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Yu Peng
School of Pharmaceutical Sciences, South-Central Minzu University, Wuhan 430074, China

Yuruo Gong
School of Pharmaceutical Sciences, South-Central Minzu University, Wuhan 430074, China

Congwei Wang
School of Pharmaceutical Sciences, South-Central Minzu University, Wuhan 430074, China

Dujuan Shi
School of Pharmaceutical Sciences, South-Central Minzu University, Wuhan 430074, China

Jinyan Zhang
School of Pharmaceutical Sciences, South-Central Minzu University, Wuhan 430074, China

G Wei Kevin Zhang
School of Pharmaceutical Sciences, South-Central Minzu University, Wuhan 430074, China

Xinzhou Yang
School of Pharmaceutical Sciences, South-Central Minzu University, Wuhan 430074, China
ORCID: 0000-0003-1697-2923

Xiaojun Li
School of Pharmaceutical Sciences, South-Central Minzu University, Wuhan 430074, China

Correspondence:
Dr. Xiaojun Li
School of Pharmaceutical Sciences, South-Central Minzu University, Wuhan 430074, China
Email: lxj@mail.scuec.edu.cn

de-*O*-methyllasiodiplodin from *Ludwigia hyssop folia* causes death of human liver cancer cells through the mitochondrial apoptotic, Akt/NF-κB and STAT3 pathway *in vitro*

Yu Peng, Yuruo Gong, Congwei Wang, Dujuan Shi, Jinyan Zhang, Wei Kevin Zhang, Xinzhou Yang, Xiaojun Li

ABSTRACT

(+)-(R)-de-*O*-methyllasiodiplodin (DOML), isolated from the Chinese herbal medicine *Ludwigia hyssop folia*, has great potential for development in pharmacological research on hepatocellular carcinoma (HCC). In our study, the CCK-8 assay, morphological observation, flow cytometry (also known as Annexin V-FITC/PI double staining), as well as Western blotting were adopted to study the anti-liver cancer activity and mechanisms of DOML on HepG2 and HuH-7 cells. The research exhibited that DOML dose- and time-dependently reduced the cell viability of HCC cells. DOML treatment resulted in changes in cell morphology, such as irregular edges, reduced volume, and decreased adhesion were observed under the microscope. Flow cytometry analysis indicated that apoptosis is the major form of cell death. In addition, blocking autophagy and necroptosis pathways couldn't alleviate DOML-induced apoptosis. Protein expression levels of Bax, activated Caspase-3 and Caspase-9, and PARP were increased, while Bcl-2 protein levels were reduced by DOML treatment, which suggested that the mitochondrial apoptotic pathway may be involved in DOML-induced cell death. Moreover, the expression of NF-κB and the phosphorylation of Akt and STAT3 decreased with the increase of dosage, suggesting that the apoptotic mechanism might be related to the Akt/NF-κB and STAT3 signaling pathways. All these results indicate that DOML has the potential effects of anti-hepatoma.

Keywords: Anti-Cancer; de-O-methyllasiodiplodin; Apoptosis; Mitochondrial Pathway; Akt/NF-κB; STAT3.

INTRODUCTION

Hepatocellular carcinoma (HCC), which is a malignant liver tumor, can be divided into two categories, primary and secondary, accounting for 4.7% of the global cancer incidence in 2018, ranking sixth [1]. accounting for 8.2% of the total deaths during the same period. It is the fourth leading cause of death worldwide [2]. And in recent years, the morbidity and mortality have also shown an upward trend, which seriously threatens human life and health [3]. The more thorough method for liver cancer treatment is surgery. However, because the indications for surgical resection are more stringent and cannot meet the needs of all patients, and if there is too little resection, liver cancer cells may remain and cause recurrence. Therefore, it is required to completely remove the tumor to the maximum extent, so that the margin is free of residue [4]. Choi *et al* [5]. believe that radiotherapy is a reliable and effective method for the treatment of hepatocellular carcinoma, which depends on the degree of disease and patient characteristics. In order to further improve the survival rate and treatment methods, improve the quality of life of patients, and prolong the survival time of patients, it is necessary to pay attention to the research of anticancer drugs and find suitable clinical anticancer drugs as soon as possible.

The natural products are rich in species, diverse in structure, and have a variety of biological activities, which are important sources for the discovery of new bioactive compounds today. In addition, natural products have the characteristics of large output and low cost, so they have great advantages in drug development and application. A large number of natural products with anti-cancer activity have been found, among which plant-derived natural products are rich in resources [6]. The application of natural products for cancer chemotherapy is indeed of great value for development and utilization.

Ludwigia hyssop folia (G. Don) Exell is a plant belonging to the genus Ludwigia in the family Agavaceae, distributed in southwestern China. The whole herb is used to clear heat and detoxify and decompose muscles. It can be used to treat colds, sore throats, and sores. At present, there are few reports about the chemical composition of Ludwigia hyssop folia, and only the anti-tumor effect of pipeline has been reported [7]. In this study, a macrolide compound, (+)-(R)-de-O-methyllasiodiplodin,

was isolated from *Ludwigia hyssop folia*. It is known that macrolide compounds occupy a very important position in the antibiotic family. At present, macrolide compounds are known to occupy a very important position in the antibiotic family, and they are favored by clinicians because of their precise efficacy, strong antibacterial activity, resistance to drugs resistance, and broad antibacterial spectrum [8]. It was found in previous preliminary experiments that it had a certain inhibitory effect on laryngeal cancer cells [9]. so, we also performed tests on liver cancer cells, and the results proved that it has great research value. In this experiment we evaluated the cytotoxic activity of DOML. Further research shows that DOML can inhibit the proliferation of HCC cells in a dose- and time-dependent manner, and has less damage to human-derived normal liver cells L_{02}. The low toxicity of DOML can be considered to meet the basic requirements for becoming a potential liver cancer chemotherapy drug. In order to further verify the potential of DOML as an anticancer agent, we studied its anticancer mechanism.

MATERIALS AND METHODS

Cell culture

Liver cancer cell lines HepG2 (CCTCC, China) and Huh-7 (Porcello, China), and normal liver cell line L02, all of which were originated from humans. After cells thawing, HepG2, Huh-7 and L_{02} cells were separately cultured in a HG-DMEM (Gibco, USA) medium supplemented 10% fetal bovine serum and 1% penicillin/streptomycin. Above-mentioned cells were maintained in 96- or 6-well plates and cultured at 37°C and 5% CO_2 in a humidified incubator.

Antiproliferative activity by CCK-8 assay

The cells were inoculated into 96-well plates at 1×10^5 cells/well and cultured until they reached 85% confluence. At the beginning, dissolved DOML with DMSO to prepare a certain concentration as a stock solution, and then dilute to the desired concentration (0, 2, 8, 12, 16 and 20 µmol/L) with serum-free HG-DMEM. After serum starvation overnight, the cells were respectively treated with specific concentrations of DOML for 24 h. After cultivation, CCK-8 assay (Best Bio, Shanghai, China) was performed according to the kit instructions. Each well was added with 10 µl of CCK-8, and maintain the plates at 37 °C for 1- 4 h. Consequently, the optical density (OD) value in well was examined individually at 450 nm with a Microplate Reader (BIO-RAD, USA). The formula, cell viability (%) = $(OD_{sample} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100$, was adopted to calculate the cell viability. The IC_{50} value of DOML, the concentration of DOML corresponding to 50% cell inhibition rate, was estimated by the IBM SPSS Statistics Software.

Observation of morphological changes

Seed cells in 6-well plates, and the seeding density was 2.5×10^6 cells/well. After serum starvation overnight, they were exposed to different concentrations of DOML (0, 2, 10 and 20 µmol/L). Before treatment, DOML were dissolved and diluted. After 24 h of drug treatment, the cell morphology was observed under an inverted phase contrast microscope and photographed with a 10 × magnification camera.

Hoechst 33258 staining

Seed HepG2 cells in 6-well plates, and the seeding density was 2.5×10^6 cells/well. After starvation with serum overnight, the cells were cultured with specific concentrations of DOML (0, 2, 10 and 20 µmol/L) for 24 h. After cultivation, remove the original medium, and add cell fixative consisting of 75% methanol and 25% glacial acetic acid was added to per well for 15 min. Then, in the dark, staining the cells for 15 min with Hoechst 33258 (Bey time, Shanghai, China). A fluorescence microscope (excitation wavelength 350 nm, emission wavelength 460 nm, Sopot ICX41, Ningbo, China) was employed to observe and photograph the cell morphology at 40 × magnification.

Wound healing assay

Seed cells in 6-well plates, and the seeding density was 2.5×10^6 cells/well. Then the pipette tips (10 µl) were used to make six scratch wounds in each well and washed with PBS to remove the detached cells. After respectively incubated with different concentrations of DOML (0, 2, 10 and 20 µmol/L) scratch wounds of the cells were photographed at 10 × magnification immediately as 0 h under an inverted phase contrast microscope (Leica, Nussle, Germany). Following 24 h of drug treatment, scratch wound images of the same field were photographed. ImageJ software was applied to calculate the area of scratches.

Seed cells in 6-well plates, and the seeding density was 2.5×10^6 cells/well. After serum starvation overnight, they were exposed to specific concentrations of DOML (0, 2, 10 and 20 µmol/L) for 24 h. Following that, the cells were cleaned twice with PBS and digestion with edad-free trypsin. The cells were collected by centrifuged at 300 × g for 5 min at 4°C. After cleaning twice with PBS under the above centrifugation conditions, discard PBS and add 400 µl of 1 × Annexin V binding buffer (Best Bio, Shanghai, China) to suspend the cells. Add 5 µl Annexin V-fluorescein isothiocyanate (FITC) staining solution to the cell suspension, mix gently, and incubate at 4°C in the dark for 15 min. After adding 10 µL of propidium iodide (PI) staining solution, gently mix and incubate at 4°C in the dark for 5 min, and then immediately detect it by flow cytometry and analyzed by Flowood V_{10} software.

JC-1 fluorescent staining

Seed cells in 6-well plates, and the seeding density was 2.5×10^6 cells/well. After serum starvation overnight, they were exposed to specific concentrations of DOML (0, 2, 10 and 20 µmol/L) for 24 h. Following that, the cells were cleaned once with PBS and digestion with trypsin. Before treatment, JC-1 staining working solution should be prepared. Dilute JC-1 (Yaseen, Shanghai, China) by adding 8 mL of ultrapure water per 50 µl of JC-1 (200 ×), thoroughly dissolved and mixed JC-1 with vigorous shaking, and then add 2 mL of JC-1 staining buffer (5 ×) and mixed. Resuspend the collected cells in 0.5 mL of cell culture solution, add 0.5 mL of JC-1 staining working solution, mix by inverting several times and place in a cell culture incubator at 37°C for 20 min. In the process of incubating, dilute 5× JC-1 staining buffer with distilled water to make 1× JC-1 staining buffer appropriately, and put it in an ice bath. Afterward, centrifuge at 600 × g at 4°C for 3 min. Discard the supernatant, taking care not to remove the pellet by suction. Wash twice with JC-1 staining buffer (1 ×) under the above-mentioned centrifugation conditions. After resuspension with an appropriate amount of JC-1 staining buffer (1 ×), immediately detect it with a flow cytometer and analyzed by Flowood V_{10} software.

Western blotting

Seed cells in 6-well plates, and the seeding density was 2.5×10^6 cells/well. After serum starvation overnight, they were exposed to specific concentrations of DOML (0, 2, 10 and 20 µmol/L) for 24 h. Following that, cells were cleaned 2 times in PBS. Then, cells were lysed by adding RIPA containing PMSF and Postop and incubated for 30 min on ice. After centrifuged at 12,000 rpm for 10 min at 4°C, the supernatant containing protein was collected and measured using a Bradford Protein Assay Kit (Bey time, Shanghai, China). After adding loading buffer and heating for 5 min at 95°C to make protein denaturation, separated equal amounts of protein with a 10% SDS-PAGE gel at 80 V until bromophenol blue runs to the lower edge of the electrophoresis tank. Subsequently, transferred the protein to PVDF membrane by transfer membrane device at 300 mA current for 1.5 h. the membranes were blocked in One Step Western Blocking

Buffer for 5 min at room temperature, and then the PVDF membranes were washed 3 times for 5 min each in TBST. Primary antibodies were incubated at 4℃ over-night and the corresponding secondary antibodies were incubated at room temperature for 2 h. After cultivation of antibody, the membranes were washed 3 times for 5 min each in TBST. After the developer was added, under the Bio-Rad gel imager, the protein on the PVDF membranes was appeared and photos are scanned and saved.

Other possible death pathway tests

The cells were inoculated into 96-well plates at 1×10^5 cells/well and cultured until they reached 85% confluence. After serum starvation overnight, block HepG2 cells with autophagy inhibitors: 3-methyladenine (3-MA) and chloroquine (CQ) and necrosis inhibitors: necrostatin-1 (Nec-1) and necro sulfonamide (NSA) for 30 min in advance, and then DOML (final concentration: 20 μmol/L) was added for 24 h. After cultivation, CCK-8 assay was performed according to the kit instructions. Each well was added with 10 μl of CCK-8, and maintain the plates at 37℃ for 1- 4 h. Then, the OD value was examined and the cell viability was calculated as in the section of antiproliferative activity by CCK-8 assay in this paper.

Statistical analysis

Data for all results were presented as mean ± standard deviation (SD) of at least three trials individually. GraphPad Prism 5.0 software was utilized for statistics and analysis. One-way analysis of variance (ANOVA) was adopted to analyze significant differences and P-values < 0.05 were considered it (*p < 0.05, **p < 0.01 or ***p < 0.001).

RESULTS

DOML can induce human liver carcinoma cells death

As displayed in Fig. 1, the chemical structure of the research object DOML, and the high performance liquid chromatography (HPLC) graph showing the purity of DOML were presented.

The CCK-8 assay was employed to test the capacity of DOML in inhibiting HCC cells proliferation. HepG2 and HuH-7 cells were respectively incubated with 0, 2, 8, 12, 16 and 20 μmol/L of DOML for 12, 24 and 48 h, and the cell viability was calculated. In these results (Fig. 2), we found that DOML dose-dependently and time-dependently enhanced inhibition rate of treating HepG2 and HuH-7. It was noted that DOML can inhibit the proliferation of HepG2 cells well in 12 h (IC_{50} = 14.95 ± 1.84 μmol/L), 24 h (IC_{50} = 12.50 ± 1.825 μmol/L) and 48 h (IC_{50} = 10.55 ± 4.526 μmol/L). Similarly, in HuH-7 cells, and the IC_{50} in 12 h (IC_{50} = 12.66 ± 1.270 μmol/L), 24 h (IC_{50} = 11.00 ± 0.745 μmol/L) and 48 h (IC_{50} = 10.24 ± 0.529 μmol/L) were displayed in Fig.2B and C. The human normal liver cell line L02 was cultured under the same conditions as HCC cells to evaluate toxic effects. And we observed that its antiproliferative ability to L02 is relatively weak. Therefore, based on the IC_{50} value, DOML of 0, 2, 10 and 20 μmol/L was selected as the concentration gradient, which is mainly used for subsequent assay predominantly.

DOML can inhibit the migration of HepG2 and HuH-7 cells

One of the hallmarks of cancer invasion is collective cell migration. Quantitative wound healing is a powerful method used to assess the migratory capacity of cancer cells. In this paper, DOML's influence upon liver cancer HepG2 and HuH-7 cells migration was determined through a wound healing assay. The results showed that DOML could effectively inhibit the migration of liver cancer HepG2 and HuH-7 cells time- and dose-dependently (Fig. 3A and B).

Apoptosis is the main form of cell death caused by DOML

We were aware of the potential of DOML on death in HCC cells. In order to investigate the manner of death induced by DOML, we compared the morphological changes of DOML-treated HCC cells with those of the untreated group under a phase contrast microscope and a fluorescence microscope. The photos obtained under a phase-contrast microscope show that typical apoptotic features of the cell shape, such as shrinkage, deformation, and flotation, are gradually observed with concentration gradients (Fig. 4A and D). When apoptosis occurs, the morphological characteristics of cells undergo multi-stage changes. For example, the first is that cells shrink in size, lose connections, and separate from surrounding cells. Apoptosis usually involves a single cell, and even a few cells occur asynchronously. In addition, the degree of apoptosis of L_{02} cells was much lighter than that of HepG2 and HuH-7 cells, except for the highest concentration group, which was no different from that of the untreated group.

We further observed nuclear morphological changes of HepG2 and HuH-7 cells. In the later period, the nuclear membrane nucleoli are destroyed by the cell membrane to form a vesicle, and the apoptotic cell remains can be divided into several apoptotic bodies [10]. After Hoechst 33258 staining (Fig. 4B and E), the cells in the blank group were intact and uniform in color. Apoptosis was observed in the chromatin-concentrated drug group, divided into blocks, and bright apoptotic bodies appeared, particularly in the high-dose group.

To examine and confirm whether DOML can induce apoptosis of liver cancer cells at 24 hours, the Annexin V-FITC/PI double staining kit was adopted to stain cells, and flow cytometry was employed to analyze. Take Annexin V-FITC and PI as the horizontal axis and the vertical axis, respectively. The lower and upper right quadrant represent early and late apoptotic cells, separately. The lower left quadrant represents normal cells, and the upper left quadrant represents mechanically damaged cells or necrotic cells. The percentage of apoptotic HepG2 cells was significantly higher after incubation with different concentrations (10, 20 μmol/L) of DOML (Q2 + Q3: 17.43%; 53.20%) than that of the control group (Q2 + Q3: 9.22%). The proportion of apoptotic HuH-7 cells was also remarkably higher after treated with concentrations (20 μmol/L) of DOML (Q2 + Q3: 23.2%;) than that of the control group (Q2 + Q3: 13.54%). In addition, the percentage of apoptotic cells after incubation at a low concentration (2 μmol/L) was not significantly different from that of the control group. (Fig. 4C and F).

DOML induced HepG2 and HuH-7 cells apoptosis through mitochondrial pathway

During apoptosis, mitochondria release cytochrome C into the cytoplasm, triggering the downstream Caspase cascade, thereby inducing apoptosis. The release of cytochrome C requires the regulation of members of the Bcl-2 protein family. The down-regulation of the anti-apoptotic protein Bcl-2 and the up-regulation of the pro-apoptotic protein Bax are usually expressed by the ratio of Bcl-2/Bax, and the decrease in the ratio of Bcl-2/Bax represents the promotion of apoptosis. Similarly, the Caspase family is an indispensable part in this process and plays an important role. Activation of Caspase is the key to the final execution of apoptosis in the apoptotic pathway [11]. PARP splicing is considered to be an important indicator of apoptosis, and is also generally considered to be an indicator of Caspase 3 activation. Treatment of DOML with different concentrations (2, 10, 20 μmol/L) in two liver cancer cells raised the expression of lysed PARP and diminished the expression of Caspase-3 and Caspase-9 (Fig. 5A and B). In addition, DOML increased Bax expression while decreased Bcl-2 expression in HepG2 and HuH-7 cells.

JC-1, a fluorescent probe, is adopted to determine mitochondrial membrane potential (△Ψm) broadly. In the early stages of apoptosis, the mitochondrial membrane potential will decrease [12]. The results of measuring mitochondrial membrane potential showed that with 24 h of treatment, HepG2 cells increased the intensity of green fluorescence in the cytoplasm as DOML concentration increased,

while the intensity of red fluorescence in the mitochondria decreased (Fig. 6). This further proved that DOML induced HepG2 cell apoptosis through mitochondrial pathway.

Effect of DOML on Akt/NF-κB and STAT3 signalling pathway in HepG2 and HuH-7 Cells

Akt/NF-κB signalling pathway is an important signalling pathway in cells, and the activation of NF-κB signalling pathway is very important in the happening and blooming of various tumours [13,14]. Researches have displayed that Akt can activate the downstream NF-κB signalling pathway after phosphorylation, phosphorylate IκB, release NF-KB/p65 into the nucleus and combine with the target sequence to regulate the expression of anti-apoptotic genes [15,16]. thereby inhibiting tumour cell apoptosis. Western blot assay exhibited that DOML can inhibit the expression of NF-κB and p-Akt in a dose-dependent way without inhibiting the protein level of total Akt. (Fig. 7A and B). This indicated that Akt/NF-κB signalling pathway was involved in DOML-induced apoptosis of liver cancer cells.

Signal transducers and activators of transcription 3 (STAT3) is a transcription factor that plays an important role in regulating the signal transduction events mediated by cytokines and growth factors. In normal status, STAT3 was involved in many activities including survival, proliferation, differentiation and apoptosis [17]. STAT3 has been reported to regulate genes that inhibit the apoptotic pathway, which is critical for ultimately contributing to tumour development. Western blot analysis suggested that DOML inhibited the expression of p-STAT3 in a dose-dependent manner without inhibiting the protein level of total STAT3 (Fig. 8A and B). This revealed that STAT3 signalling pathway may be involved in DOML-induced apoptosis of liver cancer cells.

Autophagy and necrosis are not the main ways in which DOML causes HepG2 cell death

To further investigate whether there are other ways in which DOML can cause HepG2 cell death, we used 3-MA and chloroquine, inhibitors of autophagy; Nec-1 and NSA, inhibitors of necroptosis. The cell viability after was not significantly alleviated by the above-mentioned inhibitors (Fig. 9).

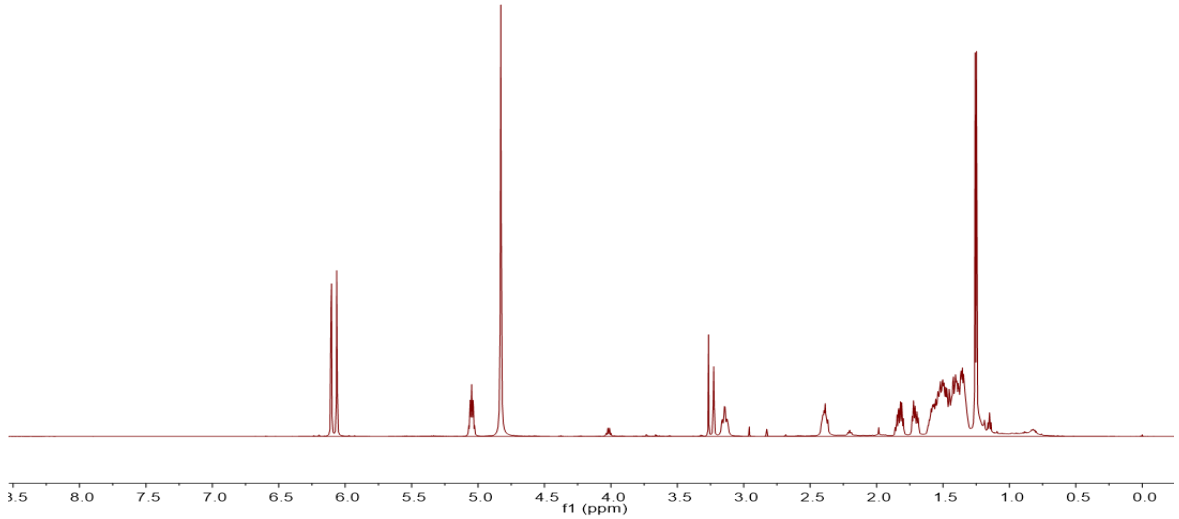

Figure 1: [1]. H NMR spectrum (600 MHz, CD3OD) of DOML

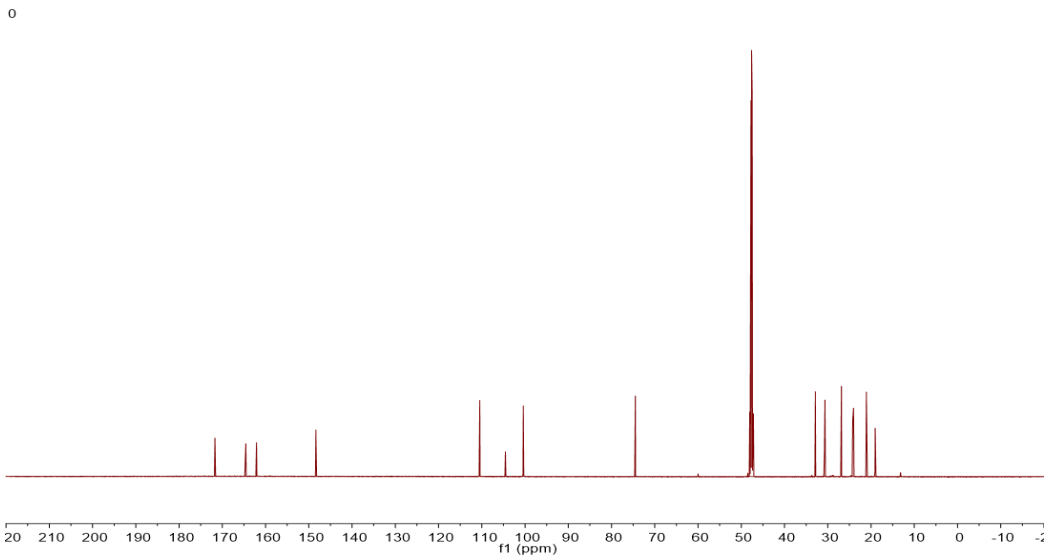

Figure 2: [13]. C NMR spectrum (150 MHz, CD3OD) of DOML

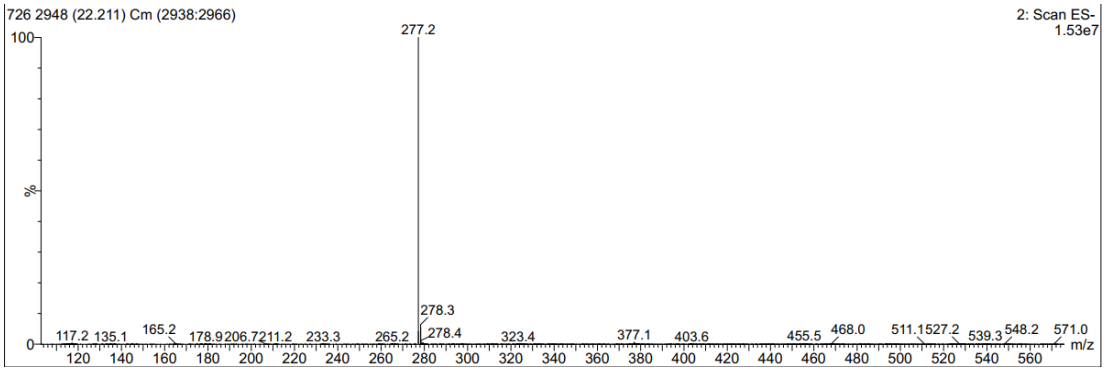

Figure 3: Mass spectrum of DOML

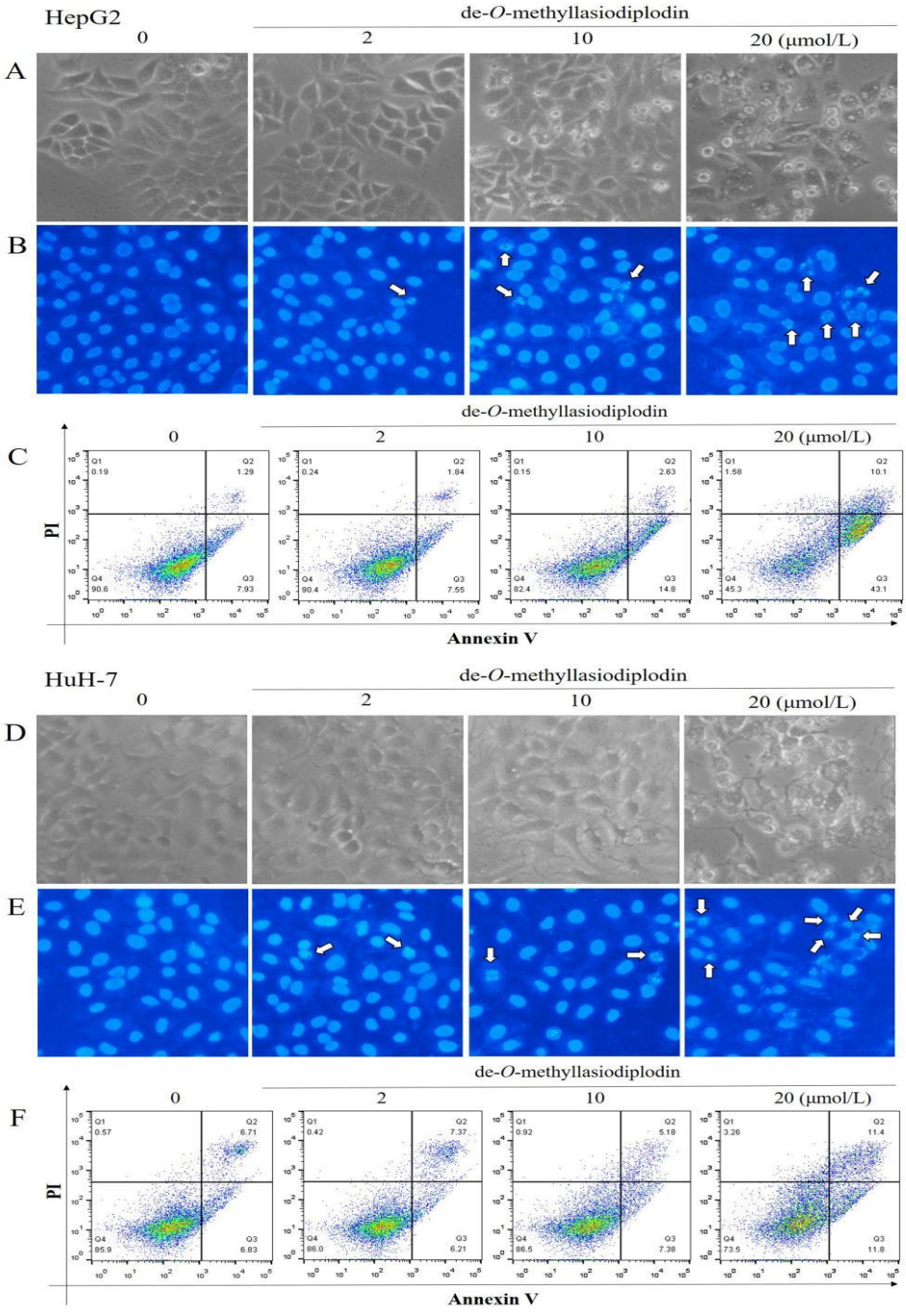

Figure 4: DOML induced apoptosis in HepG2 and HuH-7 cells. (A, D) Two liver cancer cells were cultured with specific concentrations of DOML for 12 h, and the cell morphology was photographed under an inverted phase contrast microscope (80×); (B, E) After staining with Hoechst 33258, the morphology of two liver cancer cells was photographed under an 80× fluorescence microscope; (C, F) Two liver cancer cells were handled with DOML for 24 h and then stained with Annexin V-FITC/PI double staining and then analyzed by flow cytometry

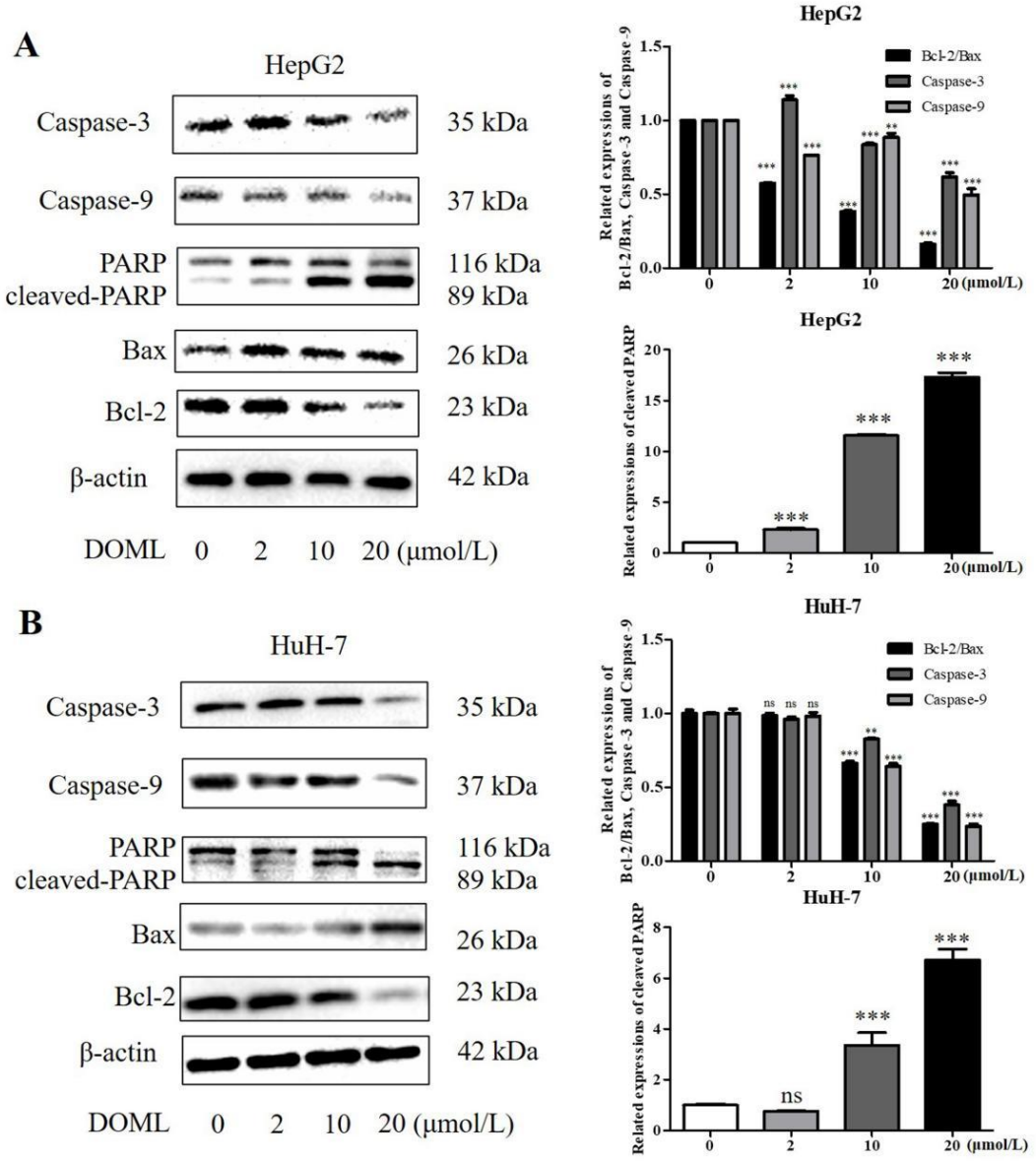

Figure 5: DOML induced apoptosis in two liver cancer cells via the mitochondrial pathway. (A, B) The expressions of mitochondrial pathway-related apoptosis proteins Bcl-2/Bax, Caspase-3, Caspase-9 and cleaved PARP in two liver cancer cells were detected by Western blotting. β-actin as internal control. (*p < 0.05, **p < 0.01 or ***p < 0.001)

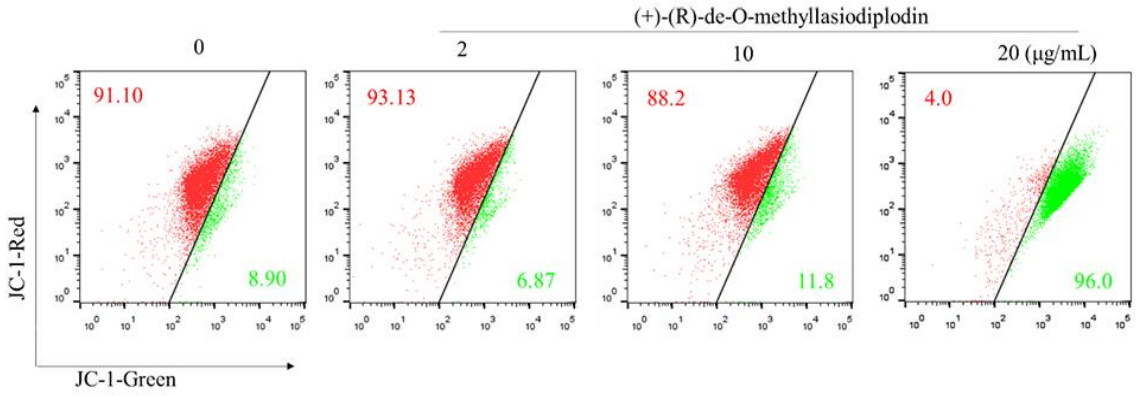

Figure 6: HepG2 cells were treated with DOML for 24 h and then stained with JC-1 fluorescent staining and then evaluated by flow cytometry

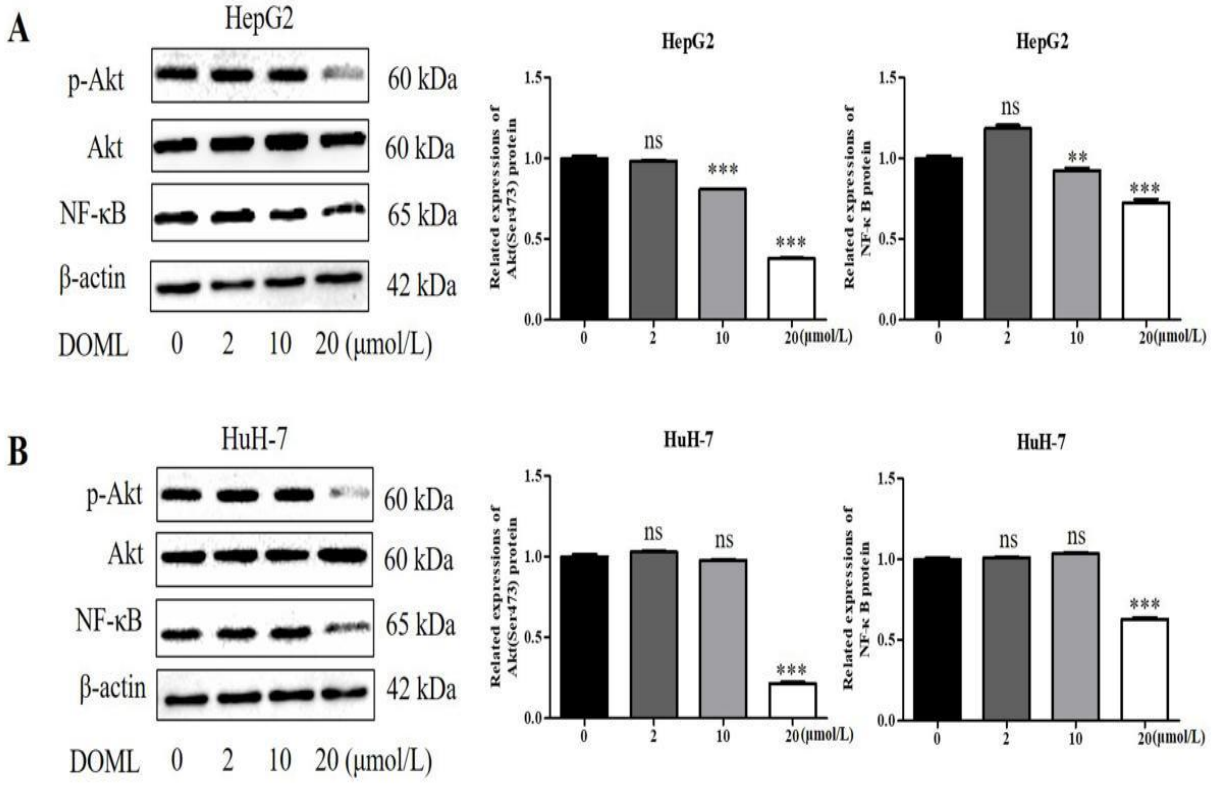

Figure 7: Regulation of Akt/NF-κB signaling pathway by DOML. (A) The relative expression of p-Akt and NF-κB were detected by Western blotting in HepG2 and HuH-7 cells incubated with DOML for 24 h. β-actin as internal control. (*p < 0.05, **p < 0.01 or ***p < 0.001)

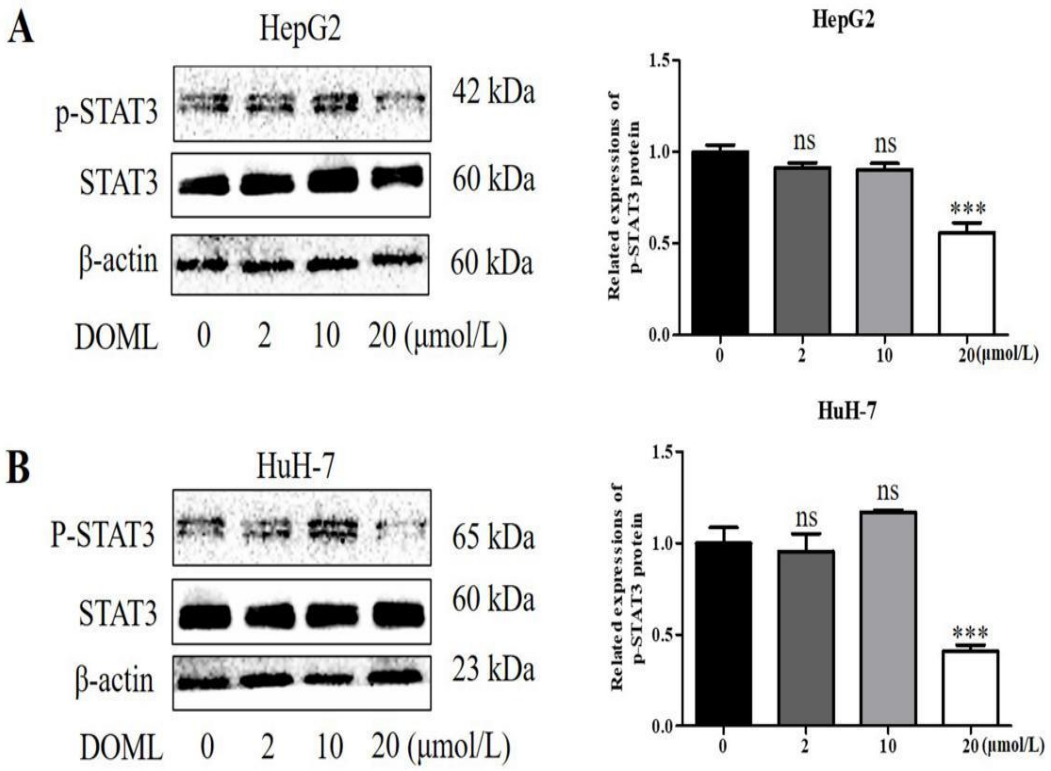

Figure 8: Regulation of STAT3 signaling pathway by DOML. (A, B) The relative expression of p-STAT3 were detected by western blotting in HepG2 and HuH-7 cells incubated with DOML for 24 h. β-actin as internal control. (*p < 0.05, **p < 0.01 or ***p < 0.001)

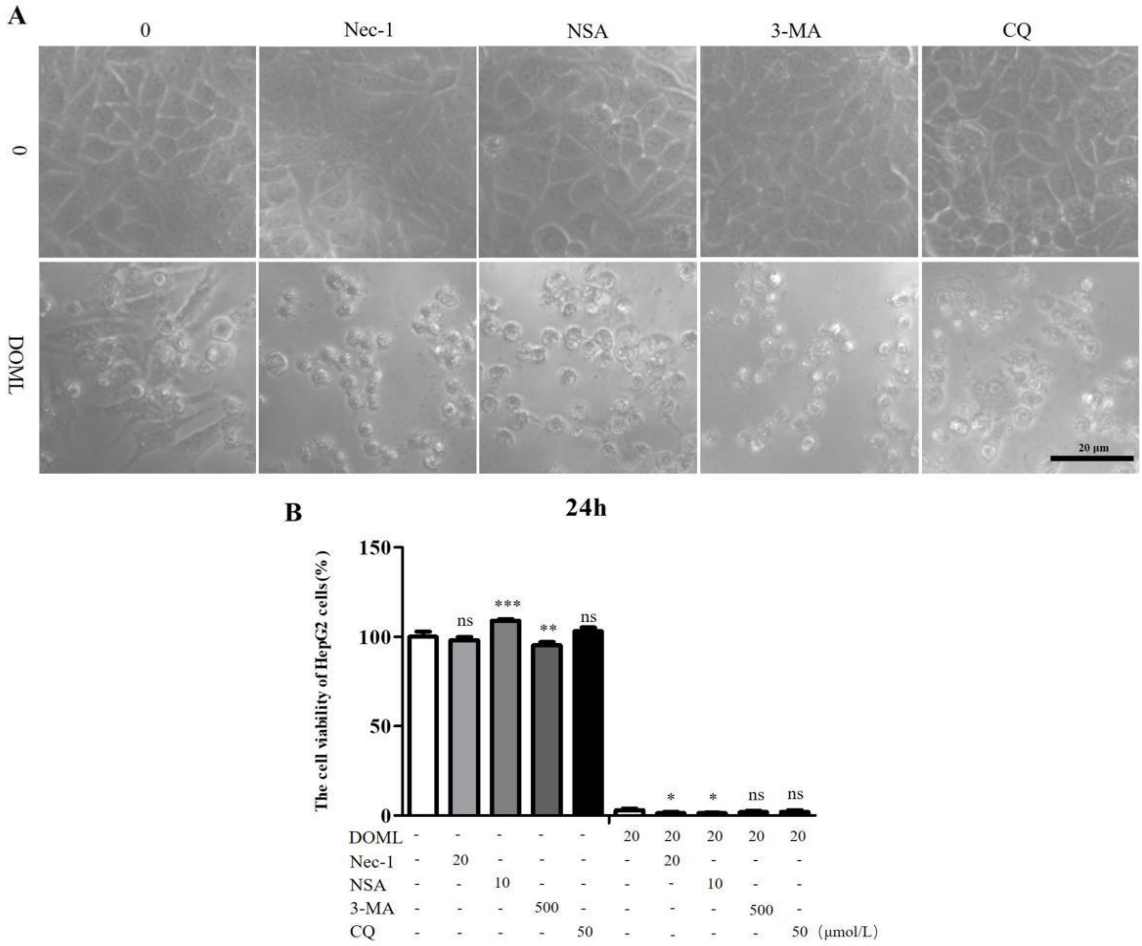

Figure 9: (A) Observation of morphological changes and apoptosis in HepG2 cells with DOML and different kinds of inhibitors; (B) HepG2 cells were treated with 20 μmol/L of DOML and different kinds of inhibitors. After 24 h, cell viability was examined by CCK-8. (*P < 0.05, **P < 0.01 or ***P < 0.001)

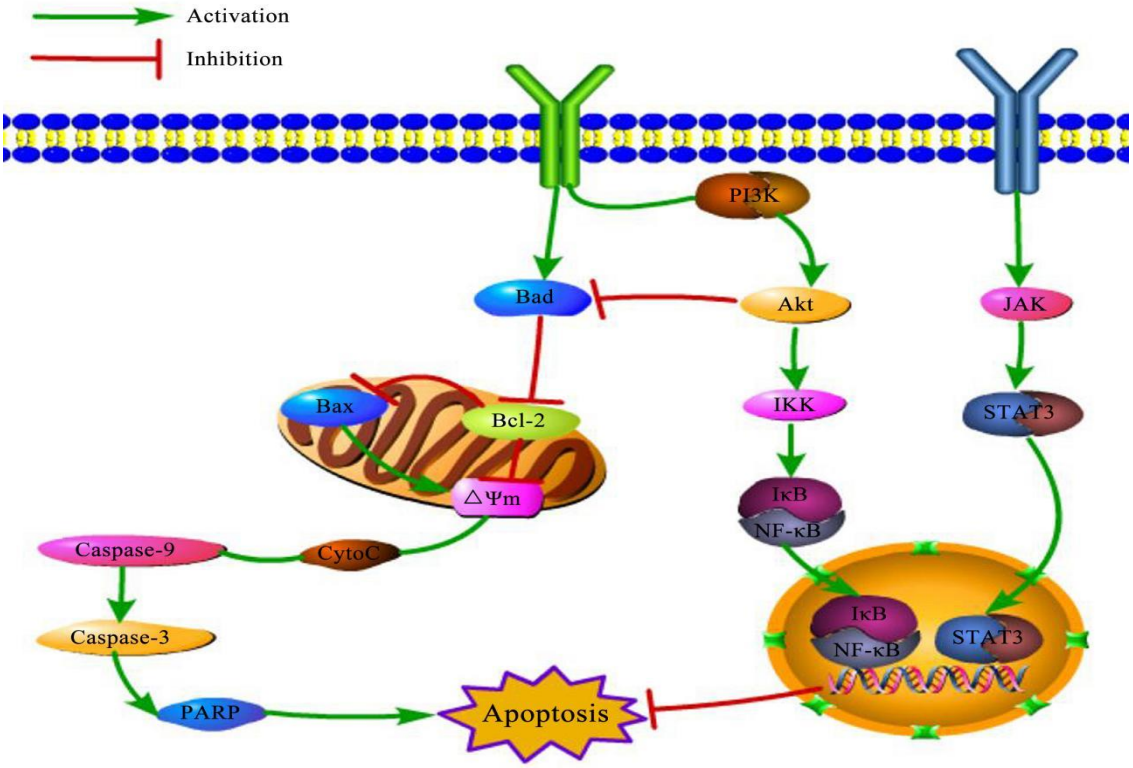

Figure 10: The mechanism of DOML exerts its efficacy in liver cancer cells

DISCUSSION

Natural products play an important role in the treatment of cancer and the development of drugs. About 57% of anticancer drugs in clinical application are directly or indirectly derived from natural products [18]. As a traditional Chinese medicine, Ludwigia hyssop folia is currently only used in a few areas and is not widely used in clinical applications. Moreover, the previous literatures mostly focused on the research of chemical composition, and few studies combined with pharmacological effects. In our initial research, we screened several parts of Ludwigia hyssop folia and found that DOML showed strong cytotoxic activity on HCC cells [12]. It shows that the application of Ludwigia hyssop folia in medicine has a lot of research space.

In our experiments, we found that DOML can effectively inhibit the proliferation of human liver cancer HepG2 and HuH-7 cells. In the wound healing assay, compared with the DOML-untreated group, the wound surface area of the DOML treatment group also has poor recovery ability, which can effectively inhibit the metastasis of cancer cells. As demonstrated by the above results, DOML has very good antitumor activity.

The uncontrolled regulation of liver cell proliferation and the inactivation of apoptosis mechanisms, leading to the unlimited growth of tumours are the main factors of carcinogenesis, so inhibiting the growth of hepatocytes and inducing apoptosis is one of the effective treatment strategies for liver cancer. The results showed that the cell morphology changed significantly after administration. It can be seen that HCC cells have irregular edges, reduced volume, decreased adhesion, concentrated chromatin, and bright spots (withered Dead body). Annexin V-FITC/PI dual staining also confirmed that DOML can induce apoptosis in HCC cells.

In addition to apoptosis, there are many ways of cell death such as autophagy, necrosis, necroptosis, etc. Autophagy is an intracellular degradation pathway regulated by genes and evolutionarily conserved [19]. Necroptosis, which is a form of death that is both regulated by death signals and has necrotic-like structural features [20]. Compared with apoptosis, necroptosis does not form apoptotic bodies and chromatin does not condense. Compared with necrosis, necroptosis is regulated by multiple genes and is a regular way of cell death. In this experiment, we used autophagy inhibitors and necroptosis inhibitors to explore whether autophagy or necroptosis participated in DOML-induced HepG2 death, the results showed that the inhibitory effect of DOML on HepG2 was not reduced after the inhibitor was used, and the inhibitory effect was aggravated after the necroptosis pathway was inhibited. So, for the time being we speculate that the death of HepG2 caused by DOML is not caused by autophagy and necroptosis.

Anticancer drugs can induce tumour cell apoptosis through multiple signalling pathways [21]. Mitochondria-mediated endogenous apoptotic pathway is the main pathway for programmed cell death in mammals. In this research, the possible molecular mechanisms of DOML against liver cancer were explored. And the expression of key proteins in DOML-treated HepG2 and HuH-7 cells at different concentrations were detected by Western blotting. The results displayed that DOML decreased the expression of Caspase-9 and Caspase-3 with increasing dose, increased the expression of cleaved PARP, and meanwhile, decreased the ratio of Bcl-2/Bax. In addition, the JC-1 results of HepG2 cells showed that mitochondrial membrane potential also decreased with increasing dose. Therefore, we speculated that DOML may induce apoptosis in HepG2 cells through the mitochondrial pathway.

In the mitochondrial pathway, the release of cytochrome C [22]. an active substance related to apoptosis in the mitochondria, from the mitochondrial outer membrane (MOMP) is also a key link, which is mainly regulated by Bcl-2 family proteins. Among the members of the Bcl-2 family, there are proteins that inhibit apoptosis, such as Bcl-2, Bcl-XL, and there are also proteins that promote apoptosis, such as Bid, Bax, Bak, etc [23]. Cytochrome C can form apoptotic bodies with Caspase-9, Caspase-9 self-shear activation [24]. activate Caspase-3 and other members in the presence of d ATP and ATP, making apoptosis progress go on. Therefore, we hypothesized that when cells are stimulated by DOML, they will cause the release of cytochrome C leading to a decrease in mitochondrial membrane potential.

The Akt signalling pathway is one of the important signal transduction pathways in cells. Akt activated by PI3K can activate or inhibit its downstream target proteins Bad, Caspase-9, NF-κB, etc. through phosphorylation, and is an important anti-apoptotic regulator [25]. NF-κB is the most important nuclear transcription factor that mediates intracellular signal transmission. In most cells, NF-κB directly binds to its inhibitory protein IκB in the form of homodimers or heterotrimers to form trimers. The complex exists in many types of cells in an inactive form. When the cell is stimulated, there is a signal to induce activation of IκB kinase (IKK), causing the degradation of IκB, allowing NF-κB to quickly enter the nucleus and contact with DNA, thereby regulating the expression of downstream genes. By inducing the anti-apoptotic factor Bcl-2 protein family, starting the X-chromosome linked inhibitor of apoptosis protein (XIAP) promotes the activation of the oncogene c-Myc, and then develops resistance to apoptosis information, and is closely related to the occurrence and development of tumors [26]. The results of Western blot are consistent with our speculation that DOML can induce HCC cells apoptosis by inhibiting Akt/NF-κB signalling pathway.

STAT3 can be activated by different growth factors, hormones, and cytokines. When activated, it forms a dimer, subsequently, translocate to the nucleus to bind to specific DNA as a transcription factor, and directly acts on the promoter of the downstream target gene Surviving to exercise gene transcription regulation, and promote the activation of Surviving [27,28]. Activated Surviving directly inhibits the most important link in cell apoptosis, namely the Caspase cascade and the release of Cyt-c, which hinders the process of cell apoptosis [29].

DOML is a macrolide compound. Macrolide drugs are usually applied in anti-inflammatory and antibacterial, and still have a lot of research space in anti-cancer. Macrolide compounds have unique structures and novel mechanisms of action, and have considerable potential in the development of new drugs and clinical applications [30].

Our current pharmacological research shows that DOML extracted from the traditional Chinese herb Ludwigia hyssop folia has the ability to inhibit the migration of HCC cells and induce its apoptosis, but has little toxicity to normal liver cells. In terms of mechanism, we have now concluded that DOML induces apoptosis in liver cancer HepG2 cells by activating mitochondrial apoptosis signalling pathway. Our results provide evidence that DOML may be used as a potential NF-κB, Akt and STAT3 inhibitor in targeted chemotherapeutic drug development.

CONCLUSION

In our study, de-*O*-methyllasiodiplodin induced human hepatoma cells apoptosis *in vitro* through mitochondrial apoptosis, Akt/NF-κB and STAT3 pathways, and it can be regarded as a potential anti-HCC agent.

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Author Contributions

Yu Peng and Yuruo Gong: conceptualization, data curation, formal

Analysis, methodology and writing – original draft; Congwei Wang and Dujuan Shi: investigation and resources; Jinyan Zhang: the plant material and performed the chemical experiments; Wei Kevin Zhang: validation and software; Xiaojun Li and Xinzhou Yang: funding acquisition, project administration, supervision and writing – review & editing.

Conflict of Interest

None declared.

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