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Anti-proliferative and apoptosis-inducing activities of juglone in LS-174T cells

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Abstract

Anti-proliferative and apoptosis-inducing effects of juglone in LS-174T cells were investigated. In this study, we showed that juglone inhibited the proliferation of LS-174T cells in a time and dose-dependent manner, treatment of juglone resulted in the activation of caspase-9 and 3, decrease of Bcl-2. N-acetylcysteine significantly attenuate LS-174T cell death induced by juglone ($p < 0.001$). In addition, NAC could reverse caspase-3 and 9 activation, increase expression of Bcl-2 protein. Taken together, these findings indicated that juglone-mediated oxidative injury may act as upstream change, trigger ROS release, Bcl-2 modulation, caspase activation, and consequently leading cell apoptosis in LS-174T cells. In conclusion, these findings suggest that juglone may be an effective way for treating human cancers.

Introduction

Quinones represent a broad category of widely distributed quinoid compounds in nature. Many quinones have been associated with a range of biological activities, including anti-cancer activity (Babula et al., 2007). Juglone (5-hydroxy-1,4-naphtha-quinone) (Figure 1) is a quinone found in the roots, leaves and bark of walnut trees (Inbaraj et al., 2004; Varga et al., 1996).

The bark, branches and exocarp of the immature green fruit of walnut trees have been used to treat gastric cancer, liver cancer, lung cancer and other types of cancer for a long time in China (Liu et al., 2004). Juglone has cytotoxic properties when administered to cell cultures (Inbaraj et al., 2004; Kamei et al., 1998; Rippmann et al., 2000; Kiran et al., 2009; Ji et al., 2009.) and it possesses antiviral, antibacterial and antifungal properties (Clark et al., 2006; Inbaraj et al., 2004). In addition, juglone promotes generation of hydrogen peroxide (Inbaraj et al., 2004), block of K⁺ channel (Varga et al., 1996) and inhibition of transcription (Chao et al., 2001). Furthermore, benzobijuglone, a novel cytotoxic compound from *Juglans mandshurica*, could

induce apoptosis in HeLa cells (Li et al., 2007).

In this paper, we investigated the anti-proliferative and apoptosis-inducing effects of juglone in LS-174T cells. The work reported here is focused on which components of apoptosis pathway were involved in cell death, and whether reactive oxygen species (ROS) have a role in LS-174T cells death induced by juglone. Although some groups have studied the *in vitro* cytotoxic activity of juglone against cancer cell lines (Kamei et al., 1998; Segura-Aguilar et al., 1992), the exact mechanism remains doubtful. Therefore, we investigated the cytotoxic potential of juglone and its underlying mechanisms.

Materials and methods

Materials

Juglone was kindly gifted by Dr. Ma Zhiqiang. The purity of juglone was measured by HPLC and determined to be 97%. Juglone was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution. The concentration of DMSO was kept below 0.3% in all the cell



cultures and did not exert any detectable effect on cell growth. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33258, RNase A, Proteinase K and N-acetyl-L-cysteine (NAC) were purchased from Sigma Chemical (USA). Cell Death Detection ELISA^{PLUS} was purchased from Roche (Roche Molecular Biochemicals, Germany). Western blotting antibodies against caspase-3, 9 and alkaline phosphatase conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (USA). Antibody against Bcl-2 was purchased from Abcam (Cambridge, MA). Antibodies against PARP and caspase-3 colorimetric activity assay kit were purchased from Beyotime Institute of Biotechnology (Haimen, Jiangsu).

Cell culture

LS-174T, human colon adenocarcinoma cell line (ATCC CL-188), were cultured in RPMI-1640 medium (Hyclone, Logan, UT) supplemented with 10% heat inactivated (56°C, 30 min) fetal calf serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Zhejiang), 2 mM glutamine (Gibco, Grand Island, NY), and maintained at 37°C with 5% CO₂ in a humidified atmosphere.

Cell growth inhibition test

The inhibition of cultured cells was determined by MTT test (Rasu Azhar et al., 2012). In brief, after incubation with juglone (0-200 μM) for indicated time period, LS-174T cells (5 × 10⁴/well) in 96-well plate were washed once with PBS and MTT (20 μL of 5 mg/mL in PBS) was added to each well. The cells were further incubated at 37°C for 4 hours, and DMSO (150 μL) was added to dissolve the formazan crystals. Absorbance was measured at 490 nm with micoplate reader (BioRad 680, Hercules, CA).

Nuclear damage observed by Hoechst 33258 staining

After treated by 50 μM juglone for 36 hours, LS-174T cells were harvested by centrifugation at 1,000 × g for 5 min, washed two times with PBS and fixed with 3.7% paraformaldehyde at room temperature for 2 hours. The fixed cells were washed with PBS and stained with Hoechst 33258 solution for 10 min at room temperature, then observed with fluorescence microscopy.

Determination of DNA fragmentation by agarose gel electrophoresis

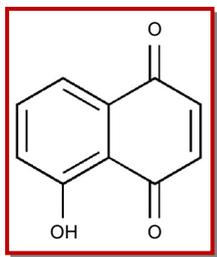


Figure 1: The chemical structure of juglone

LS-174T cells, both adherent and floating, were collected by centrifugation at 1,000 × g for 5 min. The cell pellets were suspended in cell lysis buffer (Tris-HCl 10 mM pH 7.4, edetic acid 10 mM pH 8.0, Triton-100 0.5%) and kept at 4°C for 30 min. The lysate was centrifuged at 25,000 × g for 20 min. The supernatant was incubated with 20 mg/mL RNase A (2 μL) at 37°C for 1 hours, then incubated with 20 mg/mL proteinase K (2 μL) at 37°C for 1 hours. The supernatant was mixed with 5 M NaCl (20 μL) and isopropanol (120 μL) at -20°C overnight, then centrifuged at 25,000 × g for 15 min. After drying, DNA was dissolved in TE buffer (Tris-HCl 10 mM pH 7.4, edetic acid 1 mM pH 8.0) and separated by 2% agarose gel electrophoresis at 100 V for 50 min.

Enzyme-linked immunosorbent assay for apoptosis

The Cell Death Detection ELISA kit (Roche Molecular Biochemicals) was employed to quantify DNA fragmentation on the basis of antibody detection of free histone according to the protocol of manufacture.

Western blot analysis of protein expression

Western blot analysis was performed as follows. Briefly, LS-174T cells treated with different concentrations of juglone for 36 hours were harvested, rinsed twice with cold PBS, and resuspended in lysis buffer, including Hepes 50 mM pH 7.4, 1% Triton-X 100, sodium orthovanada 2 mM, sodium fluoride 100 mM, edetic acid 1 mM, egtazic acid 1 mM, PMSF 1 mM, aprotinin 0.1 g/L, leupeptin 0.01 g/L, then lysed in 4°C for 1 hours. After 13,000 × g centrifugation for 10 min, the protein content of supernatant was determined using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA). Equal amounts of protein (30-40 μg) was subjected to 12% SDS polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% defatted milk, and then incubated overnight with the appropriate primary antibody at dilutions specified by the manufacturer, followed by incubation at room temperature for 1 hour with the corresponding alkaline phosphatase conjugated secondary antibody at 1:500-1000 dilution in TBST. Bound secondary antibody was detected using an BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China)

Determination of caspase-3 activity

The activity of caspase-3 was determined using the caspase-3 activity kit. Briefly, LS-174T cells were seeded in culture flasks (1 × 10⁶ cells/flask) and allowed to attach overnight. After treating the cells with juglone (12, 25, 50 and 100 μmol/L) or medium for 36 hours, cell lysates were prepared by incubating 2 × 10⁶ cells in 100 mL lysis buffer for 15 min on ice. Cell lysates were centrifuged at 20,000 × g for 15 min at 4°C. Supernatants were collected and added to an ice-cold

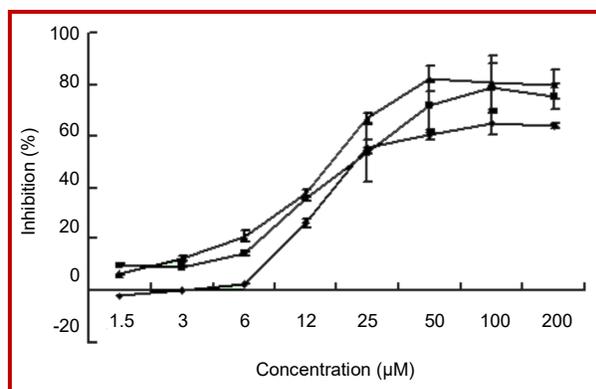


Figure 2: Juglone induces growth inhibition

The cells were exposed to juglone at concentrations ranging from 0-200 μM and incubated for 24 hours (\blacklozenge), 36 hours (\blacksquare) and 48 hours (\blacktriangle). The inhibitory ratio was measured by MTT assay. Data represent the mean \pm SD of three independent experiments

centrifuge tube. A blank solution containing 90 mL reaction buffer and 10 mL Ac-DEVD-pNA and the sample solution for each group including 80 mL reaction buffer, 10 mL sample and 10 mL Ac-DEVD-pNA were incubated in a 96-well microplate for 2 hours at 37°C. Caspase-3 activity was measured at 405 nm using a microplate reader (Bio-Rad 680, Hercules, CA). The active unit of caspase-3 was calculated. One unit is the amount of enzyme that will cleave 1.0 nmol/L of the colorimetric substrate Ac-DEVD-pNA per hour at 37°C under saturated substrate concentrations.

Statistical analysis

All experiments were repeated at least three times. The results from treated and untreated control cells were

compared using the Student's t-test to assess statistical significance.

Results

To determine the inhibition of LS-174T cells exposed to juglone, cells were treated with juglone of different concentrations, ranging from 0 to 200 μM for 24, 36 and 48 hours. Inhibition of LS-174T cell proliferation was in a time- and dose-dependent manner (Figure 2). The IC_{50} dose of juglone for 24, 36 and 48 hours was approximately 56, 30 and 22 μM , respectively.

Morphological changes were observed by fluorescence microscopy. Nuclear morphological changes were observed by Hoechst 33258 staining. In control group, cells were round in shape and stained homogeneously. After 24 hours treatment with 50 μM juglone, blebbing nuclei and granular apoptotic bodies appeared (Figure 3A).

To further verify apoptosis induced by juglone, the Cell Death Detection ELISA kit was employed to quantify cytoplasmic histone-associated-DNA-fragments (by measuring optical density). The results showed that juglone induced significant apoptotic cell death at 25 and 50 μM (Figure 3B). In addition, DNA fragmentation became obvious after 25, 50 and 100 μM juglone treatment for 36 hours on agarose gel electrophoresis (Figure 3C), which further confirmed juglone induced cell apoptosis.

To investigate whether caspase-3 and 9 participated in juglone-induced cell apoptosis, expressions of procaspase-3 and -9 by western blot analysis were

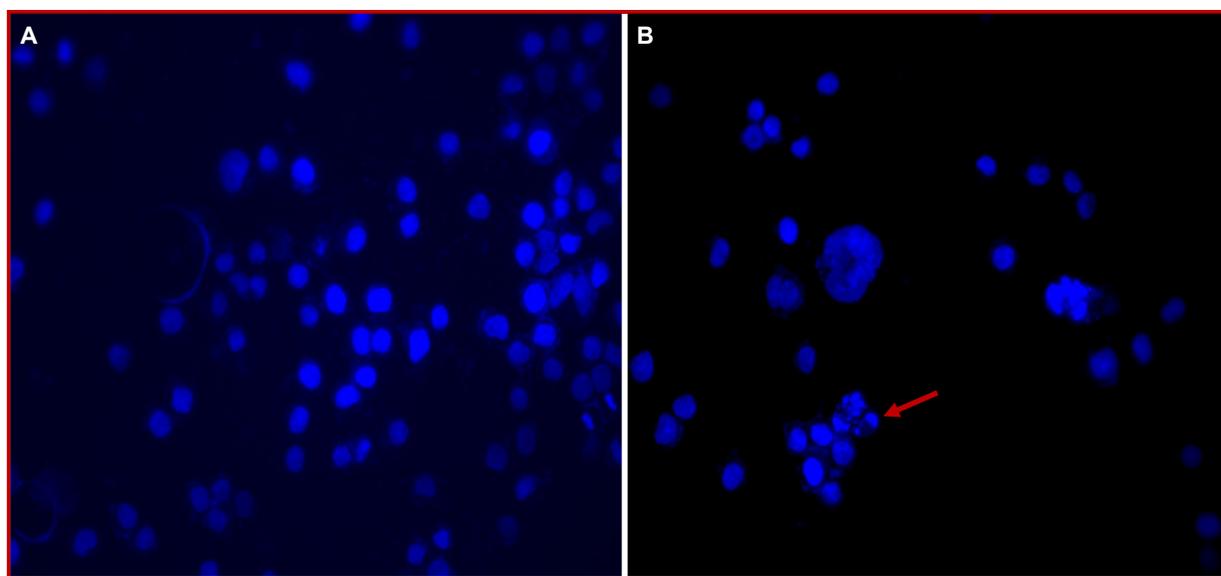


Figure 3A: Juglone-induced apoptotic morphological changes

LS-174T were incubated in the medium alone for 36 hours (A) or in the medium containing 50 μM juglone for 36 hours (B). Arrows indicates fragmented nuclei

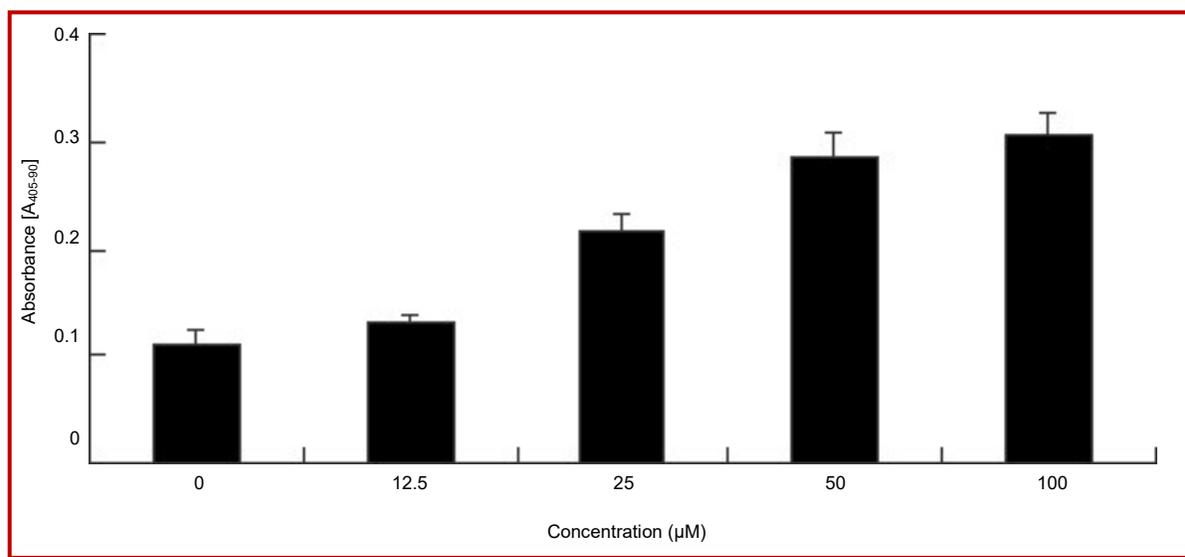


Figure 3B: Pro-apoptotic effect of juglone determined by ELISA quantification of DNA fragmentation expressed as optical density (OD) values

LS-174T cells were incubated in the medium alone or in the medium containing 12.5, 25, 50 and 100 µM juglone for 36 hours (** and *** stands for $p < 0.01$ and $p < 0.001$ compared with 0 µM, respectively)

performed. After incubation with juglone of indicated concentrations, expression of procaspase-3 and -9 protein was decreased, indicating caspase-3 and -9 activation (Figure 4A).

To further determine whether caspase 3 participated juglone induced apoptosis in LS-174T cells, Western blot analysis was performed to examine the expressions of caspase 3 substrate, PARP, and caspase-3 activity

was also determined. Figure 4B showed that the expression of minor PARP (85 kDa) protein after cleavage was up-regulated by juglone, indicating that caspase-3 is activated. Caspase-3 activity was increased in a dose-dependent manner (Figure 4C). These results correspond to the activation of caspase-3 as shown in Figure 4A.

Mitochondrial Bcl-2 family is a series of proteins that regulate apoptosis. We measured the expressions of Bcl-2 by Western blot analysis. After incubation with juglone, expression of Bcl-2 protein was decreased (Figure 5).

The generation of ROS may be relate to apoptosis induced by juglone, therefore, we investigated the induction of apoptosis by juglone to determine if it was associated with ROS generation. To accomplish this, LS-174T cells were pre-treated for 1 hour with 0.4, 2 and 10 mM NAC, a commonly used reactive oxygen intermediate scavenger. The cells were then treated with 50 µM juglone for 36 hours. As shown in Figure 6A, pre-treatment with NAC (0.4, 2 and 10 mM) significantly blocked the inhibition induced by juglone ($p < 0.001$). Furthermore, pre-treatment with 5 mM NAC inhibited procaspase-3 and -9 activation that was observed in LS-174T cells in response to juglone treatment. Additionally, we also found that pre-treatment with 5 mM NAC reversed juglone-induced Bcl-2 down-regulation (Figure 6B)

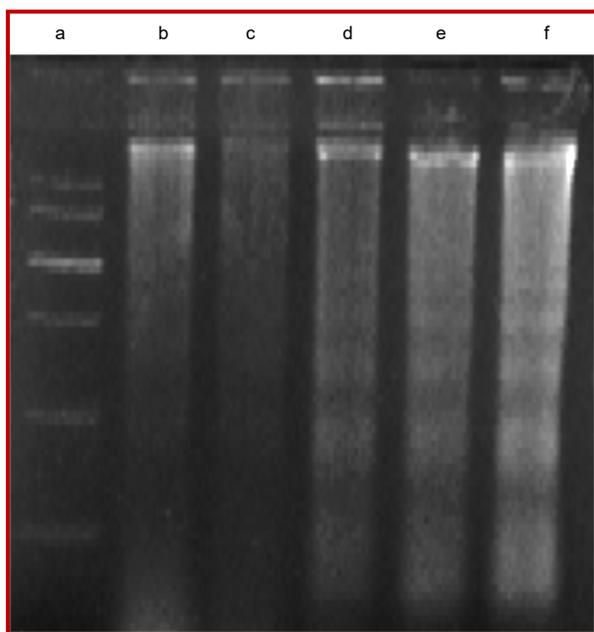


Figure 3C: DNA fragmentation induced by juglone in LS-174T cells

a: marker. b-f: LS-174T cells were treated with juglone 0, 12, 25, 50, 100 µM for 36 h. DNA was isolated by agarose gel electrophoresis and analyzed by ethidium bromide staining

Discussion

Walnut has been used in traditional medicines for various ailments and some extracts of walnut are also

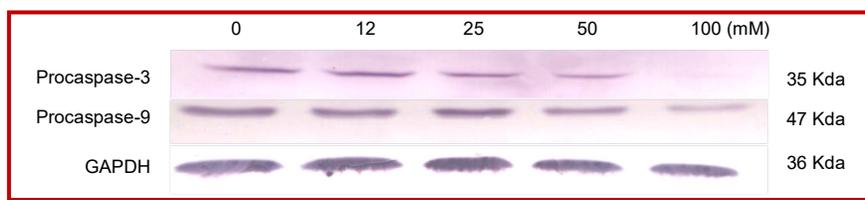


Figure 4A: The expression of procaspase-3 and procaspase-9 in 0, 12, 25, 50, 100 μ M juglone-treated LS-174T cells. The cells were treated with juglone 36 hours. Procaspase-3 and procaspase-9 were analysed by western blot. GAPDH was used as an equal loading control

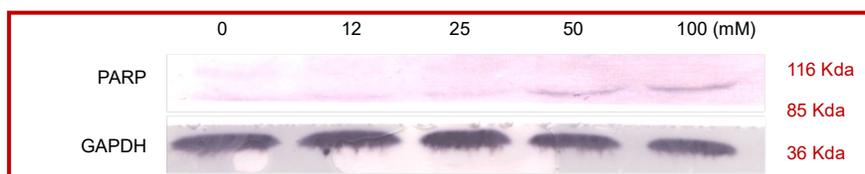


Figure 4B: The expression of PARP in 0, 12, 25, 50, 100 μ M juglone-treated LS-174T cells. The cells were treated with juglone 36 hours. PARP were analysed by Western blot. GAPDH was used as an equal loading control

reported to possess anticancer properties (Duke et al., 1998). However little has been reported about the mechanisms underlying the cytotoxic potential of juglone in LS-174T cells. In the present study, we found juglone, a main active ingredient in walnut, strongly inhibited the growth of LS-174T cells in a concentration- and time-dependent manner. Earlier, Segura-Aguilar and co-workers (1992) compared the effect of juglone and other quinones on human leukemic (HL-60) cells and doxorubicin-resistant human leukemic (HL-60R) cells and concluded that multidrug resistance that develops in the doxorubicin-resistant HL-60R cells had no effect on the cytotoxicity of juglone indicating its therapeutic possibilities (Segura-Aguilar et al., 1992). In addition, we did observe juglone-induced typical chromatin condensation and DNA fragmentation shown by Hoechst 33258 staining and ethidium

bromide staining.

Caspases play an important role in apoptosis (Kwon et al., 2003). Caspase-3 is activated either by extrinsic pathway (death receptor mediated) or by intrinsic pathway (mitochondria dependent pathway) (Verhagen et al., 2000). Caspases are proteases that are activated during apoptosis, and cleave substrates such as PARP (Lazebnik et al., 1994). PARP (116 kDa), a DNA repair enzyme, is probably best characterized caspase substrate, which is cleaved during apoptosis to a 24 kDa and a 85 kDa fragment representing the N-terminal DNA-binding domain and the C-terminal catalytic subunit, respectively. During apoptosis, PARP is selectively cleaved by several caspases, especially by caspase-3 (Lazebnik et al., 1994; Kaufmann et al., 1993). Detection of a 85 kDa or 24 kDa caspase cleavage

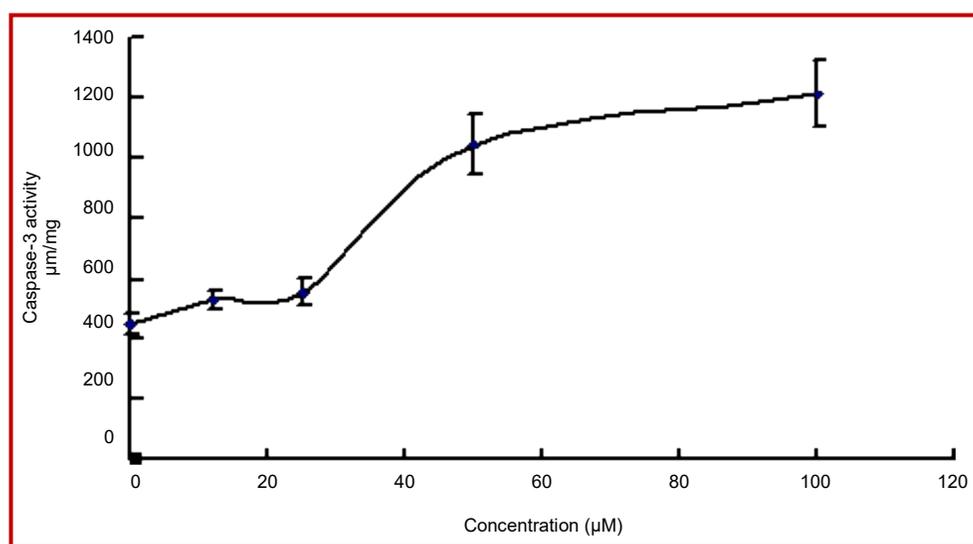


Figure 4C: Caspase-3 activity of juglone-treated LS-174T cells. The cells were treated with 0, 12, 25, 50, 100 μ M juglone for 36 hours (**stands for $p < 0.01$ compared with 0 μ M)

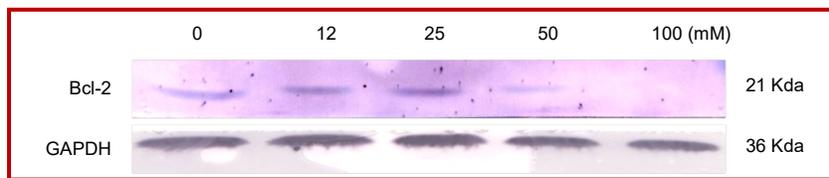


Figure 5: The expression of Bcl-2 in 0, 12, 25, 50, 100 μ M Juglone-treated LS-174T. The cells were treated with juglone 36 hours. Bcl-2 were analysed by western blot. GAPDH was used as an equal loading control

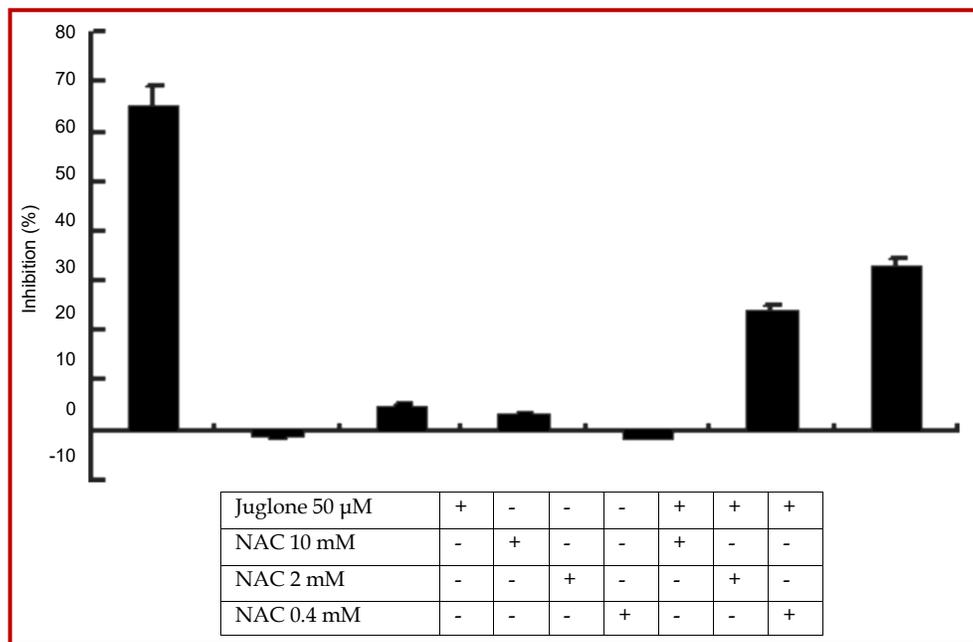


Figure 6A: Effect of NAC on juglone -induced cell death

One hour prior to the addition of 50 μ M juglone, LS-174T cells were treated with NAC (0.4, 2 and 10 mM), then incubated for 36 hours. The inhibition was measured by MTT method. $\bar{x} \pm s$, n = 3 (***)stands for $p < 0.001$

fragment of PARP was shown to be a hallmark of apoptosis. In this paper, PARP minor 85 kDa fragment expression was increased in a time dependent manner, and caspase-3 activity was also up-regulated in a concentration-dependent manner after treatment with juglone, which further confirmed that caspase-3 is activated in juglone induced cell apoptosis.

It is widely accepted that alteration in mitochondrial structure and function play an important role in caspase-9 dependent apoptosis through releasing cytochrome *c*, which interacts with Apaf-1 and procaspase-9 to form the apoptosome. Then caspase-9 was activated, which in turn cleaves and activated caspase-3, the executioner caspase, which cleaves PARP and activates endonucleases leading to DNA fragmentation. To verify whether mitochondrial pathway participates in juglone-induced cell apoptosis, procaspase-9 protein was determined. The activation of caspase-9 in juglone-induced cell apoptosis strongly suggests an involvement of a mitochondrial pathway.

The Bcl-2 family of proteins serves as critical regulators of pathways involved in apoptosis (Adams et al., 1998).

The main protagonists are suggested to be anti-apoptotic Bcl-2 and pro-apoptotic Bax. If the concentration of Bcl-2 is enough to complex with at least half of Bax, then apoptosis is prevented (Burlacu et al., 2003). In this paper, the Bcl-2 protein in juglone-treated LS-174T cells was down-regulated. This result suggested that the mitochondrial pathway of cell death might be involved in juglone -induced LS-174T cells death.

Induction of apoptosis by compounds such as arsenic trioxide (As_2O_3) (Jing et al., 1999; Chen et al., 1998), tumor necrosis factor- α (TNF- α) (Larrick et al., 1990), ceramide (Quillet-Mary et al., 1997) and erbstatin (Shimizu et al., 1996) involves the production of ROS. In order to determine whether ROS participates juglone-induced cells apoptosis, a water-soluble antioxidant, N-acetylcysteine (NAC) was used. NAC, a sulfhydryl group donor, serves as a precursor of GSH synthesis (Lauterburg et al., 1983) and inhibits the formation of extracellular reactive oxygen intermediates (Nakano et al., 1995). In this paper, juglone-induced cytotoxicity was markedly decreased by ROS scavenger NAC. In addition, activation of caspase-3 and caspase-9 was

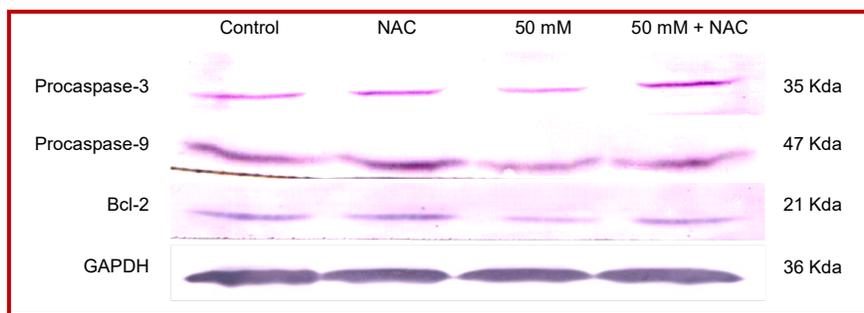


Figure 6B: Effect of NAC on juglone-induced procaspase-3 and procaspase-9 activation and Bcl-2 degradation. The cells were treated with 50 μ M juglone for 36 h in the presence or absence of 5 mM NAC, followed by western blot analysis for detection of procaspase-3, procaspase-9 and Bcl-2 expressions. GAPDH was used as an equal loading control

reversed by NAC and down-regulation of bcl-2 protein was also reversed, suggesting plausible role of ROS in juglone-induced apoptosis.

Conclusion

Our *in vitro* studies suggested that juglone could inhibit growth of LS-174T cells in a dose- and time-dependent manner. In addition, juglone was shown to induce apoptosis. This apoptotic response was associated with the down-regulation of Bcl-2 and activation of caspase-3 and -9. Taken together, juglone may be a promising chemopreventive and chemotherapeutic agent against colon adenocarcinoma.

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