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Metabolites of *Lycium barbarum* L. from *Lactobacillus acidophilus* as Anti-hepatocellular carcinoma agents: induce apoptosis

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Abstract

The fruit of *Lycium barbarum* L., also known as Gouqi, is a well-known Chinese herbal medicine with various biological activities. Gouqi has a long history of consumption in fermented milk products. In current study, five novel Gouqi metabolites by *Lactobacillus acidophilus* (GMLs) were structural identified and anti-neoplastic potency against hepatocellular carcinoma (HCC) was further explored. Mechanistic study revealed that GML-4 (methyl 2-(benzyloxy)-2-(2-(2,4-dimethoxybenzamido)acetamido)acetate) blocked the HepG2 cells in G0/G1-phase, as indicated by the decreased expressions of Cyclins and CDKs, and increased expressions of p21 and p27. Further, GML-4-induced cell apoptosis, as indicated by Caspases activation and phosphorylation of AKT/mTOR/S6K1/4E-BP1 signaling pathways. Of note, these findings suggest that GML-4 might be a potential lead compound candidate for the management of anti-HCC.

Keywords: *Lactobacillus acidophilus*; *Lycium barbarum* L.; Liver cancer; AKT/mTOR/S6K1/4E-BP1

1 Introduction

As reported, the incidence and mortality of digestive tumor account for a considerable proportion of malignant tumor. Hepatocellular carcinoma (HCC), also called malignant hepatoma, is one of the deadliest cancers due to its complexities, re-occurrence after surgical resection, metastasis and heterogeneity[1]. HCC is the sixth most common cancer and rank third in cancer-related deaths globally considering malignancies, severity, treatment challenges and 5-year survival rate (< 5 %) among cancer patients[2]. Multiple factors trigger the initiation and progression of HCC including chronic alcohol consumption, viral hepatitis B and C infection, metabolic disorders and age[3]. Systemic therapies using small molecule drugs to target various signaling pathways following surgical resection and liver transplantation have been applied particularly where locoregional therapy such as transcatheter arterial chemoembolization has failed. Among the various multikinase inhibitors used for systemic treatment of HCC, sorafenib deserves special mention as it is the only approved drug for treatment of advanced HCC[4]. Thus, there is an urgent need to explore other molecular targeted-agents for HCC.

The AKT/mTOR/S6K1/4E-BP1 cascades plays a significant role in cancer cells survival[5]. AKT promotes cell survival through the phosphorylating of substrates that leads to a decrease in the activity of pro-apoptotic proteins or increase in the activity of anti-apoptotic proteins. Once AKT activated, it can phosphorylate a diverse array of substrates, including the mammalian target of rapamycin (mTOR). Also, activated mTOR mediates the phosphorylation of the eIF4EBP1 (Eukaryotic Translation Initiation Factor-4E-Binding Protein-1) and the ribosomal protein S6K1 (S6 Kinase)[6].

4EBP1 can repress the activity of the eIF4F (eukaryotic Initiation Factor-4) complex. Phosphorylation of 4EBP1 by mTOR reduces its affinity for eIF4E, and then the two proteins dissociate from each other physically[6, 7]. eIF4E is then able to associate with the other components of eIF4F, which include the large scaffolding protein, eIF4G (Eukaryotic Translation Initiation Factor-4-Gamma), the adenosine triphosphate dependent RNA helicase eIF4A (Eukaryotic Translation Initiation Factor-4A), and eIF4B (Eukaryotic Translation Initiation Factor-4B), to form an active complex. Besides, ribosomal S6 kinase 1 (S6K1) is another downstream component of mTOR signaling pathway as a key mTOR effector of cell growth and proliferation in cancer cells. The phosphorylation of S6K1 is often used as an indicator for mTOR activity in laboratory and clinical research studies, and the S6K1 gene amplification or over expression of S6K1 has been associated with a poor prognosis in tumor patients[8].

The probiotics is a group of active microorganisms that exerts beneficial potency to the host, and are mainly added to the functional food and dietary supplements[9]. The complex microbial population residing in the human gastrointestinal tract consists of commensal, potential pathogenic and beneficial species[10, 11]. As reported previously, probiotics were widely used to treat irritable bowel syndrome(IBS)[12], acute diarrhea, immunomodulation, cancer and other diseases[13, 14]. *Lycium barbarum* is a kind of perennial Solanaceous deciduous shrubbelonging to the genus Lycium. Orange-red colored fruit of *Lycium barbarum* also called Gouqizi, has long been favorite in China, and is becoming more and more popular in the western countries due to its functions of ‘nourishing the kidney and producing essence, retard the aging process[15], treating the tumor, nourishing the liver and brightening eyes[16]. It has been widely used as health-giving food for 2300 years. Phenolic derivatives, as one of main bio-active components of Gouqizi, have been proved to possess a large variety of biological activities such as antivirus, immunomodulation, hypoglycemic, antioxidant, hypolipidemic and anti-tumor effects[17, 18]. In current study, we fermented juice extracted

from the Gouqizi using *Lactobacillus acidophilus* and investigated the novel phenolic derivatives associated with anti-cancer potency in HCC cells.

2 Materials and Methods

2.1 Materials and reagents

The fresh mature fruits of Gouqizi (5.0 kg) were purchased from Shenzhen Kailian Health Biotechnology Co., Ltd. (the plant was cultivated in Ningxia Huizu Autonomous Region, P. R. China). The reagents PI and JC-1 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). PierceTM BCA Protein Assay Kit was obtained from Thermo Fisher Scientific (Rockford, IL, USA). MTT and RIPA buffer were purchased from Beyotime (Shanghai, China). Both the normal and tumor cell lines were acquired from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). DMEM medium, penicillin-streptomycin (Pen Strep), fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). The DMSO was purchased from Beyotime (Shanghai, China). All the antibodies were purchased from Cell Signaling Technology (CST, Beverly, MA, USA). The other chemicals used in the current study were purchased from Aldrich or Adamas without any further purification.

2.2 Bacterial strain and Fermentation

The lactic acid bacteria strains used in current study, *Lactobacillus acidophilus* were provided by China General Microbiological Culture Collection Center. Beijing, China (CGMCC Number: 1.1878).

Initially 100 µL of frozen *Lactobacillus acidophilus* stock was inoculated into 10 mL MRS broth (Difco) and incubated for 24 h at 37°C. Then, 100 µL of the 24 h grown strain was re-inoculated into 10 mL MRS broth for 24 h at 37°C. 90 mL of the juice was put into a 125 mL Erlenmeyer flask; pH adjusted to 6 using NaOH and inoculated using 10 mL of the broth culture. Fermentation was carried out at 37°C and 60 mL samples were taken out at 48 h. The samples were centrifuged at 15,000 g for 15 min and then prepared for the further assays.

2.3 GMLs isolation and cell viability assay

After centrifugation and subsequent freeze-drying of the solvent, the resulting residue (14.6 g) was suspended in water (100 mL) and partitioned dichloromethane (6 × 1 mL, Grüssing, Filsum, Germany), successively. The CH_2Cl_2 soluble fraction was subjected to silica gel CC and eluted with CH_2Cl_2-MeOH (100:1 to 1:100, v/v) to yield four fractions (A1-A4). Fraction

A1 (10.1 g) was separated using a silica gel CC (hexane-ethyl acetate, 10:1 to 2:1, v/v ≈ 100% MeOH) to yield four subfractions (B1-B4). Subfraction B3 (3.1 g) was subjected to RP C18-MPLC (10% methanol to 100% methanol) to yield another four subfractions (C1-C4). Compounds **1** (25.2 mg) and **2** (11.4 mg) were purified from subfraction C3 by preparative HPLC (acetonitrile-H_2O, 5:5). Subfraction C2 was subjected to preparative HPLC (acetonitrile-H_2O, 4:6) to yield four subfractions (D5-D8). Subfraction D6 was further purified by HPLC using 50% acetonitrile in H_2O to generate compound **3** (12.6 mg). Compound **4** (11.9 mg) and **5** (11.7 mg) were obtained from subfraction D8 by HPLC using 40% acetonitrile.

All the cell lines were grown in specific media supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). The cells were grown in a 5% CO_2 humidified atmosphere in incubators maintained at 37°C.

The cytotoxicity of samples was determined by the MTT method[19]. Briefly, an aliquot of 100 μl of cells (1×10^5/mL) was mixed with GMLs or alone with medium (negative control) in the 96-well plates for 48 h. MTT was dissolved in PBS at 5 mg/mL and filtered. 10 μL MTT (5 mg/mL) was added into each well and incubated for another 4 h at 37°C. The absorbance of the converted dye in living cells was measured at a wavelength of 570 nm. IC_{50} values were determined by the nonlinear multipurpose curve fitting program GraphPad Prism.

2.4 Apoptosis and cell cycle assays

After GML-4 treatments, cells were incubated with Annexin V (10 μg/mL, Invitrogen) for 10 min under the dark, which were then subjected to FACStarplus flow cytometer analysis. Annexin V percentage was recorded[20].

After the applied GML-4 treatments, cells were collected and fixed with 70% ethanol overnight. Afterwards, propidium iodide (PI) (10 μg/mL, Invitrogen) was added. The DNA contents of PI-stained cells were analyzed by a flow cytometry (FACStarplus, BD). G0/G1, S and G2/M phase ratio was recorded[21].

2.5 Western blotting

HepG2 cells were harvested via RIPA lysis buffer (Biyuntian, Nanjing, China). Protein concentration was determined via Bradford reagent (Bio-Rad, Hercules, CA). Aliquots of 40 μg lysates per sample were separated by SDS-PAGE gels, and were transferred to the PVDF membranes (Millipore, Beijing, China). Afterwards, indicated primary and secondary

antibodies were added to the blots. ECL reagents (GE Healthcare, Beijing, China) were applied to visualize the targeted protein bands[22]. *β*-actin was used as an internal standard of process control.

2.6 Statistical analysis

All data are expressed as the mean±standard deviation (SD) of three independent experiments. Statistical significance was assessed using Student's t-test (for comparisons of two treatment groups) or one-way ANOVA (for comparisons of three or more groups). *p*-values < 0.05 were considered statistically significant.

3 Results and Discussion

3.1 GMLs inhibited growth of cancer cells

Finally, five novel GMLs (**Fig. 1**) were obtained and identified. The purity was detected by HPLC >98%. The GMLs were dissolved in DMSO (10.0 mM) and stored at 4℃ as a stock solution.

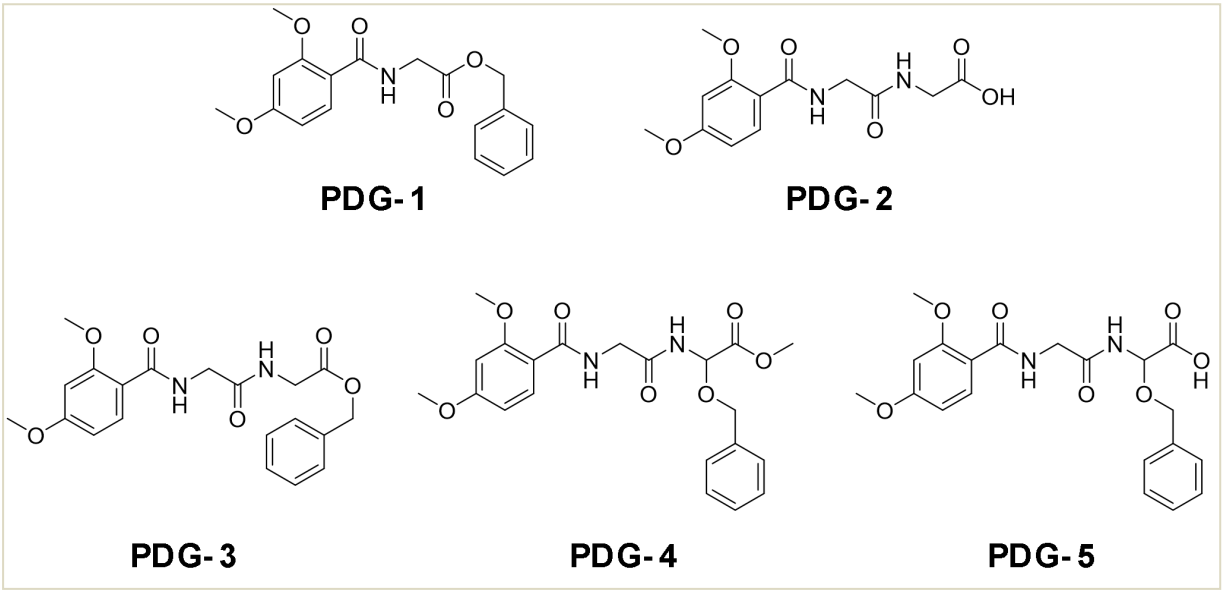

Fig. 1 Structures of GML-1~5

Table 1 Anti-proliferative activity of GML-1~5

Compounds	IC_{50} (μM)		
	MHCC97H	**Huh-7**	**HepG2**
GML-1	162 ± 16.7	147 ± 18.1	104 ±9.95
GML-2	175 ± 18.4	> 200	> 200
GML-3	> 200	150 ±17.2	112 ±11.8
GML-4	154± 17.1	127 ±13.4	86.3 ± 7.72
GML-5	162 ±15.8	158 ±17.0	> 200

IC_{50} values are shown as mean ± standard error of the mean (SD), from at least three independent experiments.

The Anti-proliferative activity of GMLs in the experimental cancer cell lines was detected and the results were list in **Table 1**. The GMLs performed excellent anti-proliferative activity in the hepatocellular carcinoma cells. Interestingly, GML-4 potently inhibited the growth of the three experimental HCC cells. Further, GML-4 potently inhibited the growth of HepG2 cells with a lowest IC_{50} value of 86.3 ± 7.72 μM, after 48 h treatment.

The cytotoxicity of GML-4 in normal cell lines from healthy tissues were detected. The cytotoxicity of GML-4 in human gastric epithelial cell line GES-1 and human liver cell line HL-7702 are at a much higher level compared to the tumor cell lines, in which the IC_{50} values are 8.31 ± 0.79 mM to GES-1 and 6.64 ± 0.66 mM to HL-7702. Since GML-4 performed a significant enhancing anti-proliferative activity in HepG2 cells, together with the fact that HCC is one of the most common malignancies. This is the reason why we chose HepG2 cells to explore the inducing apoptosis potency and the underlying mechanisms.

3.2 GML-4 induced apoptosis and activated the Caspases

The necrosis is a form of traumatic cell death that results from acute cellular injury; while apoptosis is a highly regulated and controlled process that confers advantages during the life-cycle. Different from the necrosis, cell apoptosis produces cell fragments, known as apoptotic bodies. Phagocytic cells can phagocyt and remove the cells quickly before the content spills to the surrounding cells and causes damage to the adjacent cells[23].

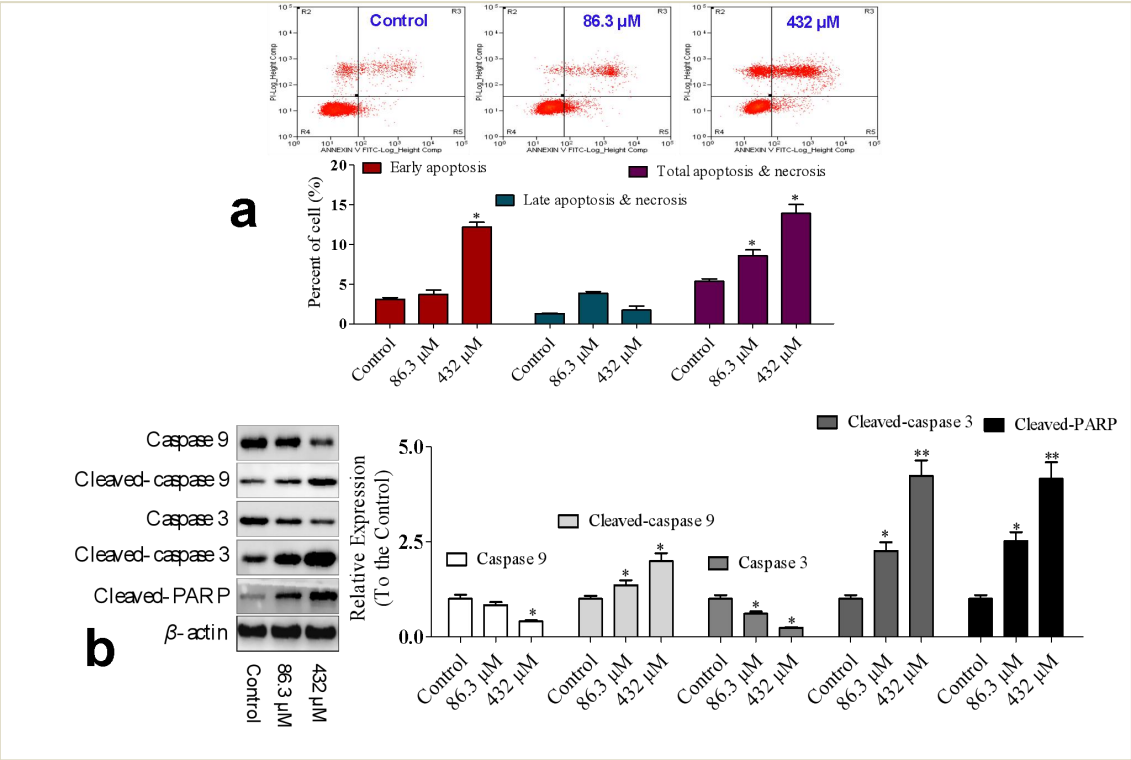

Fig. 2 GML-4 induces Caspase-dependent apoptosis in HepG2 cells

Representative scatter diagrams. (**A**) Flow cytometric analysis of GML-4-induced apoptosis in HepG2 cells using Annexin V-FITC/PI staining. Cells in the lower right quadrant (Annexin V^+/PI^-) represent early apoptotic cells, and those in the upper right quadrant (Annexin V^+/PI^+) represent late apoptotic cells. (**B**) GML-4 activates Caspase cleavage in a concentration-dependent manner. Equal amounts of whole-cell extracts were separated by 10% SDS-PAGE, electrotransferred onto PVDF membranes, and analyzed by western blotting using the indicated antibodies against proteins related to Caspase-dependent apoptosis. *β*-actin was used as a loading control. The data are expressed as the mean ± SD of 3 independent experiments. $^*p< 0.05$, $^{**}p< 0.01$, compared with the control group.

The MTT assay results suggested that GML-4 inhibited the proliferation of HepG2 cells. Subsequently, flow cytometry analysis was used to explore whether the GML-4 performed the anti-proliferative activity was by inducing apoptosis. Propidium iodide (PI) and Annexin-V are used as the dyes. As shown in **Fig. 2a**, compared with the control group, the GML-4 increased the apoptosis by 3.34% and 8.56%, respectively. The results provided a visual evidence for the anti-tumor property of GML-4.

The Caspase is a family of cysteine proteases that play a pivotal role in the apoptosis. Caspase 3 is involved in the apoptotic process, where it is responsible for chromatin condensation and DNA fragmentation. Caspase 9 is an initiator Caspase. The initiated Caspase 9 will go on to

Cleave procaspase 3 and 7[24]. In other hand, PARP is a family of proteins involved in a number of cellular processes involving mainly DNA repair and programmed cell death. When PARP is cleaved by enzymes such as Caspases or cathepsins, typically the function of PARP is inactivated[25, 26]. In order to disclose how GML-4 induced cell apoptosis, we detect the expressions of Caspase 3 and 9, Cleaved caspase 3 and 9 and Cleaved poly (ADP-ribose) polymerase (PARP) by western blotting. We found that GML-4 decreased the expressions of Caspase 3 and 9, respectively; while in the mean time increased the expressions of Cleaved caspase 3, 9 and Cleaved PARP (**Fig. 2b**). Of notice, GML-4 increased the ratios of Cleaved caspase 3(9)/Caspase-3(9) and the expression of Cleaved PARP obviously. Therefore, these data support that GML-4 induces cell apoptosis via the adjustment of Caspase 3, 9, and PARP, which closely participate in programmed cell death.

3.3 GML-4 induced cell cycle arrest and regulated cell cycle regulatory proteins

According to the content of DNA in cells, the main mode of cell proliferation which will go through three stages of cell cycle: G0/G1, S and G2/M in turn. Tumor is a periodic disease, and the disorder of cell cycle regulation can lead to excessive cell proliferation and ultimately the uncontrolled growth of tumors[27]. Therefore, induction of cell cycle arrest plays a fatal role in anti-tumor agent development.

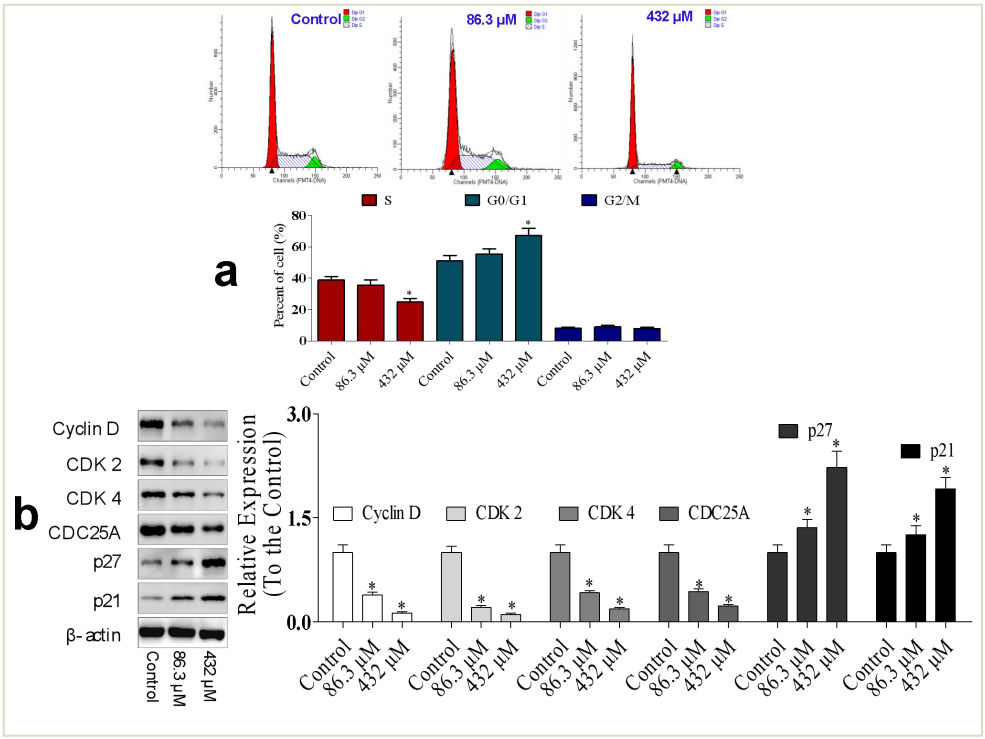

Fig. 3 GML-4 induces G0/G1 cell cycle arrest in HepG2 cells

(**A**) Cell cycle profiles were measured by flow cytometry following treatment of the cells with various concentrations of GML-4 for 48 h. (**B**) HepG2 cells were treated with various concentrations of GML-4. Western blotting was performed to detect p27, p21, cyclin D, CDK2, CDK4 and CDC25A. *β*-actin was used as a loading control. The data are expressed as the mean ± SD of 3 independent experiments. $^{*}p< 0.05$, $^{**}p< 0.01$, compared with the control group. Blots were quantified using Image *J* software.

To establish whether GML-4 inhibited cell growth by interrupting the cell cycle progress, cellular DNA was analyzed and stained with propidium iodide (PI). The cells were analyzed using flow cytometry, and the profiles were shown in **Fig. 3a**. Interestingly, increases in the G0/G1 population were observed in cells stimulation with GML-4, and we inferred that HepG2 cells can be significantly blocked in G0/G1 phase. With GML-4 treatments, the populations of the G0/G1 were increased by 4.26% and 14.2%, respectively. Usually, the ensuing G0/G1 phase starts when DNA synthesis commences; when this phase is complete, all of the chromosomes have been replicated, i.e., each chromosome has two (sister) chromatids. If DNA can not be replicated, cell cycle will be stopped at this stage[28]. Based on the current result, it was suggested that GML-4 may inhibit the DNA replication and make the cell cycle stop in G0/G1 phase. This fact suggests that the cell cycle arresting is one of the primary mechanisms responsible for the anti-tumor activities of GML-4.

To further investigate the molecular basis by which GML-4 inhibited the G0/G1 transition in tumor cells, we treated cells with GML-4 and then analyzed the expression of proteins involved in cell cycle regulation. We found that GML-4 treatment inhibited cyclin D expression and reduced the expression of CDK2 and CDK4; in contrast, p21 and p27 were increased in HepG2 cells (**Fig. 3b**). The expression level of cell division cycle 25A (CDC25A), which acts as an upstream regulator of the CDK/cyclin complex, was significantly inhibited by GML-4.

3.4 GML-4 induced Δψm collapse and suppressed the Cyt c in HepG2 cells

The mitochondrion-dependent pathway is the most common apoptotic pathway in vertebrate tumor cells[29]. To investigate the mechanism underlying GML-4-induced HepG2 cell apoptosis, we measured the Δψm in HepG2 cells.

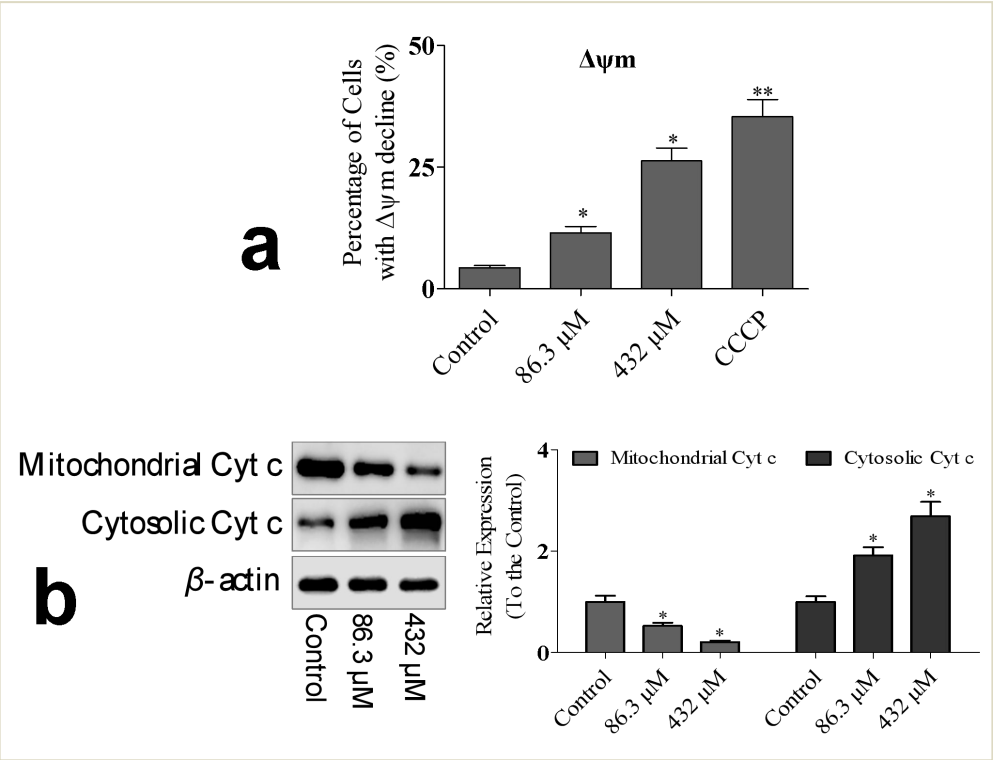

Fig. 4 GML-4 induces apoptosis via the mitochondrial pathway in HepG2 cells

(**A**) Decrease in mitochondrial potential induced by GML-4. After treatment with GML-4, the cells were stained with JC-1 for 15 min and analyzed by flow cytometry. (**B**) HepG2 cells were treated with various concentrations of GML-4. Western blotting was performed to detect cytosolic and mitochondrial levels of the pro-apoptotic proteins cytochrome c. The data are expressed as the mean ± SD of 3 independent experiments. $^{*}p< 0.05$, $^{**}p< 0.01$, compared with the control group. Blots were quantified using Image *J* software.

As shown in **Fig. 4a**, the cells suffered with Δψm decline increased from 4.34% to 11.5% and 26.4%, respectively, suggesting that GML-4 induced the Δψm declines in HepG2 cells in a concentration dependent manner.

Mitochondrial dysfunction, as indicated by the dissipation of Δψm, could subsequently cause the release of cytochrome c (Cyt c) from mitochondria into the cytosol[30]. Therefore, we evaluated the cytosolic and mitochondrial Cyt c levels. Western blot analysis showed that treatment with GML-4 for 48 h led a striking increase in the expression of cytosolic Cyt c and a decrease in the expression of mitochondrial Cyt c, compared with the control group (**Fig. 4b**).

3.5 GML-4 inhibits phosphorylation of AKT/mTOR/S6K1/4E-BP1 signaling pathway in HepG2 cells

AKT/mTOR/S6K1/4E-BP1 are the major anti-apoptotic pathways that confer the survival advantage and resistance to cancer cells against various chemotherapeutic agents[31]. We first investigated the potential effect of GML-4 on constitutive AKT and mTOR activation in HepG2 cells.

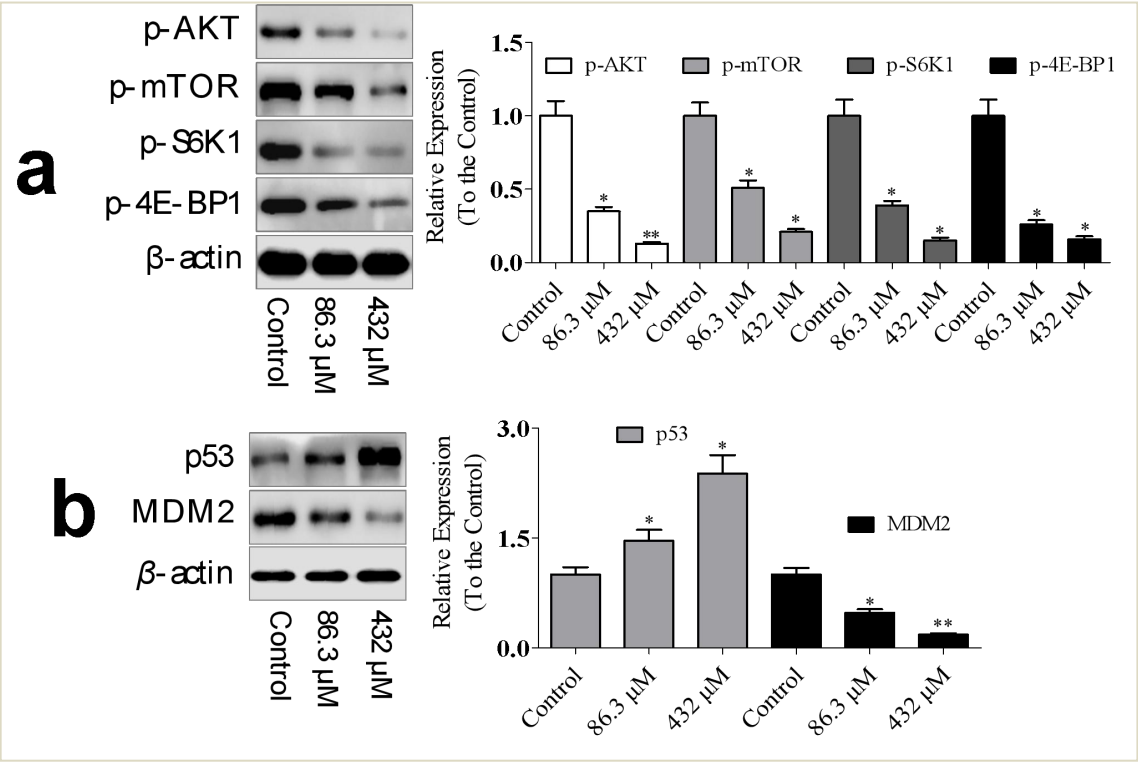

Fig. 5 GML-4 suppresses phosphorylation of AKT, mTOR, S6K1, 4E-BP1 and regulates the states of p53/MDM2 in HepG2 cells

HepG2 cells (1×10^6 cells/well) were treated with various indicated concentrations of GML-4. Thereafter, equal amounts of lysates were analyzed by Western blotting analysis using antibodies against p-AKT, p-mTOR, p-S6K1, p-4E-BP1, p53 and MDM2. Graphs represent band intensities of indicated proteins. All data were expressed as mean ± SD. *$p < 0.05$ vs. Control, **$p < 0.01$ vs. Control.

As shown in **Fig. 5a**, we interestingly found that the constitutive activation of both AKT and mTOR kinases was strongly suppressed by GML-4 in HepG2 cells. In order to determine which downstream signaling molecules are involved in GML-4-mediated AKT and mTOR inactivation, we further examined the effects of GML-4 on the phosphorylation of S6K1 and

4E-BP1 in HepG2 cells. As shown in **Fig. 5a**, S6K1 was constitutively active in the cells and the treatment with GML-4 significantly suppressed this phosphorylation. We also observed that GML-4 clearly repressed the constitutive phosphorylation of 4E-BP1.

We found that GML-4 suppressed the expression of phospho-AKT in HepG2 cells, which further indicates the possibility that GML-4 could interfere with TORC2 (the mTOR-rictor complex that phosphorylates AKT at Ser473). AKT is also known to modulate the NF-κB transcription factor through the phosphorylation of p65 to enhance the transcriptional activity of NF-κB. NF-κB activation is also known to modulate the expression of various cell survival, proliferative, metastatic, and invasive gene products. Thus, it is clear from our cumulative results that the simultaneous inhibition of AKT/mTOR/S6K1/4E-BP signaling cascade along with NF-κB can significantly contribute to the anti-cancer effects of GML-4 in tumor cells.

Previous reports indicate that the aberrant activation of mTOR is closely linked with tumorigenesis. Recently, specific mTOR inhibitors have shown great promise in clinical trials for the treatment of various malignant tumors[32]. We for the first time found that GML-4 repressed constitutive mTOR activation in HepG2 cells. Thus, it is possible that targeting the mTOR signaling pathway with various agents derived from natural sources can be a useful therapeutic strategy both for the prevention and treatment of HCC. Since the phosphorylation of 4EBP1 results in cell cycle arrest, invasion, and angiogenesis in tumor cells, it can be considered a good molecular target for cancer therapy. Our results also indicate, that GML-4 inhibited constitutive 4EBP1 activation, which is a direct downstream target of mTOR.

3.6 GML-4 regulated the p53/MDM2 states

The p53 is a tumor suppressor which plays significant roles of inducing cell cycle arresting, DNA repair, senescence, and apoptosis. While MDM2 (murine double minute 2) is the main endogenous negative regulator of it. This oncoprotein MDM2 binds p53 and negatively regulates p53 activity by direct inhibition of p53 transcriptional activity and enhancement of p53 degradation via the ubiquitinproteasome pathway. Hence, an appealing therapeutic strategy for many wild-type p53 tumors with over expressed MDM2 is to restore p53 activity via inhibiting the p53/MDM2 interaction[33].

Therefore, we detected whether GML-4 can up-regulate the p53 expression, and down-regulate the MDM2 expression. Of interest, GML-4 up-regulated p53 expression, and

down-regulated MDM2 expression, respectively. 432 μM GML-4 treatment changed the p53/MDM2 ratio from 0.31 (control group) to 4.18 (**Fig. 5b**). Based on the results, we concluded that GML-4 may also regulate the p53/MDM2 states to promote cancer cell apoptosis.

4 Conclusion

In conclusion, we demonstrated that GML-4 from *Panax ginseng* leaves has a growth-inhibitory effect on HepG2 cells *in vitro*. We revealed that GML-4 induces tumor cell apoptosis through the Caspase-dependent mitochondrial pathway and via G0/G1 phase arrest by regulating G0/G1 checkpoint proteins. Additionally, GML-4 induced Caspase-dependent apoptosis, regulated the states of p53/MDM2 and inhibited phosphorylation of AKT/mTOR/S6K1/4E-BP1 signaling pathway. However, further studies are required to identify the specific molecular targets of GML-4 and the signaling pathways affected *in vivo*. In addition, whether GML-4 exhibits the similar effects on other cellular systems still remains to be further investigated. Nevertheless, our results shed some light on the mechanisms behind the effect of GML-4 on HCC and suggest that GML-4 could be a potential candidate for the development of novel treatment strategies for HCC.

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Compliance with ethical standard

The authors certify that there is no conflict of interest with any individual/organization for the present work. All the data was available and was got by our own experiments.

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