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Piperlongumine Induces Apoptosis in Human Melanoma Cells Via Reactive Oxygen Species Mediated Mitochondria Disruption

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ABSTRACT

Malignant melanoma is a devastating skin cancer due to its severe drug resistance and prompt metastasis. Piperlongumine is an anti-inflammation and tumor-suppressing natural product with defined structure. While numerous studies revealed exceptional inhibitory effects of piperlongumine on several carcinomas, few investigations were performed on melanoma. Therefore, the present study investigated the anti-tumor effects of piperlongumine on human melanoma cells in vitro, and explored the mechanisms of action. Results from cytotoxicity and proliferation studies demonstrated that piperlongumine inhibited cell growth in melanoma cell lines A375, A875, and B16-F10 in a dose- and time-dependent manner. Flow cytometric analysis showed that piperlongumine obstructed cell cycle progression at G2/M phase and induced apoptosis in A375 cells. Mechanistic investigations illustrated that piperlongumine promoted reactive oxygen species production and decreased mitochondrial membrane potential. In addition, piperlongumine was reported to interfere with the expression of p21, p27, cleaved caspases-3, Bax, Bcl-2, and p-Jun N-terminal kinase (JNK), which are typical regulators associated with cell proliferation, intrinsic apoptosis, and JNKs pathway. Taken together, these results strongly suggested that piperlongumine inhibits cell growth and induces apoptosis in human melanoma cells via ROS mediated mitochondria disruption and JNKs pathway, and piperlongumine may exert promising potential for patients suffering from malignant melanoma.

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Introduction

Malignant melanoma is among the most deadly types of skin cancers, and the occurrence is increasing rapidly despite the continuing improvements in radiation protection from the sun (1,2). In 2014, around 73,870 new cases of melanoma were diagnosed in the United States, accounting for 4.6% of all new cancer cases (3). The persistent rising incidences lead to the astonishing estimation that approximately 10,000 people will die from melanoma-related metastatic diseases in the United States alone each year (1). Although significant progress including immune checkpoint blockade approaches and oncogene-targeted therapies were reported to be effective in treating a subset of melanoma, the overall prognosis remains rather unsatisfactory (4–6). Five-year survival rates of patients with stage-IV melanoma were less than 5% (7). Therefore, new candidates with potential anti-tumor activity and low toxicity are urgently needed to treat metastatic melanoma.

Drug development is an expensive, time-consuming and incremental process. Exploiting new applications for existing medications is becoming a more economical and efficient approach in pharmaceutical industry (8). In addition, emerging evidences demonstrated that natural compounds present in the diet or as the supplements could notably alter the pathogenesis and progression of carcinomas (9,10). Recently, the protective roles of nutritional ingredients in metastatic melanoma have drawn massive attentions, leading to a new horizon of melanoma chemoprevention (11). Grape seed proanthocyanidins were shown to relieve UV-related skin irritation and suppress cell migrations of highly metastasis-specific melanoma cell lines A375 and Hs294t. Natural polyphenol resveratrol, rosmarinic acid from rosemary, and fig latex were reported to reduce proliferation of various melanoma cell lines. These observations suggested that dietary natural products might serve as a cost-effective adjuvant therapy for melanoma treatment.

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Piperlongumine (Figure 1) is a natural alkaloid compound isolated from the long pepper (*Piper longum*) commonly grown in southeastern Asia and southern India. Piperlongumine and its derivatives are valuable natural products possessing anti-platelet aggregation, anti-inflammatory, and anti-tumor properties (12–15). The pharmacokinetic profiles and therapeutic potential of piperlongumine have been particularly documented, while no obvious adverse effects or toxicities were reported in humans (16). Thus, the functions of piperlongumine were intensively investigated under a variety of pathological conditions including arthritis, lupus nephritis, hyperlipidemic, autoimmune encephalomyelitis, and neurodegenerative diseases (17–20). Piperlongumine was reported to increase the level of reactive oxygen species (ROS) and apoptotic cell deaths in bladder, colon, breast, pancreatic, osteosarcoma, and lung cancer cells (15). Recent studies have demonstrated that piperlongumine exerts anticancer activity in breast, colon, ovarian, and prostate cell lines (21–24). In addition, piperlongumine was able to induce selective cell death in head-and-neck cancer cells through ROS rand c-Jun N-terminal kinases (JNKs) pathways (25). However, the precise anti-tumor effects of piperlongumine on malignant melanoma have not been fully elucidated.

Therefore, the present study focused on investigating the activities and mechanism of piperlongumine in typical melanoma cell lines. The reported results demonstrated that piperlongumine directly induces apoptosis and exerts anti-tumor effects in human melanoma cells. The corresponding mechanism involves promoting cell cycle arrest at G2/M phase, increasing caspase-3 cleavage, interfering with Bcl-2 and Bax regulation, increasing ROS levels, and disrupting mitochondrial membrane potential. These data supported possible future applications of piperlongumine in treating malignant melanoma.

Materials and Methods

Chemicals and Reagents

Piperlongumine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), rhodamine 123 (RH123), dichloro-dihydro-fluorescein diacetate (DCFH-DA), and Hoechst33342 were obtained from Sigma (St Louis, MO).

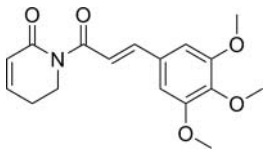

Figure 1. Chemical structure of piperlongumine.

Piperlongumine was synthesized and supplied by Sigma. The product number for piperlongumine is SML0221, and the CAS number for piperlongumine is 20069-09-4. The primary antibody for caspase-3, p-JNK, and JNK was bought from Cell Signaling Technology Company (Beverly, MA). The primary antibodies for Bcl-2, Bax, p21, and p27 were purchased from Abcam (Cambridge, UK). The Annexin V-FITC Apoptosis Detection Kit was acquired from KeyGen Biotech (Nanjing, China).

Cell Cultures

Cells were maintained in a humidified incubator at 37°C, 5% CO_2. A375, A875, and B16-F10 cells were cultured in DMEM medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and 1% antibiotics (penicillin and streptomycin). Authentications were conducted for cell lines. Cells without piperlongumine treatment were used as a vehicle group.

Cell Proliferation and Colony Formation Analysis

Cell viability evaluation after piperlongumine treatment was performed by the MTT assay. Briefly, the exponentially growing cells were seeded in 96-well plates and cultured for different durations. Following the treatment, a volume of 20 μl of MTT solution (5 mg/ml) was added to each well and incubated for an additional 2–4 h at 37°C. Then, the formazan salt was dissolved with 50 μl 20% sodium dodecyl sulfate (SDS) solution overnight. The absorbance of each well was measured with Spectra MAX M5 microplate spectrophotometer (Molecular Devices) at a wavelength of 570 nm, and the median inhibitory concentration (IC_{50}) was calculated.

To investigate the survival of melanoma cells treated with piperlongumine, the cells were plated (6–10 $\times$ 10^2 per well) in a six-well plate and incubated overnight at 37°C. After exposure to various concentrations of piperlongumine for 48 h, the cells were cultured for another 12 days with fresh medium and subjected to a clonogenic assay.

Morphological Analysis by Hoechst Staining

Morphological changes associated with apoptosis in A375 cells were studied by Hoechst 33342 staining. Briefly, the cells were plated onto 18-mm cover glass in six-well plates for 24 h, and then treated with piperlongumine for another 24 h. Following the treatment, cells were rinsed with cold PBS and fixed in paraformaldehyde solution for 20 min. The cells were stained with

Hoechst 33342 solution (5 μg/ml) followed by PBS washing and examination under fluorescence microscope (Zeiss, Axiovert 200, Germany) to identify the nuclear morphology of apoptotic cells.

Flow Cytometric Analysis

To detect the apoptosis inducing effect of piperlongumine, Annexin V-FITC apoptosis detection kit were used. Briefly, after treatment with different concentrations of piperlongumine for different durations, melanoma cells were harvested and washed twice with ice-cold phosphate-buffered saline (PBS). Following centrifugation, cells were stained with Annexin V-FITC and propidium iodide (PI) in the dark, and then analyzed with FCM (Becton-Dickinson, USA).

Analysis on ROS Levels and Mitochondrial Membrane Potential ($\Delta\Psi$)

After exposure to different concentrations of piperlongumine for 12 h, cells were incubated with PBS containing with DCFH-DA (10 μM) at 37°C for about 20 min in the dark and then measured by flow cytometer. ROS-mediated mitochondrial disruption in piperlongumine-treated cells was confirmed by comparing the ROS induction in cells treated with or without 5 mM N-acetyl cysteine (NAC) for 2 h before exposure to 2.5 μM piperlongumine for 12 h, and intracellular ROS levels were measured by flow cytometer. Cells staining with RH123 were utilized to evaluate the changes in mitochondrial membrane potential ($\Delta\Psi$). Cell culture and piperlongumine treatment were done as described above. The harvested melanoma cells were washed with cold PBS after incubation with RH123 (5 μg/ml) at 37°C for 30 min in the dark and then measured by flow cytometer.

Western Blot Analysis

Cells were lysed in RIPA buffer after treatment with piperlongumine. Equal amounts of total proteins from each sample were subjected to SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. After electrophoresis, the membranes were blocked with 5% milk for 2 h at room temperature and incubated overnight at 4°C with the respective primary anti-bodies followed by the incubation of relevant secondary anti-body. The immunostaining signal was detected by the enhanced chemiluminescence system (Amersham, Piscataway, NJ).

Statistical Analysis

Data were represented as means ± SD from three independent technical replicates. One-way ANOVA analysis of variance followed by Tukey's post-hoc test was used to assess the statistical significant difference between groups. Statistically significant P values were labeled as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Results

Piperlongumine Exerts Cytotoxic Effects in Melanoma Cells

In order to assess the anti-tumor effects of piperlongumine, the overall cell viability was first evaluated in three representative melanoma cell lines. MTT assays were performed to investigate the cytotoxic effects of piperlongumine on A375, A875, and B16-F10 cell lines. 0.1% DMSO and varying concentrations of piperlongumine (0.625 μM, 1.25 μM, 2.5 μM, 5 μM, 10 μM, and 20 μM) were incubated with cells for 12 h, 24 h, and 48 h, respectively, followed by addition of 20 μl of MTT solution (5 mg/ml). The absorbances at 570 nm were then recorded and analyzed. The percent growth inhibition was calculated based on the A_{570} ratio of treated cells vs. 0.1% DMSO (Figure 2A). When piperlongumine was present in the cell culture, the A_{570} gradually decreased with increasing concentration. When piperlongumine incubation duration was beyond 24 h, significant impact on A_{570} and percent growth inhibition was observed ($P < 0.01$). These results demonstrated that piperlongumine was cytotoxic against melanoma cell lines. Since an apparent dose-dependent effect was reported at 0.6 μM, 2.5 μM, and 2.5 μM, these three concentrations of piperlongumine was recruited for the following investigations.

Piperlongumine Inhibits Proliferation of Melanoma Cells

Apart from cytotoxic evaluations, clonogenic assays were conducted to further determine the effect of piperlongumine on the proliferation of A375 cells. After exposure to gradient concentrations of piperlongumine (0.6 μM, 1.2 μM, and 2.5 μM) for 48 h, the cells were cultured for another 12 days with fresh medium and subjected to a clonogenic assay. Significant inhibitions of colony formation were observed in A375 cells (Figure 2B). When the concentration of piperlongumine was at 2.5 μM, single digit colony formation was observed. In the presence of 0.6 μM, 1.2 μM, and 2.5 μM of piperlongumine, the colony formation decreased 52.6%, 92.6%, and 99.6%, respectively. These results suggested that piperlongumine

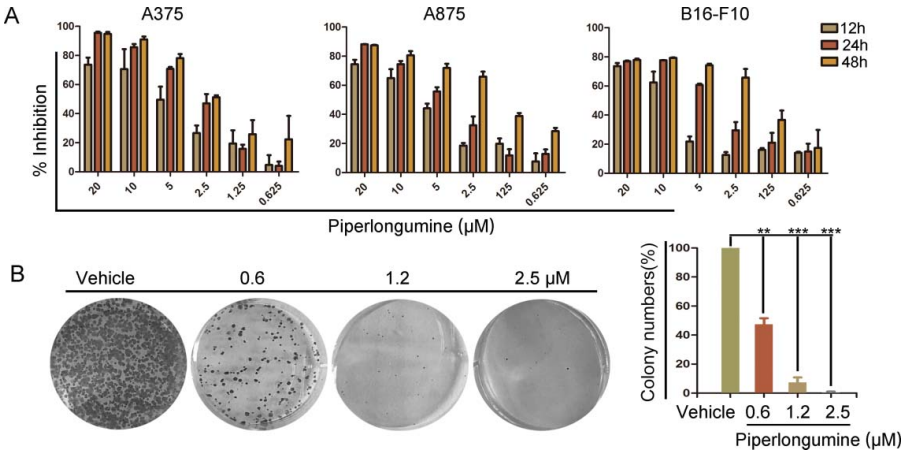

Figure 2. Piperlongumine inhibited cell growth and colony formation in human melanoma cell lines in vitro. A: A375, A875, and B16-F10 cells were treated with concentrations of piperlongumine ranging from 0.6 μM to 20.0 μM for 12 h to 48 h and cell viability was determined by MTT assay. B: Effects of varying concentrations of piperlongumine on colony formation of A375 cells after treatment for 12 days (left). Statistical results of colony forming assays in A375 cells were presented as surviving colonies (percentage of untreated control). Data were expressed as mean ± SD, and significant *P* values were labeled as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (right).

substantially inhibited the growth of melanoma A375 cells in a concentration-dependent manner.

Piperlongumine Induces G2/M Phase Arrest in Melanoma Cells

Abnormal cell proliferation typically occurs when cell cycle is stalled at G0/G1, S, or G2/M phase. In order to thoroughly clarify the details for the growth inhibitory effects of piperlongumine, PI was utilized to stain and quantify the content of nucleus DNA, and flow cytometric analysis was performed to analyze cell cycle distributions in A375 and A875 cells. After treatment with varying concentrations of piperlongumine (0.6 μM, 1.2 μM, and 2.5 μM) for 48 h, G2/M phase arrest was observed evidently in a concentration-dependent manner, which was accompanied by a decrease in cells with G0/G1 and S DNA content (Figure 3). When A375 cells were treated with 2.5 μM of piperlongumine, cells at G0/G1 phase dropped from 43.97% to 31.95%, cells at S phase decreased from 30.49% to 22.88%, and cells at G2/M phase significantly increased from 25.22% to 44.85%. When A875 cells were treated with 2.5 μM of piperlongumine, cells at G0/G1 phase dropped from 49.86% to 34.04%, cells at S phase decreased from 28.53% to 26.2%, and cells at G2/M phase significantly increased from 21.97% to 39.93%. These combined results demonstrated that 2.5 μM of piperlongumine induced an apparent 1.8-fold increase in G2/M phase arrest in both A375 and A875 cells, leading to disrupted cell cycle, growth inhibition and eventually anticancer effects in melanoma cells.

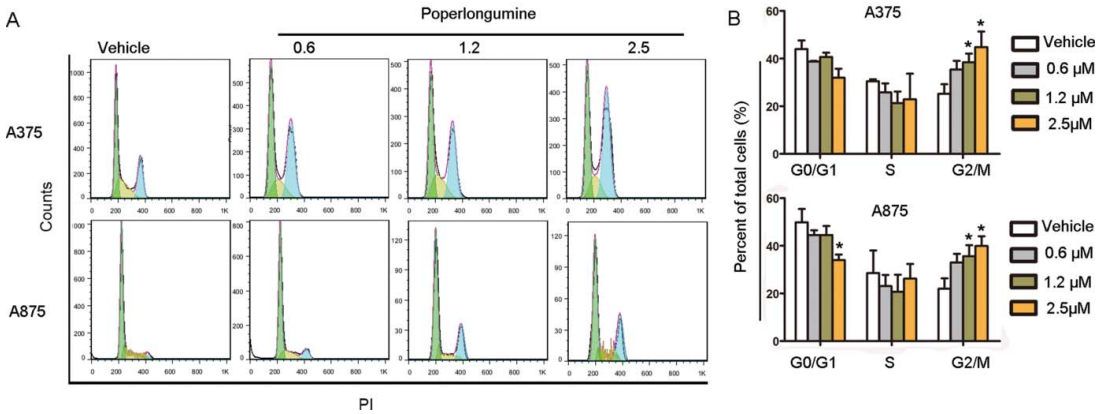

Figure 3. Piperlongumine-induced G2/M arrest in melanoma cells. A: A375 and A875 cells were incubated with piperlongumine for 48 h, and subjected to cell cycle analysis by flow cytometric analysis. B: Quantified histograms demonstrated the effects of piperlongumine on cell cycle distribution. Data were expressed as mean ± SD, and significant *P* values were labeled as $^{*}P < 0.05$.

Piperlongumine Triggers Apoptosis of A375 Melanoma Cells

Anticancer treatments often result in various kinds of cell death, including apoptosis, mitotic catastrophe, autophagy, and necrosis. In order to examine whether piperlongumine-induced cytotoxicity and growth inhibition were associated with programmed apoptotic cell death, morphological analysis was first carried out using Hoechst 33342 staining in A375 cells. The A375 cells exhibited prominent apoptotic characteristics, including bright blue fluorescent condensed nuclei, reduction of cell volume and nuclear fragmentation (Figure 4A). To further verify the morphological observations, flow cytometry was utilized to determine the portions of apoptotic cells in the presence of piperlongumine. A375 cells were incubated with varying concentrations of piperlongumine (0.6 μM, 1.2 μM, and 2.5 μM) for 48 h, followed by PI staining and Annexin V-FITC/PI dual labeled fluorescence staining (Figure 4B, C and E). Dose-dependent changes in the percentage of apoptotic cells were observed when cells were exposed to increasing concentrations of piperlongumine for 48 h. When piperlongumine concentrations were at 0.6 μM, 1.2 μM, and 2.5 μM, the percentages of surviving cells were 84.8%, 70.5%, and 58.2%, respectively. These results suggested that piperlongumine triggered apoptosis in A375 melanoma cell in a concentration-dependent manner. Time-dependent changes in the percentage of apoptotic cells were demonstrated when cells were treated with 2.5 μM piperlongumine for 12 h, 24 h, and 48 h (Figure 4D and F). With the increasing durations of treatment, cells in early apoptotic stage went from 19.8% in 12 h to 23.9% in 24 h, and dropped to 11.0% in 48 h, while cells in late apoptotic stage increased with increasing treatment time, from 9.8% in 12 h to 36.9% in 48 h.

Piperlongumine Increases ROS Level and Disrupts Mitochondrial Membrane Potential

While piperlongumine was suggested to increase ROS levels in various types of cancer cells, whether piperlongumine exerts similar effect in melanoma cells is not explored. Accordingly, the effect of piperlongumine on ROS production in melanoma cells was investigated. A375 cells were incubated with varying concentrations of piperlongumine (0.6 μM, 1.2 μM, and 2.5 μM) for 12 h, followed by staining with ROS reactive DCFH–DA and flow cytometric analysis (Figure 5). Comparing to the control, when piperlongumine concentrations were at 0.6 μM, 1.2 μM, and 2.5 μM, the ROS levels increased 1.3-fold, 3.4-fold, and 5.6-fold, respectively (Figure 5A and D). These results suggested that piperlongumine significantly increased ROS level in A375 melanoma cell in a concentration-dependent manner. Pretreatment with the antioxidant NAC distinctly inhibited piperlongumine-mediated ROS induction (Figure 5B and E). In the presence of both 5 mM NAC and 2.5 μM piperlongumine, the ROS induction was 4.6-fold lower than cells treated with 2.5 μM piperlongumine alone, while it was not significantly different from the vehicle, suggesting that the effect of piperlongumine was mediated via ROS.

A critical step in the apoptosis is the damage of mitochondria by elevated ROS. In order to investigate the effect of piperlongumine on mitochondrial membrane potential ($\Delta\Psi$) in A375 melanoma cells, a mitochondria-specific and voltage-dependent dye RH123 was utilized. A dose-dependent reduction in $\Delta\Psi$ was observed when cells were exposed to increasing concentrations of piperlongumine (Figure 5C). When the concentration of piperlongumine was at 0.6 μM, 1.2 μM, and 2.5 μM, 10%, 24%, and 44% reduction in the $\Delta\Psi$ was observed, respectively (Figure 5F). When piperlongumine was at 2.5 μM, the loss of mitochondrial membrane potential was significantly ninefold higher than the control. These results demonstrated that piperlongumine was able to promote ROS production and trigger mitochondria disruption.

Piperlongumine Interferes with Expression of Proliferation, Apoptosis, and ROS Regulators

Inhibition of cell proliferation is typically associated with the upregulation of p53 downstream protein p21 and p27. In order to validate the growth inhibitory effect of piperlongumine on A375 cells, Western blot analysis was performed. Increasing expressions of p21 and p27 were observed with increasing concentrations of piperlongumine (Figure 6A). In the presence of 2.5 μM of piperlongumine, the relative expression of p21 and p27 increased 6.3-fold and 5.7-fold, respectively, supporting that piperlongumine significantly upregulated p21 and p27 expression and inhibited proliferation of A375 cells.

Since the present study demonstrated that piperlongumine was able to induce apoptosis in A375 cells, further characterization of the mechanistic pathways was first investigated by analyzing the activation of specific caspase cleavage cascades including cleaved caspase-3, Bax, and Bcl-2. Following exposure to piperlongumine in A375 cells, apparent increases in the protein level of cleaved caspase-3 were observed, and the expressions of Bcl-2 and Bax were significantly influenced in a concentration-dependent manner (Figure 6B). In the presence of 0.6 μM, 1.2 μM, and 2.5 μM of piperlongumine, the level of cleaved caspase-3 increased 1.3-fold, 3.7-fold, and 3.5-fold, respectively. When the concentration of

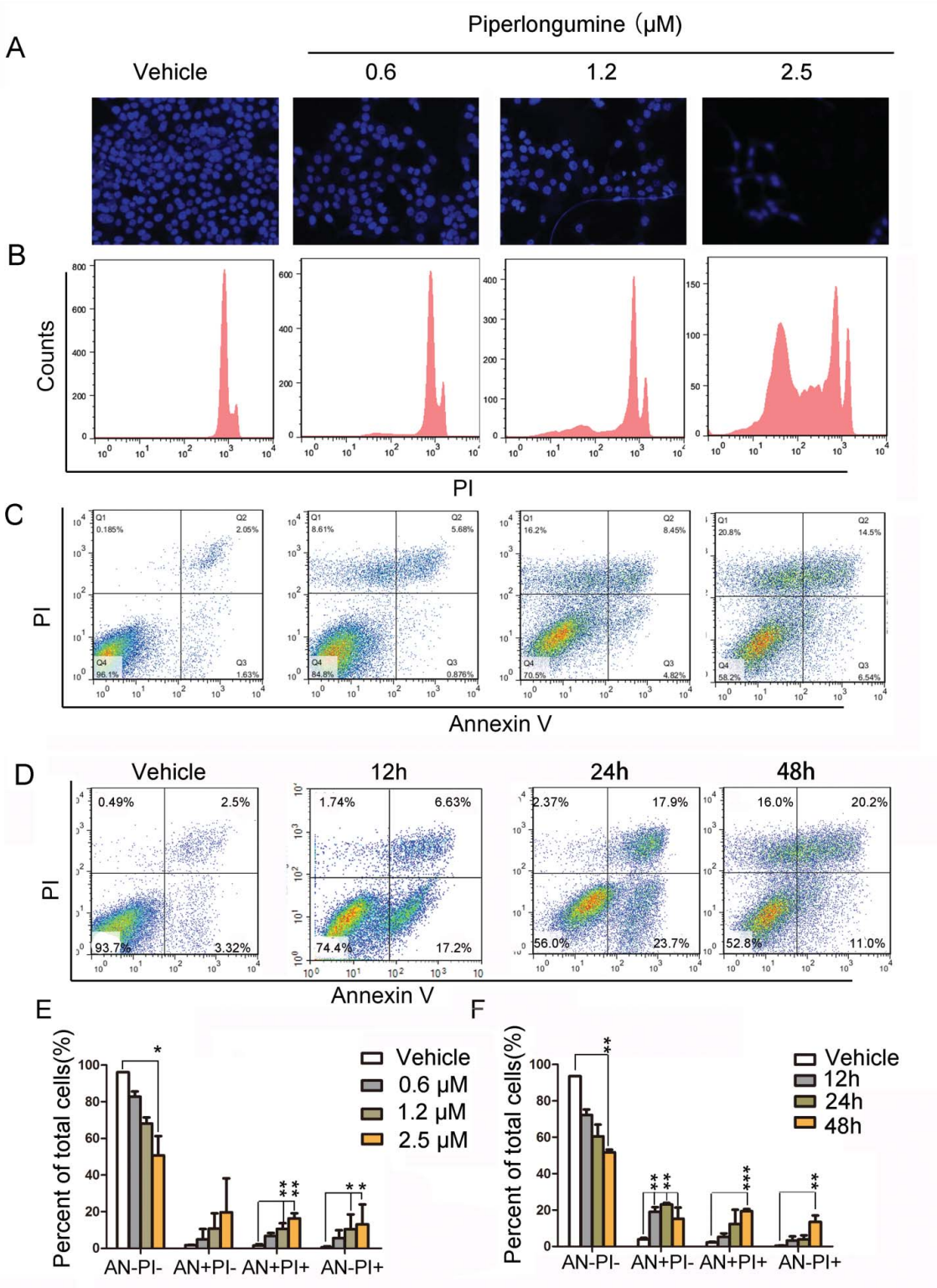

Figure 4. Piperlongumine-induced apoptosis in A375 cells. A: Fluorescence microscopy analysis of Hoechst33342 stained A375 cells after incubation with varying concentrations of piperlongumine (×20). B: A375 cells were treated with piperlongumine with increasing concentration for 48 h, and then analyzed by flow cytometry after PI-staining. C: A375 cells were treated with piperlongumine for 48 h and apoptosis was examined by flow cytometry after Annexin V/PI staining. The Annexin V positive population was considered apoptotic cells. D: A375 cells were treated with 2.5 μM piperlongumine for 12 h, 24 h, and 48 h. Apoptosis was examined by flow cytometry after Annexin V/PI staining. E and F: Quantified histograms represented the proportions of apoptotic cells, including the early apoptotic cells and the late apoptotic cells. Data were expressed as mean ± SD, and significant *P* values were labeled as $^{*}P < 0.05$, $^{**}P < 0.01$.

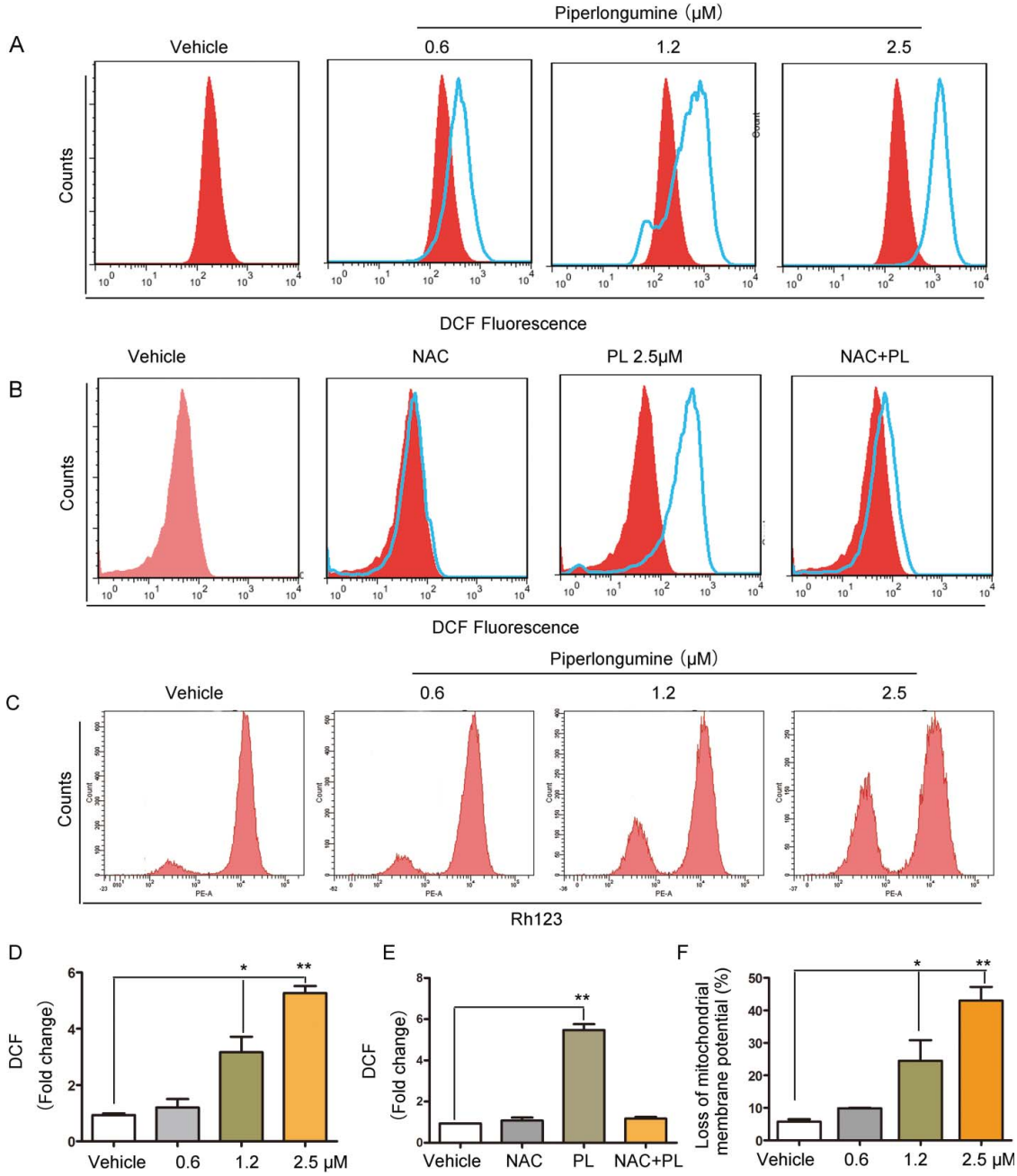

Figure 5. Piperlongumine increased ROS production and disrupted mitochondrial membrane potential ($\Delta\Psi$) in A375 cells. A: A375 cells were treated with varying concentrations of piperlongumine. The harvested cells were stained with DCFH–DA followed by flow cytometric analysis. Quantification of ROS was also shown compared with the control. B: Cells were pretreated with or without 5 mM NAC for 2 h before exposure to 2.5 μM piperlongumine for 12 h, and intracellular ROS levels were measured by flow cytometer. C: The mitochondrial membrane potential ($\Delta\Psi$) was evaluated by flow cytometric analysis after treatment with piperlongumine at varying concentrations for 48 h. The quantified values were shown in panels D, E, and F. Data were expressed as mean ± SD, and significant *P* values were labeled as $^{*}P < 0.05$, $^{**}P < 0.01$.

piperlongumine was at 2.5 μM, the expression of anti-apoptotic Bcl-2 decreased 2.1-fold, while the expression of pro-apoptotic Bax increased 2.2-fold, indicating that piperlongumine induced apoptosis was activated through Bcl-2/Bax related pathway.

In addition, the mechanism behind piperlongumine-induced ROS production and mitochondria disruption was investigated in A375 cells (Figure 6C). In the presence of 0.6 μM, 1.2 μM, and 2.5 μM of piperlongumine, the relative expressions of phosphorylated p-JNK were upregulated 1.6-fold, 2.1-fold, and 3.3-fold, respectively, while the level of non-phosphorylated JNK were not significantly altered, suggesting that piperlongumine was able to substantially

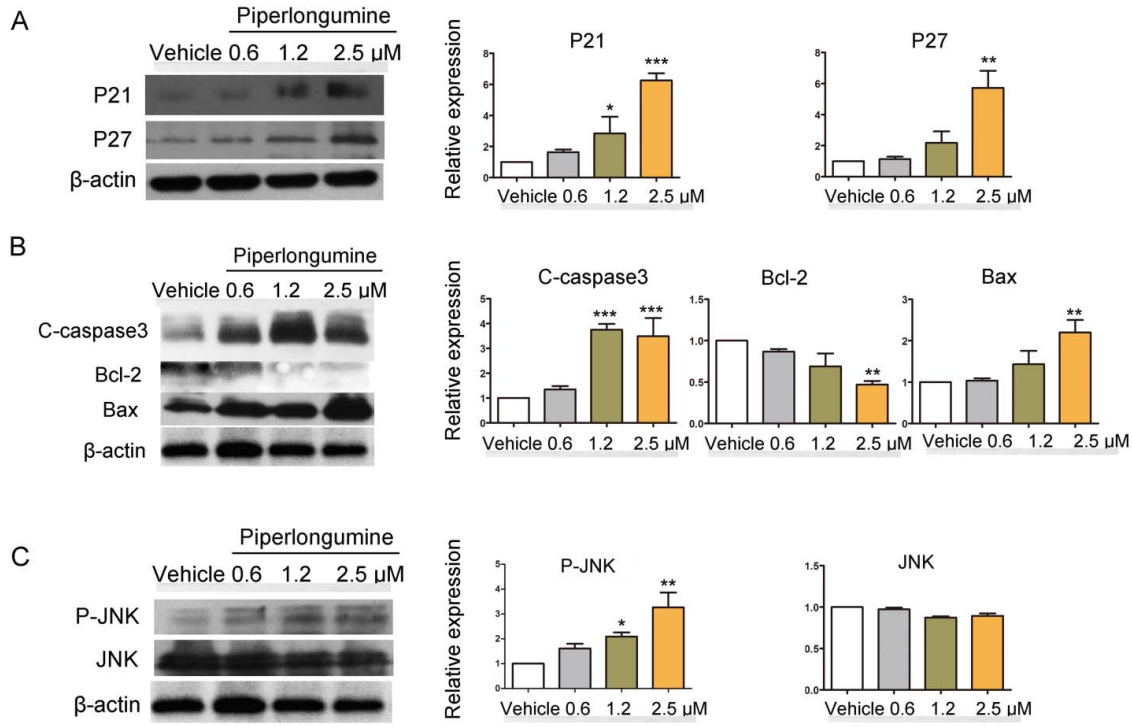

Figure 6. Piperlongumine interfered with the protein expressions in proliferation, apoptosis, and oxidative stress-related JNK activation. When A375 cells were exposed to varying concentrations of piperlongumine for 48 h, Western blot analysis was performed to evaluate the effects of piperlongumine on the expression of A: P21 and P27; B: C-caspases 3, Bcl-2, and Bax; C: phospho-JNK (p-JNK) and total JNK. The relative expression of target protein was compared the house keeping β-actin and quantified values were also shown. Data were expressed as mean ± SD, and significant *P* values were labeled as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

promote JNK phosphorylation. The combined results from this study implied that piperlongumine could increase ROS level, disrupt mitochondrial membrane potential, and induce apoptosis though both Bcl-2/Bax and JNKs pathways in A375 cells.

Discussion

Melanoma is highly malignant with considerable metastatic potential and drug resistance, and the incidence is increasing rapidly (26). Since metastatic melanoma is one of the deadliest skin cancers, identifying new applications of existing pharmaceuticals and natural products for melanoma might be a cost-effective therapeutic strategy. *Piper longum*, known as long pepper, is a traditional Chinese medicine with anti-inflammatory and anticancer properties. Being an active ingredient of the medicinal long pepper, piperlongumine is widely studied under a variety of pathological conditions (12–13). The pharmacokinetic evaluations of piperlongumine have been carried out and the safety profiles were documented, which laid the foundations for exploring novel applications of piperlongumine (27). Recent studies reported promising effects of piperlongumine on various types of cancer, while the anti-tumor capacity of piperlongumine on melanoma and the mechanisms of action need further researches.

In this study, the anti-tumor activity and corresponding mechanism of piperlongumine were investigated in human melanoma cell lines. Piperlongumine was shown to inhibit cell growth and proliferation of melanoma cell lines in a dose- and time-dependent manner in vitro. In addition, piperlongumine promoted cell cycle arrest at G2/M phase and induced apoptosis. Mechanistic studies demonstrated that piperlongumine induced apoptosis was associated with elevation of ROS levels and disruption of mitochondrial membrane potential. Furthermore, piperlongumine significantly interfered with the expression of various proliferation and apoptosis regulators including p21, p27, cleaved caspases-3, Bax, Bcl-2, and p-JNK. These findings strongly supported that piperlongumine was able to induce apoptosis in melanoma cells via pathways involving ROS mediated mitochondria disruption.

In cancer therapy, targeting cell replication and inducing cell cycle arrest is one of the major strategies to suppress cell growth and trigger apoptosis for many anticancer drugs (28–32). Here, piperlongumine was reported to promote cell cycle arrest in melanoma cells at G2/M phase. This observation was consistent with

that of a previous study on ovarian cancer cells (23). However, in triple-negative breast cancer cells, piperlongumine induced G0/G1 phase arrest, suggesting that cell cycle arrest induced by piperlongumine is cell type-dependent (33). As deregulation of cell cycle progression play an important role in the treatment of cancer, the effectiveness of piperlongumine toward human melanoma cells might partly be due to the ability to induce cell cycle arrest at G2/M phase.

Apoptosis, the programmed cell death, plays a fundamental role in the development and maintenance of tissue homeostasis and the elimination of unwanted or damaged cells (28,34). Induction of apoptosis is usually a promising strategy to treat cancers (35–37). The data in this study demonstrated that piperlongumine was able to induce apoptosis of A375 cells in a dose- and time-dependent manner. In various apoptotic pathways, caspase-3 is a key participant and the cleavage of caspase-3 results in chromatin condensation, DNA laddering, and initiation of apoptosis (38,39). The induction of apoptosis intensively involves Bcl-2 family proteins, including pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 (40). In this study, the results showed that the occurrence of piperlongumine-induced apoptosis was accompanied by significant decrease in expressions of cleaved caspases-3 and Bax, and distinct increase in protein levels of Bcl-2, indicating a Bcl-2-/Bax-related apoptotic pathway. In addition, ROS was believed to be responsible for apoptotic cell deaths in multiple cancer cells through JNKs pathway (15,25). The production of ROS could intensively disrupt mitochondrial transmembrane potential ($\Delta\Psi$) and damage the mitochondrial integrity, which in turn lead to apoptosis eventually (41,42). In our study, substantial elevation in ROS level and significant reduction in $\Delta\Psi$ was observed by flow cytometric analysis following piperlongumine treatment. And JNK was distinctly phosphorylated in the presence of piperlongumine, suggesting the effect of piperlongumine-induced ROS production was associated with JNKs pathway. Therefore, ROS-mediated mitochondria disruption might, at least in part, answer for the eventual apoptosis of melanoma cells treated with piperlongumine.

In conclusion, the present study illustrated that piperlongumine was able to induce apoptosis in human melanoma cell lines via ROS-mediated mitochondria disruption in a concentration-dependent manner, supporting promising anti-tumor potential in malignant melanoma treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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