4°C. The concentrated samples were stored at −20°C.

Phenol–sulfuric acid method to determine polysaccharide content

A glucose solution (100 μg/mL) was prepared in distilled water and subsequently diluted to 40–90 μg/mL. A solution of 5% phenol solution (1 mL), sugar solution (1 mL), and H_2SO_4 (5 mL) was prepared, and its absorbance was measured after 15 min at 488 nm. The polysaccharide contents of GFW (100 μg/mL) and GFW-GF (100 μg/mL) were estimated using sugar analysis and compared with the glucose standard solutions.

Size fractionation by gel filtration

Purified GFW was size-fractionated by gel filtration using a BioLogic LP System (Bio-Rad, USA) on a HiLoad 16/60 Superdex 200 prep grade column (GE Healthcare, USA). GFW was eluted with phosphate-buffered saline (PBS) at a flow rate of 0.6 mL/min. Fractions were evaluated for proteins by absorbance at 280 nm to obtain purified bioactive compound (GFW-GF) for use in further experiments.

Molecular size analysis

The molecular masses were estimated by size fractionation through a HiLoad 16/60 Superdex 200 prep grade column using a gel-filtration standard (Bio-Rad, USA) in PBS. The molecular masses (Mr) of GFW-GF were estimated from a linear fit to the plot of log Mr versus elution volume generated with the standards.

Cell line and cell culture

Human HCC Hep3B, HA22T, and Huh7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, USA) supplemented with 10% fetal bovine serum (Invitrogen, USA) and 1% penicillin–streptomycin solution (Invitrogen, USA). The cells were incubated at 37°C in 5% CO_2.

MTS assay

Cell viability was assessed using the MTS/PMS (Sigma, USA) ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2 H-tetrazolium, inner salt)/phenazine methosulfate) assay. Hep3B cells were plated in

a 96-well plate at 4×10^3 cells/well. The cells were treated with control solution or various concentrations of GFW and GFW-GF. After incubation for 3 days, 20 μL MTS solution (2 mg/mL) was added to each well, and cells were incubated for 3 h. The absorbance was measured at 490 nm in a microplate reader 550 model (Bio-rad, USA).

Colony formation assay

Hep3B cells were cultured overnight in a 25-T flask at a density of 4×10^3 cells. GFW and GFW-GF were freshly prepared at concentrations of 6 μg/mL and added to the flask. The cells were allowed to grow for 14 days to form colonies. The colonies were fixed in methanol for 15 min at room temperature, and each well was stained with 0.5% crystal violet for 1 h at room temperature. The colonies were then counted.

Wound healing assay

To measure cell motility, a wound-healing assay was performed. Cell migration was determined using culture inserts (Ibidi, Germany). Hep3B cells were seeded on each 6-well dish inside an Ibidi culture insert (4×10^4 cells per side) and incubated at 37°C in a humidified atmosphere with 5% CO_2. Cells were allowed to attach for 12 h, and then the culture inserts were gently removed. Hep3B cells were treated with 6 μg/mL GFW for 24 h and observed under a microscope (Olympus, Japan). Images were processed and analyzed using Image Plus software.

Cell cycle analysis

Apoptosis was analyzed by detecting propidium iodide (Sigma, USA) with flow cytometry. Hep3B cells (5×10^5) were cultured overnight in 10-cm dishes and subsequently incubated with 6 μg/mL GFW or 4 μg/mL GFW-GF for 24 and 48 h. Cells were trypsinized and fixed in ice-cold 70% ethanol at −20°C overnight. The fixed cells were washed twice in ice-cold PBS, resuspended in staining buffer (20 μg/mL propidium iodide (Sigma, USA), 0.1% Tween 20 (Sigma, USA), and 0.2 mg/mL RNase A (Sigma, USA) in PBS), and incubated at room temperature for 30 min in the dark. The stained nuclei were analyzed using flow cytometry (FACSort Instrument and Analysis Software; ModFit LT, USA), and cells were analyzed by cell cycle distribution and apoptosis.

DsRed-LC3 analysis

DsRed-LC3 vector were kindly provided by Dr. H. Y. Chen (Institute of Biomedical Sciences of Academia Sinica). 3-Methyladenine (3-MA) was purchased from Calbiochem (Calbiochem, USA). DeRed-LC3 vector were transfected into Hep3B by FuGENE® Transfection Reagent (Roche Applied Science, USA). After 24 h, cells were treated with DMEM (Control), 3-MA (5 mM), GFW (6 μg/mL), GFW-GF (4 μg/mL), GFW + 3-MA, or GFW-GF + 3-MA for 12 h then fixed in 4% formaldehyde for 10 min. Fixed cells were washed thrice with PBS, stained with 4′, 6-diamidino-2-phenylindole (Sigma, USA) and observed under a fluorescence microscope (Olympus, Japan).

Tumor xenograft study

Five-week-old male BALB/c athymic nude mice (LASCO, Taiwan) were housed in sterile laminar flow rooms with 12 h light–dark cycles at 19°C–23°C and 40–60% humidity in the Laboratory Animal Centre of China Medical University. All individuals were randomly assigned to two groups of five mice each ($n = 5$ per group) and subcutaneously inoculated with Hep3B and Huh7 cells (2×10^6 cells/mL) suspended in 200 μL serum-free DMEM and Matrigel (BD Bioscience Pharmingen, USA) in the right and left flanks. GFW (10, 20, and 50 mg/kg body weight) was administered orally seven times per week for 42 days beginning 7 days after implantation. Tumors were measured with calipers, and mice were weighed twice per week. Tumor volume was calculated as length × width × width and expressed as mm^3. At the end of the experiment, mice were sacrificed, and tumors were immediately removed, weighed, and fixed in 4% paraformaldehyde (Merck, Germany). All protocols involving animals were approved by China Medical University and the Institutional Animal Care and Use Committee; experiments involving animals were performed in accordance with the relevant approved guidelines and regulations.

Immunohistochemical staining

Tumor tissues were fixed in paraformaldehyde and embedded in paraffin. Immunostaining was performed on 5-mm-thick sections. After antigen retrieval, endogenous peroxidase was blocked with 3% H_2O_2 solution. Slides were washed in distilled water and incubated in PBS for 5 min. Normal goat sera were used to block non-specific binding. Slides were then incubated sequentially with anti-proliferating cell nuclear antigen (PCNA) antibodies, biotinylated secondary antibody, and horseradish peroxidase (HRP)-conjugated streptavidin. All sections were analyzed by light microscopy.

Western blot analysis

Whole-cell lysates and tumor tissues were lysed in RIPA (10 mM Tris-Cl, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.1% sodium dodecyl sulfate (SDS), and 0.5% deoxycholate) or M-PER (Thermo Scientific, USA) lysis buffer containing protease inhibitors (Roche Applied Science, USA) and phosphatase inhibitors (Roche Applied Science, USA). Each sample, containing 40 μg protein, was loaded on an 8–15% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane. The membrane was blocked in Tris-buffered saline containing 1% Tween 20 (TBST) and 5% nonfat milk for 1 h at room temperature, followed by incubation with the following primary antibodies overnight at 4°C: Beclin-1, LC3A, LC3B, Atg12, Atg5, Atg7, Atg3, Bcl-2, PI3K p85 (19H8), Akt, p44/42 MAP kinase (Thr180/Tyr182), SAPK/JNK, p-PI3K p85 (Tyr458), p-Akt (Ser473), p-p44/42 MAP kinase (Thr202/Tyr204), p-SAPK/JNK (Thr183/Tyr185), cleaved caspase-3, cleaved caspase-9, beta-actin, and PCNA (Cell Signaling, USA). The membrane was then washed three times for 10 min each with TBST and incubated with

HRP-conjugated secondary antibodies for 1 h at room temperature. The membranes were developed using an enhanced chemiluminescence detection system, HRP substrate (Millipore, USA), and ImageQuant LAS-4000 Chemiluminescence and Fluorescence Imaging System (FujiFilm, Japan).

Statistical analysis

Results are presented as mean ± SD. All results were derived from at least three independent experiments. Statistical analysis was performed using a Student's *t*-test to compare differences in values between the control and experimental groups. $P < 0.05$ was considered statistically significant.

Results

GFW inhibits proliferation of human HCC Hep3B cells

To determine the effects of extraction temperature on the potency of GF extracts against HCC, GF was extracted at different temperatures (4°C, 25°C, 50°C, and 100°C) for 12 h. GF extracted at 4°C had the highest potency against Hep3B cells (Figure 1(a)). In addition, GFW inhibited the growth of Huh7 and HA22T HCC cell lines (Supplementary Figure 1(a)). Treatment of Hep3B cells with various concentrations of GFW for 72 h inhibited cell growth significantly from 7.813 to 125 μg GFW ($P < 0.05$) (Figure 1(b)) with an IC_{50} of 6 μg/mL. Long-term effects of GFW on the growth of Hep3B cells were further assessed by a colony formation assay. After 14 days of treatment with 6 μg/mL GFW,

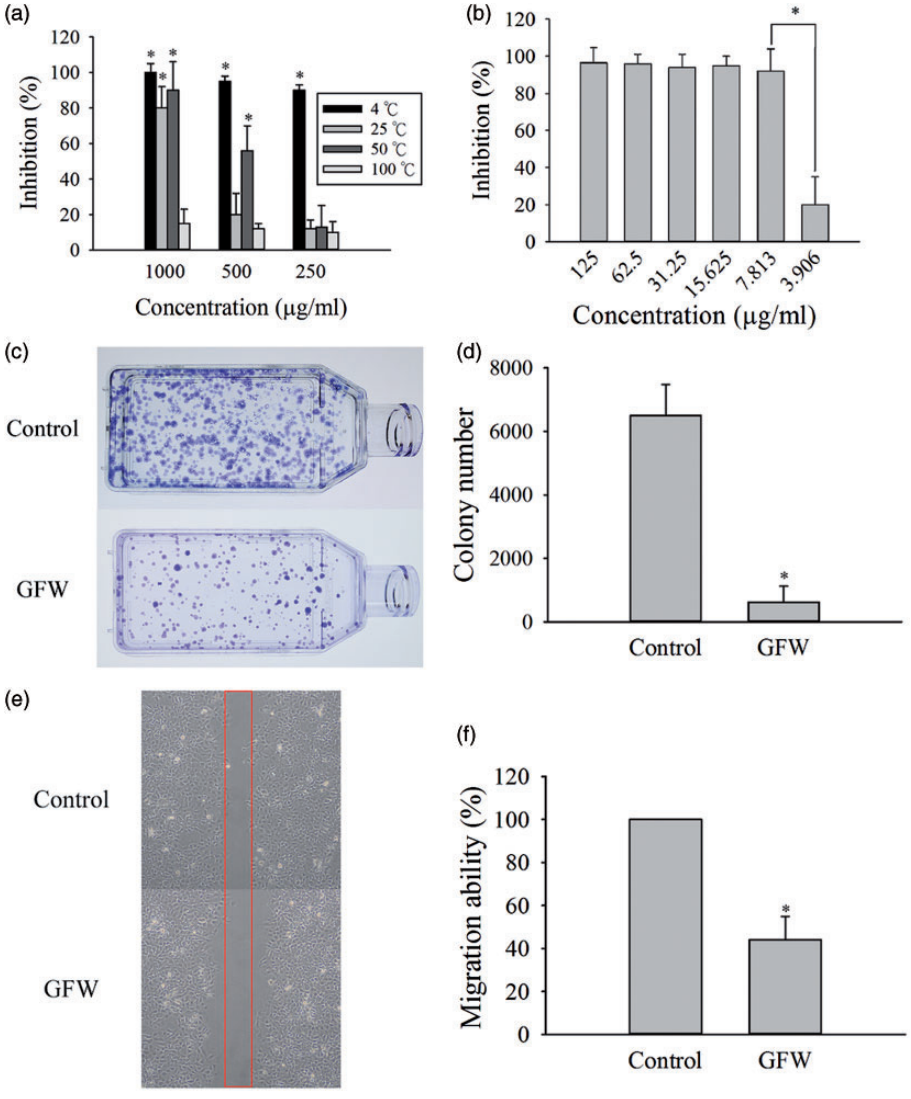

Figure 1 Effects of GFW on cell viability in Hep3B cells. (a) Hep3B cells were cultured in 96-well plates and treated with 1000, 500, or 250 μg/mL GFW extracts isolated at different temperature (4°C, 25°C, 50°C, and 100°C) for 72 h. Cell viability was assessed with an MTS/PMS assay. (b) Hep3B cells were cultured in 96-well plates and treated with varying concentrations of GFW (3.9–125 μg/mL) for 72 h as indicated. Cell viability was assessed with an MTS/PMS assay. (c) Effects of treatment with 6 μg/mL GFW for 14 days on colony formation in Hep3B cells. Hep3B cells were cultured overnight in a 25-T flask at a density of 4×10^3 cells and cultured for 14 days before staining with crystal violet. (d) Quantification of colony numbers from colony-forming assays of untreated Hep3B cells (control) and cells treated with GFW. (e) Representative images showing wound healing assays for Hep3B cells treated with 6 μg GFW and an untreated control after 24 h. (f) Quantification of migration ability from wound healing assays of untreated Hep3B cells (control) and cells treated with GFW. Results are the mean ± SD of three independent experiments performed in triplicate. *Indicates statistically significant ($P < 0.05$) compared with control. (A color version of this figure is available in the online journal.)

colony numbers were significantly ($P < 0.05$) lower in GFW-treated cells (Figure 1(c) and (d)). GFW also inhibited HCC migration by 50% compared with control cells (Figure 1(e) and (f)). These results indicated that GFW potently reduced the proliferation, colony formation, and mobility potential of Hep3B cells.

GFW induces cell cycle arrest and apoptosis

To determine whether GFW inhibited cell growth by inducing cell cycle arrest and apoptosis, Hep3B cells were treated with 6 μg/mL GFW for 24 and 48 h, and cell cycle progress was analyzed by flow cytometry. We found increased levels of sub-G1 stage (apoptosis) cells with GFW treatment, which indicated apoptosis was induced in HCC cells (Figure 2(a)). The apoptosis ratios ranged from 18% and 81% and were statistically significantly compared with controls ($P < 0.05$). S phase arrest was found when cells were treated with GFW for 24 h (32%) and 48 h (44%). The ratio of cells in S phase significantly increased after GFW treatment, and the percentage of cells in G1 phase was reduced compared with control cells. Previous studies showed that apoptosis in cancer cells is regulated by multiple signaling pathways such as the Akt and extracellular signal-regulated kinase (ERK) signaling pathways.[29,30] Western blot analysis showed that GFW decreased the

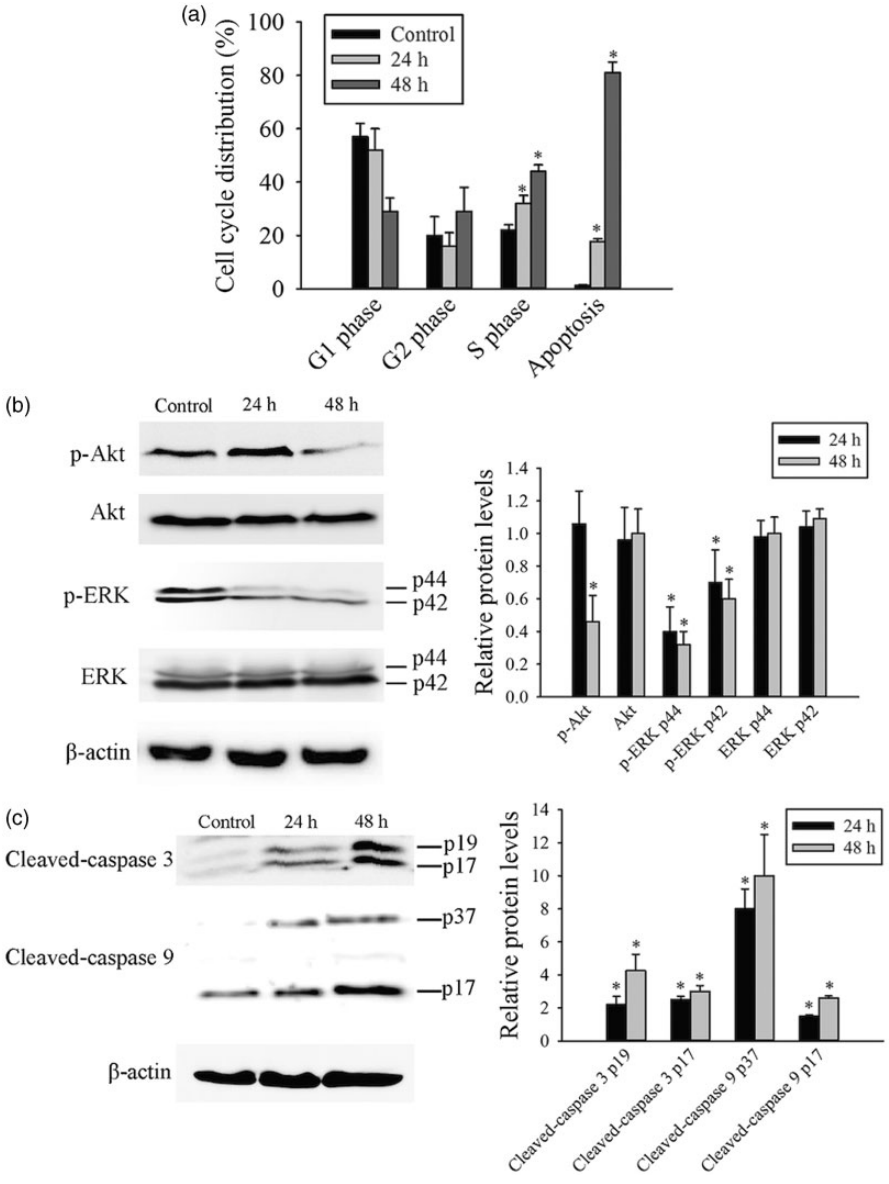

Figure 2 GFW induced S phase arrest and cell death by inhibiting the Akt and ERK pathways and activating caspase-3 and caspase-9 in Hep3B cells. (a) Flow cytometry showed the cell cycle distribution after treatment with 6 μg/mL GFW for 24 and 48 h. (b) Western blotting analysis of the expression levels of Akt, p-Akt (Ser-473), ERK, and p-ERK (Thr202/Tyr204) in Hep3B cells treated with 6 μg/mL GFW for 24 and 48 h. Beta-actin served as a loading control. (c) Cleaved caspase-3 and cleaved caspase-9 were monitored by Western blotting after Hep3B cells were treated with 6 μg GFW for 24 and 48 h. Beta-actin served as a loading control. One representative of three different Western blot experiments with similar results is shown. Results are the mean ± SD of three independent experiments performed in triplicate. *Indicates statistically significant ($P < 0.05$) compared with control.

activation of Akt (Ser473) and ERK (Thr202/Tyr204) (Figure 2(b)) but increased the expression levels of apoptosis-related molecules caspase-3 and caspase-9 (Figure 2(c)).

GFW induces autophagy in Hep3B cells

Growing evidence suggests that autophagy plays a critical role in the development of cancer;[26,31,32] we, therefore, investigated whether GFW could induce autophagy in Hep3B cells. We found an increased number of cytoplasmic vacuoles (autophagosomes) when Hep3B cells were treated with 6 μg/mL GFW for 12 h (Figure 3(a)). LC3 is a major regulator of autophagy pathways.[33] A hallmark of autophagy is the conversion of LC3A to LC3B by proteolytic cleavage and lipidation, which is essential for the formation of autophagosomes.[34] To ascertain whether GFW induces autophagy in Hep3B cells, we transiently transfected Hep3B cells with DsRed-LC3 before incubation with 6 μg/mL GFW for 12 h and performed fluorescence imaging (Figure 3(b)). Our results indicated that GFW treatment induced autophagy relative to the control. 3-MA is a PI3K inhibitor known to inhibit autophagy on the autophagosome membrane.[35] Autophagy induction by GFW was reduced with 3-MA, indicating that PI3K is likely involved in the autophagic response to GFW. The expression levels of autophagy-related proteins LC3A, LC3B, Atg3, Atg5, Atg7, Beclin-1, and Bcl-2 were determined by Western blot (Figure 3(c)). The results showed significant increases in LC3A, LC3B, Atg3, Atg5, Atg7, and Beclin-1 but a reduction in Bcl-2 for cells treated with GFW compared with the control cells. These results suggest that GFW induces autophagy by modulating autophagosome formation in Hep3B cells.

GFW induced autophagy by inhibiting PI3K and activating JNK

To identify the signal transduction pathway leading to autophagy in GFW-treated cells, the phosphorylation levels of PI3K and JNK were monitored. The results demonstrated that the expression levels of p-PI3K (Tyr458) were significantly lower in the GFW-treated cells than in the untreated control cells (Figure 3(d)). On the other hand, previous studies have correlated autophagic cell death with increased levels of phosphorylated JNK. Our results showed increased phosphorylation of JNK (Thr183/Tyr185) upon GFW treatment. Thus, GFW inhibited

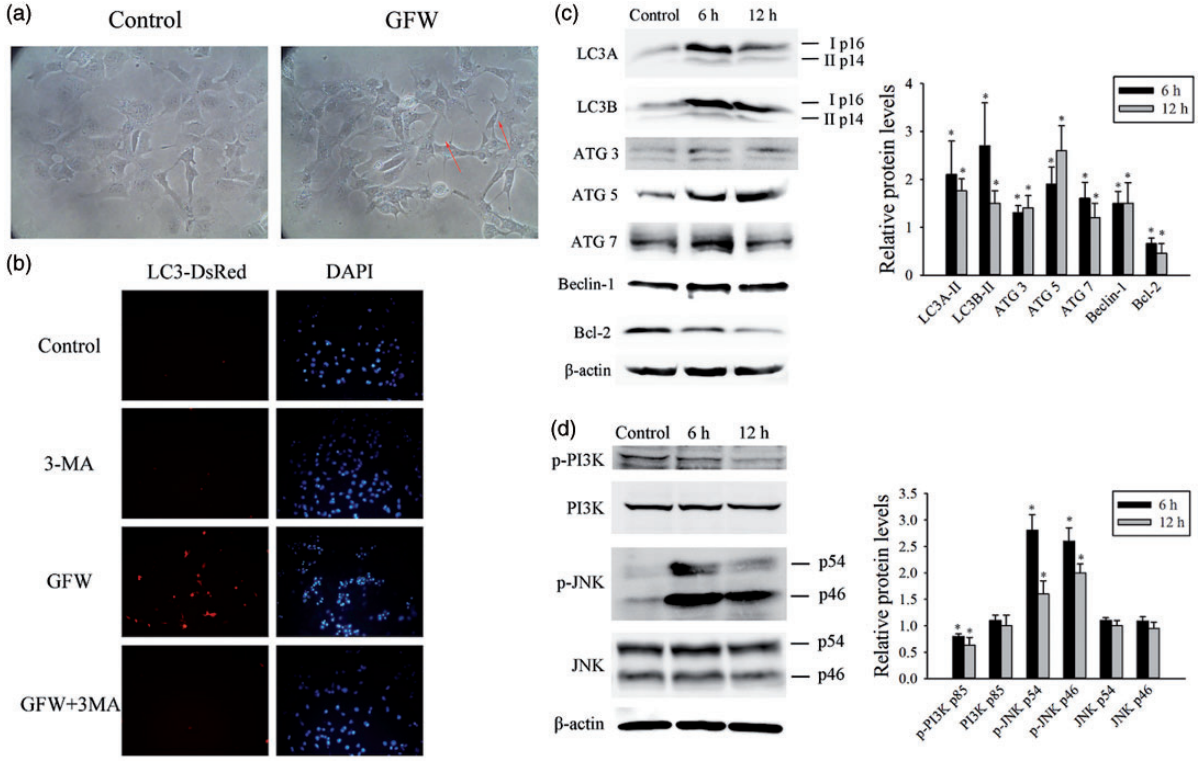

Figure 3 Autophagic cell death induced by GFW in hepatocellular carcinoma Hep3B cells. (a) Cells were cultured in complete medium and treated with 6 μg/mL GFW for 12 h. Representative images were obtained by phase contrast microscopy. Red arrows indicate cytoplasmic vacuoles. (b) Redistribution of DsRed-LC3. Hep3B cells were stably transfected with a DsRed-LC3 fusion construct and cultured in complete serum media with or without 6 μg/mL GFW in the presence or absence of autophagy inhibitor 3-methyladenine (3-MA) for 12 h. Cells were visualized under a fluorescence microscope to examine the expression of LC3. (c) Western blotting to examine the expression of autophagy-related proteins. Cell lysates from controls and cells treated with 6 μg/mL GFW were subjected to SDS-PAGE and immunoblotted with LC3A, LC3B, Atg3, Atg5, Atg7, Beclin-1, and Bcl-2 antibodies. Beta-actin served as a loading control. (d) Western blotting to examine the expression of autophagy signaling protein. Cell lysates from controls and cells treated with 6 μg/mL GFW were subjected to SDS-PAGE and immunoblotted with p-PI3K (Tyr458), PI3K, p-JNK (Thr183/Tyr185), and JNK antibodies. Beta-actin served as a loading control. One representative of three different Western blot experiments with similar results is shown. Results are the mean ± SD of three independent experiments performed in triplicate. *Indicates statistically significant ($P < 0.05$) compared with control. (A color version of this figure is available in the online journal.)

phosphorylation of PI3K (Tyr458) but activated JNK (Thr183/Tyr185) in Hep3B cells.

Effects of GFW on the growth of xenograft tumors in nude mice

The *in vivo* antitumor activity of GFW was evaluated using Hep3B cell xenografts in nude mice (Figure 4(a)). Gastric gavage of GFW (50 mg/kg/day) for 6 weeks significantly reduced the tumor volume (Figure 4(b)) and tumor weight (Figure 4(c)) compared with the control group. We also examined GFW on the growth of Huh7 xenograft tumors in nude mice. Gastric gavage (20 mg/kg/day) or intra-peritoneal administration (10 mg/kg/day) of GFW significantly reduced Huh7 xenograft tumor volume (Supplementary Figure 1(b) and (c)). The intra-peritoneal administration of GFW caused a remarkable suppression of tumor growth, which is significantly greater than gastric gavage of GFW (Supplementary Figure 1(b)). The immunohistochemical staining of PCNA showed that administration of GFW decreased the proliferation of cancer cells (Figure 4(e)). There was no difference in body weight in the GFW-treated group compared to the control group (Figure 4(d)), indicating low GFW toxicity at the curative dose. Our results demonstrated the *in vivo* antitumor efficacy of GFW against HCC in a mouse model without any apparent sign of toxicity. Next, we examined changes in the levels of autophagy- and apoptosis-related proteins. We observed increased levels of LC3B and caspase-3 and decreased levels of phosphorylated Akt (Ser473) and ERK (Thr202/Tyr204)

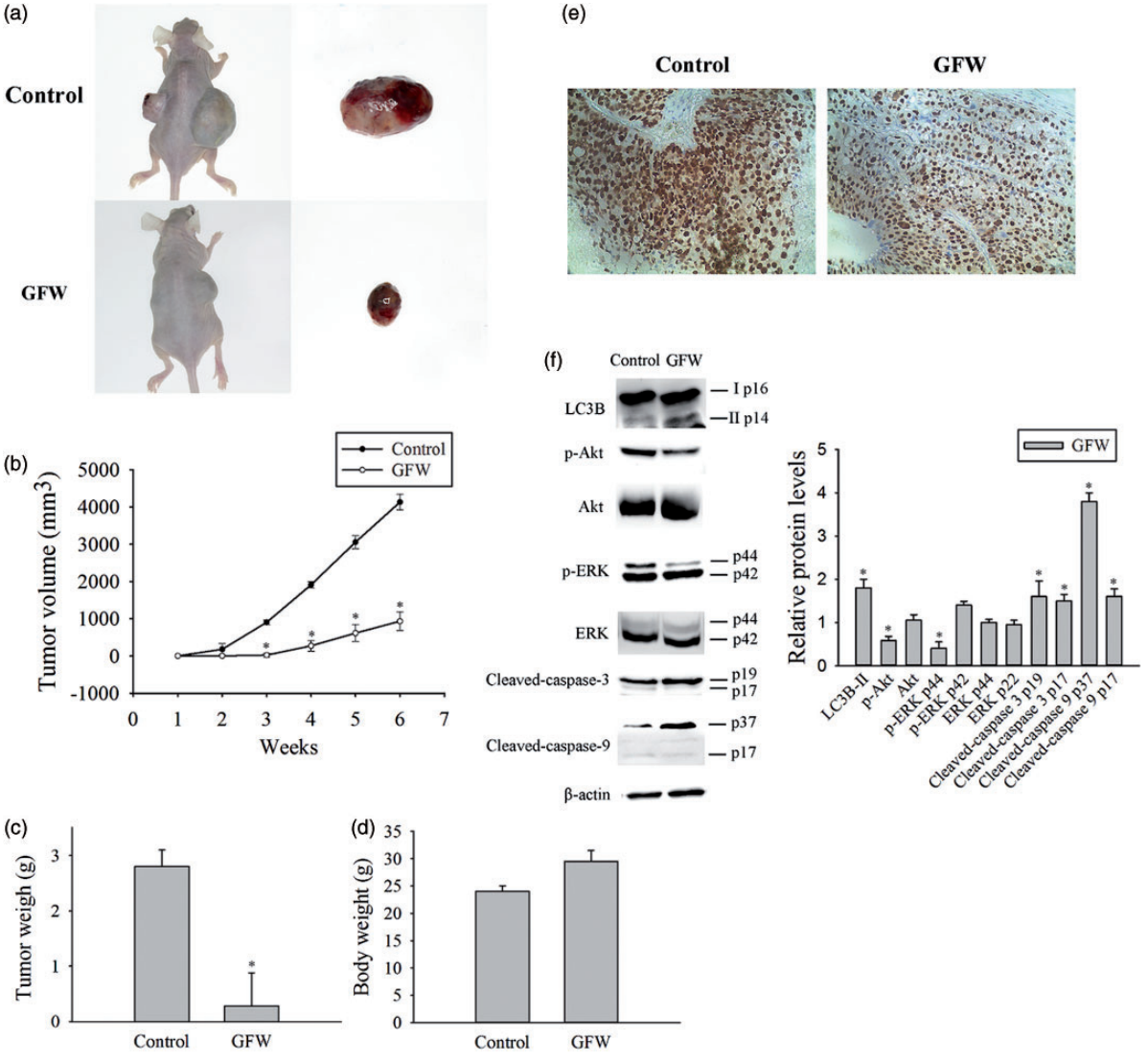

Figure 4 GFW inhibits Hep3B xenograft growth *in vivo*. (a,b) Hep3B cells were injected into the flanks of nude mice. Subsequently, 50 mg/kg GFW was administered orally every day for 6 weeks. On day 43, the tumors were excised and subjected to further analyses. (c) Tumor weights of the nude mice in the GFW-treated and control groups were shown. (d) There was no apparent change in body weight between control and GFW-treated animals. (e) Tumor sections were stained with an anti-PCNA antibody to detect proliferating cells. (f) Western blotting analysis of LC3B, Akt, p-Akt (Ser-473), ERK, p-ERK (Thr202/Tyr204), cleaved caspase-3, and cleaved caspase-9 from tumor homogenates, with beta-actin as a protein loading control. One representative of three different Western blot experiments with similar results is shown. Results are the mean ± SD of three independent experiments performed in triplicate. *Indicates statistically significant ($P < 0.05$) compared with control. (A color version of this figure is available in the online journal.)

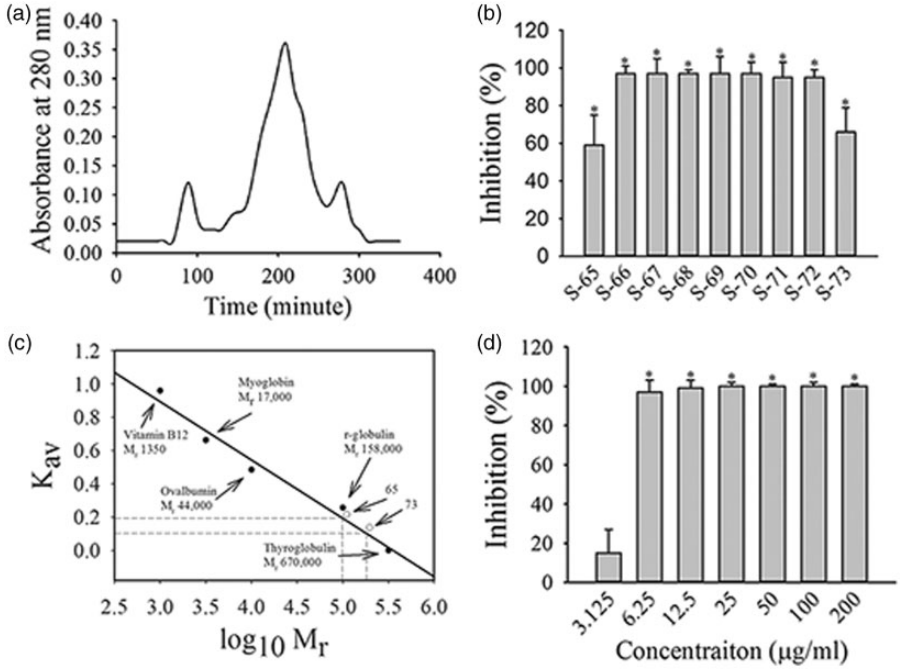

Figure 5 Isolation of active fractions of GFW by gel filtration chromatography. (a) Size-exclusion gel filtration chromatogram from a HiLoad 16/60 Superdex 200 prep grade column. Fractions were eluted with PBS at a flow rate of 0.6 mL/min, and 1-mL fractions were collected. (b) Hep3B cells were cultured in 96-well plates and treated with 32 μg/mL active fractions 65–73 (GFW-GF) for 72 h as indicated. Cell viability was assessed with an MTS/PMS assay. (c) Size exclusion chromatography of purified GFW. The elution profiles of GFW and proteins of known gel-filtration standards (thyroglobulin: 670,000 Da; bovine γ-globulin: 158,000 Da; chicken ovalbumin: 44,000 Da; equine myoglobin: 17,000 Da; and vitamin B-12: 1350 Da) from a HiLoad 16/60 Superdex 200 column are shown. Elution of the gel-filtration standards was monitored by UV absorption at 280 nm (open circles). (d) Hep3B cells were cultured in 96-well plates and treated with varying concentrations of GFW (3.125–200 μg/mL) for 72 h as indicated. Cell viability was assessed with an MTS/PMS assay. Results are the mean ± SD of three independent experiments performed in triplicate. *Indicates statistically significant ($P < 0.05$) compared with control.

(Figure 4(f)). Thus, these results suggest that GFW shows *in vivo* antitumor efficacy by inducing autophagy and apoptosis in a mouse model.

Isolation of active fractions of GFW by gel filtration chromatography

To identify the active fractions of GFW, the extract was fractioned by gel filtration chromatography, and 1-mL fractions were collected for analysis (Figure 5(a)). The bioactive fractions were determined by an MTS/PMS assay. We found cell growth was significantly decreased upon treatment with fractions 65–73 (GFW-GF) ($P < 0.05$) (Figure 5(b)). Using a gel-filtration standard, we found that the polysaccharide (65–73) in GFW-GF had a molecular weight range from 91 to 198 kDa (Figure 5(c)). The IC_{50} for pooled fractions 65–73 was 4 μg/mL (Figure 5(d)). Previous studies indicate that most of the GF anticancer effect arises from polysaccharides.[11,16] We used the phenol–sulfuric acid method to estimate the total polysaccharide contents of GFW and GFW-GF, which were 47% ± 6.6 and 53% ± 15.2, respectively.

GFW-GF induced apoptosis and autophagy in Hep3B cells

To elucidate whether GFW-GF inhibits cell growth by inducing apoptosis, we examined the effects of GFW-GF on cell cycle progression by flow cytometry. GFW-GF increased the percentage of sub-G1 cells and induced cell cycle arrest at S phase in Hep3B cells, which is comparable with GFW (Figure 6(a)). Compared with the control group, Akt and ERK activation were decreased, while the expression levels of cleaved caspase-3 and cleaved caspase-9 increased in the GFW-GF group (Figure 6(b)). We next assessed whether GFW-GF induced autophagy similar to the effects observed for GFW in Hep3B cells. Representative images of Hep3B cells treated with 4 μg/mL GFW-GF for 12 h are provided in Figure 6(c), with arrows indicating the various cytoplasmic vacuoles that might be autophagosomes. To further confirm the induction of autophagy, we transiently transfected Hep3B cells with DsRed-LC3, an autophagy sensor, and incubated them with 4 μg GFW-GF for 12 h before imaging with fluorescence microscopy. Our results showed that GFW-GF treatment induced autophagy more than the control (Figure 6(d)). We then measured the levels of autophagy-related proteins LC3A, LC3B, Atg3, Atg5, Atg7, Beclin-1, and Bcl-2 in Hep3B cells. Our results showed that cells treated with GFW-GF exhibited significant increases in LC3A, LC3B, Atg5, Atg7, and Beclin-1 protein levels compared with control cells (Figure 6(e)). To identify the signal transduction pathway leading to autophagy in GFW-GF-treated cells, we measured changes in the activation of p-PI3K (Tyr458) and p-JNK (Thr183/Tyr185) in Hep3B cells treated with GFW-GF and found that their expression levels were significantly lower and higher, respectively, in the GFW-GF-treated cells than in the untreated control cells (Figure 6(f)). These results suggest

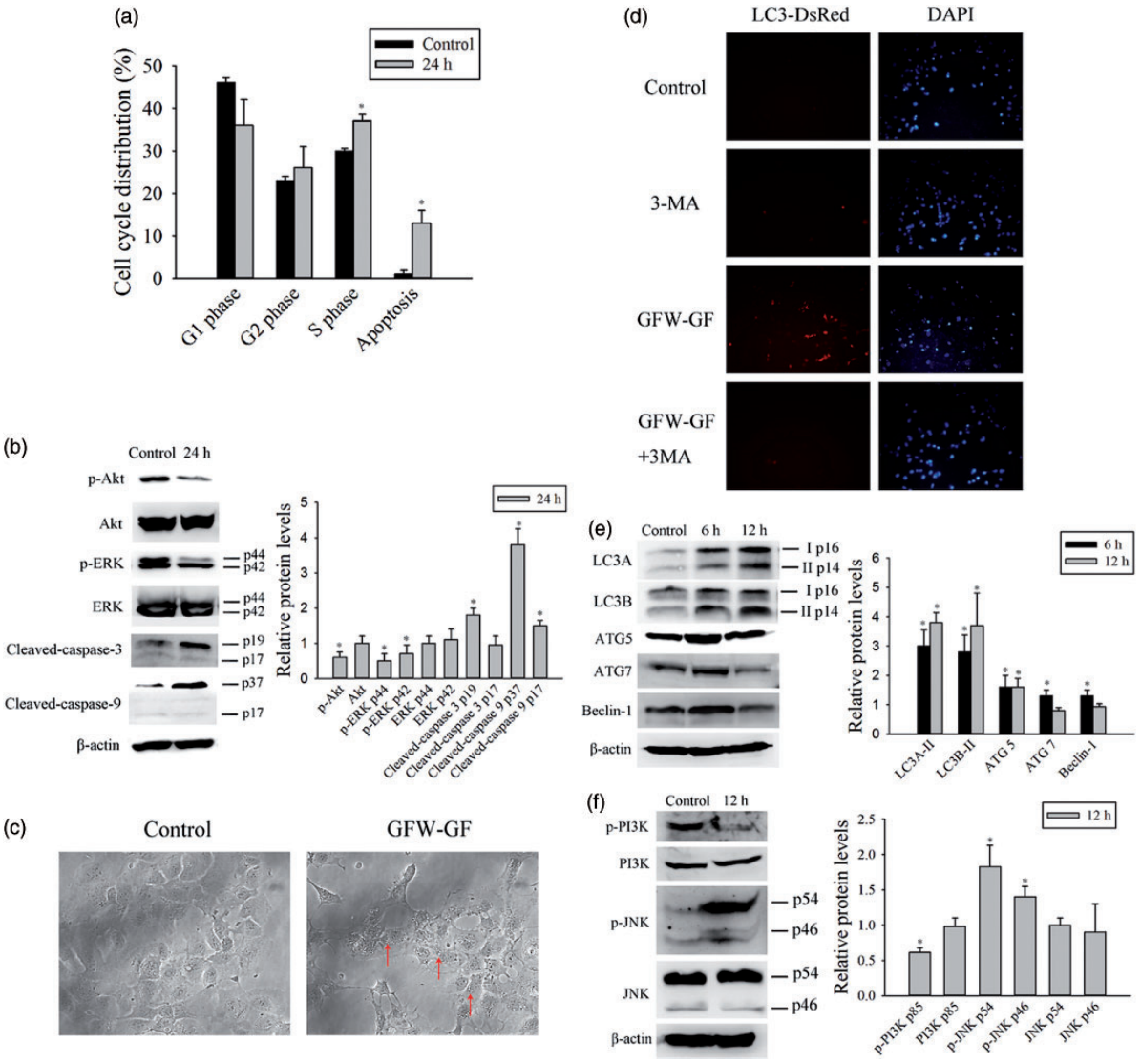

Figure 6 Apoptotic and autophagic cell death induced by GFW-GF in HCC Hep3B cells. (a) Flow cytometry shows the cell cycle distribution for cells treated with 4 μg/mL GFW-GF for 24 h. (b) Western blotting analysis of p-Akt (Ser-473), Akt, p-ERK (Thr202/Tyr204), ERK, cleaved caspase-3, and cleaved caspase-9 in Hep3B cells treated with 4 μg/mL GFW-GF for 24 h. Beta-actin served as a loading control. (c) Cells were cultured in complete medium and treated with 4 μg/mL GFW-GF for 12 h. Representative images were obtained by phase contrast microscopy. Red arrows indicate cytoplasmic vacuoles. (d) Redistribution of DsRed-LC3. Hep3B cells were stably transfected with a DsRed-LC3 fusion construct and cultured in complete serum media and with or without 4 μg/mL GFW-GF in the presence or absence of autophagy inhibitor 3-methyladenine (3-MA) for 12 h. Cells were visualized under a fluorescence microscope to examine the expression of LC3. (e) Western blotting to examine the expression of autophagy-related proteins. Cell lysates from controls and cells treated with 4 μg GFW-GF was subjected to SDS-PAGE and immunoblotted with LC3A, LC3B, Atg5, Atg7, and Beclin-1 antibodies. Beta-actin served as a loading control. (f) Western blotting to examine the expression of autophagy signaling protein. Cell lysates from controls and cells treated with 4 μg/mL GFW were subjected to SDS-PAGE and immunoblotted with p-PI3K (Tyr458), PI3K, p-JNK (Thr183/Tyr185), and JNK antibodies. Beta-actin served as a loading control. One representative of three different Western blot experiments with similar results is shown. Results are the mean ± SD of three independent experiments performed in triplicate. *Indicates statistically significant ($P < 0.05$) compared with control. (A color version of this figure is available in the online journal.)

that GFW-GF can induce autophagy by inhibiting PI3K and activating JNK in Hep3B cells.

Discussion

HCC is the most common human cancer in both developed and developing countries and is increasingly associated with cancer-related deat.[36] Chemoprevention has gained increasing attention because it is effective and avoids the side effects of cancer treatment.[37] However, the current lack of successful chemopreventive agents for HCC emphasizes the need to evaluate new and effective agents for HCC prevention and treatment.

Mushrooms have historically been used worldwide for their anticancer effects. Over the past decades, studies in China, Taiwan, Japan, and the United States have increasingly demonstrated the potent and specific properties of mushroom extracts for the prevention and treatment of cancer.[11,38–40] Compounds derived from GF have been tested against several major types of cancer, including colon, breast, gastric, and liver cancers.[39,41–43] In recent years, anticancer studies have concentrated on the isolation of bioactive polysaccharides from mushrooms.[40] An extract from GF called the Maitake D-fraction is marketed as a dietary supplement worldwide and contains β-glucan. In 1998, the US Food and Drug Administration authorized a phase 2

study to investigate a new drug application of the Maitake D-fraction for breast and prostate cancers.[11] Numerous studies have shown that β-glucans can nonspecifically activate the host immune system.[44] Several recent studies have shown the mechanisms of GF-induced apoptotic cell death. Wang et al.[45] found the GF polysaccharide inhibited Akt signaling pathways in HepG2 cells, which caused apoptotic cell death. In another elegant study, Ito et al.[39] demonstrated that water-soluble extract from GF induced caspase-3-dependent apoptosis in human gastric cancer cell lines.

In a study of hot and cold-water extracts of *Ulva reticulata*, *Ulva lactuca*, *Ulva rigida*, and *Ulva fasciata*, the uronic acid, sulfate, ash contents were higher in hot-water polysaccharides compared to cold-water polysaccharides whereas protein contents were lower in hot-water polysaccharides. Xylose content (7.6-fold higher in *Ulva fasciata*) were higher in hot-water polysaccharide than in cold-water polysaccharide.[46] Cold-water fraction polysaccharide extracted from *Lentinus edodes* mushroom consisted of $(1 \rightarrow 3)$ and $(1 \rightarrow 4)$ linkage of glucose and galactose. Galactose in cold-water polysaccharides was highly branched at O-3 and O-4 residues. Hot-water fraction polysaccharides revealed $(1 \rightarrow 4)$, $(1 \rightarrow 6)$-linked glucopyranosyl residues and were branched at O-4 and O-6.[47] There are huge differences in the polysaccharide and protein contents between cold- and hot-water extracts, which cause diversity in the biological activities. It has been shown that the biological activities of polysaccharides are closely associated to their structures including monosaccharide compositions, molecular weight, degree of branching, solution conformation, and the main chain and branches.[48,49] In our results, cold-water extracts of GF provides better anti-hepatoma effects than hot-water extracts.

Herein, we showed that GFW and GFW-GF effectively inhibit Hep3B cancer cell growth *in vitro*, concomitant with induction of autophagy, cell cycle arrest, and apoptotic cell death. Furthermore, GFW inhibits tumor cell growth in nude mice by inducing apoptosis. Drug resistance is one of the major causes of therapeutic failure in cancer, and many studies have indicated chemotherapy-resistant cancer defects in the apoptosis pathway.[50,51] Activation of the caspase-related protein cascade plays an important role in apoptosis.[52] Caspase-3 and caspase-9 are crucial facilitators of mitochondrion-mediated apoptosis.[53] Our results showed that caspase-3 and caspase-9 levels were significantly increased and anti-apoptotic BCL-2 levels were decreased after GFW and GFW-GF treatment (Figures 2(c) and 6(c)). Therefore, our data indicated that GFW and GFW-GF inhibited HCC proliferation *in vitro* and *in vivo* by inducing apoptosis.

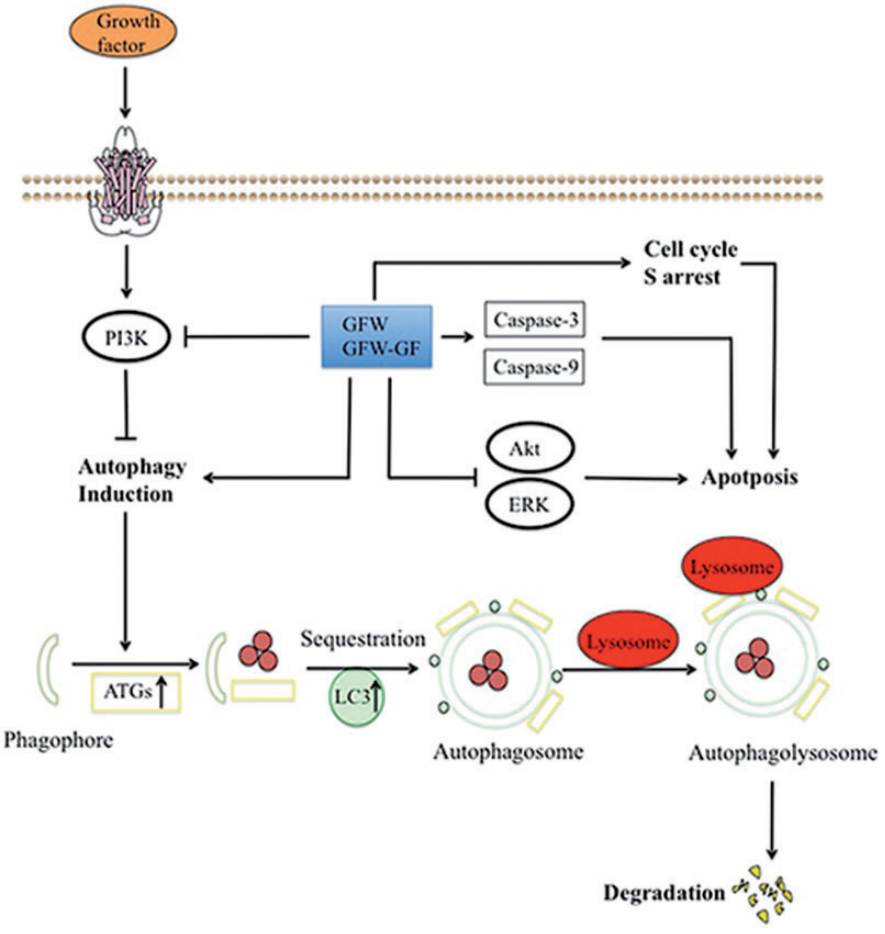

Figure 7 Schematic presentation of the molecular mechanism underlying the anticancer effects of GFW and GFW-GF in hepatocellular carcinoma cells. (A color version of this figure is available in the online journal.)

Autophagy is an important physiological process of programmed cell death and an important conserved catabolic process involving the degradation of abnormal cellular organelles and proteins in living cells.[22,54] The role of autophagy in cancer remains somewhat controversial and appears to be quite divergent in the pre- and post-malignant states. In the present study, we also found that GFW and GFW-GF promoted autophagy in Hep3B cells using microscopic DsRed-LC3 analysis and Western blotting (Figures 3 and 6). Furthermore, a number of signaling pathways are involved in autophagy, including the PI3K and JNK pathways.[55,56] Our results indicate that GFW and GFW-GF significantly reduced PI3K phosphorylation in Hep3B cells but significantly enhanced JNK phosphorylation. The crosstalk between autophagy and apoptosis is complicated. Generally, autophagy inhibits the induction of apoptosis, and apoptosis-associated caspase proteins activation turns off the evolution of autophagy. However, there are many reports indicating that both of these processes occur simultaneously to induce both autophagy and apoptosis in cancer cells.[57] It has also been reported that induction of autophagy promoted the activation of apoptosis.[58] We hypothesized that these two key processes of cell death initiated by GFW and GFW-GF were coordinated with important molecules such as PI3K, JNK, and Bcl-2. We found that GFW and GFW-GF activated autophagy earlier than apoptosis, suggesting that autophagy is upstream of apoptosis. GFW and GFW-GF are combined with autophagy and apoptosis to enhance the anticancer effect.

Nude mice are generally considered to be ideal experimental animals to establish human tumor models. The tumor introduced by xenograft remains with the original tissue shape, immunological characteristics and sensitivity to antineoplastic agents, and the original functions of the tumor would remain.[59] In this study, the HCC Hep3B xenograft tumor model was used to evaluate the *in vivo* effect of GFW on HCC in nude mice. The results showed that the tumor growths were inhibited by GFW administration, suggesting that GFW could significantly intervene and control Hep3B tumor growth *in vivo*. In general, our *in vitro* and *in vivo* studies could be helpful to assess the effect of GFW on HCC cells.

In summary, the present study showed that GFW and GFW-GF inhibited cell proliferation, induced S phase arrest, activated the mitochondria-dependent apoptotic pathway, and promoted autophagy through the PI3K and JNK signaling pathways in Hep3B cells (Figure 7). Furthermore, our *in vivo* results showed that GFW inhibited cancer growth, activated the mitochondria-dependent apoptotic pathway, and promoted autophagy. These results demonstrate that GFW and GFW-GF may represent remarkable cytotoxic agents at low concentrations *in vitro* and *in vivo*. From a standpoint of better cancer therapy, GFW and GFW-GF can serve as a promising anticancer agent that target both autophagy and apoptosis.

Author contributions: KRL and LW designed the research project; CHL performed the laboratory work, conducted the experiments, analyzed the data generated from the experiments, conducted statistical analysis, and drafted the manuscript; and KRL and LW participated in the critical analysis of the data and revisions of the manuscript. All authors read and approved the final manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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