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GRAPHICAL ABSTRACT
Juglone induces apoptosis and autophagy via modulation of mitogen-activated protein kinase pathways in human hepatocellular carcinoma cells

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Abstract

Juglone (JG), a naturally-occurring naphthoquinone of Manchurian walnut (*Juglans mandshurica*) was shown to inhibit proliferation in various tumor types. However, the molecular mechanisms of JG on the induction of apoptosis and autophagy in HepG2 cells have not been examined. Herein, we investigated that JG could inhibit cell proliferation by induction of G2/M phase arrest. Also, occurrence of apoptosis was closely related with loss of mitochondrial membrane potential, the changes of apoptosis-related proteins after treatment with JG. In addition, we found that JG caused autophagy, as evidenced by increased expressions of LC3-II and Beclin-1. Interestingly, inhibition of JG-induced autophagy by 3-methyladenine (3-MA) and wortmannin (WT) significantly decreased apoptosis, whereas the apoptosis inhibitor z-VAD-fmk slightly enhanced autophagy. Furthermore, the induction of autophagy and apoptosis was associated with activation of MAPK family members (p38 and JNK) and production of reactive oxygen species (ROS). Both JNK inhibitor (SP600125) and ROS scavenger (N-acetylcysteine, NAC) could attenuate JG-induced autophagy and apoptosis. However, the p38-specific inhibitor SB203580 enhanced autophagic and apoptotic death. Moreover, the ROS scavenger NAC prevented phosphorylation of both p38 and JNK. Collectively, our data revealed that JG induced G2/M phase arrest, apoptosis, and autophagy through the ROS-dependent signaling pathway.

*Keyword*: Juglone, Human hepatocellular carcinoma cell, reactive oxygen species, apoptosis, autophagy, MAPK
1. Introduction

Hepatocellular carcinoma is a disastrously malignant neoplasm and has been the third leading cause of cancer death in the world [1]. Nowadays, there are various therapeutic approach including chemotherapy, intervening therapy and surgical interventions [2]. Cumulative evidences have indicated that traditional Chinese medicines seem to have great potential in prevention and treatment of tumor, which attracts more and more interests in the world [3,4]. *Juglans mandshurica Maxim*, widely distributed from Europe and Asia, has been used as a traditional medicine for treatment of cancer, especially in China and Korea [5]. As a major bioactive component of naphthoquinones, juglone (5-hydroxy-1, 4-naphthoquinone) is seperated from the roots, leaves, and fruits of *J. manshurica Maxim* and has been demonstrated to inhibit proliferation of a variety of tumor cells through the activation of apoptotic caspase cascade and ROS production, such as human gastric cancer cell (SGC-7901), human leukemia cell (HL-60) and human colon carcinoma cell (HCT-15) [6]. Juglone was also proven to inhibit normal fibroblastcell proliferation through the degradation of p53 [7]. Recently, juglone had been found to induce apoptosis through different mechanisms, in C6 rat glioma cells *via* reducing the invasiveness [8], in cervical cancer Hela cells *via* c-Jun N-terminal kinase/c-Jun pathway [9], and in Glioma stem-like cells *via* ROS-p38 pathway [10]. Additionally, juglone could potentiate TRAIL-induced melanoma cells apoptosis, which was partially mediated through the ROS-p38-p53 pathway [11].

According to morphological distinctive forms of cells, there are two major types of programmed cell death (PCD), apoptosis and autophagy [12]. Apoptosis, as the Type-1 form of PCD, is regulated by numerous processes, and leads to a series of specific morphological changes including cell membrane blebbing, cell shrinkage,
chromatin condensation and DNA fragmentation [13]. In addition, apoptotic cell death is tightly dependent on activation of caspases, which plays an important role in chemotherapy of various tumors [14]. Autophagy is an evolutionarily conserved cellular degradation system that sequesters unfolded proteins and/or unnecessary cellular contents into lysosomes for degradation, which is important for maintaining a normal physiological state of cells [15]. During autophagy, with the extension and elongation of double membrane vesicles, autophagosomes are formed firstly. Subsequently, the fusion of autophagosomes and lysosomes leads to the formation of the autophagosome where damaged cellular components are degraded [16]. Recent accumulating evidences indicate that autophagy could act as a double-edged sword. In some cellular settings, it provides nutrients and energy, and prevents cell death from starvation or stress. Conversely, it can also enhance the therapeutic effect by collaborating with apoptosis or as a back-up mechanism to accelerate the death or type II cell death [17]. Currently, the functional relationship between apoptosis and autophagy remain complex and controversial. Therefore, further researches are required for the interconnection of apoptosis and autophagy, which may be promising for cancer therapy.

ROS, as active molecules of oxygen, is a by-product of cell metabolism including the superoxide anion ($O_2^{−}$), the hydroxyl radical (HO·) and hydrogen peroxide ($H_2O_2$) [18]. At normal physiological conditions, the generation and elimination of ROS keep homeostasis in the body. However, the excessive generation of ROS in response to anti-tumor drugs causes cellular damage to the lipids, proteins and DNA [19]. Moreover, ROS plays a critical role in induction of both apoptotic and autophagic cell death [20], through the activation of the mitogen-activated protein kinases (MAPKs) including JNK, p38 and ERK1/2 [21]. JNK, a stress-activated protein kinase, often
involves in a plethora of cellular events including apoptosis and autophagy in response to oxidative stress [22]. Studies have demonstrated that p38 MAPK is associated with apoptosis, cell cycle arrest, growth inhibition and differentiation. Besides regulating apoptosis, p38 MAPK is crucial to mediation of autophagy in response to chemotherapeutic substances [23]. Overwhelming evidence has unequivocally unraveled that ERK also contributes to the regulation of apoptosis and autophagy [24]. However, the role of ROS induced JG in autophagy and apoptosis and the interaction between autophagy and apoptosis in HepG2 cells remain unclear. Hence, the aim of present study was to examine the inhibitory effect of JG on HepG2 cells in vitro, and elucidate the molecular mechanisms of crosstalk between apoptosis and autophagy in JG-induced cell death. Overall, our data provided a foundation for further researches on JG for HCC treatment.
2. Materials and methods

2.1. Reagents and antibodies

Juglone (purity \( \geq 98\% \)) was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). A 10 mM stock solution of JG was prepared in dimethyl sulfoxide (DMSO) and stored at \( -80 \, ^\circ\text{C} \).

Polyclonal antibodies against cleaved caspase-3, poly (ADPribose) polymerase (PARP), LC3B, p21, JNK, phospho-JNK (Thr183/Tyr185), p38 MAPK, phospho-p38 MAPK (Thr180/Tyr182), p42/p44 MAPK, phospho- p42/44 MAPK (Thr202/Tyr204), Actin and inhibitor compounds z-VAD-fmk (a caspase inhibitor), U0126 (ERK inhibitor), SP600125 (JNK inhibitor), SB203580 (p38 MAPK inhibitor) and Ad-GFP-LC3B were purchased from Beyotime Institute of Biotechnology (Beijing, China). Antibodies against phospho-Cdc2, Cdc2, phospho-Cdc25C, Cdc25C, cyclin B1, phospho-Chk2, and Chk2 were purchased from Cell Signaling Technology (Beverly, MA, USA). Dimethyl sulfoxide (DMSO), N-acetyl-L-cysteine (NAC), Wortmannin (WT) and MTT were obtained from Sigma (St. Louis, MO, USA). Other reagents and chemicals were purchased from Beijing Chemical Reagents Co. (Beijing, China). Secondary antibodies were obtained from Beyotime Institute of Biotechnology (Beijing, China). Deionized water was purified by a Milli Q Water Purification system from Millipore (Millipore Corp., Bedford, MA). Polyvinylidene fluoride (PVDF) membrane was purchased from Millipore (Millipore Corp., Bedford, MA).

2.2. Cell culture and proliferation assay

Human hepatoma HepG2 cells were purchased from the CBCAS (Cell Bank of the Chinese Academic of Sciences, Shanghai, PR China) and the normal human cell
lines HL-7702 cells was generous gifts by Professor GC Sui. The cells were cultured in DMEM (Invitrogen) containing 10% (v/v) fetal bovine serum (Hyclone) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) (Hyclone) at 37 °C in a humidified 5% CO₂ incubator.

The cell viability of HepG2 and L02 was measured via MTT assay. Cells were plated into 96-well plates (5 × 10³ cells/well) 24 h before treatment. Then, cells were treated with various concentrations of JG for 12, 24 or 48 h, followed by incubating with 5 mg/ml of MTT working solution for 4 h at 37 °C. After added 100 µl of DMSO to dissolve the crystals, the absorbance of each well at 570 nm was measured. Three replicates were carried out for each of the different treatments.

2.3. Colony-formation assay

Cells (500 cells/well) were seeded in 6-well plates and allowed to grow for 24 h. After attachment, cells were pretreated with different concentrations of JG for 24 h at 37 °C. Thereafter, the medium was discarded and incubated for an additional 14 days in complete medium. After that, cells were fixed with 4% paraformaldehyde for 15 min, washed with PBS, and stained with crystal violet for 10 min. Finally, the dye was washed with PBS. The clones with more than 50 cells were counted under an ordinary optical microscope.

2.4. Flow cytometric analysis

The intracellular accumulation of ROS, mitochondrial membrane potential and apoptosis rate were measured by flow cytometric as previously described [25,26].

2.5. Cell cycle analysis

For cell cycle analysis, HepG2 cells (1 × 10⁶ cells/well) were seeded in six-well
plate for 24 h at 37 °C. Then, cells were exposed to JG (0, 10, 20 and 30 µM) for 24 h. After treatment, cells were centrifuged and washed with PBS followed by suspension in 800 µL PBS, 200 µL CyStain (Partec GmbH, Germany). The cell cycle arrest was measured with flow cytometry and analyzed by FloMax software.

2.6. Nuclear staining with Hoechst 33258

DNA condensation and nuclear fragmentation was determined by the method as described [26].

2.7. Western blotting

Proteins were extracted and immunoblotted as previously described [25].

2.8. Evaluation of fluorescent LC3 puncta

HepG2 cells were seeded in 6-well plates (NEST) and grown to 50%-60% confluence, then cells were infected with Ad-GFP-LC3B at 10 MOI and incubated in medium containing 2% FBS for 12 h. Next, the medium were replaced by complete culture medium. After 24 h, cells were treated with or without different concentrations of JG for different times. Immunofluorescence images were obtained by Cytation 5 Cell Imaging Multi-Mode Reader (Biotek, USA).

2.9. DNA fragmentation assay

DNA fragmentation was measured by agarose gel electrophoresis after JG treatment (0–30 µM) according to the manufacturer's protocol (Beyotime Institute of Biotechnology, China) [25].

2.10. Monodansylcadaverine & Acridine Orange staining

For monodansylcadaverine (MDC) staining, after treatment with various
concentrations of JG for indicated time, cells were washed with ice-cold PBS, and incubated with 50 µM of MDC at 37 °C for 20 min. The stained cells were washed and immediately analyzed under a fluorescence microscope. For Acridine Orange (AO) staining, after designated treatments, cells were washed with PBS and stained by 1 µg·mL⁻¹ AO in PBS at 37°C for 15 min. Then cells were washed three times with PBS and visualized with a fluorescence microscope.

2.11. Statistical analysis

All results were expressed as mean values ± standard deviation (n = 3). Differences between groups were calculated by one-way ANOVA. An analysis of ANOVA variance with a Tukey post hoc test was used for multiple comparisons. All statistics were calculated using the STATISTICA program (StatSoft, Tulsa, OK). Correlations were calculated using the ReglinP function and inverted Student’s t test. p < 0.05 was considered as statistically significant.
3. Results

3.1. JG inhibited the proliferation of HepG2 and triggered cell cycle arrest at the G2/M phase.

In order to investigate whether JG could affect cell growth, MTT assay was used. As shown in Fig. 1A and S1A, we found that JG significantly decreased the cell viability in a time and dose-dependent manner. The IC\textsubscript{50} values of JG were 44.36 µM (12 h), 29.04 µM (24 h) and 20.43 µM (48 h) for HepG2 cells, respectively. However, JG exhibited little toxicity to the normal human hepatic (L02) cells compared with HepG2 cells. In addition, colony-formation assay demonstrated that the number of colonies treated by JG was significantly reduced when compared with untreated cells (Fig. 1B and S1B). These results indicated that JG had a strong ability to inhibit the HepG2 cells proliferation.

To further verify the relationship between cell cycle and cell proliferation inhibition, we performed flow cytometry analysis to examine the effect of JG on cell cycle distribution. As shown in Fig. 1C, exposure of HepG2 cells to JG at concentrations ranging from 10 to 30 µM for 24 h led to the accumulation at G2/M phase, accompanied with the decreased radio of G0/G1 phase. Furthermore, the expressions of cell cycle-regulated proteins were monitored by western blotting. As shown in Fig. 1D and S1C, Treatment with JG increased the protein levels of Chk2, phospho-Chk2, phospho-Cdc2, p21 and phospho-Cdc25C, while decreasing the protein level of Cdc2. The amount of Cyclin B1 was slightly lower at concentrations of 20 and 30 µM. The above data indicated that JG was involved in G2/M phase
arrest by altering G2/M cell cycle specific regulator.

3.2. JG induced apoptosis of HepG2 cells

Considering cell cycle arrest will more likely result in cell apoptosis. Therefore, apoptosis induced by JG was initially investigated through Hoechst 33258 staining. As shown in Fig. 2A and S2A, condensed and fragmented nuclei were gradually increased after treatment with JG for 24 h, whereas untreated cells did not appear. Consistent with above findings, flow cytometric analysis indicated that the number of late apoptotic cells was significantly increased after treatment with JG in a dose-dependent manner (Fig. 2B and S2B). Next, we found that mitochondrial membrane potential (MMP) was remarkably altered in dose- and time- dependent manner after JG treatment (Fig. 2C and S2C). To elucidate whether apoptosis was induced through the mitochondrial signaling pathway, we investigated the expression of apoptosis-related proteins by western blotting. As shown in Fig. 2D and S2D, JG caused a dramatic decrease in the protein level of Bcl-2, while Bax, cleaved-PARP and cleaved-caspase 3 protein levels were significantly increased. In order to further clarify above findings, cells were pretreated with z-VAD-fmk (a pan caspase inhibitor) in the following experiments. We found that z-VAD-fmk could remarkably block JG-induced loss of viability and apoptosis (Fig. 2E, S2E and S2F).

3.3. JG triggered autophagy in HepG2 cells.

As z-VAD-fmk could only reduce part of the cell death caused by JG, recent several studies have shown that autophagy contributes to cell death in a caspase-independent model [27], we next determined whether autophagy was
induced in HepG2 cells after JG treatment. As shown in Fig. 3A and S3A, JG treatment led to a significant increase in GFP-LC3 puncta formation and the accumulation of autophagic vacuoles labeled by AO and MDC in both dose- and time-dependent manner compared with untreated cells. Moreover, western blotting results also confirmed that an increasing level of LC3B-II protein was observed in a concentration- and time- dependent manner after JG treatment (Fig. 3B and S3B).

3.4. Autophagy contributed to JG-induced cell apoptosis

Autophagy is a quite complex mechanism in the regulation of physiological and pathophysiological processes [17]. Besides that, the interconnection between apoptosis and autophagy is ambiguous. First of all, 3-methyladenine (3-MA), an inhibitor of autophagy, was used in the following experiments. As shown in Fig. 4A and S4A, treatment with 3-MA significantly abolished AO-labeled AVOs, MDC-labeled AVs and GFP-LC3 puncta formation. Moreover, JG-induced apoptotic effects were also blocked in the presence of 3-MA and WT compared to JG treatment alone (Fig. 4B, C and S4B, C). Subsequently, we investigated the effect of apoptosis inhibition on autophagy. Inhibition of apoptosis by z-VAD-fmk resulted in a slight increase of GFP-LC3 puncta (Fig. 4A and S4A). In addition, western blotting analysis also demonstrated that suppression of apoptosis by z-VAD-fmk led to a slight enhancement of LC3B-II expression (Fig. 4D and S4D). To further confirm that coactivation of autophagy and apoptosis was involved in JG-induced cell death, cell viability was analyzed in presence of autophagy and apoptosis inhibitors. Interestingly, JG-induced cell death was almost entirely recovered after
combination of z-VAD-fmk and 3-MA (Fig. 4E). Thus, our data indicated that autophagy inhibition decreased cell death, while inhibition of apoptosis might enhance autophagy.

3.5. JG-induced autophagy was partially attributed to the activation of p38 MAPK and JNK pathways

Recently, a series of studies have shown that MAPKs pathways are involved in regulation of autophagy and apoptosis [21-24]. Therefore, western blotting was used to analyze the expression of MAPKs, including ERK1/2, JNK and p38 MAPK. Fig. 5A and S5A revealed that an increase in phosphorylation of p38 MAPK and JNK was found in the dose- and time-dependent manner after JG treatment, but not ERK1/2. To further verify whether these pathways were involved in JG -induced autophagy and apoptosis, selective inhibitors U0126 (an upstream inhibitor of ERK1/2), SP600125 (a JNK inhibitor) or SB203580 (a p38 MAPK inhibitor) were used in subsequent experiments. As shown in Fig. 5B, C and S5B, the loss of cell viability and apoptosis were blocked after pretreatment with SP600125, while these were dramatically increased in the presence of SB203580. In addition, western blot analysis demonstrated that SB203580 not only up-regulated the expression of cleaved-PARP, cleaved caspase-3, LC3-II and Beclin-1, but also enhanced the accumulation of GFP-LC3 puncta and formation of AVOs and AVs. However, pretreatment with SP600125 only suppressed LC3-II, Beclin-1 expression, GFP-LC3 puncta accumulation, AVOs and AVs formation, which had no effects on the cleaved levels of both PARP and caspase-3 (Fig. 5D, E and S5C, D).
3.6. ROS was involved in JG-induced apoptosis and autophagy

Several reports had provided that JG induced apoptosis in various cell lines through generation of reactive oxygen species (ROS) [6]. Therefore, the level of ROS was measured by flow cytometry. As showed in Fig. 6A and S6A, ROS generation was obviously accumulated in time- and dose-dependent manner when cells were treated with JG. In order to confirm whether ROS plays any roles in JG-induced autophagy and apoptosis, the antioxidant N-acetylcysteine (NAC) was used. Pre-treatment with NAC effectively suppressed JG-induced generation of ROS and reduction of MMP (Fig. 6B and S6B, 6C). Then, MTT assay and Annexin -FITC/PI assay illustrated that NAC could remarkably attenuate cell death caused by JG (Fig. 6C and S6D), which were consistent with the results obtained from western blot (Fig. 6D and S6E). Furthermore, we examined the effect of ROS on JG-induced autophagy. Addition of NAC almost abrogated the JG-induced AVOs and AVs formation, GFP-LC3 puncta accumulation, and LC3-II expression (Fig. 6D, E and S6E). Additionally, the phosphorylation of p38 MAPK and JNK also was attenuated in the presence of NAC (Fig. 6F and SG).
4. Discussion

Hepatocellular carcinoma is the most common type of liver cancer in both developed and developing countries. Natural products have long been a fertile source for cancer and more than 1000 plants were found to possess significant anticancer properties treatment drugs [28]. JG, a naturally-occurring compound of the traditional Chinese medicine herb, has been reported to reduce the proliferation of a variety of tumor cells. In the present study, our data revealed that JG could significantly suppress the growth of HepG2 cells through induction of G2/M phase arrest and apoptosis. Moreover, it was the first to found that JG was also able to cause autophagy, which contributed to JG-induced cell death, through activating ROS-mediated p38 and JNK pathway.

Cell cycle regulation plays a key role in cell proliferation, which requires a range of cyclin-dependent kinases (CDKs) or proteins involved. It has been reported that natural agents are able to cause the imbalance of cell cycle progression at a specific checkpoint such as G0-, S-, or G2/M-phase arrest [29, 30]. Cdc2 interacting with cyclin B, which is suppressed by the phosphorylation of cdc2, usually regulates the transition from G2 to M phase [31]. For the onset of mitosis, Cdc25C is known as a dual specificity phosphatase, activated for de-phosphorylation of Cdc2. Chk1 and Chk2, the checkpoint kinases, could activate Cdc25C, suppressing Cdc25C activity [32]. Our study indicated that JG caused G2/M phase arrest via upregulation of phospho-Chk2, Chk2, phospho-Cdc25C, phospho-Cdc2, p21 and downregulation of Cdc2, Chk2. Similar phenomena were also observed with plumbagin, another
naphthoquinone from *Plumbago zeylanica* L., which induced G2/M in breast cancer cells [33].

Apoptosis is known as a programmed cell death process, which plays a central role in regulating proliferation of cancer cells and formation of tumor in response to pro-apoptotic stimuli, including chemotherapy, γ-irradiation and so on [34,35]. Previous studies showed that loss MMP played an important role in JG-induced cell apoptosis [36, 37]; Ji et al pointed that JG could potently inhibit the growth and induce apoptosis of SGC-7901 cells by the activation of the mitochondrial death pathway [36]. To human leukemia cell HL-60, JG induced apoptosis by releasing of Cyt c, Smac and AIF from mitochondria and elevating the ratio of Bax/Bcl-2 [37]. In the current study, we found that JG could significantly reduce MMP in a concentration- and time-dependent manner. Moreover, the flow cytometric analysis of Annexin V/PI double staining cells demonstrated an obvious increase in apoptosis proportion in JG-treated HepG2 cells. Recently, it has been shown that JG inhibited the growth of TSCs in gliomas through the activation of ROS-p38-MAPK pathway *in vitro* [10]. Lu et al stated that JG regulated the expression of Bax, CytC, Fas, FasL and Caspase-3 by activating JNK/c-Jun pathway, thus inducing apoptosis of cervical cancer cells [9]. However, our data showed JG-induced apoptosis was dependent on the activations of PARP and caspase-3 and the changes of Bcl-2 and Bax protein expression. Interestingly, the pan-caspase inhibitor z-VAD-fmk dramatically blocked JG-induced cell death, but not entirely prevented the cell death, suggesting that other caspase-independent pathways involved in JG-induced apoptosis.
Autophagy, an evolutionarily conserved intracellular degradation mechanism, has been a novel therapeutic target for chemotherapy drugs in treatment of various cancers. Recent a growing body of studies has been reported that various natural compounds have the capability to induce autophagy. Some important examples are resveratrol [38], quercetin [39] and plumbagin [40], all of which have been shown to induce autophagy in various cancer cells. To the best of our knowledge, this study is the first to demonstrate that JG induces autophagy in HepG2 cells. In this study, we found an enhanced formation of AVs and AVOs, increased accumulation of GFP-LC3 puncta and upregulated expression of LC3-II, implying the occurrence of autophagy in the HepG2 cells after JG treatment. Both apoptosis and autophagy are essential cellular degradation pathways that are often induced by similar stimuli and regulated by similar pathways [17]. However, the effects of autophagy, pro-survival or pro-death, are controversial in cancer. For example, quercetin can induce a pro-survival autophagy in esophageal squamous carcinoma cells and gastric cancer cells [39], whereas resveratrol or plumbagin induces an autophagic cell death chronic myelogenous leukemia cells or in human non-small cell lung cancer cells, respectively [38]. In summary, autophagy and apoptosis can cooperate, antagonize, or assist each other to differentially influence the fate of the cell. In the present study, inhibition of autophagy by pre-treatment with 3-MA and WT diminished JG-induced cell death. Intriguingly, the pharmacological inhibition of both autophagy and apoptosis by combination of 3-MA and z-VAD-fmk markedly decreased JG-induced cell death, suggesting JG-induced autophagy was a pro-apoptotic
Recently, there is accumulating evidence revealing that ROS, especially through the mitochondria, not only play critical roles in induction of apoptosis but are also as the potent regulators for autophagy [41]. Consistent with most of previous studies, our results showed that both the autophagic markers (Beclin-1 and LC3-II) and apoptotic markers (caspase-3 and PARP) were suppressed by the ROS inhibitor NAC in JG-treated cells. Accumulating evidence has indicated that autophagic cell death is triggered by the MAPKs [16, 42]. Additionally, recent studies have suggested that JG could activate ERK1/2, p38 MAPK, and JNK1/2 [9, 10, 11, 43]. In HepG2 cells, we found that treatment with JG resulted in activation of JNK and p38, but did not change ERK phosphorylation. Inhibition of p38 MAPK with SB203580 notably promoted JG-induced autophagy and apoptosis. However, inhibition of JNK with SP600125 only impaired JG-induced autophagy. ROS is involved in activation of the downstream signaling pathways, such as PI3K/Akt and MAPK [44, 45]. Therefore, we presume that ROS production may be responsible for the activation of MAPK upon JG treatment. The results showed that pre-treatment with NAC prevented JG-induced phosphorylation of JNK and p38 MAPK, suggesting that induction of ROS might activate JNK and p38 MAPK, and lead to apoptosis and autophagy in HepG2 cells.

In conclusion, our study, for the first time, demonstrated that JG could effectively suppress viability of HepG2 cells, induce cell cycle arrest at G2/M phase, and subsequently cause apoptotic and autophagic cell death. In addition, JG triggered
apoptosis and autophagy via the activation of ROS-mediated JNK and p38 signaling pathway. These findings contribute to the development a promising agent for the treatment of human HCC.
Conflict of Interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

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**Legends to Figures**

**Fig. 1.** Effect of JG on the proliferation and cell cycle distribution in the human hepatic cell line.

(A) HepG2 cells were treated with indicated concentrations of JG for 12 h, 24 h and 48 h, and the viability was measured by MTT assay. (B) Colony-formation assay was performed with and without JG treatment, and the colonies were counted. (C) The distribution of cell cycle was assessed by flow cytometry. The percentage of cell cycle distribution (mean ± S.D.) is from three replicate wells and representative of three separate experiments. (D) The expressions of cell cycle-regulated proteins were analyzed by western blot. Relative expression in the intensities of protein bands were measured by quantitative software. Data shown are mean ± SD (n=3), *p < 0.05 and **p < 0.01 vs control.

**Fig. 2.** Effect of JG on HepG2 cell apoptosis.

Cells were treated with JG (0, 10, 20, and 30 µM) for 24 h. (A) Apoptotic nuclear changes were stained with Hoechst 33258 and observed under a microscopy. (B) Cell apoptosis was analyzed by Annexin V-FITC/PI staining and flow cytometry. The histograms illustrated the percentage of cell apoptosis from three independent experiments. Cells were treated with JG (30 µM) for different hours or with indicated concentrations for 24 h. (C) The mitochondrial membrane potential was analyzed using fluorescent probe JC-1 and presented as bar diagrams, and (D) apoptosis-related proteins were determined by western blot, Actin was used as control. Relative expression in the intensities of protein bands were measured by quantitative software (E) HepG2 cells were pre-treated with z-VAD-fmk for 2 h before JG treatment for a further 24 h. Cell apoptosis was analyzed by flow
cytometry and presented as bar diagrams. Each bar represents the mean ± SD of three independent experiments. *p < 0.05 and **p < 0.01 vs control, #p < 0.05 and ##p < 0.01 versus JG treatment.

**Fig. 3.** Effect of JG on autophagy induction in HepG2 cells.

(A) HepG2 cells were infected with Ad-GFP-LC3B for 24 h, and then incubated with JG (30 µM) for different hours (Upper pane) or treated with various concentrations of JG for 24 h (Bottom panel). The cells were visualized under a fluorescent microscope, JG -treated cells displayed a pattern of GFP-LC3 expression, indicating formation of autophagosomes. (B) Cells were incubated with JG (20 µM) for indicated hours or treated with various concentrations of JG for 24 h. Autophagy-related proteins, LC3 and beclin1, were analyzed by western blot and presented as bar diagrams. Relative expression in the intensities of protein bands were measured by quantitative software. *p < 0.05 and **p < 0.01 vs control.

**Fig. 4.** Involvement of autophagy in JG -induced cell death.

HepG2 cells were infected with Ad-GFP-LC3B for 24 h, and then cells were pretreated with 3-MA or z-VAD-fmk for 2 h before JG treatment for a further 24 h. (A) GFP-LC3 puncta was assessed by fluorescence microscopy. (B) Cell viability was assessed by MTT assay. (C) Cell apoptosis was analyzed using Annexin V-FITC/PI by flow cytometry. The histograms indicate that apoptosis proportion from three separate experiments. (D) The expression of autophagy and apoptosis -regulated proteins was assessed by western blot and presented as bar diagrams. Actin was used as loading control. Relative expression in the intensities of protein
bands were measured by quantitative software. (E) Combination of 3-MA or z-VAD-fmk was added to cells 2 h before JG treatment. After 24 h, cell viability was determined. Data are presented as means ± SD (n=3). *p < 0.05 and **p < 0.01 vs control, #p < 0.05 and ##p < 0.01 versus JG treatment.

**Fig. 5.** Involvement of JNK1/2 and p38 MAPK in JG-induced autophagy and apoptosis.

(A) Cells were treated with various concentrations of JG for 24 h or incubated with JG (30 µM) for different hours. Levels of p-JNK, JNK, p-p38, p38, p-ERK1/2 and ERK1/2 were analyzed by western blotting. Cells were pretreated with U0126 (ERK1/2 inhibitor, 20 µM), SB203580 (p38 MAPK inhibitor, 20 µM), or SP600125 (JNK1/2 inhibitor, 40 µM) for 2 h followed by treatment with or without JG for 24 h. (B) Cell viability was measured by MTT assay. (C) The percentages of apoptotic cells were evaluated by flow cytometry. (D) GFP-LC3 puncta was assessed by fluorescence microscopy. (E) Apoptosis-related proteins (cleaved PARP and cleaved caspase-3), autophagy-related proteins (LC3 and Beclin1) and phospho-p38, phospho-JNK were analyzed by western blotting and presented as bar diagrams. Actin was used as loading control. Relative expression in the intensities of protein bands were measured by quantitative software. Values represent the mean ± SD of at least three independent experiments. *p < 0.05 and **p < 0.01 vs control, #p < 0.05 and ##p < 0.01 versus JG treatment.

**Fig. 6.** Roles of ROS production in JG-induced autophagy and apoptosis in HepG2 cells.
(A) Cells were treated with various concentrations of JG for 24 h or incubated with JG (30 µM) for different hours. The level of ROS was determined by flow cytometry. Quantitative analysis of ROS generation was shown in histograms. Cells were pretreated with NAC for 2 h, and then treated with JG (30 µM) for 24 h. (B) The mitochondrial membrane potential and (C) The percentages of apoptotic cells were evaluated by flow cytometry and presented as bar diagrams. (D) Autophagy and apoptosis-regulated proteins was assessed by western blot. Actin was used as loading control. (E) GFP-LC3 puncta was assessed by fluorescence microscopy. (F) HepG2 cells were pre-treated with 5 mM NAC for 2 h followed by treatment with JG. The expression of phospho-p38 and phospho-JNK was analyzed by western blot analysis. Actin was used as loading control. Relative expression in the intensities of protein bands were measured by quantitative software. The results were expressed as mean ± SD of three independent experiments. *p < 0.05 and **p < 0.01 vs control, #p < 0.05 and ##p < 0.01 versus JG treatment versus JG treatment.
Fig. 1.

(A) Cell viability (%) vs. Juglone (µM).

(B) Number of colonies vs. Juglone (µM).

(C) Cell cycle distribution (%) vs. Juglone (µM).
Fig. 2.

(A) Control
Juglone 10 μM
Juglone 20 μM
Juglone 30 μM

(B) Apoptosis rate (%) vs. Juglone (μM)

(C) FL1 count (%) vs. time
       vs. Juglone (μM)
Fig. 3.

(A) GFP-LC3

Control 3-MA z-VAD-fmk

Juglone Juglone+3-MA Juglone+z-VAD-fmk

(B) Relative expression levels (% of control)

LC-3 α Beclin-1

0 h 3 h 6 h 12 h 24 h

Relative expression levels (% of control)

LC-3 α Beclin-1

JG 0 μM JG 10 μM JG 20 μM JG 30 μM
Fig. 4.

(A) Control 3-MA z-VAD-fmk

GFP-LC3

Juglone Juglone+3-MA Juglone+z-VAD-fmk

(B) Cell viability (%)

0 10 20 30

Juglone (μM)

Juglone
Juglone+3-MA

(C) Apoptosis rate (%)

0 10 20 30 40

Juglone (30 μM)
3-MA (2.5 mM)
Fig. 5.

(A) Relative expression levels (% of control)

(B) Cell viability (%)

(C) Apoptosis rate (%)
Fig. 6.

(A) DCF fluorescence (%) against time (0 h, 3 h, 6 h, 12 h, 24 h) and juglone concentration (0, 10, 20, 30 μM).

(B) FL1 count (%) with juglone (30 μM) and NAC (5 mM) treatment combinations.

(C) Apoptosis rate (%) with juglone (30 μM) and NAC (5 mM) treatment combinations.
Highlights

(1) JG-induced ROS production caused oxidative damage to mitochondria and DNA.

(2) JG kills HepG2 cells through the induction of apoptosis.

(3) JG triggers autophagy, which contributes to JG-induced cell death.

(4) The autophagic cell death was dependent on ROS generation and the activation of p38 MAPK and JNK pathways.

(5) JG is a potential therapeutic agent for the treatment of HCC.