Daphnoretin induces reactive oxygen species-mediated apoptosis in melanoma cells

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Abstract. Research suggests that daphnoretin exhibits a diverse array of antitumor mechanisms and pharmacological activities. However, there is no definitive explanation for the antitumor mechanisms of daphnoretin in malignant melanoma. In the present study, MTT and colony formation assays demonstrated that daphnoretin significantly inhibited the proliferation of melanoma A375 and B16 cells. Following treatment with daphnoretin, apoptotic bodies were observed in A375 and B16 cells via Hoechst 33258 staining. Furthermore, western blot analysis revealed that the apoptosis-related proteins cleaved caspase-3, cleaved caspase-9, Bax, cytochrome *c* and apoptotic protease-activating factor 1 were significantly upregulated, while the expression levels of caspase-3, caspase-9 and Bcl-2 were downregulated in A375 and B16 cells. Flow cytometry and fluorescence microscopy revealed that daphnoretin induced higher levels of reactive oxygen species (ROS). Therefore, the results of the present study indicated that daphnoretin induced ROS-mediated mitochondria apoptosis in human (A375) and murine (B16) malignant melanoma cells.

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Introduction

Melanoma, also known as malignant melanoma, is characterized by the abnormal proliferation of melanocytes in the skin (1). Melanoma accounts for ~2% of all skin cancer cases, but due to its strong invasiveness, causes the majority of skin cancer-associated deaths (2). The incidence rate and degree of malignancy in melanoma are high, with seriously impacts on human health (3). Melanoma is less common than other types of skin cancer, but more likely to invade nearby tissues and metastasize to other regions of the body (4). Furthermore, the rate of melanoma occurrence has increased considerably over the past few decades (5). Currently, surgery, chemotherapy and radiotherapy are the primary treatment options (6), and in the early stages of melanoma, the lesion can be successfully resected (7). Although clinical treatments are becoming increasingly more refined, a number of disadvantages remain, including the various side effects of radiotherapy and chemotherapy. Due to the frequent emergence of drug resistance, currently-available chemotherapeutics do not effectively treat patients with advanced melanoma.

As a result of good efficacy, mild side effects, low toxicity and its numerous targets, Traditional Chinese Medicine has been used to treat various cancer types (8). Consequently, the identification of effective anti-melanoma drugs from Chinese plant medicine is of great clinical significance. Traditional Chinese Medicine comprises complex components with low drug resistance (9), and leading antitumor components with novel structures and significant pharmacological activity have been confirmed (10,11). Some of these components act on multiple targets simultaneously or cooperatively, making it possible to screen multi-target therapeutic drugs that meet clinical requirements (12,13). Daphnoretin (14), which is extracted from *Wikstroemia indica* (15), has been proven to exert significant antitumor effects. Nevertheless, the mechanism of its antitumor properties in melanoma remains unclear, thus prompted the evaluation of daphnoretin treatment in the present study.

To determine the possible intrinsic mechanism of daphnoretin-induced melanoma cell apoptosis, the antiproliferative effect of daphnoretin on melanoma A375 and B16 cells was examined using MTT and colony formation assays. Subsequently, the apoptotic rate and the levels

of apoptosis- associated proteins were determined by Hoechst 33258 staining and western blotting. In addition, the ROS levels in daphnoretin-treated A375 and B16 cells were detected using DCFH-DA probes. These findings may promote further research into novel treatments for melanoma.

Materials and methods

Cell culture and reagents. Human A375 and murine B16 malignant melanoma cells were purchased from the cell banks of the Chinese Academy of Sciences. Daphnoretin (cat no. X-051-191008; purity, ≥98%) was purchased from Chengdu Herbpurify Co., Ltd. DMEM high-glucose culture medium was procured from HyClone (Cytiva), and supplemented with 10% FBS and 1% penicillin-streptomycin (Beijing Solarbio Science & Technology Co., Ltd.). The cells were maintained in a humidified incubator at 37˚C (5% CO_2).

MTT assay. Cells were seeded into 96-well plates at a density of $1x10^5$ cells/ml and cultured for 24 h, after which different concentrations of daphnoretin (0, 10, 20, 30, 40, 50, 60, 70 and 80 μg/ml) were added for a further 24 h. Next, 20 μl MTT (5 mg/ml) was added to each well and the cells were incubated for an additional 4 h before the media were discarded. Then, 150 μl DMSO was added, and the plates were agitated for 10 min at room temperature. The absorbance value was determined using a microplate reader (Infinite 200 Pro; Tecan Group, Ltd.) at 490 nm (16).

Colony formation assay. Cells in the logarithmic phase were harvested with 0.25% trypsin and homogenized. The cells were resuspended in DMEM medium with 10% FBS and then seeded into 6-well plates at 200 cells/well. Once adherent proliferation was established, the cells were treated with different doses of daphnoretin (0, 20, 40 and 60 μg/ml) and incubated for 24 h. On the second day, the media were replaced, and the cells were continuously cultured in a 37˚C (5% CO_2); the media were then replaced once every three days for 2-3 weeks. The cells were fixed with 4% paraformaldehyde solution (Beijing Solarbio Science & Technology Co., Ltd.) for 10 min at 4˚C, and then stained with 1% crystal violet for 10 min at room temperature (both Beijing Solarbio Science & Technology Co., Ltd.). The cells were observed under a fluorescence microscope (DMI3000B, Leica Microsystems GmbH) and images were captured.

Hoechst 33258 staining. To observed changes in morphology, cells were seeded into 6-well plates ($2x10^5$ cells/ml) and incubated for 24 h, after which different concentrations of daphnoretin (0, 20, 40 and 60 μg/ml) were added. After 24 h, 4% paraformaldehyde solution was added at 4˚C. After 10 min, the cells were washed twice with PBS and stained with Hoechst 33258 for 10 min at room temperature. Images were then captured using a fluorescence microscope (17).

Flow cytometric apoptosis analysis. Apoptosis was detected by flow cytometry. The cells were seeded into 6-well plates ($2x10^5$ cells/ml) and incubated for 24 h. Then, daphnoretin was added at different concentrations (0, 20, 40 and 60 μg/ml). The cells were collected after 24 h, and 10 μl propidium iodide

was added for 10 min, followed by 5 μl Annexin V-FITC for a further 5 min (both at room temperature). Flow cytometry was performed using a FACSCanto II flow cytometer (Becton, Dickinson and Company) (18). The apoptosis rate was analyzed using FACSDiva 6.1.3 software (Becton, Dickinson and Company).

ROS detection. Cells were seeded into 6-well plates at a density of $1.8x10^5$ cells/ml, and then incubated for 24 h. Different concentrations of daphnoretin were administered (0, 20, 40 and 60 μg/ml) for 24 h, after which the cells were digested with 0.25% trypsin (Beijing Solarbio Science & Technology Co., Ltd.). DCFH-DA (Beijing Solarbio Science & Technology Co., Ltd.) was diluted with serum-free medium (1:1,000) to a final concentration of 10 μM, and 1 ml was added per well prior to incubation at 37˚C for 20 min. After washing three times with serum-free medium, the cells were analyzed by flow cytometry or photographed under a fluorescence microscope.

Western blot analysis. Cells were seeded into 100-mm cell culture dishes with daphnoretin administered (0, 20, 40 and 60 μg/ml) and incubated for 24 h. The cells were then collected and washed twice with PBS. RIPA buffer (cat. no. R0010; Beijing Solarbio Science & Technology Co., Ltd.) was added to lyse the cells, and the mixture was kept on ice for 30 min. The lysate was then centrifuged at 13,000 x g for 4 min at 4˚C, and the protein concentration was measured using BCA protein assay kit, and equalized between samples using 4X loading buffer. The mass of protein loaded per well was 80 μg. The samples were run on a 12% SDS-PAGE gel, transferred to PVDF membranes (EMD Millipore) and then blocked with 5% milk for 1 h at room temperature. Diluted primary antibodies against the following targets were then added overnight at 4˚C: β-actin, (cat. no. TA-09; 1:2,000; OriGene Technologies, Inc.); caspase-3 (cat. no. ab13847; 1:1,000; Abcam); cleaved caspase-3 (cat. no. 9661S; 1:1,000; Cell Signaling Technology, Inc.); caspase-9 (cat. no. Ab202068; 1:2,000; Abcam); cleaved caspase-9 (cat. no. 20750S; 1:1,000; Cell Signaling Technology, Inc.); apoptotic protease-activating factor 1 (Apaf-1; cat no. 5088S; 1:1,000; Cell Signaling Technology, Inc.); cytochrome *c* (cat. no. ab110325; 1:2,000; Abcam); Bcl-2 (cat. no. ab196495; 1:1,000, Abcam); Bax (cat. no. ab182734; 1:1,000; Abcam); PI3K (cat. no. 60225-1; 1:5,000, ProteinTech Group, Inc.); phospho (p-)PI3K (cat. no. ab182651; 1:500; Abcam); Akt (cat. no. 9272S; 1:1,000; Cell Signaling Technology, Inc.); and p-Akt (cat. no. 4060S; 1:2,000; Cell Signaling Technology, Inc.). The membranes were washed four times for 5 min each (with 1X TBST on a shaking platform), and then diluted secondary antibodies against the following targets were added overnight at room temperature: Peroxidase-conjugated affinipure goat anti-mouse IgG (cat. no. ZB-2305; 1:30,000; OriGene Technologies, Inc.) and goat anti-rabbit IgG (cat. no. ab6721; 1:20,000; Abcam). Membranes were washed four times for 5 min with 1X TBST. Tweezers were used to move each membrane to a culture dish, and ECL chemiluminescence working buffer (cat. no. 34580; Thermo Fisher Scientific, Inc.) was carefully pipetted over each membrane. Finally, the membranes were visualized using a BioSpectrum® Imaging System (Ultra Violet Products, Ltd.).
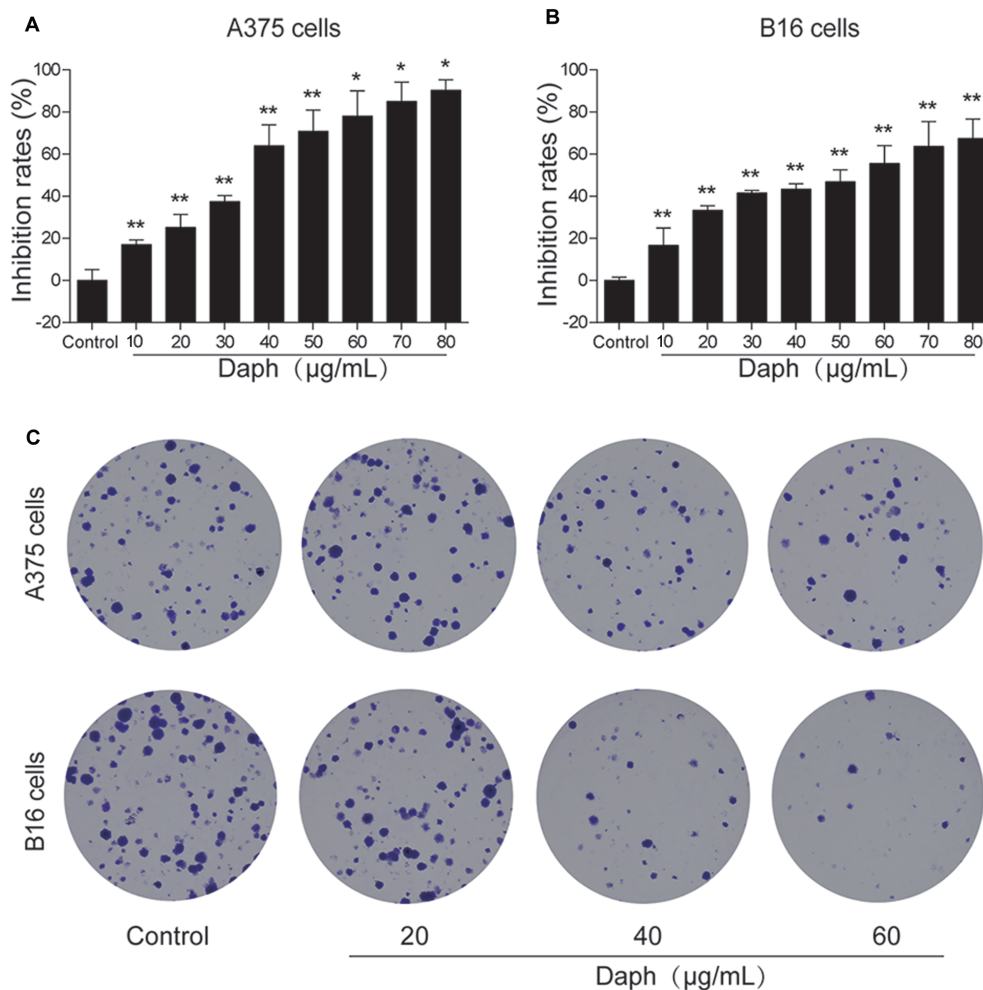
Figure 1. Daphnoretin inhibits the proliferation and colony-formation capacity of melanoma cells. MTT was used to determine the effects of different concentrations of daphnoretin on (A) A375 and (B) B16 cells after 24 h; cell viability of A375 or B16 cells. (C) Colonies were stained with crystal violet and observed under a microscope. Data are expressed as the mean ± SD of three independent experiments. *P<0.05 and **P<0.01 vs. control. Daph, daphnoretin.

ImageJ v1.43 software (National Institutes of Health) was used for densitometry.

Statistical analysis. The data are presented as the mean ± standard deviation, and each experiment was repeated at least three times. One-way ANOVA or Student's t-test followed by Bonferroni's post hoc test were performed to compare the differences between groups using GraphPad Prism 6.0 (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Daphnoretin inhibits A375 and B16 cell proliferation and colony formation. An MTT assay was performed to evaluate the antitumor effect of daphnoretin in melanoma A375 and B16 cells. A 24-h treatment with daphnoretin significantly suppressed cellular proliferation, and the IC_{50} values for A375 and B16 cells were 37.81 and 53.46 µg/ml, respectively (Fig. 1A and B). Based on the results of the previous experiment, a colony formation assay was conducted; the results suggested that the number of A375 and B16 cell colonies gradually decreased compared with that of the control group

(Fig. 1C). These results indicated that daphnoretin inhibited cell proliferation.

Daphnoretin induces A375 and B16 cell apoptosis. Hoechst 33258 staining was conducted to assess A375 and B16 cell apoptosis. Under a fluorescence microscope, the formation of apoptotic bodies was observed (Fig. 2A and D). To determine the mechanism of apoptosis in A375 and B16 cells following daphnoretin treatment, flow cytometry was used to quantify cells positive for annexin V-FITC and PI. Daphnoretin significantly increased the apoptotic rate of A375 and B16 cells in a dose-dependent manner (Fig. 2B and E). Moreover, the apoptotic rates of daphnoretin-treated A375 and B16 cells were significantly increased compared with those of the corresponding control groups (Fig. 2C and F). These findings suggested that daphnoretin induced apoptosis in A375 and B16 melanoma cells.

Daphnoretin induces melanoma cell apoptosis in a caspase-dependent manner. The effects of daphnoretin on the levels of apoptosis-related proteins were evaluated using A375 and B16 cells. Western blotting revealed that the protein levels of caspase-3 and -9 were both significantly decreased,
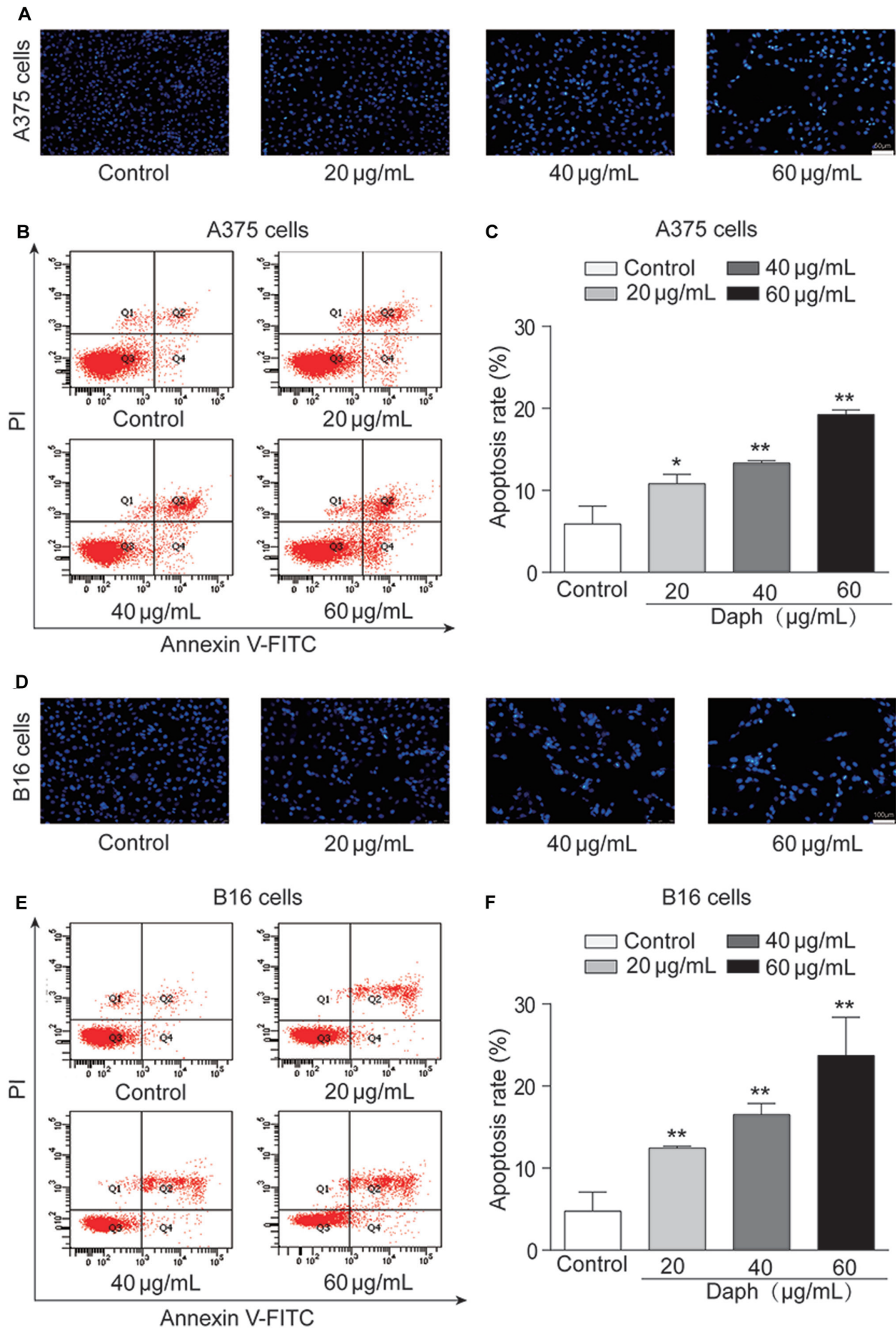
Figure 2. Daphnoretin induces A375 and B16 melanoma cell apoptosis. (A) Morphological changes in daphnoretin-treated A375 cells by Hoechst 33258 staining. (B) Apoptotic rate of daphnoretin-treated A375 cells was determined by flow cytometry. (C) Statistical analysis of the apoptosis rate of A375 cells after daphnoretin treatment. (D) Morphological changes in daphnoretin-treated B16 cells by Hoechst 33258 staining. (E) Apoptotic rate of daphnoretin-treated B16 cells was determined by flow cytometry. (F) Statistical analysis of the apoptosis rate of B16 cells after daphnoretin treatment. Data are expressed as the mean ± SD of three independent experiments. *P<0.05 and **P<0.01 vs. control. Daph, daphnoretin.
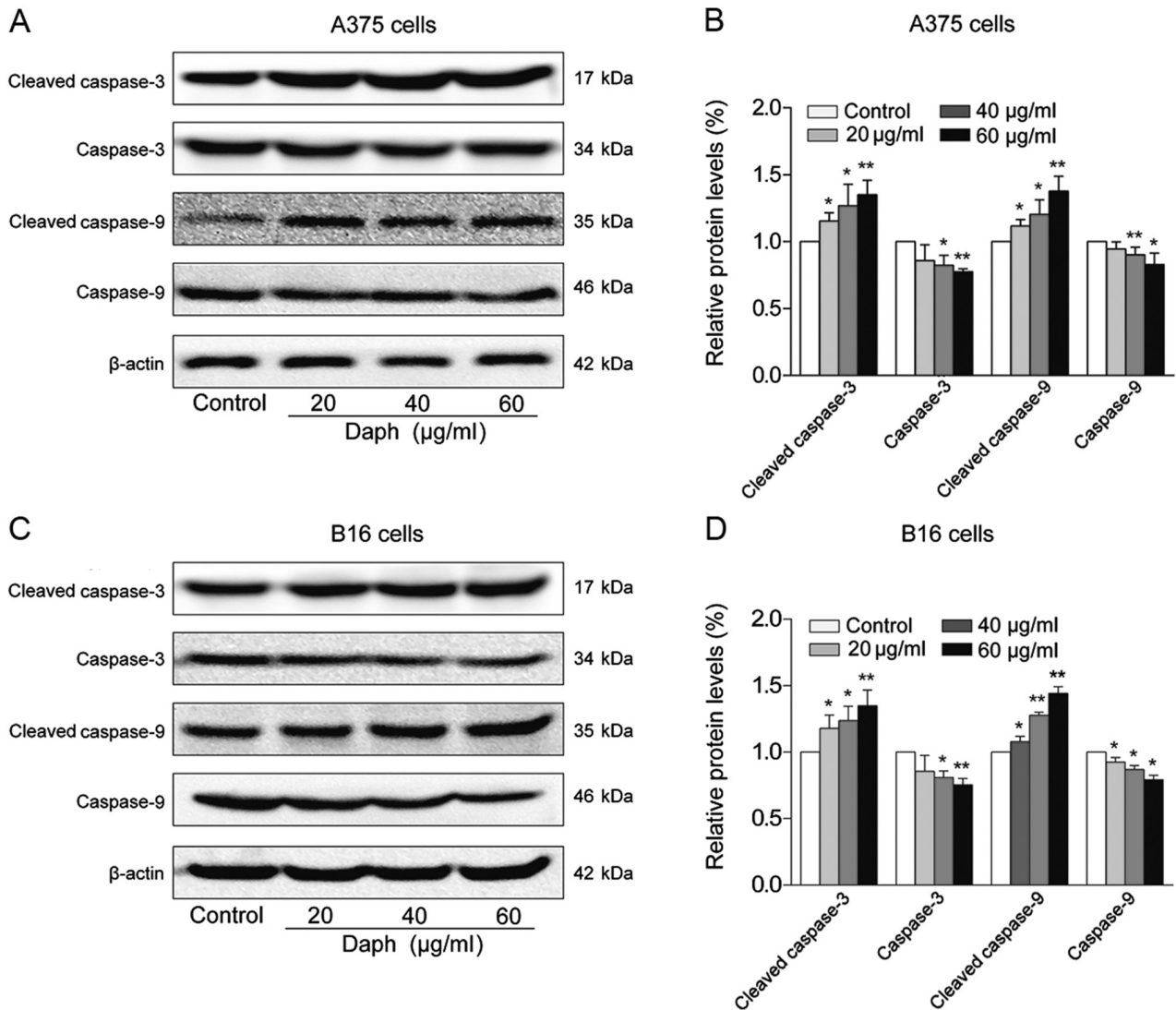
Figure 3. Daphnoretin induces melanoma cell apoptosis via a caspase-dependent mechanism. (A) Expression levels of cleaved caspase-3, caspase-3, cleaved caspase-9 and caspase-9 in daphnoretin-treated A375 cells were measured by western blotting. (B) Statistical analysis of protein levels in A375 cells following daphnoretin treatment. (C) Expression levels of cleaved caspase-3, caspase-3, cleaved caspase-9 and caspase-9 in daphnoretin-treated B16 cells were quantified by western blotting. (D) Statistical analysis of the protein levels after daphnoretin treatment. Data are expressed as the mean ± SD of three independent experiments. *P<0.05 and **P<0.01 vs. control. Daph, daphnoretin.

while those of cleaved caspase-3 and -9 were dose-dependently increased in daphnoretin-treated A375 cells (Fig. 3A and B). Similarly, the expression levels of caspase-3 and -9 were upregulated, and those of cleaved caspase-3 and -9 were downregulated in daphnoretin-treated B16 cells, compared with the control group (Fig. 3C and D). These results suggested that daphnoretin induced apoptosis in A375 and B16 cells by a caspase-dependent mechanism.

Daphnoretin induces melanoma cell apoptosis via the Bax/Bcl-2 pathway. The expression levels of another set of apoptosis-related proteins were measured by western blotting. The data showed that the levels of Bcl-2 were upregulated, whereas Bax, cytochrome *c* and Apaf-1 were downregulated in daphnoretin-treated A375 cells (Fig. 4A and B). Similarly, the levels of Bcl-2 increased, while those of Bax, cytochrome *c* and Apaf-1 decreased in B16 cells following treatment with daphnoretin (Fig. 4C and D). These results suggested that

daphnoretin induced apoptosis in A375 and B16 cells by a Bax/Bcl-2-dependent pathway.

Daphnoretin increases the level of ROS production in melanoma cells. ROS plays a regulatory role in the process of apoptosis induced by various antitumor drugs (19). It has been confirmed that the increase in ROS may induce apoptosis (20). In the present study, the level DCF-associated green fluorescence was gradually increased in A375 and B16 cells exposed to daphnoretin (Fig. 5A and C). In addition, the flow cytometry results showed that 24 h after daphnoretin treatment, the level of ROS in A375 and B16 cells dose-dependently increased compared with the corresponding control group (Fig. 5B and D). The data indicated that daphnoretin increased the level of ROS in melanoma cells.

Daphnoretin regulates the PI3K/Akt signaling pathway in A375 and B16 cells. The PI3K/Akt signaling pathway is an
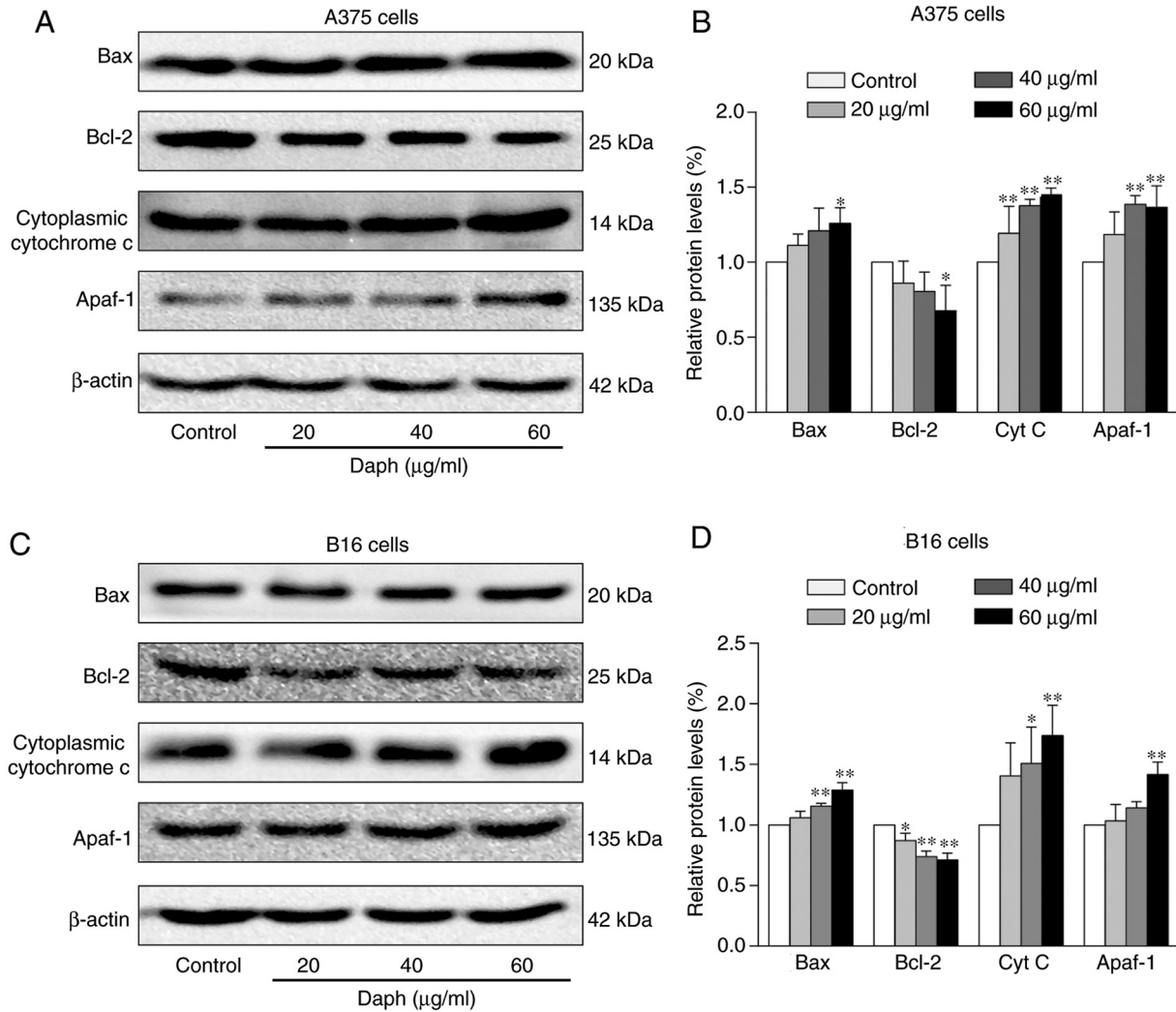
Figure 4. Daphnoretin induces melanoma cell apoptosis via a Bax/Bcl-2 dependent pathway. (A) Expression levels of Bax, Bcl-2, cytoplasmic cytochrome c and Apaf-1 in daphnoretin-treated A375 cells were measured by western blotting. (B) Statistical analysis of the protein levels of Bax, Bcl-2, cytoplasmic cytochrome c and Apaf-1 in A375 cells after daphnoretin treatment. (C) Expression levels of Bax, Bcl-2, cytoplasmic cytochrome c and Apaf-1 in daphno-retin-treated B16 cells were quantified by western blotting. (D) Statistical analysis of the protein levels after daphnoretin treatment. Data are expressed as the mean ± SD of three independent experiments. *P<0.05 and **P<0.01 vs. control. Daph, daphnoretin; Cyt C, cytochrome c; Apaf-1, apoptotic protease-activating factor 1.

important pathway closely related to tumor genesis and devel-opment, thus the protein levels of PI3K/Akt were assessed by western blotting. The results showed that the protein expres-sion levels of PI3K and Akt were increased, whereas p-PI3K and p-Akt were decreased in daphnoretin-treated A375 and B16 cells (Fig. 6A-D). These results indicated that daphnoretin regulated the PI3K/Akt signaling pathway.

Discussion

Daphnoretin exerts antitumor effects in specific tumor types. A study demonstrated that daphnoretin induced apoptosis in human osteosarcoma cells by triggering G_2/M cell cycle arrest and activating the caspase-3 pathway (21). In another study, daphnoretin was shown to induce the mitochondria-mediated apoptosis of HeLa cells (22). Daphnoretin has also been reported to influence the proliferation and apoptosis of A549 lung cancer cells *in vitro* (23). Moreover, daphnoretin was found to inhibit the proliferation, invasiveness and migration

of colon cancer HCT116 cells, and to promote apoptosis by regulating Akt signaling pathway activity (24). The results of the present study demonstrated that daphnoretin inhibited proliferation and promoted apoptosis via the ROS-mediated mitochondrial apoptosis and the PI3K/Akt signaling pathways.

In the present study, daphnoretin was first found to signifi-cantly inhibit the proliferation of A375 and B16 melanoma cells in a concentration-dependent manner. To further confirm the mechanisms associated with the daphnoretin treatment, the formation of apoptotic bodies was observed in A375 and B16 cells following Hoechst staining, and the cellular apoptotic rate was significantly increased compared with the control group. These findings suggest that daphnoretin induced apoptosis in melanoma A375 and B16 cells.

Apoptosis is a complex process in which various protein signals are transmitted through several different path-ways (25). Caspases, which are the convergence points of multiple apoptotic pathways, regulate the final execution of apoptosis (26,27). Thus, caspase activation is an essential link
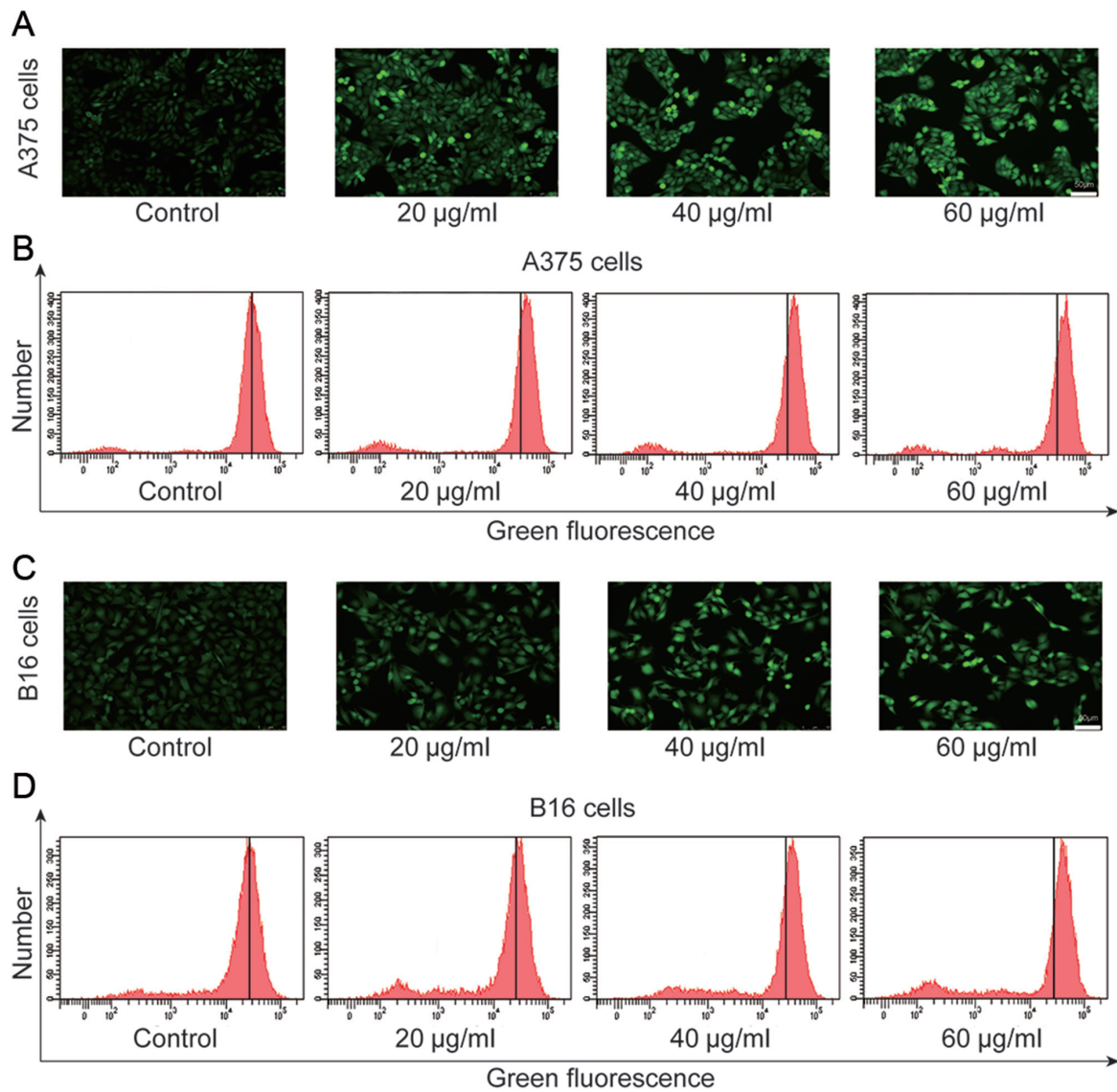
Figure 5. Daphnoretin induces ROS-mediated apoptosis in melanoma A375 and B16 cells. (A) Morphological changes in daphnoretin-treated A375 cells. (B) ROS content of daphnoretin-treated A375 cells was measured by flow cytometry. (C) Morphological changes in daphnoretin-treated B16 cells. (D) ROS content of daphnoretin-treated B16 cells was measured by flow cytometry. ROS, reactive oxygen species.

in apoptotic signal transduction (28). Under the stimulation of apoptotic signals, cytochrome c is released into the cytoplasm and combines with Apaf-1 to activate the caspase-9 precursor; activated caspase-9 then cleaves the caspase-3 proenzyme, which activates caspase-3, thus promoting its cleavage. Finally, a caspase cascade reaction is triggered, and apoptosis is induced (29,30). The present findings indicate that the levels of caspase-3 and -9 were significantly decreased, whereas the levels of cleaved caspase-3 and -9 were markedly increased compared with the control group.

Several studies have suggested that the Bcl-2 family plays a vital role in the apoptotic process (31,32). The expression and regulation of Bcl-2 family proteins are essential in signal transduction pathways and apoptosis. The pro-apoptotic protein Bax, and the anti-apoptotic protein Bcl-2, are essential members of the Bcl-2 family, and their coordinated action plays an important role in apoptosis (33). Bcl-2 and Bax usually exist as heterodimers, which jointly regulate apoptosis (34,35). When the expression of Bcl-2 is high, a large number of heterodimers with

Bax are formed, inhibiting the activity of Bax and preventing apoptosis; when the expression of Bax exceeds a certain point, Bax homodimers predominate, which induces apoptosis (35,36). The present results showed that the expression levels of Bax increased, while those of Bcl-2 decreased, suggesting that the Bcl-2 family regulates daphnoretin-induced apoptosis. Previous studies have shown that the Bcl-2 family members release cytochrome c and activated various caspases (37,38). Therefore, the results of the present study indicate that daphnoretin induces the activity of the Bcl-2 family proteins, and promote the release of cytochrome c and Apaf-1, thereby activating caspase-9 and -3, and ultimately inducing apoptosis.

An impairment of the ROS balance is associated with cancer cell proliferation inhibition. Compared with normal untransformed cells, ROS levels are increased in cancer cells (39). As signaling molecules in cellular regulation, ROS play an important role in apoptosis by regulating apoptotic factors (40). Deoxynivalenol induces the cytotoxicity of IPEC-J2 cells in a dose-dependent manner, in
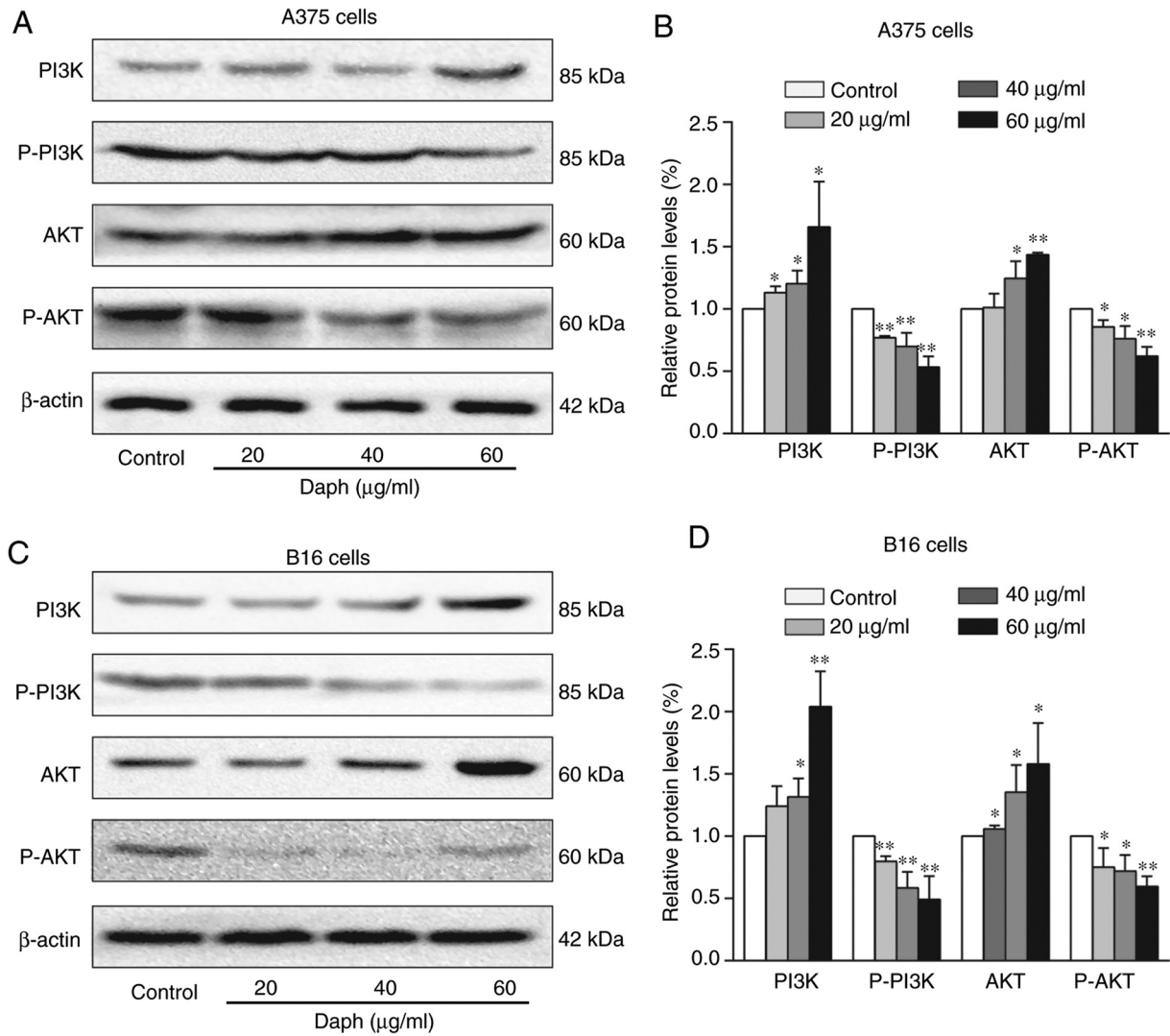
Figure 6. Daphnoretin regulates the PI3K/Akt signaling pathway in A375 and B16 cells. (A) Levels of PI3K, p-PI3K, Akt and p-Akt in daphnoretin-treated A375 cells were measured by western blotting. (B) Statistical analysis of the protein levels in A375 cells following daphnoretin treatment. (C) Levels of PI3K, p-PI3K, Akt and p-Akt in daphnoretin-treated B16 cells were assessed by western blotting. (D) Statistical analysis of the protein levels in daphnoretin-treated B16 cells. Data are expressed as the mean ± SD of three independent experiments. *$P<0.05$ and **$P<0.01$ vs. control. Daph, daphnoretin; p-, phosphorylated.

which apoptosis is potentially the result of increased ROS production (41). After cells receive pro-apoptotic signals, ROS generation increases, which promotes Ca^{2+} influx, upregulates the expression of Bax, initiates mitochondrial permeability transition pore formation, and activates caspases, which ultimately leads to apoptosis. A previous study revealed that ROS-induced apoptosis may be related to the ROS-dependent upregulation of Bax, and a reduction in Bcl-2 expression (42,43). Another study indicated that the activation of caspase-3 was regulated by ROS (44). To investigate these mechanisms in A375 and B16 cells treated with daphnoretin, ROS generation was measured by flow cytometry, which revealed increased levels of ROS in A375 and B16 cells. These results indicated that daphnoretin induced ROS-mediated apoptosis in melanoma A375 and B16 cells.

ROS have also been shown to play important roles in various signaling pathways, including the PI3K/Akt, Wnt/β-catenin and MAPK pathways (45). It has been reported that genistein induces G_2/M cell cycle arrest and

apoptosis in human bladder transitional cell carcinoma T24 cells through the ROS-mediated PI3K/Akt signaling pathway (46), which is also important for regulating ROS generation in HCT116 cells (47). Therefore, the potential association between PI3K/Akt and the daphnoretin-induced inhibition of proliferation and increased apoptosis were investigated using A375 and B16 cells. The findings suggested that the levels of PI3K and Akt increased, and that those of p-PI3K and p-Akt decreased, in daphnoretin-treated A375 and B16 cells, indicating that the PI3K/Akt signaling pathway is involved in the antitumor effect of daphnoretin in melanoma cells.

In conclusion, daphnoretin markedly inhibited cellular proliferation and induced apoptosis through the ROS-mediated mitochondrial apoptosis and PI3K/Akt signaling pathways in melanoma A375 and B16 cells. These findings demonstrate that daphnoretin has the potential to be used as an anti-melanoma drug. However, the future clinical application of daphnoretin requires further investigation.

Acknowledgements

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HW, XH, DL and QZ designed the experiments. HW and XH obtained the experimental data and wrote the manuscript. HW, ML and ZP performed Hoechst 33258 staining, flow cytometric apoptosis analysis, western blot analysis and ROS detection. HW and DL interpreted and analyzed the data. HW and QZ assessed the authenticity of all the raw 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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