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Juglone potentiates BRAF inhibitor-induced apoptosis in melanoma through reactive oxygen species and the p38-p53 pathway

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Abstract. BRAF inhibitors are some of the most effective drugs against melanoma; however, their clinical application is largely limited by drug resistance. Juglone, isolated from walnut trees, has demonstrated anti-tumour activity. In the present study, it was investigated whether juglone could enhance the responses to a BRAF inhibitor in melanoma cells (A375R and SK-MEL-5R) with an acquired resistance. These cells were treated with juglone alone, BRAF inhibitor (PLX4032) alone, or juglone combined with PLX4032. It was demonstrated that the combination of juglone and PLX4032 had synergistic effects on BRAF inhibitor-resistant melanoma cells. Juglone potentiated PLX4032-induced cytotoxicity and mitochondrial apoptosis in both A375R and SK-MEL-5R cells, which was accompanied by a decline in mitochondrial membrane potential and a decrease in Bcl-2/Bax ratio. Moreover, juglone combined with PLX4032 markedly increased the intracellular level of reactive oxygen species (ROS) and activated p38 and p53, as compared with juglone alone or PLX4032 alone. Pre-treatment with N-acetyl-L-cysteine, a ROS scavenger, completely reversed the cytotoxicity induced by juglone combined with PLX4032. In conclusion, juglone potentiated BRAF inhibitor-induced apoptosis in resistant melanoma cells, and these effects occurred partially through ROS and the p38-p53 pathway, suggesting the potential of juglone as a sensitizer to BRAF inhibitors in the treatment of melanoma.

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Introduction

Melanoma is one of the most common skin cancers, but its prognosis after metastasis remains poor (1). As reported in a previous cohort study, the morbidity of melanoma in the United States, United Kingdom and Sweden will continue to increase until at least 2022 (2). Traditional treatments, including radiotherapy and chemotherapy, have low efficacy in advanced melanoma patients (3). In contrast, the discovery of BRAF inhibitors developed for BRAF gene mutations in melanoma, such as vemurafenib and dabrafenib, has largely extended progression-free-survival (PFS) compared with the PFS seen with chemotherapy. However, melanoma has been found to be highly susceptible to acquired BRAF inhibitor resistance (4,5). Thus, it is imperative to explore methods that overcome resistance to BRAF inhibitors. The progress obtained in recent years reveals that the mechanisms underlying resistance to BRAF inhibitors are multiple, complicated and unpredictable (6). To date, a combination of mitogen-activated protein kinase kinase inhibitors and BRAF inhibitors has been shown to be clinically effective (7). However, the combination of these two targeted drugs can only partially impede BRAF inhibitor resistance (8). Therefore, the development of novel agents is urgently required to overcome BRAF inhibitor resistance.

Modern drug discovery programmes are gradually placing more focus on the screening of plants or other natural products, given their ease of availability, cost-effective nature and safer toxicity profiles. Juglone (5-hydroxy-1,4-naphthoquinone) is a natural naphthoquinone that is isolated from the roots, leaves, woods and fruits of walnut trees. It is not only a drug used to treat infections, but also an anti-tumour agent (9,10). It has been reported that juglone exerts cytotoxic and genotoxic effects against B16F10 melanoma cells (11). It has also displayed radiation-sensitizing potential (12). Our previous study demonstrated that juglone potentiated tumour necrosis factor-related apoptosis-inducing ligand-induced apoptosis in human melanoma cells (13). The cytotoxic and sensitizing effects of juglone can be attributed to mechanisms such as the induction of reactive oxygen species (ROS) (11,13,14). It has been documented that inducing ROS production might reverse the resistance to BRAF inhibition (15). Therefore, it was hypothesized that juglone might eradicate melanoma resistance to BRAF inhibitors through the induction of ROS.

In the present study, the effect of juglone on BRAF-induced cytotoxicity was examined, and it was demonstrated that the underlying mechanisms were associated with ROS and the p38-p53 pathway.

Materials and methods

Chemicals and reagents. BRAF inhibitor PLX4032 was purchased from Selleck Chemicals, and juglone was purchased from Sigma-Aldrich; Merck KGaA. They were each dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO for cell culture was <0.1% (v/v). DMSO, 3-(4',5'-dimethylthiazol-2'-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and N-acetyl-L-cysteine (NAC) were obtained from Sigma-Aldrich; Merck KGaA. 2',7'-dichlorofluorescein diacetate (DCFH-DA), 4% paraformaldehyde, 5-ethynyl-29-deoxyuridine (EdU) cell proliferation kit with Alexa Fluor® 488, and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-midacarbocyanine iodide (JC-1) staining kit were purchased from Beyotime Institute of Biotechnology. JNK inhibitor (SP600125) and p38 inhibitor (SB203580) were obtained from MedChemExpress. The Annexin-V-phycoerythrin (PE) Apoptosis Detection kit (cat. c559763) was supplied by BD Biosciences. The antibodies against β-actin (cat. no. ab8226), poly(ADP-ribose) polymerase (PARP; cat. no. ab191217), survivin (cat. no. ab76424), Bcl-2 (cat. no. ab32124), Bax (cat. no. ab32503), phosphorylated (P)-p38 (cat. no. ab195049), p38 (cat. no. ab170099), P-p53 (cat. no. ab33889) and p53 (cat. no. ab179477) were obtained from Abcam, and the antibody against cytochrome *c* (cat. no. 4280) was purchased from Cell Signaling Technology, Inc.

Cell lines and culture. Human melanoma SK-MEL-5 and A375 cell lines were obtained from American Type Culture Collection and maintained in DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) containing 4 mM L-glutamine, 3.7 g/l sodium bicarbonate, 4.5 g/l glucose and 10% foetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.). The cells were maintained in a 5% CO_2 humidified incubator at 37˚C. Both SK-MEL-5 and A375 cells carrying the BRAFV600E mutation are sensitive to vemurafenib treatment (16,17). A SK-MEL-5 subline with acquired resistance (SK-MEL-5R) to PLX4032 was generated by continuous exposure of parental SK-MEL-5 cells to gradually increasing concentrations of PLX4032 (from 0.1 μM up to 1 μM) over a period of 3 months. The fold resistance was intermittently evaluated by cell viability assays and it was found that SK-MEL-5R showed higher cell viability compared with SK-MEL-5 when treated with different concentrations of PLX4032 (data not shown). A375 cells with acquired resistant to PLX4032 (A375R) were generated by the same method, with the concentration of PLX4032 increasing from 0.1 μM up to 2 μM, as previously described (18).

Cell viability assays. The cytotoxic effects of juglone and/or PLX4032 on SK-MEL-5R and A375R cells were assessed by MTT assays. Briefly, 4,000 cells in 200 μl cell culture medium/well were seeded into 96-well plates and incubated overnight at 37˚C. After NAC (0 and 2 mM), SB203580 (0 and 10 μM) or SP600125 (0 and 10 μM) pre-treatment for 1 h at 37˚C, the cells were treated with juglone (0, 5 or 7.5 μM) and/or PLX4032 (0, 5 or 10 μM) for 24 h at 37˚C. DMEM containing corresponding amounts of 0.1% DMSO were used as vehicle controls. Then, 20 μl MTT solution (5 mg/ml) was added to each well, and the cells were incubated for 2 h at 37˚C. Subsequently, the formazan crystals that formed were dissolved with 100 μl DMSO, and the optical density (OD) was measured at 570 nm on a microplate spectrophotometer (Infinite 200 PRO; Tecan Group, Ltd.). The cell viability was determined using the following formula: Cytotoxicity (%)=(OD treatment/OD vehicle control) x100. For crystal violet staining, $2x10^5$ cells/well were seeded into 6-well plates, followed by exposure to juglone (0 and 5 μM) and/or PLX4032 (0 and 10 μM) for 72 h at 37˚C. Then, the cells were fixed with 10% formalin for 10 min at room temperature and stained with 0.05% crystal violet solution (CV) in distilled water for 30 min at room temperature. Finally, the CV was removed, cells were washed twice with distilled water and images were captured using a Flatbed Scanner (Canon LiDE 220; Canon Inc.).

Cell proliferation assays. To assess the cell proliferation, an EdU assay kit was used according to the manufacturer's instructions. Briefly, $2x10^5$ cells/well were seeded in a 6-well plate and treated with juglone (0 and 5 μM) and/or PLX4032 (0 and 10 μM) for 24 h, followed by incubation with cell culture medium containing 10 μM EdU at 37˚C for 2 h. Then, the cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.3% Triton X-100 in PBS for 15 min. After washing with PBS, cells were incubated with click additive solution (supplied with the kit) for 30 min in the dark at room temperature. Finally, cells were washed with 0.3% Triton X-100 and incubated with Hoechst 33342 for 10 min at room temperature. After washing with PBS 3 times for 5 min each time, the images were captured using a fluorescence microscope (Axio Vert. A1; Zeiss GmbH) at 400x magnification and the subsequent images were analyzed using ImageJ software (v1.52 g; National Institutes of Health). The data comprised three replicates.

Apoptosis assays. Apoptosis was measured using flow cytometry. Cells were seeded into 6-well culture plates at a density of $2x10^5$ cells/well, and then treated with juglone (0 and 5 μM) and/or PLX4032 (0 and 10 μM) for 24 h at 37˚C. Next, cells were harvested, washed with PBS twice and resuspended in 1X binding buffer (supplied with kit). Finally, the cells were incubated with Annexin V-PE and 7-AAD in the dark at room temperature for 15 min, and analysed using a flow cytometer (Attune NxT; Thermo Fisher Scientific, Inc.). The data were analysed using FlowJo software V6.0 (Tree Star, Inc.).

Western blot analysis. Cells were harvested following the pre-treatment with NAC (0 and 2 mM), SB203580 (0 and 10 μM) or SP600125 (0 and 10 μM) for 1 h at 37˚C and treated with juglone (0 and 5 μM) and/or PLX4032 (0 and 10 μM) for 6 h at 37˚C. The cells were then lysed in RIPA buffer (Beyotime Institute of Biotechnology). The mixture was centrifuged at 12,000 x g at 4˚C for 15 min, and the supernatants were collected. The protein concentrations were determined using a bicinchoninic acid protein assay kit (Bio-Rad Laboratories, Inc.). Next, 30 μg cellular proteins were separated on a 10 or 12% SDS-polyacrylamide gel and electroblotted onto a PVDF membrane. Membranes were blocked with 5%

milk in Tween-20/Tris-buffered saline (TBST) for 1 h at room temperature, prior to incubation overnight at 4˚C with 1:1,000 dilution of primary antibodies against β-actin, PARP, survivin, Bcl-2, Bax, p-p38, p38, p-p53, p53, JNK, p-JNK and cytochrome *c*. Membranes were washed with TBST, followed by 1 h incubation at room temperature in 1:4,000 dilution of horseradish peroxidise-conjugated secondary antibodies (cat. nos. 7074 and 7076; Cell Signaling Technology, Inc.). After washing again, the blots were developed using Supersignal West Femto Chemiluminescent substrate (Thermo Fisher Scientific, Inc.). Images were acquired by the ChemiDoc™ MP Imaging system (Bio-Rad Laboratories, Inc.). The band intensities were semi-quantified using ImageJ software and normalized to β-actin as the loading control. The western blot assays were repeated three times.

Evaluation of ROS. ROS were detected using the fluorescent probe DCFH-DA. Briefly, the cells were seeded into 6-well culture plates at a density of $2x10^5$ cells/well, and then treated with juglone (0 and 5 μM) and/or PLX4032 (0 and 10 μM) for 2 h. The cells were then washed with PBS, incubated in serum-free medium, and loaded with DCFH-DA (10 μM). Following incubation in the dark at 37˚C for 30 min, the cells were washed with PBS and vortexed in 700 μl lysis buffer (90% DMSO:10% PBS)/well for 15 min. The cell lysis buffer was transferred into a black 96-well Immuno Plate (Thermo Fisher Scientific, Inc.), and fluorescence was detected using a fluorescence microplate reader (Infinite 200 PRO; Tecan Group, Ltd.) at an excitation wavelength of 485 nm, and an emission wavelength of 535 nm.

Analysis of mitochondrial membrane potential ($\Delta\Psi_M$). $\Delta\Psi_M$ was evaluated using a JC-1 kit. Briefly, $1x10^6$ cells seeded in a 6-well plate were cultured with juglone (0 and 5 μM) and/or PLX4032 (0 and 10 μM) for 3 h. Then, the cells were labelled with JC-1 and incubated for 20 min at 37˚C, according to the manufacturer's instructions. Cells were washed twice, resuspended and detected by flow cytometer (Attune NxT; Thermo Fisher Scientific, Inc.); the PE and FITC detection channels were selected at emission wavelengths of 530 and 585 nm, and an excitation wavelength of 488 nm. The data were analysed by FlowJo software V6.0 (Tree Star, Inc.). Mitochondrial carbonyl cyanide 3-chlorophenylhydrazone (supplied in kit) was used as a positive control. The percentage of depolarized mitochondrial was calculated as previously described (19).

Statistical analysis. All results are presented as the mean ± standard deviation of at least three independent experiments. Statistical analysis was performed by SPSS Statistics 21.0 software (IBM Corp.). Student's t-test was used for comparisons between two groups, and one-way ANOVA and subsequent Tukey's post-hoc analysis was applied for comparison of more than two independent groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Juglone sensitizes melanoma cells to PLX4032-induced cytotoxicity. MTT was used to determine the cytotoxic effects of juglone and/or PLX4032 on melanoma cells. As shown in Fig. 1A and C, the combination of juglone and PLX4032 in SK-MEL-5R and A375R melanoma cells induced a much higher cytotoxicity than juglone or PLX4032 alone ($P<0.01$). In addition, according to the MTT results, the combination index (CI) of juglone and PLX4032 cotreatment in SK-MEL-5R cells was determined using the Chou-Talalay method (20). The CI values varied from 0.34-0.65, which suggested a strong synergistic effect between juglone and PLX4032 (Fig. 1B). Crystal violet staining confirmed that the combination of juglone and PLX4032 further induced cytotoxicity to melanoma cells, compared with either juglone alone or PLX4032 alone (Fig. 1D). EdU assays also suggested that cell proliferation was further inhibited after treatment with a combination of PLX4032 and juglone (Fig. 1E and F).

Juglone potentiates PLX4032-induced apoptosis in melanoma. To further demonstrate the synergistic effect of juglone and PLX4032, flow cytometry analysis was performed to measure the percentages of both Annexin V^+/7-AAD^- (early apoptotic cells) and Annexin V^+/7-AAD^+ (late apoptotic/necrotic cells) cells. In SK-MEL-5R cells, PLX4032 alone induced only minor apoptosis. However, the combination of juglone and PLX4032 exhibited a much higher rate of apoptosis than either PLX4032 or juglone alone (Fig. 2A and C; $P<0.01$). Similar results were observed in A375R cells (Fig. 2B and C; $P<0.01$).

PARP is one of the terminal pro-apoptotic proteins. The cleaved forms of PARP are its active forms (21). Survivin, on the other hand, inhibits apoptosis (22,23). As demonstrated by western blotting, the protein expression of cleaved PARP in the juglone and PLX4032 combination group was the highest, while the protein expression of survivin was the lowest, as compared with levels in either the PLX4032 or juglone alone groups (Fig. 2D-F).

Juglone enhances PLX4032-induced apoptosis through the mitochondrial pathway. $\Delta\Psi_M$ decline is one of the earliest indicators of intrinsic apoptosis. In the present study, the fluorescent probe JC-1 was used to determine the effect of juglone on $\Delta\Psi_M$ in SK-MEL-5R cells. When the mitochondrial potential is normal, JC-1 forms a polymer in the mitochondrial matrix and exhibits red fluorescence with an emission of 590 nm; however, when the $\Delta\Psi_M$ is downregulated, JC-1 changes into a monomeric form that yields green fluorescence with an emission of 530 nm, so the PE and FITC channel were selected to measure the fluorescence intensity of JC-1 dye in the cells (24). As shown in Fig. 3A and B, juglone caused a marked decrease in $\Delta\Psi_M$ compared with the vehicle control. Moreover, the combination of juglone and PLX4032 further decreased the $\Delta\Psi_M$, and the percentage of cells with low potential was increased from 44.5% in the juglone alone group to 55.8% when cotreated with juglone and PLX4032.

The pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 serve central roles in the mitochondria-dependent apoptotic pathway; Bax can be translocated to the outer mitochondrial membrane following a death signal, where it promotes a permeabilization that favors the release of different apoptogenic factors, such as cytochrome *c* (25). In addition, the ability of Bax to form channels on the mitochondrial membrane can be inhibited by Bcl-2 (26). As demonstrated by western blot analysis (Fig. 3C), the protein expression of Bax was notably

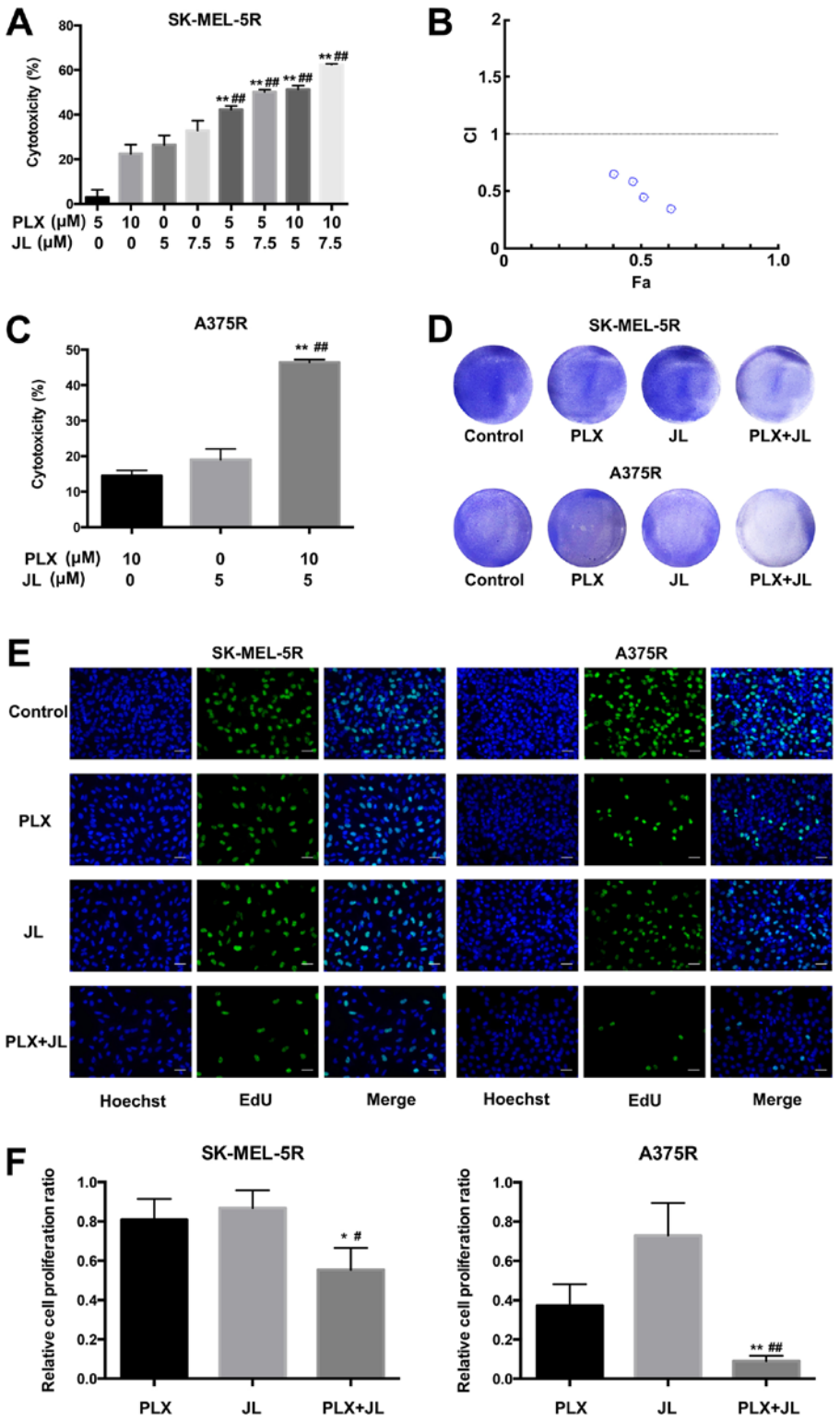

Figure 1. Juglone sensitizes melanoma cells to PLX4032-induced cytotoxicity. (A) MTT assay of SK-MEL-5R cells; SK-MEL-5R cells were treated with juglone (0, 5 and 7.5 μM) and/or PLX4032 (0, 5 and 10 μM) for 24 h. **P<0.01 vs. same concentration PLX alone, $^{\#\#}$P<0.01 vs. same concentration JL alone. (B) CI of juglone and PLX4032. (C) MTT assay of A375R cells; A375R cells were treated with juglone (0 and 5 μM) and/or PLX4032 (0 and 10 μM) for 48 h. **P<0.01 vs. PLX, $^{\#\#}$P<0.01 vs. JL. (D) Crystal violet staining; SK-MEL-5R and A375R cells were exposed to juglone (0 and 5 μM) and/or PLX4032 (0 and 10 μM) for 72 h. The cells were then fixed and stained with crystal violet solution. (E) EdU assay; cells were double-stained with EdU (green) and Hoechst 33342 (blue). Scale bar, 20 μm. (F) Relative cell proliferation rates were analyzed according to the EdU assay results. **P<0.01, *P<0.05 vs. PLX; $^{\#\#}$P<0.01, $^{\#}$P<0.05 vs. JL. CI, combination index; EdU, 5-ethynyl-29-deoxyuridine; JL, juglone; PLX, PLX4032.

increased after cotreatment with juglone and PLX4032, while no changes were seen in the juglone or PLX4032 only groups. A reduction in the Bcl-2/Bax ratio was observed after the combination drug treatment in SK-MEL-5R cells (Fig. 3D). The combination of PLX4032 and juglone also increased the protein expression of cytochrome *c* (Fig. 3C and E).

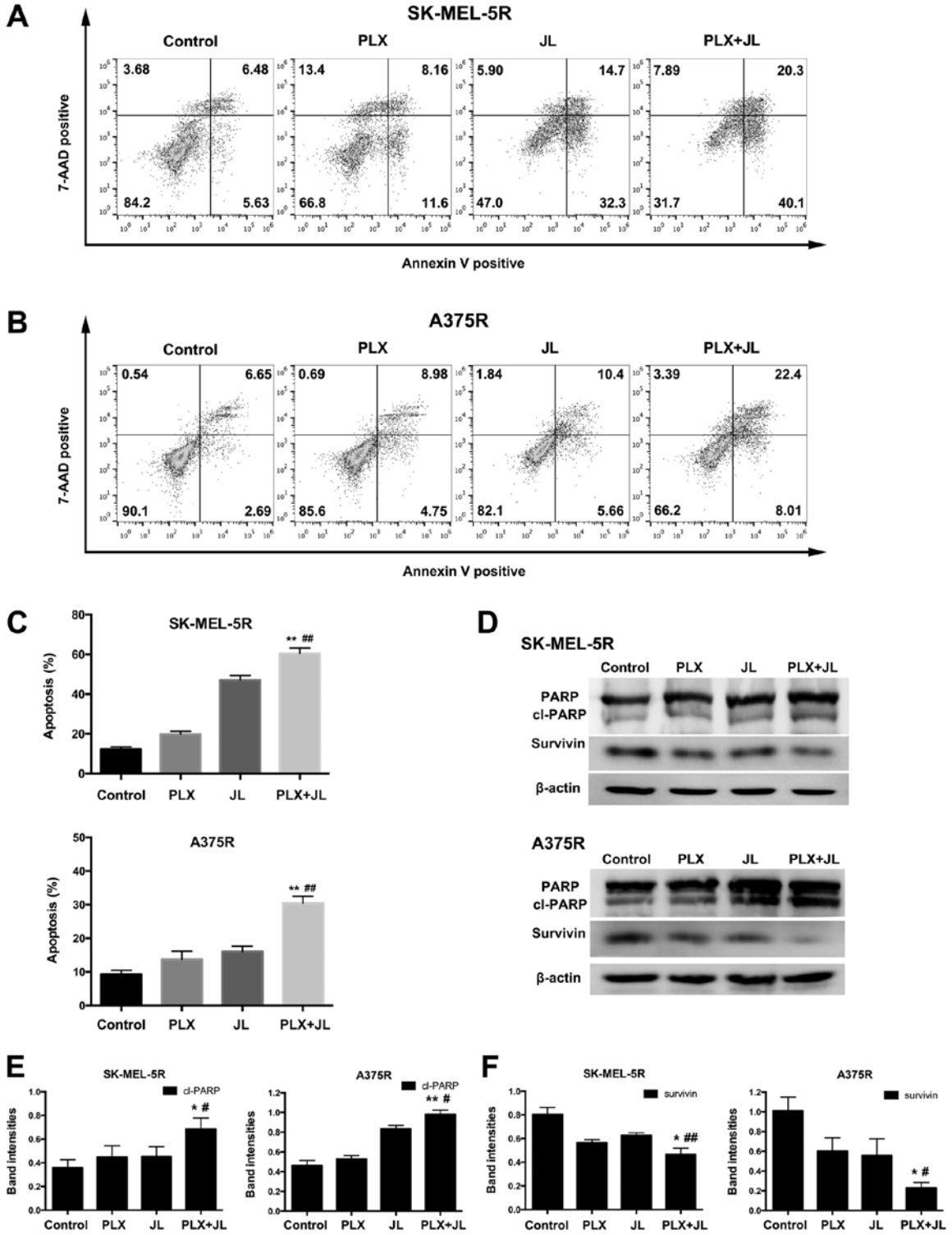

Figure 2. Juglone potentiates PLX4032-induced melanoma cell apoptosis. Flow cytometric analysis of (A) SK-MEL-5R cell apoptosis and (B) A375R cell apoptosis. (C) Quantitative presentation of early and late apoptotic cells. Cells were treated with juglone (0 and 5 μM) and/or PLX4032 (0 and 10 μM) for 24 h. Next, cells were stained with Annexin V and 7-AAD, followed by analysis in a flow cytometer. Experiments were repeated three times. **P<0.01 vs. PLX alone; $^{\#\#}$P<0.01 vs. JL alone. (D) Representative western blot images of PARP and survivin; SK-MEL-5R and A375R cells were treated with juglone (0 and 5 μM) and/or PLX4032 (0 and 10 μM) for 6 h. Semi-quantification of band intensities of (E) cl-PARP and (F) surviving in SK-MEL-5R and A3755R cells. *P<0.05, **P<0.01 vs. PLX; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. JL. JL, juglone; PARP, poly(ADP-ribose) polymerase; Cl-, cleaved.

Juglone increases the cellular ROS level and activated p38, p53 and JNK in SK-MEL-5R cells. Since mitochondrial apoptosis is associated with the generation of ROS (27), the production of intracellular ROS was examined using the oxidant-sensitive fluorescent probe DCFH-DA. The amount of ROS was analysed in cells treated with juglone and/or PLX4032 using a fluorescence microplate reader. Both PLX4032 or juglone alone increased the level of ROS. However, the level of ROS was significantly higher in the juglone and PLX4032 cotreatment group compared with the juglone or PLX4032 alone groups (Fig. 4A).

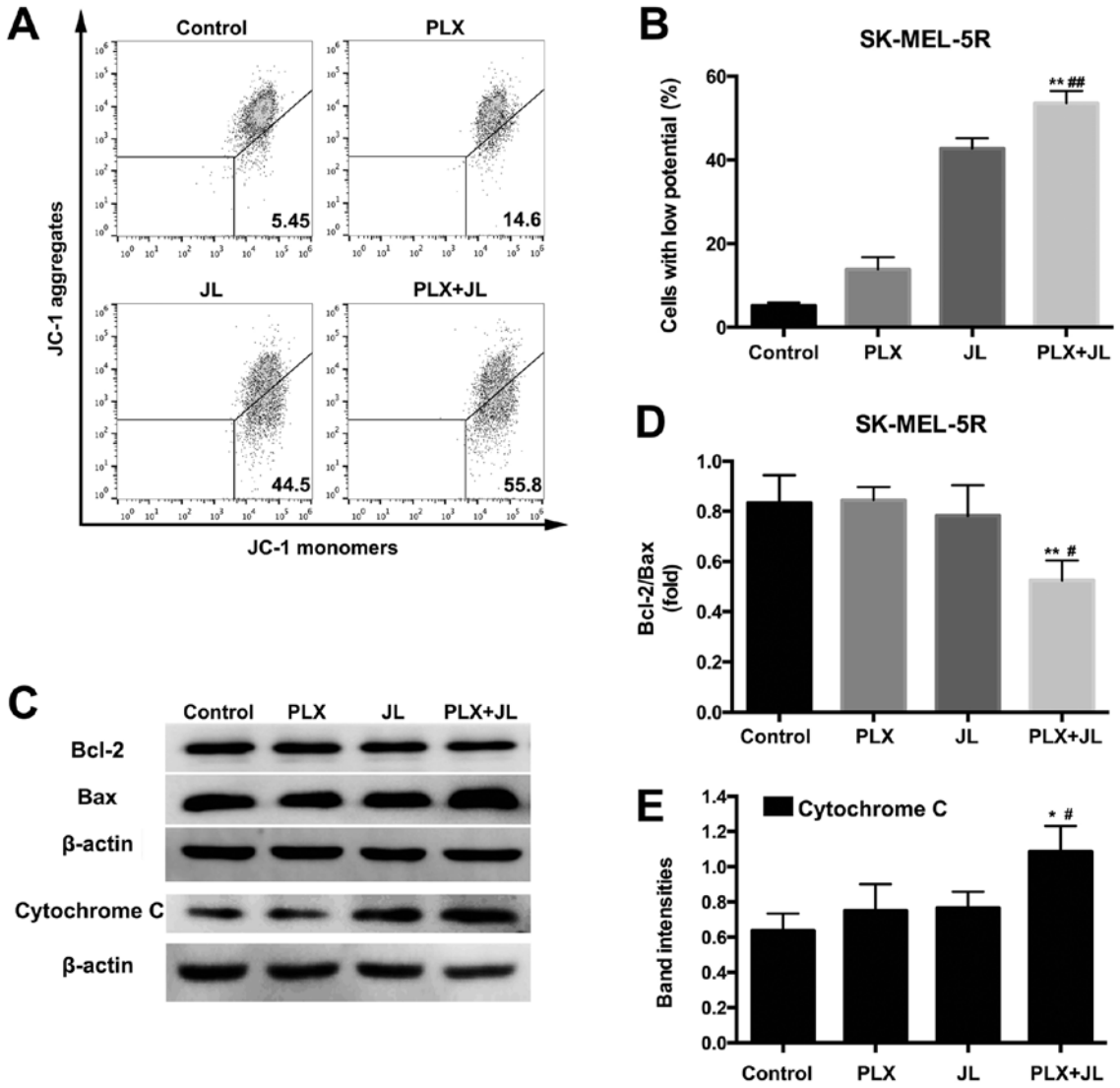

Figure 3. Juglone enhances PLX4032-induced mitochondrial apoptosis. (A) Mitochondrial membrane potential analysed by JC-1 fluorescence with flow cytometry. (B) Quantification of cells with low potential. **P<0.01 vs. PLX alone; $^{\#\#}$P<0.01 vs. JL alone. (C) Representative western blot images of Bcl-2, Bax and cytochrome *c*. (D) Bcl-2/Bax band intensity ratio. **P<0.01 vs. PLX alone; $^{\#}$P<0.05 vs. JL alone. (E) Semi-quantification of band intensities of cytochrome *c*. *P<0.05 vs. PLX; $^{\#}$P<0.05 vs. JL. JC-1, 5,5',6,6'-etrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide; PLX, PLX4032; JL, juglone.

Previous evidence has indicated that ROS can also mediate cell death via key modulators, such as p38 mitogen-activated protein kinase (MAPK) and p53 (28,29). To test whether the p38-p53 pathway was activated by juglone and PLX4032 cotreatment, western blotting was used to measure the protein expression of p38, p53 and their phosphorylated counterparts. As demonstrated in Fig. 4B, in SK-MEL-5R cells, juglone and PLX4032 cotreatment markedly increased p38 and p53 phosphorylation compared with treatment of PLX4032 or juglone alone. JNK (Thr183/Tyr185) activation was also observed after juglone and PLX4032 cotreatment.

NAC and p38 inhibitor pre-treatment partially reverse juglone and PLX4032 cotreatment-induced cytotoxicity. After cells were pre-treated with NAC (a ROS scavenger), or SB203580 (a p38 inhibitor), juglone and PLX4032 cotreatment-induced cytotoxicity was partially reversed (Fig. 4C and D). Furthermore, as demonstrated by western blotting, the protein expression of cleaved PARP in the NAC or p38 inhibitor pre-treatment group was lower than the protein expression in the juglone/PLX4032 combination group without NAC or p38 inhibitor pre-treatment (Fig. 4E-H; P<0.05). However, pre-treatment with SP600125 (a JNK inhibitor), failed to reverse the cytotoxicity induced by juglone and PLX4032 cotreatment (Fig. 4D). This was consistent with the inability of JNK inhibitor to reduce the levels of cl-PARP (Fig. 4F and H). These results suggested that ROS production and p38 activation play important roles in juglone-induced PLX4032 sensitization.

Discussion

It has been reported that juglone displays anti-tumour activities in a variety of tumour cell lines (11,14,30), and that treatment of B16F10 melanoma cells with juglone results in a concentration-dependent reduction in cell viability. For example, after 24 h exposure to 5 μM juglone, cytotoxicity was increased by only ~20% compared with the vehicle control (11). This

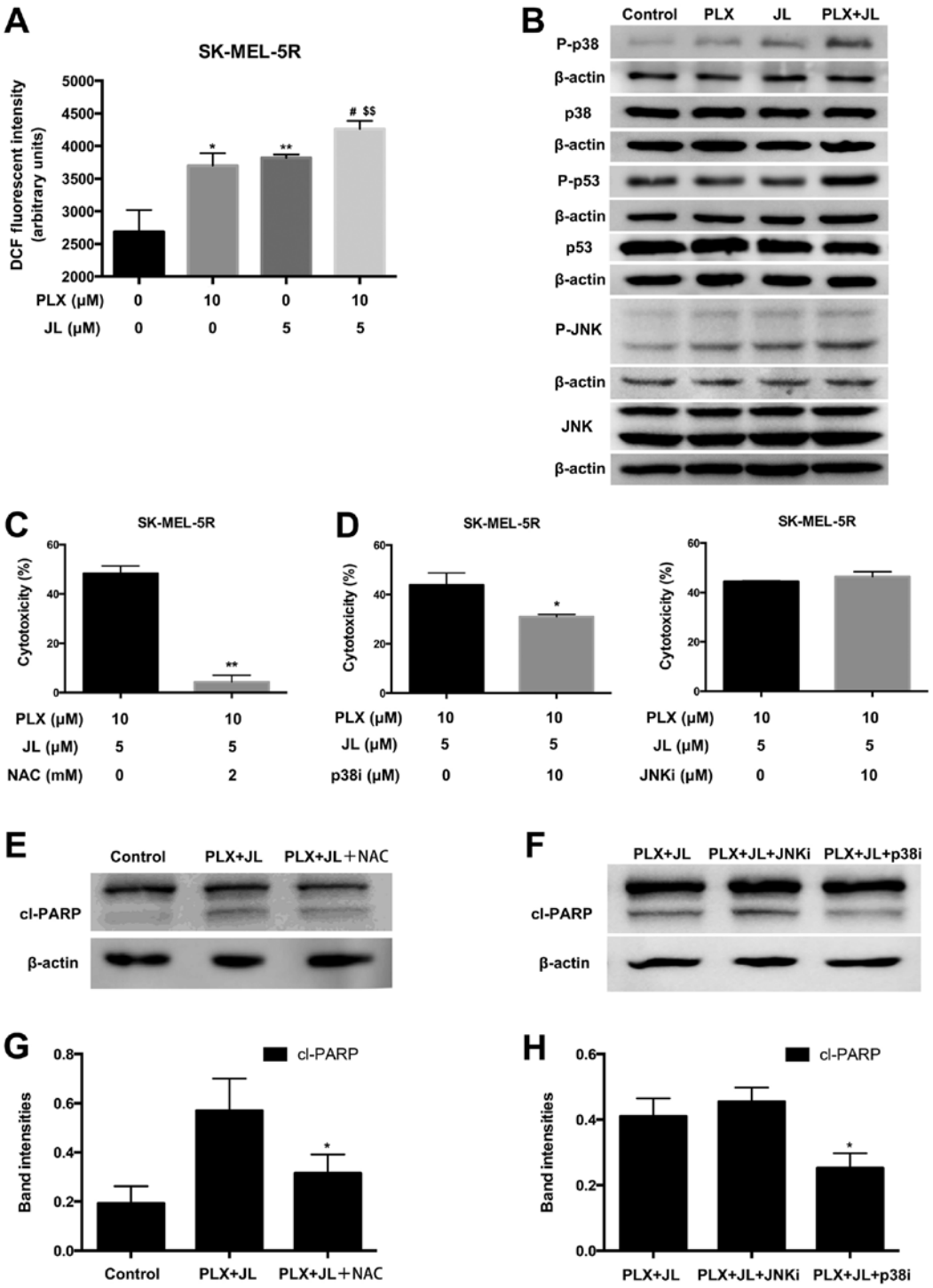

Figure 4. Juglone in combination with PLX4032 increases the production of ROS and activates p38, p53 and JNK. (A) Intracellular ROS levels measured by DCFH-DA. SK-MEL-5R cells were treated with juglone (0 and 5 μM) and/or PLX4032 (0 and 10 μM) for 2 h, fluorescence intensity was monitored by a fluorescence microplate reader. **P<0.01, *P<0.05 vs. control; $^{\#}$P<0.05 vs. PLX; $^{\$\$}$P<0.01, vs. JL. (B) Representative western blot images of p-p38, p38, p-p53, p53, p-JNK and JNK. (C) NAC pre-treatment partially reverses cytotoxic effect of juglone. SK-MEL-5R cells were pre-treated with NAC (0 and 2 mM) for 1 h, followed by juglone and PLX4032 cotreatment for 24 h. The cytotoxicity was measured using MTT assays. **P<0.01 vs. NAC non-pre-treatment. (D) p38i partially reversed the cytotoxic effect of juglone and PLX4032. SK-MEL-5R cells were pre-treated with p38i (0 and 10 μM) or JNKi (0 and 10 μM) for 1 h, followed by juglone and PLX4032 cotreatment for 24 h. Cytotoxicity was measured using MTT assays. *P<0.05. (E) Representative western blot images of cl-PARP from cells pre-treated with NAC. (F) Representative western blot images of cl-PARP from cells pre-treated with p38i and JNKi. (G) Semi-quantification of band intensities of cl-PARP from part (E). (H) Semi-quantification of band intensities of cl-PARP from part (F). *P<0.05 vs. PLX and JL cotreatment. NAC, N-acetyl-L-cysteine; PARP, poly(ADP-ribose) polymerase; DCFH-DA, 2',7'-dichlorofluorescein diacetate; DCF, 2',7'-dichlorofluorescein; JL, juglone; ROS, reactive oxygen species; P-, phosphorylated; Cl-, cleaved; p38i, p38 inhibitor; JNKi, JNK inhibitor.

is in accordance with the finding in the present study that juglone at a low concentration possesses weak activity against BRAF-mutant melanoma cells.

Juglone has displayed potent cytotoxic properties against chemo-resistant and radio-resistant tumour cell lines both *in vitro* and *in vivo* (12,31). In addition, it has been considered

as a sensitizing agent for overcoming trastuzumab resistance in human breast cancer SKBR3 cells (32). Combination therapy with a BRAF inhibitor and juglone might provide a novel strategy for overcoming BRAF inhibitor resistance. In the present study, to the best of our knowledge, it was reported for the first time that juglone enhanced the response to a BRAF inhibitor in BRAF inhibitor-resistant melanoma cells.

Apoptosis is a form of complex signalling-controlled cell death. The two main pathways of apoptosis are the extrinsic pathway and the intrinsic pathway, which is also called the mitochondrial pathway. The intrinsic pathway that initiates apoptosis involves a diverse array of non-receptor-mediated stimuli. These produce intracellular signals that cause changes in the inner mitochondrial membrane (33). The anti-cancer effects of juglone can be partially attributed to mitochondrial apoptosis (30,34). Juglone has been found to markedly alter the levels of Bcl-2 and Bax, thereby releasing cytochrome *c* into the cell cytoplasm, and inducing subsequent caspase activation and activation of PARP (30,34). In the present study, it was found that juglone potentiated BRAF inhibitor-induced apoptosis in both A375R and SK-MEL-5R cells, which was accompanied by a decline in $\Delta\Psi_M$, and a reduction of the Bcl-2/Bax ratio.

ROS are one of the stimuli that can induce the mitochondrial apoptotic pathway. An increase in ROS leads to the opening of the mitochondrial membrane permeability transition pore, which destroys the integrity of the mitochondrial membrane, inducing $\Delta\Psi_M$ loss (33). Meanwhile, damaged mitochondria produce more ROS and accelerate apoptosis. It was revealed that the cytotoxicity of juglone is generally attributed to its ability to induce redox cycling with consequent ROS, leading to oxidative stress-mediated cell death (35). Elevated ROS levels have been observed after juglone treatment in multiple human cancer lines, including glioma, gastric cancer and melanoma cells (14,30,36). In the present study, juglone alone significantly increased the levels of ROS in BRAF inhibitor-resistant melanoma cells. Moreover, the level of ROS was higher in the juglone and PLX4032 cotreatment group than in the single agent treatment groups. Pre-treatment with NAC, a ROS scavenger, partially reversed juglone and PLX4032 combination-induced cytotoxicity. Therefore, it was concluded that the synergistic effects of juglone and a BRAF inhibitor in melanoma cells were partially mediated by ROS.

In addition to disrupting the mitochondrial membrane, ROS act as secondary messengers to trigger apoptotic signals (37). The downstream molecules affected by ROS include MAPK, PI3K/AKT, phospholipase γ1, NF-κB and Janus kinase, among which the MAPK pathway is a complex family that transmits extracellular signals to the intracellular environment. MAPK pathways include ERK, JNK and p38. Unlike ERK, both JNK and p38 mediate tumour cell apoptosis. JNK and p38 and are also more stress-responsive than ERK, and are often termed stress-activated protein kinases (38). p53 is a direct downstream effector of p38 (28,39), which causes cell cycle arrest and induces cellular senescence and apoptosis (40). In the present study, it was demonstrated that the p38-p53 pathway was activated after juglone and PLX4032 cotreatment.

A previous study has demonstrated that juglone induced HeLa cells to undergo apoptosis by activating the JNK/c-Jun pathway (41). Previously, a JNK activator was reported to exert anti-tumour activity in vemurafenib-resistant melanoma cells, and this indicated that the induction of apoptosis through the activation of the JNK pathway might represent a novel strategy to overcome resistance to vemurafenib (42). In the present study, it was demonstrated that juglone and PLX4032 cotreatment activated JNK. However, unlike p38 inhibition, inhibition of JNK could not reverse the juglone-induced enhanced sensitivity to PLX4032. Therefore, this suggested that p38, and not JNK, plays a crucial role in the induction of apoptosis following juglone and PLX4032 cotreatment. Juglone has been reported to possess low cytotoxicity in human peripheral blood mononuclear cells (43). However, it is unclear whether juglone combined with a BRAF inhibitor is toxic in normal cells. Further *in vivo* studies are necessary to determine the efficacy and safety of juglone and BRAF inhibitor cotreatment in BRAF inhibitor-resistant melanoma.

In conclusion, juglone potentiated the BRAF inhibitor-induced apoptosis in BRAF inhibitor-resistant melanoma cells, and these effects were partially mediated through ROS and the p38-p53 pathway, thereby suggesting the potential of juglone as a sensitizer to BRAF inhibitors in the treatment of melanoma.

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Availability of data and materials

The datasets used and/or analysed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

JX and JW designed the research and interpreted the data; ZL and XL performed the experiments and wrote the manuscript. ML, JC and SH analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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