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Elephantopus Mollis H.B.K. Monomer EM-10 Induces G2/M Arrest, Apoptosis and Autophagy in HepG2 Cells

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Abstract

Hepatoblastoma (HB) is the common type of primary liver cancer in children. *Elephantopus mollis* H.B.K., a plant used in traditional Chinese medicine, exhibits anti-tumor activity towards many types of malignant tumors. EM-10 is a monomer that we extracted from *Elephantopus mollis* H.B.K. but its specific antitumor mechanism was still unclear. In this study, EM-10 inhibited the proliferation of HepG2 cells while its cytotoxicity on normal human liver LO_2 cells was relatively weak. We found that EM-10 induced cell apoptosis of HepG2 cells by activating the mitochondrial apoptosis pathway with the evidence that the protein expression level of Caspase-9, Caspase-3, PARP, XIAP, Bcl-2 and Bcl-xl were downregulated with increasing EM-10 concentrations, while the levels of Bax, Bad, Bak were upregulated distinctly, and Z-VAD-FMK reversed the apoptosis induced by EM-10. In addition, the results of flow cytometry showed a dose-dependent accumulation in the G2/M phases and a decrease in the G0/G1 phase, with the expression of p-Cdc2 (Tyr15), Cyclin B1, Cyclin A, and Wee1 was significantly increased after EM-10 treatment, while Cyclin D1 and cdc25c were down-regulated. Moreover, this study illustrated that the EM-10 activated JNK and p38 pathway, with the expression of p-JNK and p-p38 increased. Western blot also showed that EM-10 induced autophagy with the expression of LC3II and Beclin1 increased and the expression of p62 decreased. In summary, our findings revealed that EM-10 induced activation of the JNK and p38 pathway, cell cycle arrest and autophagy further promoted cell apoptosis in HepG2 cell lines.

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Keywords

Elephantopus Mollis; Hepatocellular Carcinoma Cells; Cell Cycle; Cell Apoptosis; Autophagy.

1. Introduction

Liver cancer is one of the most frequent cause of cancer deaths across the globe and one of the top five deadiedt cancers, with an annual percentage increase in occurrence[1, 2]. About 72 % of all liver cancer occurs in Asia, with China accounting for 47 % of the global burden[3]. The liver cancer has the poorest survival—the age-standardized 5-year relative survival is only 10.1%. Hepatoblastomas (HBs) and pediatric hepatocellular carcinomas (HCCs) together account for almost 80% of primary malignant liver tumors in children and adolescents/young adults[4]. Despite new breakthroughs in imaging technology, chemotherapy, interventional radiology, surgical techniques, and liver transplantation in the recent years, the prognostic rate of patients with advanced liver cancer still remained poor [5]. Up to now, surgical treatment and chemotherapy are the main therapies for liver cancer[6]. With the development of targeted therapies, patient outcomes have improved, but drug resistance and side effects still limit their use. Therefore, it is urgent to find effective new drugs with fewer side effects for the treatment of liver cancer.

Traditional Chinese medicines (TCMs) and natural medicine, alone or combined with chemotherapy drugs, have long been used in the synthesized management of cancer. Except for its lower toxicity, TCMs have a wealth of anticancer and chemoprotection properties [7]. And studies have demonstrated that TCMs and their extracts have been widely used in the treatment of human diseases and have achieved obvious efficacy[8], such as paclitaxel, trametes, curcumin, berberine and matriner. *Elephantopus mollis* H.B.K., a medicinal herb with diverse therapeutic properties, is mainly found in south and southwest China. In folk medicine, decoction of the plant is used to treat cough, fever, pharyngitis, hepatitis, bronchitis, nephritis, diarrhea [9]. In our previous work, we isolated 25 monomers from *Elephantopus mollis* H.B.K. and named EM-1~25 [10]. And we discovered that EM-10 had an anti-tumor effect, but the molecular mechanism remaines unclear. Therefore, in this study, we aimed to investigate the effect of EM-10 on autophagy, cell cycle and apoptosis in HepG2 cells, as well as elucidate the molecular mechanism involved.

2. Materials and Methods

2.1. Materials and Reagents

EM-10 was isolated from *Elephantopus mollis* H.B.K. (provided by the Institute of Traditional Chinese Medicine and Natural Products of Jinan University), dissolved in dimethyl sulfoxide (DMSO; Sigma, USA) at concentration of 100 mmol/L and diluted to the desired concentration using Roswell Park Memorial Institute (RPMI)-1640 medium (DMSO final concentration <0.01 %, v/v) for cell culture experiments. RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Trypsin and MTT Kit were obtained from Sigma (USA). Annexin Vfluorescein isothiocyanate (Annexin V-FITC); propidium iodide (PI) and Bradford Protein Assay kit were purchased from Beyotime Biotechnology (Nanjing, China). Polyvinylidene fluoride (PVDF) was obtained from Millipore (MA) and antibody against Bcl-2, Bcl-xL, XIAP, Bad, Bax, Bak, Caspase-3, Caspase-9, poly ADP-ribose poly merase (PARP), p21(Waf1/Cip1), p-cdc-2(Tyr15), JNK, p-JNK(Thr183/Tyr185), p38, p-p38, p53, PUMA, LC3, Beclin-1, p62, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Cell Signaling Technology (Boston, USA).

ISSN: 1813-4890

2.2. Extraction and Isolation

Elephantopus mollis H.B.K. (10 kg) was crushed into a coarse powder and heated with 95% ethanol for three times. The solution was decompressed and concentrated until it had no ethanol taste to obtain 300 g of extract, which was then suspended in water and filtered successively with petroleum ether and ethyl acetate. Six fractions (fr.1-6) were obtained by the separation of petroleum ether layer by silica gel (200 ~ 300 mesh) column chromatography and the gradient elution of petroleum ether-ethyl acetate (100:0→0:100 :0), then fr.6 was separated and purified by Sephadex LH-20 column chromatography (chloroform-methanol chromatography) and HPLC to produce compound EM-10 (16.8mg).

Data on EM-10: white powder; HR-ESI-MS*m/z*:381.1310[M+Na]$^+$(calcd for$C_{20}H_{22}O_6Na$, 381.1309); UV (MeOH) λ_{max}: 205nm; IR (KBr)ν_{max}: 2934, 1760, 1747, 1693, 1640, 1384, 1278, 1136, 1049cm^{-1}; ^{1}HNMR (500 MHz, MeOD) and ^{13}CNMR (125MHz, MeOD).

2.3. Cell Lines and Cell Cultures

Human HB cell lines HepG2 and the immortalized normal human hepatic cell line LO_2 were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI 1640 medium with 10% FBS and 100 U/ml penicillin/streptomycin. All cell lines were separately incubated at 37°C with 5% CO_2 and regularly checked for mycoplasma contamination.

2.4. Cell Viability Assay

The effect of EM-10 on cell viability was measured using the MTT assay. Briefly, HepG2 and LO2 cells were seeded in 96-well plates at a density of 5.0 × 10^4 cells/well overnight and then treated with drugs for the indicated times. The cells were co-incubated with 20 μl MTT (5 mg/mL) solution for 4 h. The media were carefully removed and 100 μL of DMSO was added to each well. The plate was left on a plate shaker for 10 min with gentle shaking at room temperature. The absorbance of each well was measured at 490nm using a microplate reader (Bio-Rad, CA). IC_{50} values were determined by the GraphPad Prism software using nonlinear regression analysis.

2.5. Colony Formation Assay

HepG2 cells were seeded in 6-well plates at a density of 1000 cells/well for 12 h and were then treated with the indicated drugs for 7 days. The cells were washed three times with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 15 min. Finally, the cells were stained in 0.5% crystal violet for 15 min. Images of the colonies were captured by a digital camera.

2.6. Annexin V-FITC/PI Double-staining Assay

HepG2 cells were seeded into 6-well plates at a density of 2.0 × 10^5 cells per well and treated with different concentrations of EM-10 for 24 h under standard culture conditions. Then, the cells were harvested, washed 3 times with cold PBS and resuspended in 195 μl staining buffer. The cells were incubated at room temperature in the presence of 10 μl Annexin V-FITC and 5 μl PI for 30 min in the dark. After the addition of 300 μl of staining buffer, the cells were immediately analyzed on a flow cytometer.

2.7. Cell Cycle Distribution Analysis

HepG2 cells were seeded into 6-well plates at a density of 2×10^5 cells per well and cultured overnight. Different concentration of EM-10 (0, 7.5, 15, 30 μmol/L） was incubated with HepG2 cells for another 24 h. After overnight fixation in 70 % ethanol at 4 °C, the cells were washed three times with ice-cold PBS and incubated for 30 min in the dark with PI solution (containing

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50 μg/ml PI and 50 μg/ml RNase). Then, the samples were immediately analyzed via flow cytometry. Approximately 12,000 cells were analyzed per sample.

2.8. Western Blot Analysis

HepG2 cells were seeded in a 6-well plate at a density of 2.0 × 10^5 cells per well and treated with various concentrations of EM-10 for 24h. The cells were washed with PBS and lysed in lysis buffer buffer on ice for 30 min. Upon 12,000 g centrifugation for 15 min at 4°C, the supernatant was collected in which protein concentration was determined by Bradford Protein Kit. Protein lysates were separated by SDS-PAGE gels electrophoresis and transferred onto PVDF membrane. The membranes were blocked with 5% skimmed milk for 1 h at room temperature and incubated with primary antibodies at 4°C overnight. After washing 3 times with TBST solution, the membranes were further incubated with certain concentration of secondary antibodies for another 1 h. Followed by washing 3 times with TBST, the membranes were covered with chemiluminescence fluid and visualized by Gel Image System.

2.9. Statistical Analysis

Three independent experiments were carried out for all the experimental results. Measurement data were expressed as the mean ± SD and evaluated using GraphPad Prism (Version 5.0 GraphPad software, San Diego, California, USA). Data were analyzed using SPSS (Version 15.0.1, Palo Alto, California) with the Independent-Samples t-Test, one-way ANOVA, and Student–Newman–Keuls (SNK) test. P<0.05 was considered statistically significant.

3. Results

3.1. Proliferation Inhibition Effect of EM-10 on HepG2 and LO_2 Cells

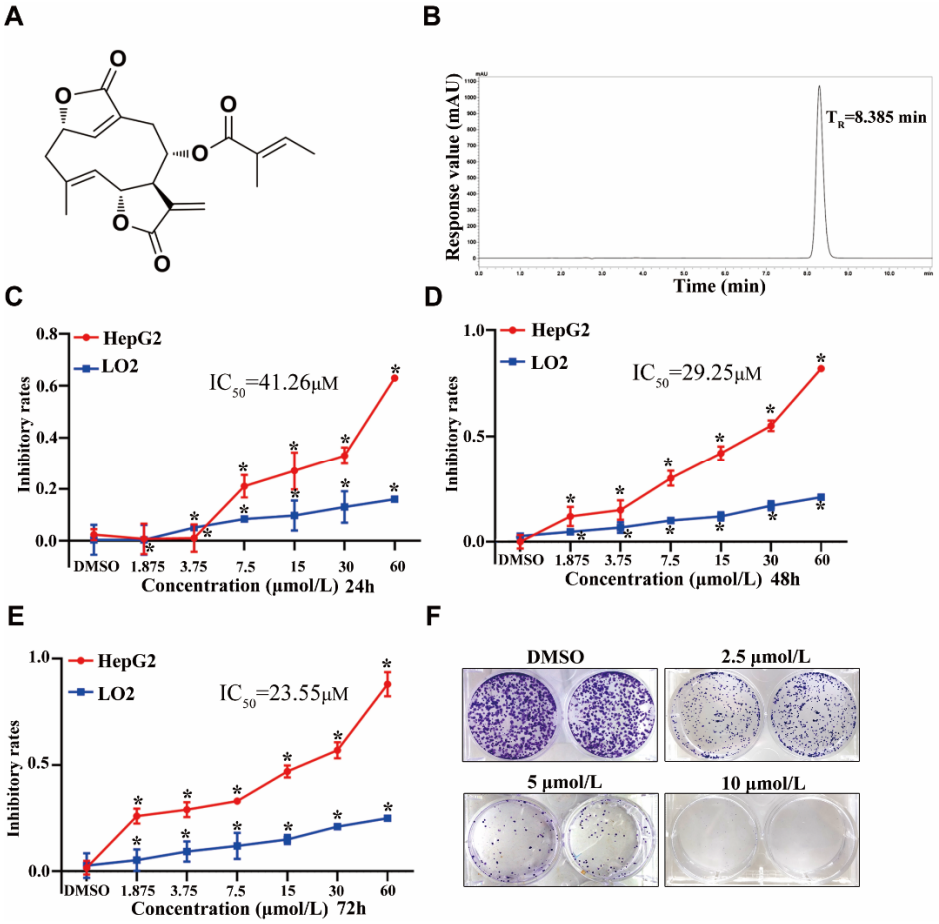

Fig 1. Cytotoxic effect of EM-10 on HepG2 and LO_2 cells

ISSN: 1813-4890

Molecular structure of EM-10. (B) The degree of purity of EM-10 used in this study was > 95 % as determined by HPLC analysis with a single peak. (C)(D)(E) HepG2 and LO_2 cells were exposed to different concentrations of EM-10 for 24, 48 and 72 h, and the cell proliferation inhibition rate was determined by MTT assay. (F) HepG2 cells were treated with EM-10 at the indicated doses for 7 days. Each value represents the result of three independent experiments.

EM-10 is a sesquiterpene lactone compound extracted and separated from *Elephantopus mollis* H.B.K., and its molecular structure was shown in Fig. 1A. The HPLC results showed only one peak for EM-10, which had a purity of over 95% (Fig.1B). The proliferation effect of EM-10 on human Hepatocellular carcinoma HepG2 cells and human hepatic LO_2 cells was analyzed by MTT assay. HepG2 cells and LO_2 cells were incubated with different concentrations of EM-10 (0, 1.875, 3.75, 7.5, 15, 30 and 60 μmol/L) for 24, 48, and 72 h, and the decrease in cell viability were shown in Fig. 1C-E. Those cells cultured with 7.5 μmol/L of EM-10 or higher showed more significant decrease in viability, and EM-10 inhibited the growth of HepG2 cells in a dose- and time-dependent manner, with the half maximal inhibitory concentration (IC_{50}) values of HepG2 cells were 41.26, 29.25, 23.55 μmol/L for 24, 48, and 72 h. The IC_{50} values of EM-10 on the normal cell line LO_2 were all above 100 μM, demonstrating that EM-10 may strongly reduce hepatocellular carcinoma cell viability while having no influence on normal cell viability. A clone formation assay confirmed the anti-proliferation effect of EM-10 on HepG2 cells, with the colony number significantly decreasing as the EM-10 dose increased (Fig. 1F).

3.2. EM-10 Induced Apoptosis Via the Mitochondrial Apoptotic Signaling Pathway in HepG2 Cells

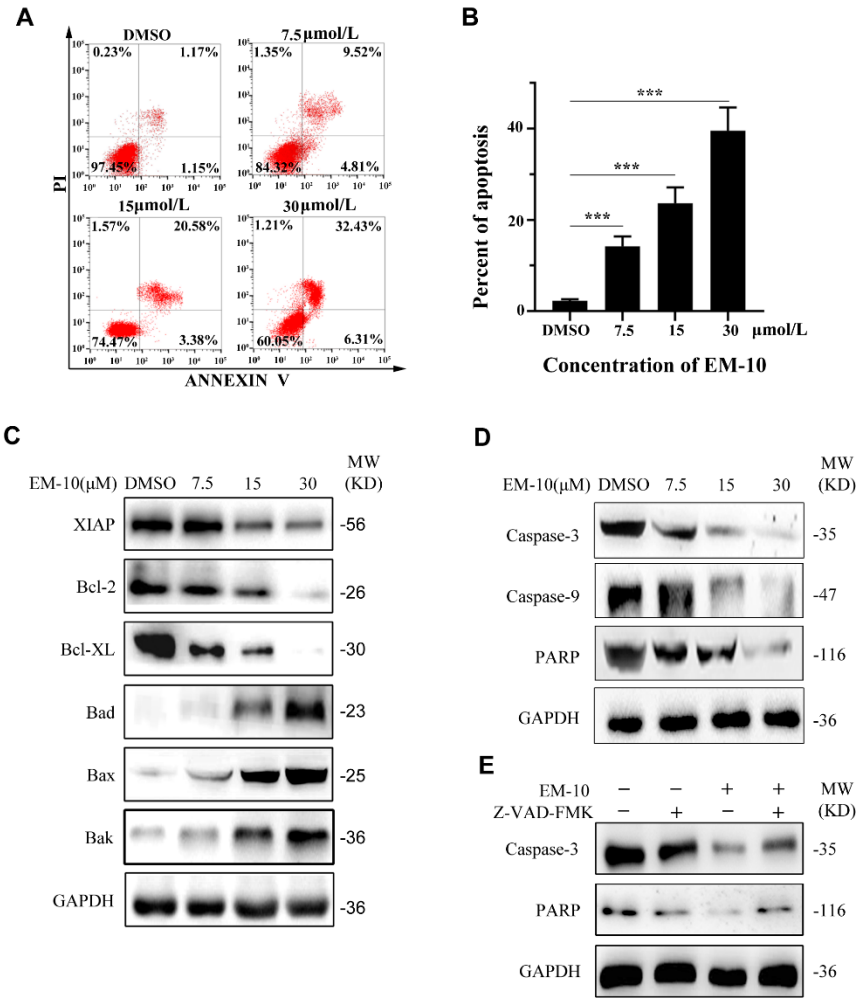

Fig 2. EM-10 induced apoptosis via the mitochondrial apoptotic signaling pathway in HepG2 cells

ISSN: 1813-4890

Annexin V-FITC/PI staining assay was performed after HepG2 cells were treated with increasing concentrations of EM-10 for 24 h. (B) The percentage of apoptotic cells was presented as the mean ± SD of triplicate determinations (vs. DMSO, ***P < 0.05). (C) The expression of Bcl-2 family members and XIAP was detected by western blot after HepG2 cells treated with the different EM-10 concentration for 24 h. (D) Cells were incubated with EM-10 (0, 7.5, 15 and 30 μmol/L) for 24 h. The expression level of apoptosis-associate proteins were analyzed by western blotting. (E) Apoptosis-associate proteins expression levels were measured by western blot following treatment with Z-VAD-FMK with or without EM-10 for 24 h.

To determine whether the proliferation inhibition of HepG2 cells was caused by by EM-10 induced apoptosis, an Annexin V-FITC/PI double-staining assay was used to detect apoptotic cells in HepG2 cells treated with different concentrations of EM-10 (0, 7.5, 15, 30 μmol/L) for 24 h. As shown in Fig. 2A-B, the apoptosis rate of EM-10 treatment groups increased with increasing concentration of EM-10 compared with the DMSO group, and the total apoptosis rate increased from 2.32% to 38.74%, indicating a dose-dependent increase in EM-10 induced apoptosis.

Apoptosis is mainly initiated through two pathways: the intrinsic pathway mediated by mitochondria and extrinsic pathway mediated by death receptor[11]. And intrinsic apoptosis is co-regulated by pro-apoptosis and anti-apoptosis proteins in the Bcl-2 family [12, 13].We further investigated whether EM-10 activated the regulation of proteins associated with apoptosis. We examined the expression levels of related proteins by western blotting and found that the protein expression level of Caspase-9, Caspase-3, PARP, XIAP, Bcl-2 and Bcl-xl were downregulated in HepG2 cell lines with increasing EM-10 (0, 7.5, 15, 30 μmol/L) concentrations, while the levels of Bax, Bad, Bak were upregulated distinctly (Fig. 2C-D). Moreover, pretreatment with Z-VAD-FMK, an inhibitor of the caspase family, significantly attenuated the EM-10 induced cell apoptosis (Fig. 2E). The above findings suggested that the EM-10 induced apoptosis in HepG2 cells through the mitochondrial signaling pathway.

3.3. EM-10 Triggered G2/M Phase Arrest of HepG2 Cells

We wondered whether EM-10 could play an antitumor role by affecting the cell cycle of HepG2 cells. Propidium Iodide staining was used to observe the effect of EM-10 on the cell cycle distribution of HepG2 cells. As expected, the results showed a dose-dependent accumulation in the G2/M phases from 3.98% to 19.45% and a decrease in the G0/G1 phase from72.78% to 50.19% as shown in Fig. 3A-C.

Cyclins and CDK complexes play an important role in the regulation of cell cycle progression, with different CDKs and cyclins regulate different phases of cell cycle. The expression of p-Cdc2 (Tyr15), Cyclin B1, Cyclin A, and Wee1 was significantly increased after EM-10 treatment, while Cyclin D1 and cdc25c were down-regulated (Fig. 3D). Taken together, we infered that the treatment of EM-10 caused the cells to undergo G2/M phase arrest.

(A) The proportion of G2 phase increased with the different concentrations of EM-10 (7.5, 15 and 30 μmol/L) for 24 h. (B) The percentage of cells in the S and G2/M phases was presented as the mean ± SD of triplicate determinations (***P < 0.05 vs. DMSO). (C) The percentage of cells in each phase of cell cycle was calculated using GraphPad Prism software. (D) HepG2 cells were incubated with different concentrations of EM-10 for 24 h, and the expression of cell cycle-associated proteins was analyzed by western blotting.

ISSN: 1813-4890

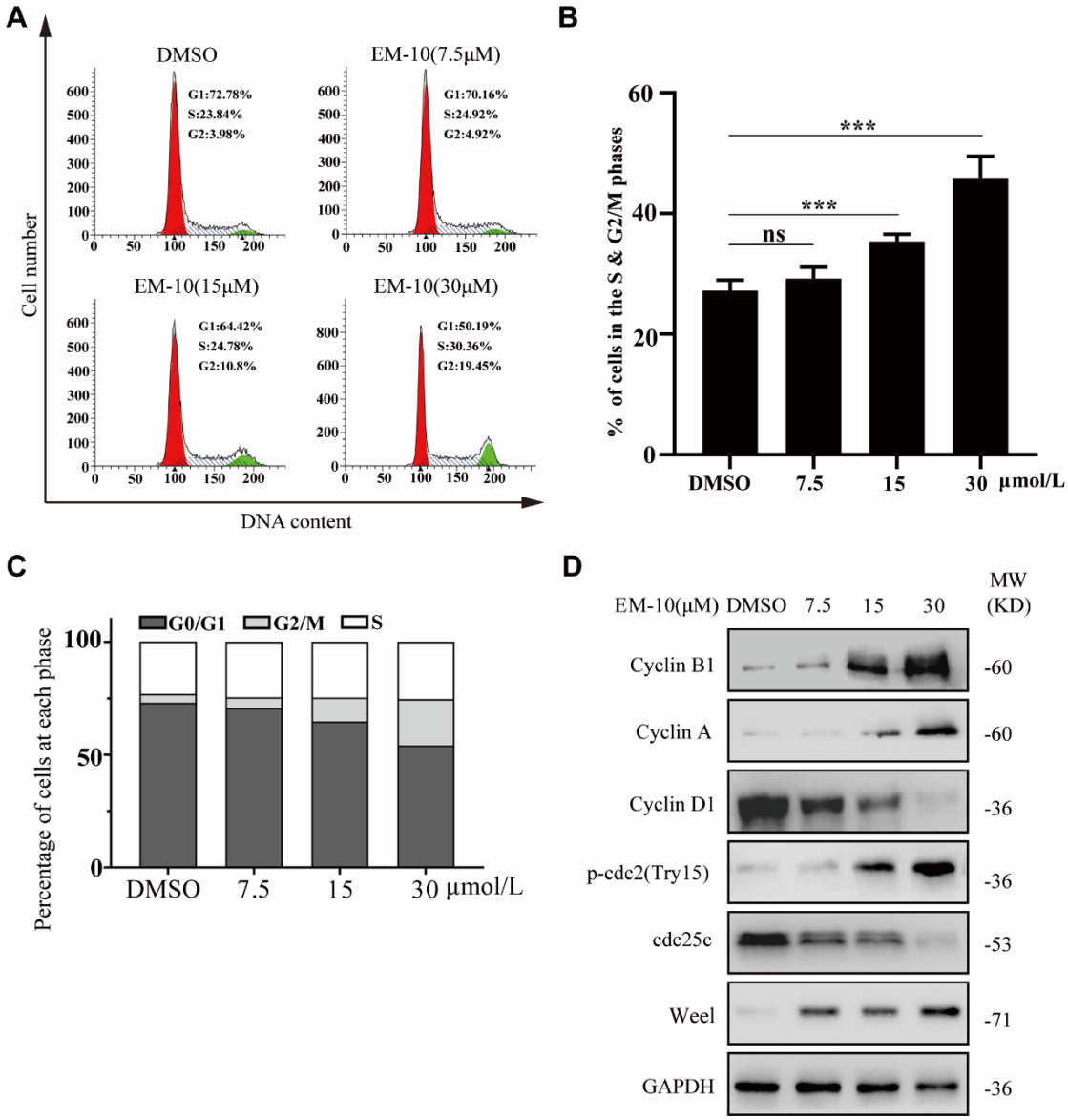

Fig 3. EM-10 triggered G2/M phase arrest of HepG2 cells

3.4. EM-10 Activated JNK and p38 Pathways in HepG2 Cells

It is well known that mitogen-activated protein kinase (MAPK) signaling pathway plays a key role in responding to extracellular signaling pathway, which involved in the regulation of multiple cell life progress. JNK and p38 pathways regulate various physiological processes including inflammatory responses, cell differentiation, cell proliferation, cell death, cell survival and expression of proteins [14]. Therefore, we further explored whether MAPK signal pathway also engaged in the apoptosis induced by EM-10. We validated the changes in the expression levels of MAPKS signaling pathway proteins. The protein expression of p-JNK and p-p38 increased as the concentration of EM-10 increased, as did the expression of its downstream protein p53, but the expression of total JNK did not change significantly (Fig. 4A). And the protein expression of PUMA was also increased. These data suggested that EM-10 could activate the JNK and p38 pathways to induce cell apoptosis.

ISSN: 1813-4890

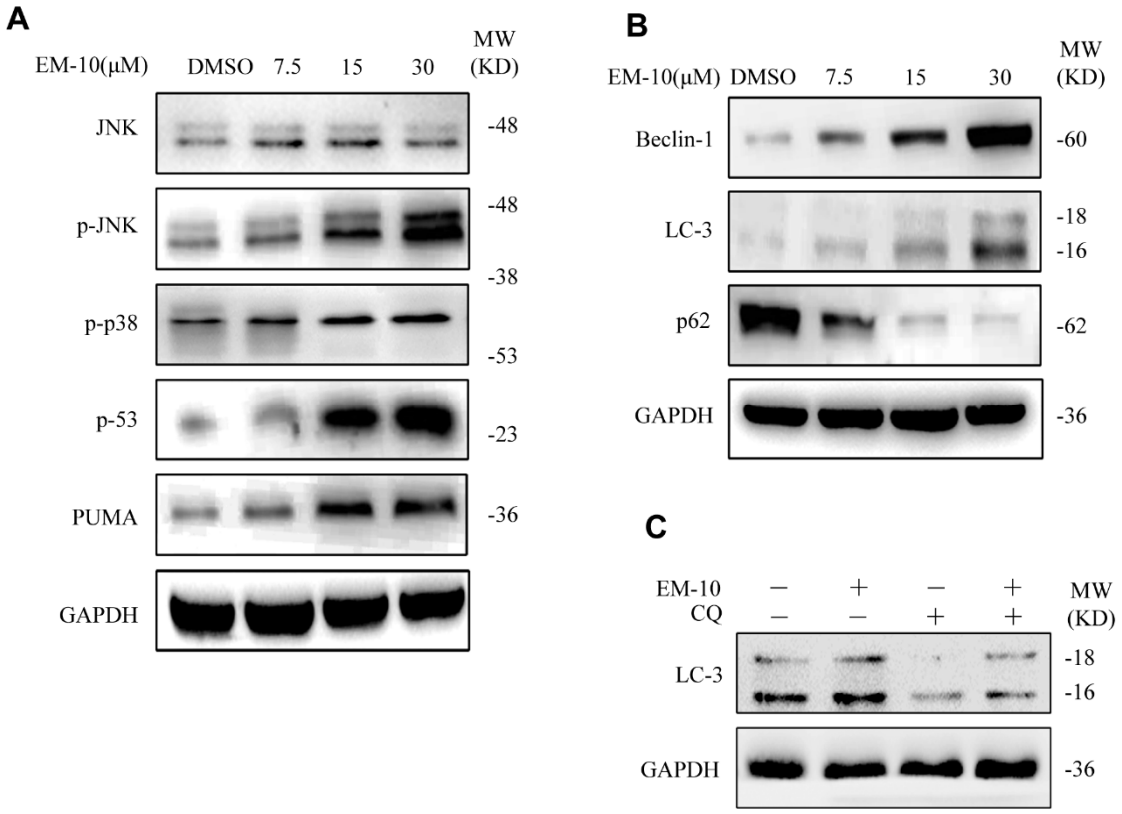

Fig 4. EM-10 activated JNK and p38 signaling pathway and induced autophagy in HepG2 cells

(A) The expression level of related proteins of JNK and p38 signaling pathway were detected by western blotting. Cells were incubated with DMSO,7.5,15,30 μmol/L EM-10 for 24 h. (B) Western blot was used to evaluate the protein expression of Beclin-1, LC3 and p62. Cells were treated with serial concentrations of EM-10 for 24 h. (C) HepG2 cells were exposed to EM-10 with or without CQ treatment. And the expression levels of autophagic marker proteins were evaluated.

3.5. EM-10 Induced Autophagy in HepG2 Cells

Autophagy is a self-protective mechanism of the cells, and the degree of autophagy can be measured by the ratio of LC3-II/LC3-I, and LC3 is regarded as a marker molecule for autophagy. In this study, we detected the expressions of autophagy associated proteins. The result showed that with the increased concentration of EM-10, Beclin-1 and ratio of LC3-II/LC3-I were increased, while p62 was decreased (Fig. 4B). In addition, Chloroquine (CQ), an established autophagy inhibitor, reversed the increased expression of LC3II induced by EM-10 (Fig. 4C). Overall, these evidence confirmed that EM-10 induced autophagy in HepG2 cells.

4. Conclusion

In summary, our findings revealed that EM-10, a monomer of *Elephantopus mollis* H.B.K., significantly inhibited the proliferation of HepG2 cells by inducing cell apoptosis via the mitochondrial signaling pathway. Moreover, EM-10 induced activation of the JNK and p38 mitogen-activated protein kinase (MAPK) pathway and autophagy further promoted cell apoptosis in HepG2 cell lines. These results indicated that EM-10 has great potential as a promising drug candidate for the future treatment of liver cancer.

ISSN: 1813-4890

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