

Juglone potentiates TRAIL-induced apoptosis in human melanoma cells via activating the ROS-p38-p53 pathway

XIAO LIU^{1*}, YAN CHEN^{2*}, YAOHUA ZHANG¹, JUAN DU¹, YUBAO LV³,
SHUMING MO⁴, YINGCHAO LIU⁵, FENG DING⁵, JINFENG WU¹ and JI LI³

¹Department of Dermatology, Huashan Hospital, Fudan University, Shanghai 200040; ²Department of Dermatology, The Affiliated Hospital, North Sichuan Medical College, Nanchong, Sichuan 637000; ³Department of Integrative Medicine, Huashan Hospital, Fudan University, Shanghai 200040; ⁴Department of Integrative Medicine, North Huashan Hospital, Fudan University, Shanghai 201907; ⁵Department of Neurosurgery, Provincial Hospital Affiliated to Shandong University, Jinan 250021, P.R. China

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Abstract. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-based cancer therapy offers promise as TRAIL can kill cancer cells without apparent toxicity towards normal cells. However, intrinsic or acquired resistance to TRAIL in several types of cancer cell has become a major challenge in TRAIL-based cancer therapy. Juglone is a natural compound isolated from walnut trees. In the present study, it was demonstrated that juglone sensitized melanoma cells to TRAIL-induced cytotoxicity by MTT and crystal violet assays. Flow cytometry analysis indicated that juglone potentiated TRAIL-induced cell death. Western blot assay demonstrated that the expressions of cleaved poly(ADP-ribose) polymerase (PARP) and cleaved caspase 3 were markedly increased in the juglone combined with TRAIL group. Exposure to TRAIL alone did not induce the production of reactive oxygen species (ROS), activation of p38 or increase of p53 in the TRAIL-resistant melanoma cells, as determined by flow cytometry and western blot analysis. However, exposure to TRAIL in combination with juglone markedly increased the production of ROS, activated p38 and increased p53, compared with the cells treated with either juglone or TRAIL alone. Pretreatment with N-acetyl cysteine, a ROS scavenger, significantly reduced the cytotoxicity of juglone in

combination with TRAIL, which further supported that ROS was involved in the juglone-induced sensitization of TRAIL. In conclusion, juglone potentiated TRAIL-induced apoptosis in melanoma cells, and these effects were partially mediated through the ROS-p38-p53 pathway. These findings suggested that juglone may be a potential sensitizer for TRAIL therapy in the treatment of melanoma.

Introduction

The incidence of melanoma in men and women has continued to increase in the last 40 years, despite stable or decreasing trends for the majority of types of cancer (1). The use of targeted drugs, including vemurafenib, dabrafenib and trametinib, and immunotherapeutic drugs, including ipilimumab, pembrolizumab and nivolumab, for melanoma treatment were approved by the US Food and Drug Administration in the last 5 years (2). All of these drugs have been shown to result in a significant increase in progression-free and overall survival rates, with long-term benefits in multiple clinical trials (2). However, these therapies have several limitations and challenges, including drug resistance, immune-related adverse events and a lack of predictive biomarkers (2). Therefore, the development of novel agents is required to overcome the limitations of currently used therapeutic agents.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of tumor necrosis factor (TNF) superfamily. TRAIL can induce the apoptosis of cells in several types of cancer without causing obvious toxicity to normal cells (3-5). Currently, recombinant human TRAIL is being assessed in clinical trials (6). However, limited therapeutic benefit has been observed and almost 50% of cancer cells, including melanoma cells, have shown resistance to TRAIL (7). TRAIL resistance has become a major challenge in TRAIL-based cancer therapy. Reactive oxygen species (ROS) are known to induce a wide range of responses, which are dependent on cell type and the levels of ROS within the cells (8,9). High levels of ROS can lead to necrotic cell death, whereas low levels of ROS have been shown to induce apoptotic cell death (8,9). It has been reported that TRAIL

Correspondence to: Dr Ji Li, Department of Integrative Medicine, Huashan Hospital, Fudan University, 12 Middle Urumqi Road, Shanghai 200040, P.R. China
E-mail: thomas19991015@163.com

Dr Jinfeng Wu, Department of Dermatology, Huashan Hospital, Fudan University, 12 Middle Urumqi Road, Shanghai 200040, P.R. China
E-mail: wujinfeng21@163.com

*Contributed equally

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exposure can induce the accumulation of ROS, activation of p38 mitogen-activated protein kinase (MAPK), enhancement of the expression of p53 and activation of caspases with subsequent apoptosis in TRAIL-sensitive cancer cells (10,11). In TRAIL-resistant cancer cells, natural ROS inducers, including curcumin (12), sulforaphane (13), baicalein (14) and icarisperone (15), have been reported to overcome TRAIL resistance in cancer cells.

Juglone (5-hydroxy-1,4-naphthoquinone), is a natural compound isolated from the roots, leaves, woods and fruits of walnut trees. It exhibits various pharmacological effects, including antiviral, antibacterial and antifungal effects (16,17). Previous studies have shown that juglone is cytotoxic towards cells in several types of cancer, including human lung cancer (A549) cells (18), human leukemia (HL-60) cells (19) and human cervical carcinoma (HeLa) cells (20). It has been documented that juglone exerts its cytotoxic effect via the production of ROS (21-23). In the present study, the effect of juglone on TRAIL-induced cytotoxicity was examined. It was shown that juglone potentiated TRAIL-induced apoptosis in melanoma cells, and that these effects were partially mediated through the ROS-p38-p53 pathway.

Materials and methods

Reagents and cell culture. Juglone was purchased from Sigma-Aldrich; Merck Millipore (Darmstadt Germany; purity >97; CAS 481-39-0; Fig. 1A) and dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO used was <0.1% (v/v). The human melanoma A2058 and MEWO cells were obtained from American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 4 mM L-glutamine, 3.7 g/l sodium bicarbonate, 4.5 g/l glucose and 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.). The cells were maintained in a 5% CO₂ humidified incubator at 37°C. Human recombinant TRAIL was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). Propidium iodide (PI) and RNaseA were supplied by Beyotime Institute of Biotechnology (Jiangsu, China). The antibodies targeting phosphorylated (p)-p38 (cat. no. 4511), p38 (cat. no. 8690), p53 (cat. no. 2527), poly(ADP-ribose) polymerase (PARP, cat. no. 9542), caspase 3 (cat. no. 9662), and GAPDH (cat. no. 5174) were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Thiazolylblue tetrazolium bromide (MTT), and N-acetyl-L-cysteine (NAC) were supplied by Sigma-Aldrich; Merck Millipore.

Cell viability assays. The cytotoxic effects of juglone and/or TRAIL on the A2058 and MEWO cells were determined using an MTT assay. Cells (4,000/200 µl/well) were seeded into 96-well plates and pretreated with juglone (0, 10 or 20 µM) for 6 h at 37°C. The cells were then washed with PBS, and the medium was replaced, following which the cells were treated with TRAIL (0 and 25 ng/ml) for 24 h at 37°C. Subsequently, 20 µl of MTT solution (5 mg/ml) was added to each well and incubated for 2 h at 37°C. The formazan crystals formed were dissolved with 100 µl of DMSO and the optical density (OD) was detected at 570 nm on a microplate spectrophotometer (BD Biosciences, San Jose, CA, USA). The cell

viability was determined using the following formula: Ratio (%) = (OD_{treatment}/OD_{vehicle control}) × 100. For the crystal violet assay, the cells (2.5 × 10⁵) were seeded into 60 mm dishes and exposed to juglone (0 and 20 µM) for 6 h. The cells were then washed with PBS and the medium was replaced, following which the cells were treated with TRAIL (0 and 25 ng/ml) for 72 h. The cells were then fixed with 10% formalin for 10 min, followed by staining with 0.05% crystal violet solution in distilled water for 30 min. Finally, the crystal violet was removed, and the cells were washed twice with distilled water. Cells were visualized using a light microscope and images of them were captured by a Flatbed Scanner (Canon LiDE 220, Canon Inc., Tokyo, Japan).

Cell death assays. The A2058 and MEWO cells were seeded at a density of 2 × 10⁵ cells/well in 6-well culture plates for 24 h. Subsequently, the cells were pretreated with juglone (0 and 20 µM) for 6 h. The cells were then washed with PBS and medium was replaced, following which the cells were treated with TRAIL (0 and 25 ng/ml) for 24 h. Following incubation, the cells were collected and fixed in 70% ethanol for 24 h at 4°C. The cells were then centrifuged at 300 × g at 4°C for 10 min and the cell pellet was resuspended in 400 µl of PBS containing RNaseA (10 mg/ml; 50 µl) and PI (2 mg/ml; 10 µl). The mixture was incubated in the dark at 37°C for 30 min and analyzed using a FACSCalibur flow cytometer (BD Biosciences). The cell death data were analyzed using FlowJo software V6.0 (Tree Star, Inc., Ashland, OR, USA). The extent of cell death was determined by evaluating the sub G1 fraction. The data comprised three replicates.

Western blot analysis. The A2058 cells were pretreated with juglone (0 and 20 µM) for 6 h. The cells were then washed with PBS and medium was replaced, following which the cells were treated with TRAIL (0 and 25 ng/ml) for 4 h. The cells were then resuspended in lysis buffer containing 150 mmol/l NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mmol/l Tris-Cl (pH 8.0), 2 µg/ml aprotinin, 2 µg/ml leupeptin, 40 mg/ml of phenylmethylsulfonyl fluoride and 2 mmol/l DTT. The mixture was centrifuged at 10,000 × g at 4°C for 15 min to remove nuclei and cell debris. The supernatants were then immediately frozen at -80°C until use. The protein concentrations were determined using a Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and 30 µg of cellular proteins were electroblotted onto a PVDF membrane following separation via 10% SDS-polyacrylamide gel electrophoresis. The immunoblot was blocked for 1 h with 5% milk at room temperature, followed by incubation overnight at 4°C with 1:1,000 dilutions of primary antibodies against p-p38, p38, p53, PARP, caspase 3 or GAPDH. The blots were washed twice with Tween 20/Tris-buffered saline (TTBS) prior to the addition of a 1:1,000 dilution of HRP-conjugated secondary antibody (cat. no. 7074, Cell Signaling Technology Inc., Danvers, MA, USA) for 1 h at room temperature. The blots were washed again with TTBS, and developed by enhanced chemiluminescence using Supersignal West Femto Chemiluminescent substrate (Pierce; Thermo Fisher Scientific, Inc.). The band intensities were quantified using UN-SCAN-IT gel analysis software (version 6; Silk Scientific, Orem, UT, USA). The OD values for the target proteins were calculated

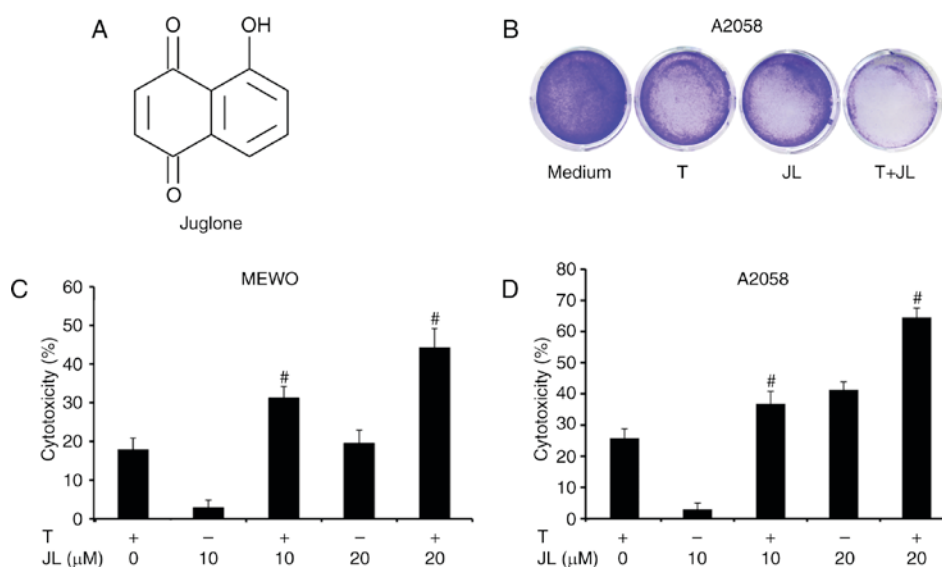


Figure 1. Juglone sensitizes melanoma cells to TRAIL-induced cytotoxicity. (A) Chemical structure of juglone. (B) Crystal violet assay. A2058 cells were exposed to juglone (0 and 20 μ M) for 6 h, following which cells were washed with PBS and treated with TRAIL (0 and 25 ng/ml) for 72 h. The cells were fixed and stained with crystal violet solution. (C) Cytotoxicity assay of MEWO cells and (D) A2058 cells. MEWO and A2058 cells were pretreated with juglone (0, 10 and 20 μ M) for 6 h, washed with PBS and medium was replaced, following which cells were treated with TRAIL (0 and 25 ng/ml) for 24 h. Thiazolylblue tetrazolium bromide solution was added and the absorbance was detected at 570 nm. [#] $P < 0.01$, vs. JL or Talone. JL, juglone; T/TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

as a proportion of the OD value for GAPDH. The western blot assays were repeated three times.

Evaluation of ROS. ROS were detected using the cell-permeable fluorescent probe 2,7-dichlorofluorescein-diacetate (H₂DCFDA; Sigma-Aldrich; Merck Millipore), a non-fluorescent compound, which is converted into highly fluorescent dichlorofluorescein (DCF) by cellular peroxides. Briefly, the A2058 cells were pretreated with juglone (0 and 20 μ M) for 6 h. The cells then were washed with PBS and medium was replaced, following which the cells were treated with TRAIL (0 and 25 ng/ml) for 4 h. Following treatment, the cells were loaded with H₂DCFDA (10 μ M) in serum-free DMEM. Following incubation at 37°C for 30 min, the cells were washed with PBS and fluorescence was monitored using flow cytometry at an excitation wave length of 488 nm and emission wavelength of 530 nm. The mean fluorescence intensity (MFI) data were analyzed using FlowJo software V6.0 (Tree Star, Inc.) and included three replicates.

Statistical analysis. All data are presented as the mean \pm standard deviation. Statistical analysis was performed by SPSS Statistics 17.0 software (SPSS Inc., Chicago, IL, USA) using one-way analysis of variance. For comparisons between two groups, Student's t-test was used. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Juglone sensitizes melanoma cells to TRAIL-induced cytotoxicity. The present study first examined the cytotoxic effects of juglone and/or TRAIL in TRAIL-resistant melanoma cells. As demonstrated by the crystal violet assay (Fig. 1B) and MTT assay (Fig. 1C and D), the MEWO and A2058 melanoma cells exhibited a low level response to

TRAIL treatment. Juglone (10 and 20 μ M) treatment alone induced cytotoxicity in a dose-dependent manner, whereas a higher level of cytotoxicity was observed in the juglone and TRAIL combination group, compared with that in the groups treated with either TRAIL or juglone alone ($P < 0.01$).

Juglone potentiates TRAIL-induced cell death. As demonstrated by the flow cytometric analysis (Fig. 2), MEWO melanoma cells were not affected by TRAIL-induced cell death (Fig. 2A and C), whereas the A2058 cells exhibited a minor response to TRAIL-induced cell death (Fig. 2B and C). Treatment with juglone (20 μ M) alone significantly induced cell death, however, the combination of juglone and TRAIL caused a higher rate of cell death, compared with that in the cells treated with either TRAIL or juglone alone (Fig. 2; $P < 0.01$).

PARP and caspase 3 are the terminal pro-apoptotic proteins. The cleaved forms of these two proteins are the active forms. As demonstrated in the results of the western blot analysis (Fig. 3), no significant changes in the expression of PARP and caspase 3 were observed in the group treated with TRAIL alone. However, juglone treatment markedly increased the expression of cleaved PARP (Fig. 3A and B) and cleaved caspase 3 (Fig. 3A and C). The levels of cleaved PARP and cleaved caspase 3 in the juglone and TRAIL combination group were higher, compared with the levels in the group treated with juglone alone (Fig. 3; $P < 0.01$). Significant decreases in the levels of pro-PARP and pro-caspase 3 were observed following combined juglone and TRAIL treatment.

Juglone and TRAIL combination treatment increases the production of ROS, and activates p38 and p53. To examine the molecular mechanism underlying juglone-induced TRAIL sensitization, the present study investigated whether juglone

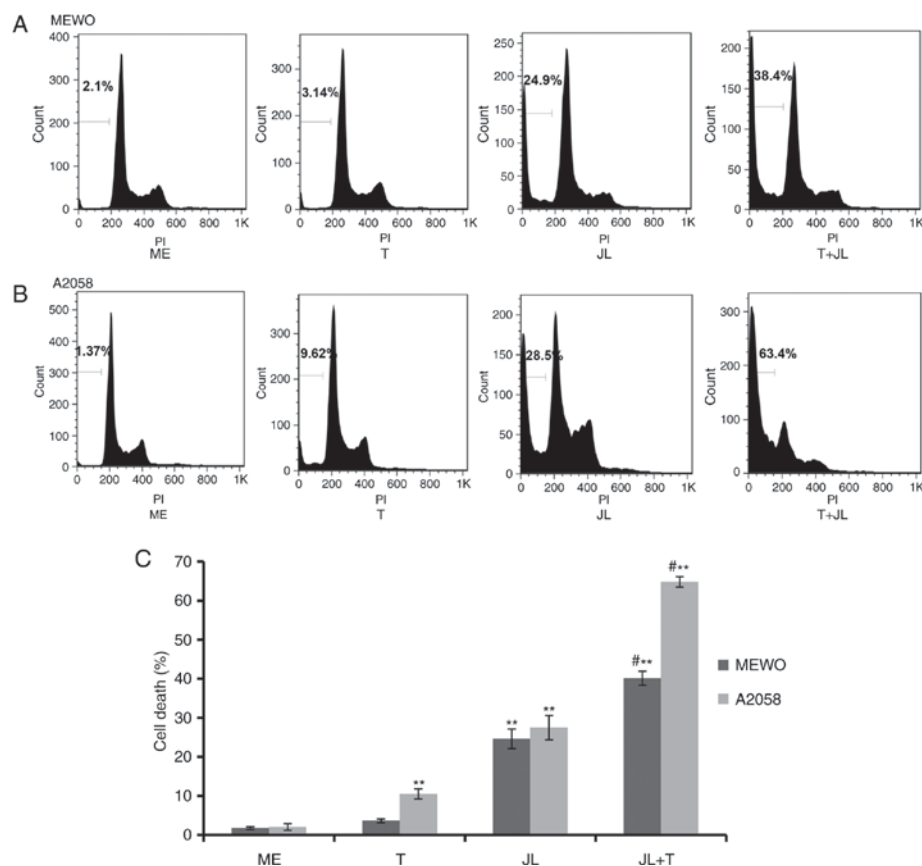


Figure 2. Juglone potentiates TRAIL-induced melanoma cell death. Representative results of (A) MEWO cell death and (B) A2058 cell death. (C) Statistical analysis of cell death data. MEWO and A2058 cell were exposed to juglone (0 and 20 μ M) for 6 h, washed with PBS and treated with TRAIL (0 and 25 ng/ml) for 24 h. Following fixation, the cells were stained with PI and analyzed in a FACSCalibur cytometer. The cell death experiments were repeated three times. ** $P < 0.01$, vs. control; # $P < 0.01$, vs. JL or TRAIL. ME, medium; JL, juglone; T/TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; PI, propidium iodide.

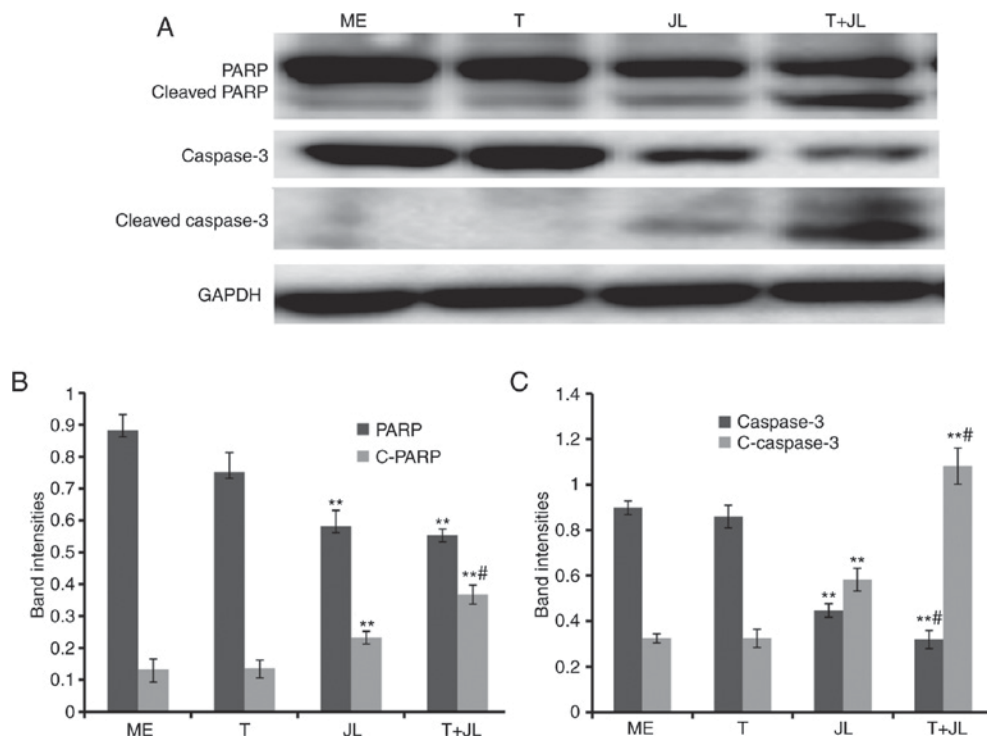


Figure 3. Juglone in combination with TRAIL increases levels of cleaved caspase 3 and cleaved PARP. (A) Representative western blot images of PARP and caspase 3. (B) Quantification of band intensities of PARP and (C) caspase 3. A2058 cells were pretreated with juglone (0 and 20 μ M) for 6 h, washed with PBS and treated with TRAIL (0 and 25 ng/ml) for 4 h. Caspase 3 and PARP were detected using western blot analysis. The band densities for target proteins are shown as a proportion of that for GAPDH. ** $P < 0.01$, vs. ME control; # $P < 0.01$, vs. JL or TRAIL alone. ME, medium; JL, juglone; T/TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; PARP, poly(ADP-ribose) polymerase; C-, cleaved.

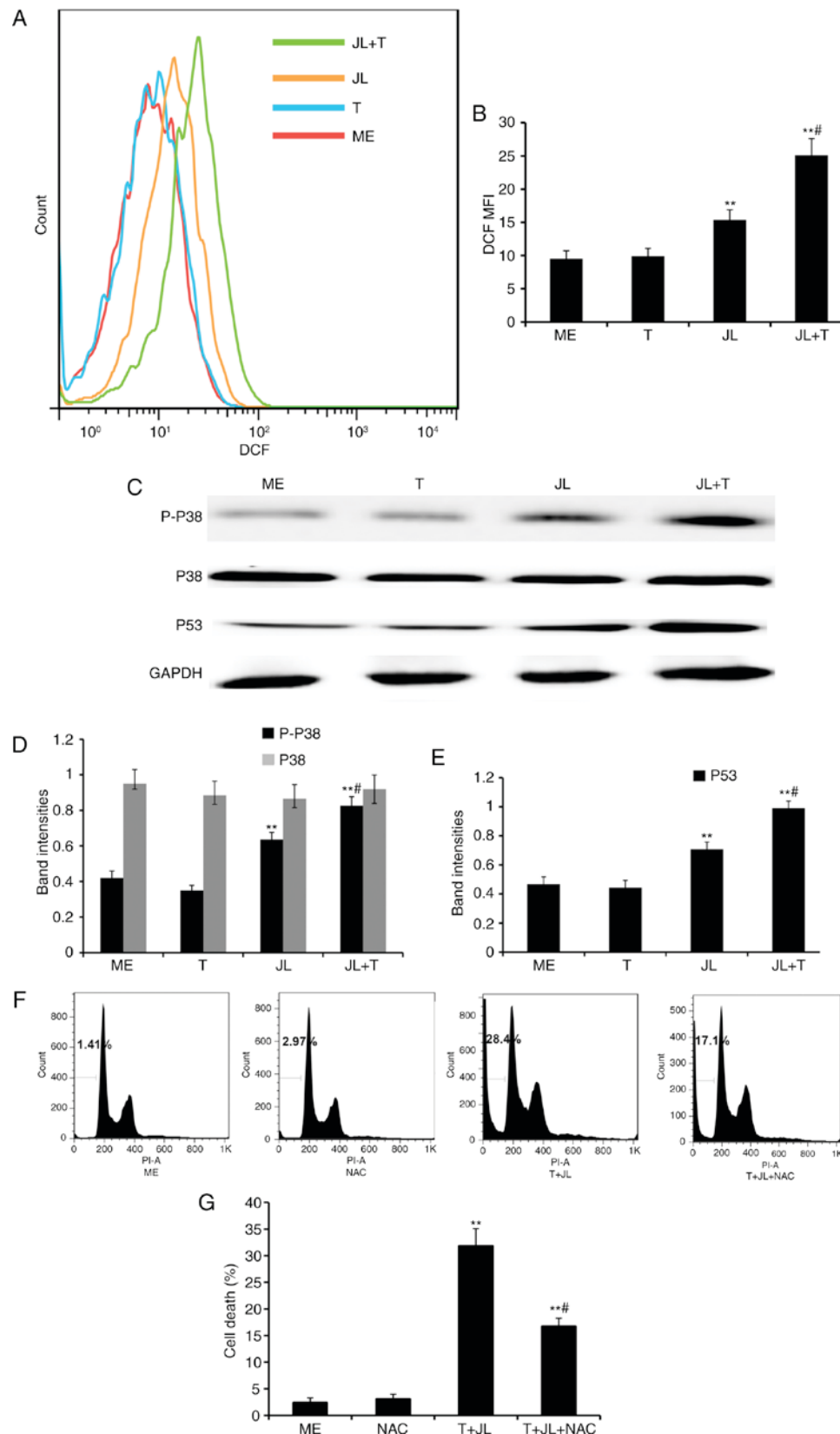


Figure 4. Juglone in combination with TRAIL increases the production of ROS, and activates p38 and p53. For the ROS assay, A2058 cells were pretreated with juglone (0 and 20 μ M) for 6 h, washed with PBS, and treated with TRAIL (0 and 25 ng/ml) for 4 h. The cells were loaded with H₂DCFDA and fluorescence was monitored by flow cytometry. MFI data were analyzed using FlowJo software V6.0. (A) Representative images of ROS MFI and (B) statistical data. For western blot analysis, the cells were treated with juglone and/or TRAIL for 4 h, total protein was extracted, and p-p38, p38 and p53 were detected. GAPDH was used as the loading control. (C) Representative western blot images of p-p38, total p38 and p53, and quantification of band intensities of (D) p-p38, total p38 and (E) p53. The band densities for target protein are shown as a proportion of that for GAPDH. For the cell death assay, A2058 cells were pretreated with NAC (0 and 2 mM) for 1 h, followed by juglone and TRAIL combination treatment for 16 h. The fixed cells were stained with PI and analyzed in a FACSCalibur cytometer, with experiments repeated three times. (F) Representative images of cell death and (G) statistical analysis. **P<0.01, vs. ME control; #P<0.01, vs. JL or T alone. ME, medium; JL, juglone; T/TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; P-, phosphorylated; ROS, reactive oxygen species; DCF MFI, dichlorofluorescein mean fluorescence intensity; PI, propidium iodide.

and/or TRAIL treatment induced the production of ROS. The ROS levels were determined 6 h following juglone and/or TRAIL treatment. As demonstrated by the flow cytometric analysis (Fig. 4A and B), TRAIL treatment alone did not increase the production of ROS, whereas juglone treatment alone significantly increased the production of ROS ($P<0.01$). The production of ROS was further increased in the juglone and TRAIL combination group, compared with the cells treated with juglone alone ($P<0.01$).

Previous evidence indicates that ROS induces cell death via the activation of p38 and p53 (10,11). As demonstrated by western blot analysis (Fig. 4C-E), treatment with TRAIL alone did not activate p38 and p53, whereas treatment with juglone alone significantly increased the levels of p-p38 and p53 ($P<0.01$). The expression levels of p-p38 and p53 were further increased in the juglone and TRAIL combination group, as compared with the group treated with juglone alone ($P<0.01$). No significant change was observed in the protein expression of total p38 following juglone and/or TRAIL treatment.

NAC pretreatment partially reverses juglone and TRAIL combination-induced cell death. As demonstrated by the flow cytometric analysis (Fig. 4F and G), treatment with juglone in combination with TRAIL for 16 h resulted in a significant increase in the rate of cell death (28.4%), compared with that in the medium control group (1.41%), whereas pretreatment with NAC, a ROS scavenger, partially reversed the juglone and TRAIL combination-induced cell death ($P<0.01$; 14.7, vs. 28.4%).

Discussion

Intrinsic or acquired resistance to TRAIL in several types of cancer cell has become a major challenge in TRAIL-based cancer therapy. In the present study, it was found that juglone sensitized melanoma cells to TRAIL-induced cytotoxicity, which was accompanied by increases in the levels of cleaved PARP and cleaved caspase 3.

It has been reported that TRAIL exposure can induce the accumulation of ROS and activation of p38 MAPK, enhance the expression of p53 and activate caspases in TRAIL-sensitive cancer cells. In the present study, it was found that TRAIL exposure did not induce the production of ROS, activation of p38 or increase in p53 in the TRAIL-resistant melanoma cells. However juglone in combination with TRAIL markedly increased the production of ROS and activation of p38, and increased the expression of p53, compared with the groups treated with either juglone or TRAIL alone. Pretreatment with NAC, a ROS scavenger, significantly reduced the cytotoxicity of juglone in combination with TRAIL, which further supported the hypothesis that ROS is involved in juglone-induced TRAIL sensitization.

The resistance of several types of cancer cell to TRAIL is partially due to decreased levels or mutations of TRAIL receptors, including death receptor 4 (DR4) and DR5 (24,25). The sensitivity of cancer cells to TRAIL can be partially restored by treatment with subtoxic concentrations of chemotherapeutic drugs through the upregulation of DR4 and DR5 (26,27). Several studies have shown that DR4 and DR5 can be upregulated by ROS and MAPKs, including

extracellular signal-regulated kinases1/2, p38 MAPK and c-Jun NH₂-terminal kinase (28-30). As juglone could increase the production of ROS and increase p-p38 and p53 protein levels, further investigations are required to determine the effects of juglone on DR4 and DR5 TRAIL receptors.

In conclusion, the present study demonstrated that juglone, a natural product from walnut trees, potentiated TRAIL-induced apoptosis in melanoma cells and that these effects were partially mediated through the ROS-p38-p53 pathway. These findings suggest that juglone maybe a potential sensitizer for TRAIL in the treatment of melanoma.

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