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# Juglone, from *Juglans mandshruica Maxim*, inhibits growth and induces apoptosis in human leukemia cell HL-60 through a reactive oxygen species-dependent mechanism

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ABSTRACT

Juglone, a major chemical constituent of *Juglans mandshruica Maxim,* is a promising anticancer agent that has shown a strong activity against cancer cells in vitro. Our previous study showed that juglone inhibited the proliferation of HL-60 cells with an IC50 value ~8 μM. To further explore the proapoptotic mechanism of juglone, we investigated the role of the reactive oxygen species (ROS) in the apoptosis induced by juglone in HL-60 cells. The generation of ROS was about 2 to 8-fold as compared to control cell after treatment with juglone (2, 4 and 8 μM) for 24 h. The glutathione (GSH) depletion was consistent with ROS generation after treatment with juglone. Reversal of apoptosis in antioxidants (NAC and catalase) pretreated cells indicated the involvement of ROS in juglone-induced apoptosis. The cleavage of PARP and procaspase-3 and -9, loss of mitochondrial membrane potential ($\triangle\Psi m$), and release of cytochrome c (Cyt c) and Smac induced by juglone were significantly blocked by NAC. NAC also prevented the inhibition the phosphorylation of Akt and mTOR proteins by juglone. Collectively, these results indicated that ROS played a significant role in the apoptosis induced by juglone in human leukemia cell HL-60.



## 1. Introduction

*Juglans manshurica Maxim* has been used in folk medicine for many years, particularly in China and India, and its therapeutic benefits have been ascribed to its content of naphtoquinones. Juglone (5-hydroxy-1,4-naphtoquinone), a naturally-occurring naphtoquinone, is collected from the roots, leaves, and bark of *J. manshurica Maxim*. It has multiple effects on cells, such as inducing DNA damage (Kamei et al., 1998; Wang et al., 2001), inhibiting transcription reduction of p53 protein levels (Chao et al., 2001) and inducing of cell death (Paulsen and Ljungman, 2005). Juglone can induce apoptosis through different mechanisms, in human gastric cancer SGC-7901 cells via the mitochondrial pathway (Ji et al., 2011), while in normal fibroblast cell through degradation of p53 (Chao et al., 2001). Additionally, it has been reported to possess anti-inflammatory, anti-viral, anti-bacterial, anti-fungal properties (Omar et al., 2000). Our previous results showed that juglone could induce apoptosis in HL-60 cells through a mitochondria-dependent pathway (Xu et al., 2010).

Reactive oxygen species (ROS), include superoxide anions ($O_2^-$), hydroxyl radicals (.OH), hydrogen peroxides ($H_2O_2$), and peroxynitrite ($ONOO^-$), those are normally generated by mitochondria and other intracellular molecules in all aerobes (Inoue et al., 2003; Zorov et al., 2006). ROS elicit diverse biological effects depending on the concentration of ROS in cells. At low concentration, ROS have been identified as a second messenger in signaling pathways and implicated in transcription regulation to promote cell proliferation. But at high concentration, ROS result in macromolecular damage, growth arrest, apoptosis, and cell death (Hussain et al., 2003; Martindale and Holbrook, 2002; Wang et al., 2004). Under normal conditions, ROS are cleared by antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase and thioredoxin and non-enzyme systems in cells. Most anticancer drugs act, at least in part, by producing reactive oxygen species (ROS) (Deeb et al., 2010; Gao et al., 2005; Ramanathan et al., 2005; Sun and Rigas, 2008). The generation of ROS has been recognized in an SGC-7901 cell treated with juglone (Ji et al., 2011). But, the role of ROS in the apoptosis induced by juglone in HL-60 cells remains ambiguous.

Hence, the aim of the present study is to investigate whether apoptosis induced by juglone in HL-60 cells is also mediated through ROS generation. Our data demonstrated that juglone induced intracellular ROS production in human leukemia cell HL-60, depleted GSH and caused oxidative stress. Pretreatment

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with antioxidants could block the annexin V-binding, cleavage of PARP and procaspase-3 and -9, loss of mitochondrial membrane potential and release of cytochrome *c* and Smac induced by juglone. NAC also prevented the inhibition of phosphorylation of AKT and mTOR proteins by juglone.

## 2. Materials and methods

### 2.1. Materials

Juglone, extracted from bark of *J. mandshruica Maxim*, was provided by Professor Yanping Chen (School of Chemistry, Jilin University). The purity of juglone used in the experiments was >95% as determined by HPLC. Juglone was applied in DMSO to 0.1 M and stored at −20 °C. The concentrations used here were freshly diluted with RPMI-1640 to final concentrations. Control was always treated with the same amount of DMSO (0.1%) as used in the corresponding experiments. Catalase was obtained from Sigma. Antibodies against pro-caspase-3, -9, PARP, AKT, p-AKT (Ser 473), mTOR, p-mTOR (Ser 2448), Cyt c and Smac were obtained from Cell Signaling Technology. Antibody β-actin was obtained from Tianjing Jingmai. NAC, GSH assay kit and Caspase-3 activity assay kit were obtained from Beyotime Institute of Biotechnology. Apoptosis kit was obtained from KeyGen Biotech Co., Ltd. RPMI-1640 medium and fetal calf serum was purchased from GIBCO.

### 2.2. Cell line and culture conditions

Human leukemic cell HL-60 was kindly provided by Dr. Li Wei (Department of Hematopathy, First Hospital, Jilin University). The cell was routinely cultured in RPMI-1640 medium, supplemented with 10% fetal calf serum. The culture was maintained at 37 °C with a gas mixture of 5% $CO_2$/95% air. All media were supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. The medium was changed every day. HL-60 cells of three to ten passages in the actively growing condition were used for experiments.

### 2.3. Measurement of ROS

The production of the reactive oxygen species (ROS) was monitored by nonfluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) as previously described (Deeb et al., 2010). DCFH-DA readily diffuses into the cell and is deacetylated by nonspecific esterases to yield nonfluorescent 2′,7′-dichlorofluorescein (DCFH). In the presence of cellular oxidizing agent, such as $H_2O_2$, DCFH is oxidized to the highly fluorescent compound dichlorofluorescein (DCF), which is trapped inside the cells (Wang and Joseph, 1999). Thus, the fluorescence intensity is proportional to the amount of ROS produced in the cells. Briefly, HL-60 cells ($5 \times 10^5$) were exposed to different concentrations of juglone for 24 h. After treatment, the cells were collected, washed twice with PBS and then incubated with DCFH-DA (10 μM) at 37 °C for 20 min in the dark. The fluorescence of DCF was detected by flowcytometry.

### 2.4. Measurement of GSH

The intracellular content of glutathione (GSH) was assessed using a GSH assay kit (Beyotime Institute of Biotechnology, China). DTNB (5,5′-dithiobis(2-nitrobenzoic acid)), known as Ellman's reagent, is developed for the detection of thiol compounds. Since DTNB and GSH react to generate the yellow-colored product, 2-nitro-5-thiobenzoic acid, GSH concentration can be determined by measuring the absorption at 412 nm. Briefly, HL-60 cells ($5 \times 10^5$) were exposed to different concentrations of juglone for 24 h. After treatment, cells were collected, and then centrifuged at 10,000*g* for 10 min. The supernatants were then added to a 96-well plate, and the assay was performed according to the manufacturer's instructions.

### 2.5. Measurement of cell viability

Cell viability was assessed by measuring their ability to metabolize 3-(4,5-dimethydiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Cheng et al., 2003). Briefly, HL-60 cells were collected and suspended in an RPMI-1640 medium at $1.2 \times 10^5$ cells/ml, and 100 μl aliquots were added to each well of 96-well flat-bottomed microtiter plates. HL-60 cells were pretreated with NAC (5 mM) for 1 h before being treated with juglone (8 μM) for 24 h in the presence or absence of NAC. Three replicate wells were used for each data point in the experiments. After incubation for the indicated intervals, 10 μl of MTT (Sigma, 5 mg/ml in PBS) solution were added to each well, and the plates were then incubated for another 4 h. The plates were centrifuged, and the supernatant was removed. Intracellular formazan crystals were dissolved by addition 100 μl of DMSO to each well, and the plates were shaken for 10 min. The absorption was measured at 570 nm with a microplate reader (SpectraMax Plus384, Molecular Devices, USA). Percentage survival was calculated as the fraction of the negative control.

### 2.6. Annexin V-FITC/PI staining

Apoptosis was determined by staining cells with Annexin V-FITC and PI labeling, because Annexin V could be used to identify the externalization of phosphatidylserine during the progression of apoptosis. This assay was modified from a previous report (Bharti et al., 2004). Briefly, HL-60 cells ($5 \times 10^5$) were pretreated with NAC (5 mM) or catalase (2000 U/ml) for 1 h before being treated with juglone (8 μM) for 24 h in the presence or absence of NAC or catalase. After treatment, the cells were collected and washed twice in ice cold PBS and suspended in 300 μl of binding buffer at $2 \times 10^5$ cells/ml. The samples were incubated with 5 μl of Annexin V-FITC and 5 μl propidium iodide in the dark for 15 min at room temperature. Finally, the samples were analyzed by flowcytometry and evaluated based on the percentage of cells for Annexin V positive.

### 2.7. Measurement of mitochondrial membrane potential ($\triangle\Psi m$)

Mitochondrial membrane potential ($\triangle\Psi$m) was measured by using the fluorescent dye, Rhodamine 123, a cell-permeable cationic dye that preferentially enters mitochondria based on the highly negative mitochondrial membrane potential. Depolarization of the membrane results in the loss of Rhodamine 123 from the mitochondria and a decrease in intracellular fluorescence in intracellular. Briefly, HL-60 cells ($5 \times 10^5$ cells) were pretreated with NAC (5 mM) for 1 h before being treated with juglone (8 μM) for 24 h in the presence or absence of NAC. After treatment, the cells were incubated with Rhodamine 123 (10 μg/ml) at 37 °C for 30 min, and the washed with PBS. The cell pellet was collected by centrifugation, and resuspended in 300 μl PBS. Fluorescence intensities of Rhodamine 123 in cells were analyzed by flow cytometry.

### 2.8. Caspase-3 activity assay

The activity of caspase-3 was determined using the Caspase-3 activity according the manufacturer's protocol. Briefly, HL-60 cells ($5 \times 10^5$ cells) were pretreated with NAC (5 mM) or catalse (2000 U/ml) for 1 h before being treated with juglone (8 μM) for 24 h in the presence or absence of NAC or catalase. After treatment, the cells were collected and rinsed with cold PBS. Cell lysates were prepared in a lysis buffer (100 μl) for 15 min on ice and then centrifuged at 18,000*g* for 10 min at 4 °C. The supernatants were collected and the total protein was quantified by the Bradford method. Assays were performed on 96-well microtitre plates by incubation of 20 μl protein of cell lysate per sample in 80 μl reaction buffer (1% NP-40, 20 mM Tris–HCl (PH 7.5), 137 mM Nad and 10% glycerol) containing 10 μl caspase-3 substrate (Ac-DEVD-pNA) (2 mM). Lysates were incubated at 37 °C for 2 h and the absorption at 405 nm was measured using a microplate reader (SpectraMax Plus384, Molecular Devices, USA). The results are represented as the percentage of change of activity compared to the untreated control.

### 2.9. Subcellular fractionation

Subcellular fraction from HL-60 cells was extracted using the mitochondrial/mytosol fractionation kit according to the manufacturer's instructions. Briefly, HL-60 cells were a pretreated with NAC (5 mM) for 1 h before being treated with juglone (8 μM) for different times in the presence or absence of NAC. After treatment, the cells were collected and suspended in a cytosol extraction buffer and incubated on ice. Then, the cells were homogenized using a tissue grinder on ice and centrifuged. The supernatant was the cytosolic fraction.

### 2.10. Western blotting

Western blot was performed for detection of Cyt *c*, Smac, procaspase-9, -3, PARP, Akt, p-Akt, mTOR and p-mTOR proteins. HL-60 cells ($5 \times 10^5$) were pretreated with NAC (5 mM) for 1 h before being treated with juglone (8 μM) for 24 h in the presence or absence of NAC. After treatment, the cells were harvested and lysed in RIPA buffer(150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl pH 7.4) for 30 min on ice. After centrifugation (13, 000*g* 4 °C, 15 min), the supernatants were loaded onto 12% polyacrylamide–SDS gel. Each lane was loaded with 40 μg of cell lysate protein. After electrophoresis, the gels were blotted onto a PVDF membrane, blocked with 5% (w/v) milk for 1 h on a shaker at room temperature, washed twice with TBS-T, and then probed overnight at 4 °C. Primary antibody binding was detected with anti-rabbit IgG conjugated to HRP, and visualized using ECL enhanced chemiluminescence.

### 2.11. Statistical analysis

The results are expressed as mean ± S.D. for three independent experiments. Statistical differences were evaluated using Student's test or one-way analysis of variance (ANOVA). *P*-values less than 0.05 were considered to be significant.