Piperlongumine induces apoptosis via the MAPK pathway and ERK-mediated autophagy in human melanoma cells

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Abstract. Piperlongumine (PL) is an amide alkaloid with diverse pharmacological effects against cancer, bronchitis and asthma; however, research on its efficacy against melanoma is lacking. The present study investigated the anticancer effects of PL on A375SM and A375P human melanoma cells. PL decreased the survival rate of A375SM and A375P cells, as shown by MTT assay, increase of apoptotic cells by DAPI staining. And PL induced apoptosis by decreasing the expression of the anti-apoptotic protein Bcl-2 and increasing that of the pro-apoptotic proteins cleaved-PARP and Bax. PL also induced apoptosis in A375SM and A375P cells via the MAPK pathway, increasing expression of the MAPK pathway proteins, phosphorylated-(p-ERK), p-JNK p-p38. These proteins were confirmed by western blot. In addition, A375SM and A375P cells treated with PL showed an increased number of acidic vesicular organelles by acridine orange staining. Also, autophagy induced by the expression of 1A/1B-light chain 3, Beclin 1and mTOR was investigated through western blot. When PL was applied following treatment with autophagy inhibitors 3-methyladenine and hydroxychloroquine, autophagy exhibited a cytoprotective effect against apoptosis in MTT assay. Pretreatment of A375P cells with the ERK inhibitor PD98059 and the JNK inhibitor SP600125 followed by treatment with PL confirmed that apoptosis and autophagy were mediated via the MAPK/ERK pathway by western blot. In summary, the present study provided empirical evidence supporting the anticancer effects of PL on human melanoma cells and indicated the potential of PL as a treatment for melanoma.

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Introduction

The incidence of cancer is rising globally because of the aging population; in 2040, new cancer cases are expected to increase by 47% from 2020 (1). In particular, the incidence of melanoma, the most fatal type of skin cancer, has doubled increased over from 1982 to 2011 and in the absence of new interventions, 112,000 new melanoma cases are predicted increasing in 2030 (2,3). Melanoma is a malignant tumor caused by abnormal proliferation of melanocytes, which produce the skin pigment melanin. Although melanoma accounts for only 1% of all skin cancers, it accounts for most skin cancer-related mortality (4,5). Effective treatment of melanoma involves surgical removal in combination with radiotherapy and chemotherapy. However, it is essential to investigate chemotherapeutic methods using natural substances to minimize cytotoxic effects on healthy cells (6-8).

Alkaloids exhibits various biological activities such as antiviral, antibacterial, anti-inflammatory anticancer, and has been developed as an anticancer drug in clinical trials for the treatment of various malignancies (9,10). Among these alkaloids, piperlongumine [PL; 5,6-dihydro-1-(2*E*)-1-oxo-3-(3,4,5-trimethoxyphenyl)-2-propenyl]-2(1H)-pyridinone] is one of the main compounds found in long pepper (*Piper longum*). Long peppers have traditionally been used in the Ayurvedic Medical System in India and folk therapy in Latin America. The dry, unripe fruit is used as a tonic and the roots are used to induce placental expulsion. It has also been used to treat tumors, malaria, viral infection, bronchitis, cough and asthma (11). Owing to its various applications and anticancer properties, studies have been conducted on the potential of PL as an anticancer agent (12,13). PL is known to have specific cytotoxicity for tumor cells and little toxicity in normal cells (14). Although PL is reported to exert anticancer effects against several types of cancer cell, including colon (15), lung (16), stomach (17), prostate (18), and pancreatic cancer (19), research on melanoma is lacking.

Apoptosis is a mechanism by which injured or cancerous cells can be removed without affecting nearby healthy cells (20). Apoptosis leads to nuclear and cytoplasmic condensation and DNA fragmentation; the cell forms an apoptotic body, a membrane-bound extracellular vesicle, and is

degraded by neutrophils, macrophages and dendritic cells (21). At the molecular level, apoptosis induction to the activation of Fas-associated death domain, deactivating the anti-apoptotic protein Bcl-2 and releasing cytochrome c from the mitochondria into the cytoplasm, where it regulates activity of Bax to cause apoptosis (22,23). In addition, PARP, which restores damaged DNA in cell stress, is inactivated by caspase-3, resulting in expression of cleaved-PARP (24).

MAPKs are serine/threonine kinases involved in apoptosis. The main MAPK pathway includes ERK, JNK and p38. Upon its activation by growth factors that induce cell differentiation and proliferation, ERK stimulates expression of anti-apoptotic proteins and suppresses apoptosis (25). JNK and p38 are activated by stress and are involved in cell survival and apoptosis. The MAPK pathway regulates biological activities, including cellular signal transduction, and serves an important role in cell death and proliferation (26).

Autophagy occurs before cell death in response to stress, such as cytotoxicity or chemotherapy. Autophagosomes, which have double membranes, are formed during autophagy (27). These autophagosomes fuse with lysosomes to form autolysosomes. Meanwhile, microtubule-associated protein 1A/1B-light chain 3 (LC3) in the cytoplasm combines with phosphatidylethanolamine to form an LC3-phosphatidylethanolamine conjugate (LC3-II), which aggregates into autophagosomes (28). mTOR serves a key role in regulating autophagy; its inactivation triggers autophagy. As regulating different signaling pathway including Beclin 1, Bcl-2 autophagy induces cell survival or death (29). there is a potential to develop anticancer agents that use these characteristics (30). Therefore, in the present study, two melanoma cell lines, A375SM and A375P, were used to assess the anticancer effects of PL in human melanoma. A375SM has higher metastaticity (31) and is highly invasive compared with other melanoma cells (32). The present study aimed to investigate the effects of PL on the survival of A375SM and A375P melanoma cells *in vitro* and whether these effects were mediated by apoptosis. The present also investigated the induction of autophagy by PL in melanoma cells and the role of autophagy and its association with the MAPK pathway.

Materials and methods

Materials and reagents. PL (Fig. 1A; purity, ≥97.0%) was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. MTT, DAPI and DMSO were purchased from Sigma-Aldrich (Merck KGaA). DMSO was used as a solvent for PL and control group was treated with DMSO alone. FITC Annexin-V detection kit was purchased from BD Pharmingen™ (BD Biosciences). Antibodies against PARP (rabbit, 1:1,000, cat. no. #9542), Bax (rabbit, 1:700, cat. no. #2772), Bcl-2 (rabbit, 1:1,000, cat. no. #4223), phosphorylated (p-)ERK (rabbit, 1:1,000, cat. no. #9102), ERK (rabbit, 1:1,000, cat. no. #9101), p-JNK (rabbit, 1:1,000, cat. no. #4668), JNK (rabbit, 1:1,000, cat. no. #9252), p-p38 (rabbit, 1:1,000, cat. no. #9211), p38 (rabbit, 1:1,000, cat. no. #9211), p-mTOR (rabbit, 1:1,000, cat. no. #2971), Beclin 1 (rabbit, 1:1,000, cat. no. #3738), LC3 (rabbit, 1:1,000, cat. no. #4108), secondary antibody rabbit IgG (rabbit, 1:1,000, cat. no. #7074), ERK inhibitor PD98059 and JNK inhibitor SP00615 were purchased from Cell Signaling Technology, Inc. β-actin (mouse, 1:1,000, cat. no. sc-47778) and secondary mouse IgG (1:1,000, cat. no. sc-516102) antibodies were purchased from Santa Cruz Biotechnology, Inc. Autophagy inhibitor 3-methyladenine (3-MA) and hydroxychloroquine (HCQ) were purchased from Selleck Chemical.

Cell culture. The melanoma cells A375SM and A375P were purchased from the Korea Cell Line Bank (Seoul, Korea). DMEM and FBS used for cell culture were purchased from Welgene, Inc. Streptomycin/penicillin was purchased from Gibco (Thermo Fisher Scientific, Inc.). The melanoma cells A375SM and A375P in DMEM containing 5% FBS and 1% streptomycin/penicillin were cultured in an incubator at 37˚C and 5% CO_2. When the cells reached 80-90% density, they were subcultured and the medium was replaced every 2-3 days.

MTT assay. MTT assay was performed to observe the effect of PL on A375SM and A375P cell viability. Cells were incubated in a 96-well plate ($2x10^4$ cells/ml) for 24 h at 37˚C. PL was added (0, 2, 4, 6, 8 or 10 μm) at 37˚C for 24 or 48 h. Half-maximal inhibitory concentration (IC_{50}) were calculated from curves constructed by plotting cell viability versus PL concentration. HCQ (15 or 20 μM), 3-MA (2.5 or 3.5 mM), PD98059 (25 μM) and SP600125 (25 μM) were added for 3 h pretreatment at 37˚C, then PL was added (4 μM) at 37˚C for 24 h. The medium was removed and 40 μl MTT solution was added for 2 h. After removing the MTT solution, 100 μl DMSO was used to dissolve formazan and the absorbance was measured at 595 nm with ELISA reader (Bio-Rad Laboratories Inc.).

DAPI staining. DAPI staining was performed to observe morphological changes in the nucleus due to nuclear condensation of apoptosis. After incubating $2x10^5$ cells/ml in 60-mm dish for 24 h, PL (0, 4 or 8 μm) was added at 37˚C for 24 h. After that, the medium containing PL was removed and cells were washed with PBS and fixed with 4% paraformaldehyde for 5 min at 20˚C. Following rinsing with PBS, DAPI solution was added (2 ml/dish) for 2 min at 20˚C and observed at x200 magnification with a fluorescence microscope (Zeiss AG).

Annexin V-PI staining. Annexin V-propidium iodide (PI) staining was performed to analyze the degree of early and late apoptosis induced by PL in melanoma cells and confirmed using FACS. A375SM and A375P cells were cultured 70-80% in a 175-cm^2 flask at 37˚C for 24 h, then PL was added at concentrations of 0, 4 or 8 μm and cultured at 37˚C for 24 h. Cells were removed from the flask using a cell scraper, centrifuged at 260 x g for 5 min, 4˚C to obtain cell pellets, washed with PBS, then centrifuged at 260 x g, 5 min, 4˚C. After suspending $2x10^5$ cells/ml in 1X binding buffer, Annexin-V and PI were added to stain for 15 min at 20˚C and measured using FACSCalibur™ flow cytometer (BD Biosciences) and BD FACSuite™ software version 1.0.6 (BD Biosciences).

Acridine orange (AO) staining. AO staining was performed to observe acid vesicular organelles (AVOs), one of the morphological characteristics of autophagy (33). A375SM and A375P cells were seeded ($2x10^5$ cells/ml) and cultured at 37˚C for 24 h. PL was added at a concentration of 0, 4 or 8 μm in a CO_2 incubator at 37˚C for 24 h, the medium containing PL

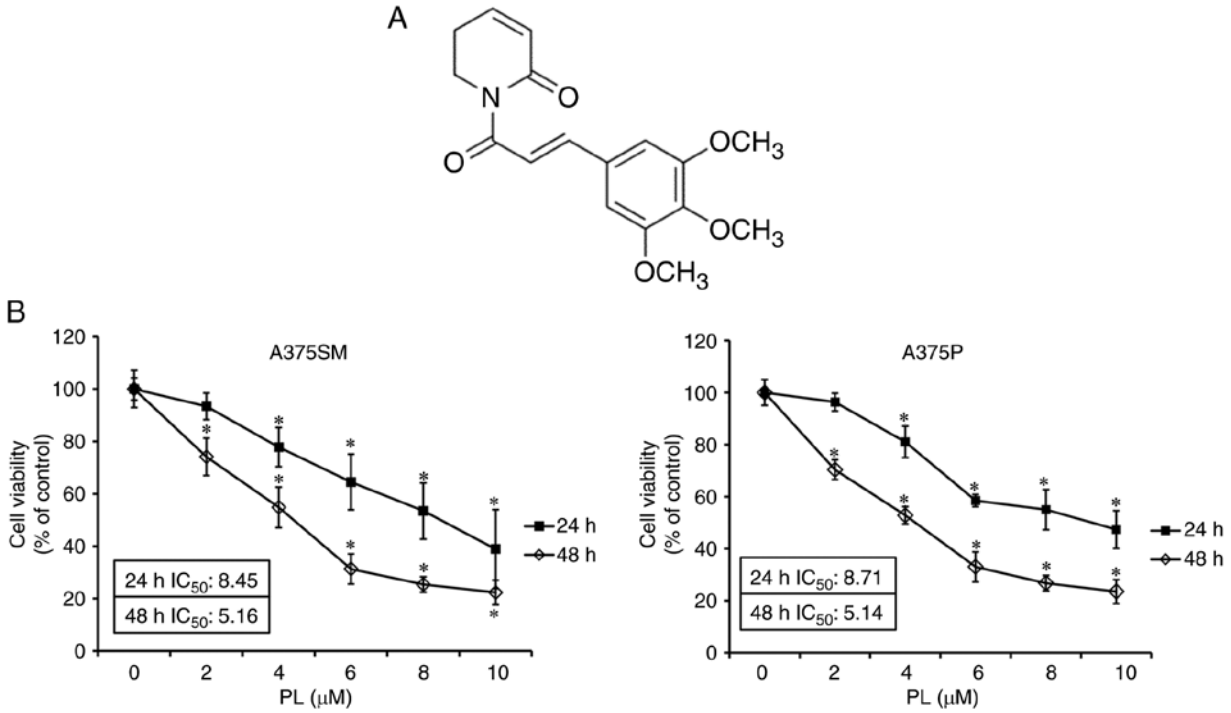

Figure 1. Effects of PL on viability in human melanoma cells. (A) Chemical structure of PL. (B) Viability of A375SM, A375P cells treated with PL was determined by MTT assay. The control group (0 μM) was treated with an equal amount of DMSO. Data are presented as mean and SD of three samples. *P<0.05 vs. 0 μM. PL, piperlongumine; IC_{50}, half-maximal inhibitory concentration.

was removed and cells were washed twice with PBS. After 4% paraformaldehyde was applied for 15 min for fixation at 20˚C, cells were washed twice with PBS and then 2 ml AO (5 μg/ml) was added for 10 min at 20˚C and cells were viewed with a fluorescence microscope at x100 magnification.

Western blot analysis. Western blot was performed to confirm expression of apoptosis-associated proteins. In a 175-cm^2 flask, A375SM and A375P were incubated 70-80% at 37˚C and 5% CO_2 for 24 h. Medium containing 0, 4 or 8 μm PL was added for 24 h at 37 ˚C. Cells were suspended using trypsin-EDTA, then centrifuged at 260 x g, 5 min at 4˚C. The cell pellet was treated with cell lysis buffer (PRO-PREP™ Protein Extraction Solution; Invitrogen; Thermo Fisher Scientific, Inc.) at 4˚C for 20 min and centrifuged at 15,920 x g, 5 min and 4˚C. The extracted protein was quantified with Bradford protein assay (Bio-Rad Laboratories Inc.). Then, 50-90 μg/lane of protein sample was loaded per lane was separated according to molecular weight using 12% SDS-PAGE and transferred to nitrocellulose membrane. Membrane was blocked with 5% skimmed milk at 20˚C for 2 h, then primary antibody was added overnight at 4˚C. Horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG was added at 20˚C for 2 h. Each protein was identified using Pierce™ enhanced chemiluminescence (ECL) western blotting substrate (Pierce; Thermo Fisher Scientific, Inc.) and quantified using Image J Launcher software version 1.52a (provided by National Center for Biotechnology Information).

Statistical analysis. All data are presented as the mean ± SD of three experiments. Comparisons between >2 groups were performed by one-way ANOVA followed by Dunnett's post hoc test. The difference between two groups was assessed by unpaired Student's t test. The data were analyzed with IBM SPSS statistics version27. P<0.05 was considered to indicate a statistically significant difference.

Results

PL decreases viability of melanoma cells. A375SM and A375P melanoma cells were incubated PL to assess survival rates using an MTT assay. Following treatment with PL, A375SM cells showed survival rates of 77.80 at 4 and 53.46% at 8 μm at 24 h, whereas A375P cells showed survival rates of 80.98 and 55.00, respectively. In addition, the IC_{50} of A375SM and A375P was 8.45 and 8.71 μm, respectively, at 24 h. A375SM cells showed survival rates of 54.84 at 4 and 25.39% at 8 μm at 48 h, whereas A375P cells showed survival rates of 52.81 and 26.78, respectively. In addition, IC_{50} of A375SM and A375P was 5.16 and 5.14 μm, respectively, at 48 h (Fig. 1B). These results demonstrated that PL decreased the survival rates of A375SM and A735P melanoma cells in a dose- and time- dependent manner. In the subsequent experiments, PL was used at 4 and 8 μm.

PL induces apoptosis of melanoma cells. DAPI staining was to confirm whether the reduction in survival rate was due to apoptosis. Fluorescence microscopy was used to identify apoptotic bodies, characterized by morphological changes in nuclear DNA and condensation of the cytoplasm (34). There was a dose-dependent increase in apoptotic cells for A375SM cells of 0.66% under the control condition, 1.45% at 4 μm PL, and 3.15% at 8 μm PL; and for A375P cells, 0.83% under the control condition; 1.19% at 4 μm PL; and 1.81% at 8 μm PL (Fig. 2A).

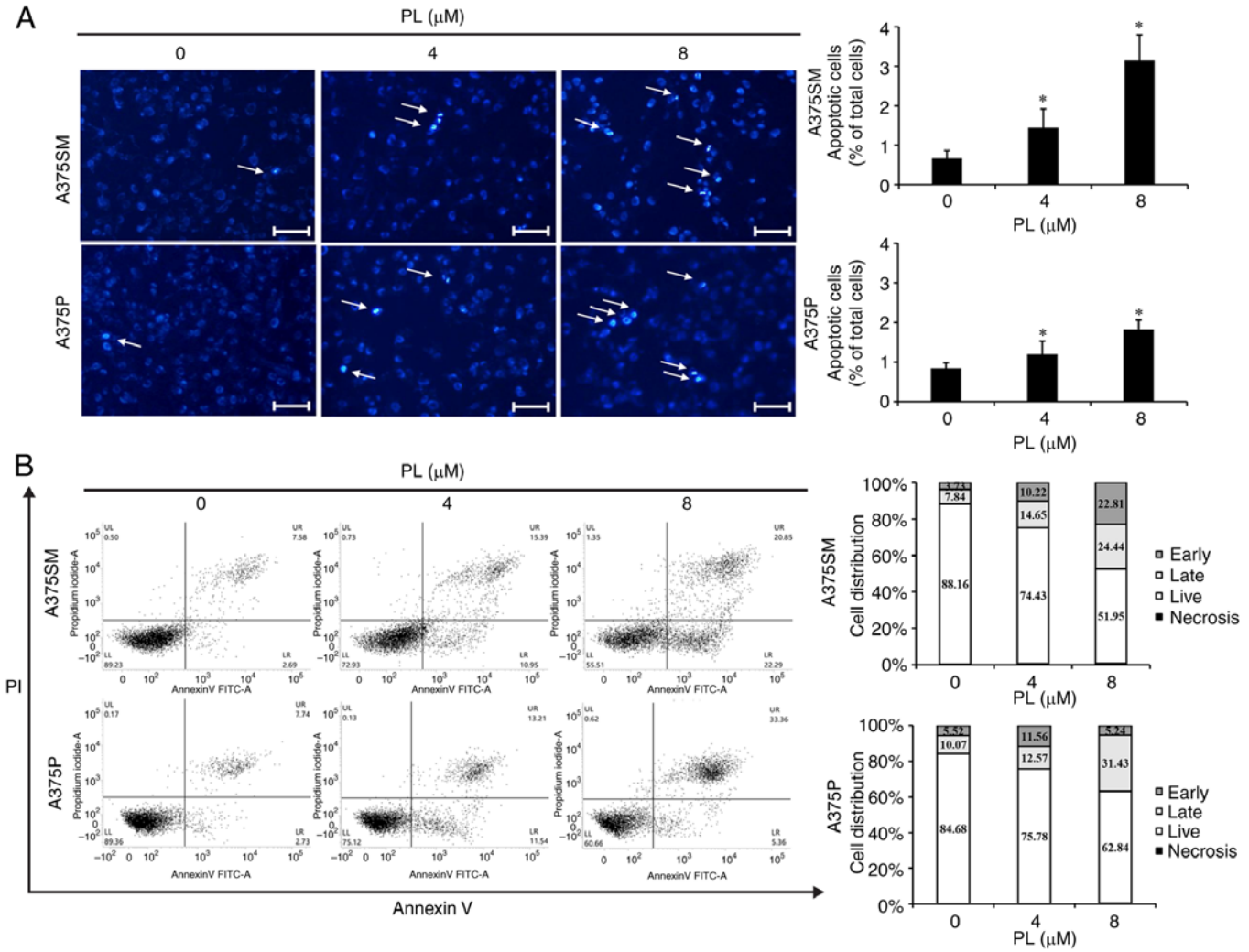

Figure 2. Effect of PL on apoptosis in human melanoma cells. (A) Fluorescence microscopic images of A375SM and A375P cells treated with PL and stained with 1X DAPI. The arrows indicate DAPI-positive (apoptotic) cells. Scale bar, 100 μm. (B) Flow cytometry of A375SM and A375P cells treated with PL 24 h. The control group (0 μM) was treated with an equal amount of DMSO. Data are presented as mean and SD of three samples. *P<0.05 vs. 0 μM. PL, piperlongumine.

Apoptosis induced by PL was measured using Annexin V-PI staining. Both A375SM and A375P cells showed dose-dependent increases in early/late apoptosis when treated with PL compared with control (Fig. 2B). These results suggested that the decrease in A375SM and A375P cell survival following treatment with PL was due to apoptosis.

PL expresses apoptosis-associated proteins in melanoma cells. Western blotting was performed to investigate expression of apoptosis-related proteins in A375SM and A375P cells following treatment with PL. When A375SM and A375P melanoma cells were treated with 0, 4 or 8 μm PL for 24 h, pro-apoptotic protein cleaved-PARP and Bax increased in a dose-dependent. Conversely, expression of the anti-apoptotic protein Bcl-2 decreased in PL-treated cells (Fig. 3). Thus, PL induced apoptosis in melanoma cells by increasing cleaved-PARP and Bax expression while decreasing Bcl-2 expression.

PL expresses MAPK pathway-associated proteins in melanoma cells. Western blotting was performed to investigate whether PL-induced apoptosis was mediated by the MAPK pathway. Compared with the control group, 4 or 8 μM PL-treated A375SM and A375P cells showed increased expression of p-ERK, p-JNK and p-p38 in a dose-dependent manner (Fig. 4).

PL induces autophagy in melanoma cells. AO staining was used to investigate whether PL induced autophagy in A375SM and A375P cells. When A375SM and A375P melanoma cells were treated with 4 or 8 μM PL for 24 h, the expression of AVOs increased compared with that in the control group (Fig. 5A and B). In addition, western blotting was performed to investigate whether PL increased the expression of autophagy-associated proteins in A375SM and A375P cells. There was an increase in LC3-II and Beclin 1 expression and a decrease in p-mTOR expression in PL-treated cells (Fig. 5C and D). These results suggested that PL induced autophagy in melanoma cells in a dose-dependent manner.

Autophagy induced by PL in melanoma cells affects apoptosis-associated protein expression. To investigate the role of autophagy, cells were pretreated with either the

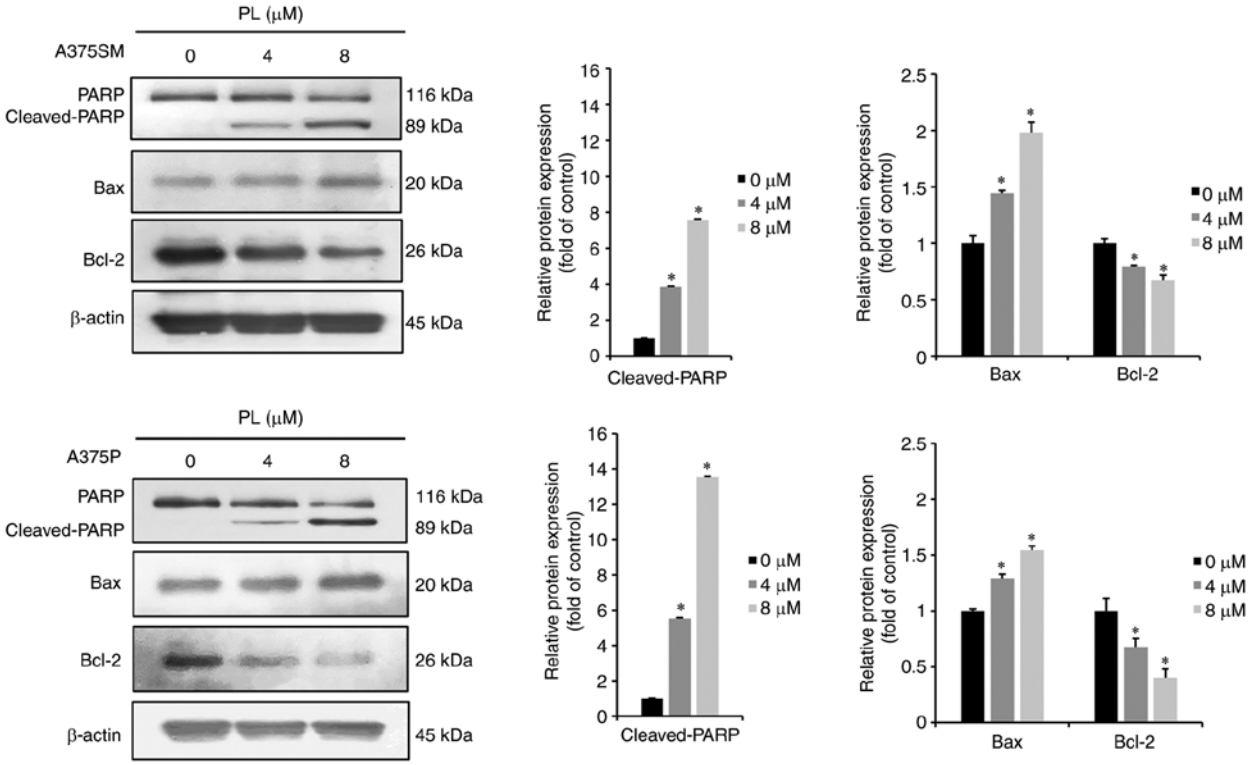

Figure 3. Effects of PL on expression of apoptosis-related protein in human melanoma cells. A375SM and A375P cells were treated with PL and protein was extracted to measure the levels of apoptotic proteins. β-actin was used as the loading control. The control group (0 μM) was treated with an equal amount of DMSO. Data are presented as mean and SD of three samples. *P<0.05 vs. 0 μM. PL, piperlongumine.

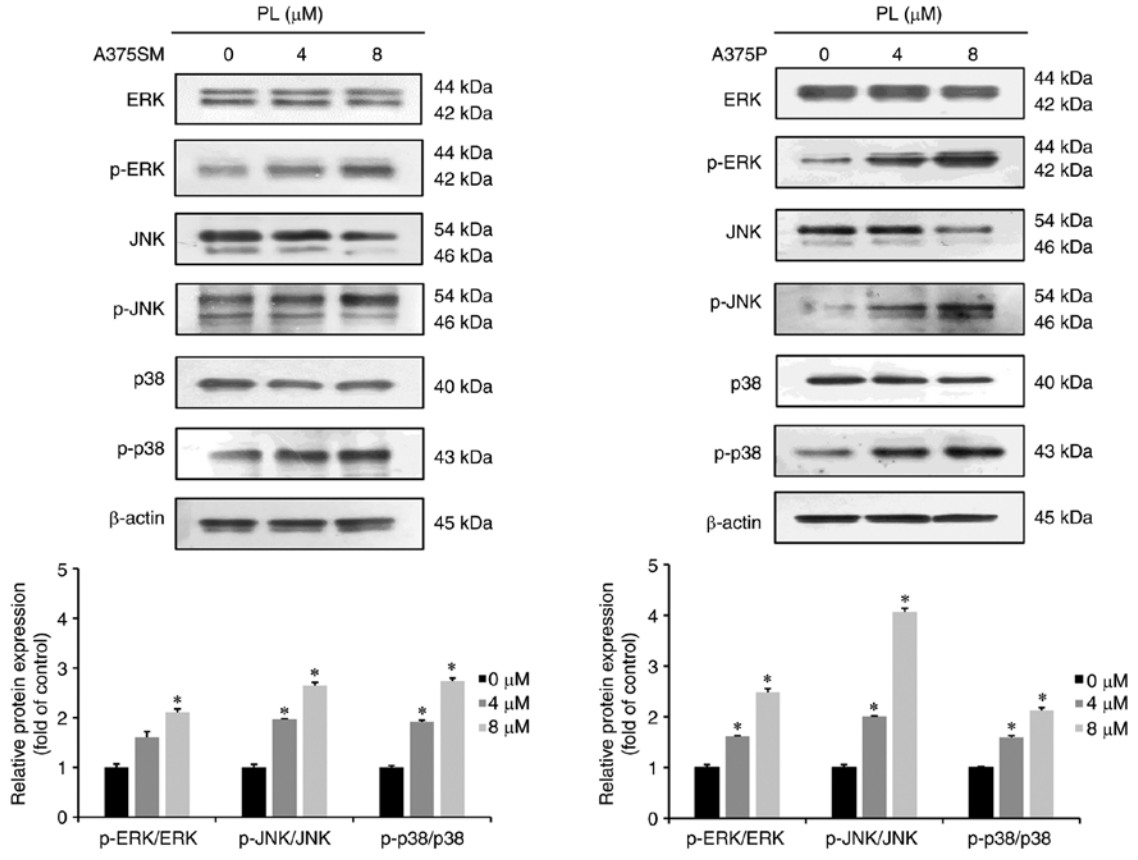

Figure 4. Effect of PL on expression of MAPK pathway-related proteins in human melanoma cells. A375SM and A375P cells were treated with PL and protein was extracted to measure levels of the MAPK pathway proteins ERK, p-ERK, JNK, p-JNK, p38 and p-p38 using western blotting. β-actin was used as the loading control. The control group (0 μM) was treated with an equal amount of DMSO. Data are presented as mean and SD of three samples. *P<0.05 vs. 0 μM. PL, piperlongumine; p, phosphorylated.

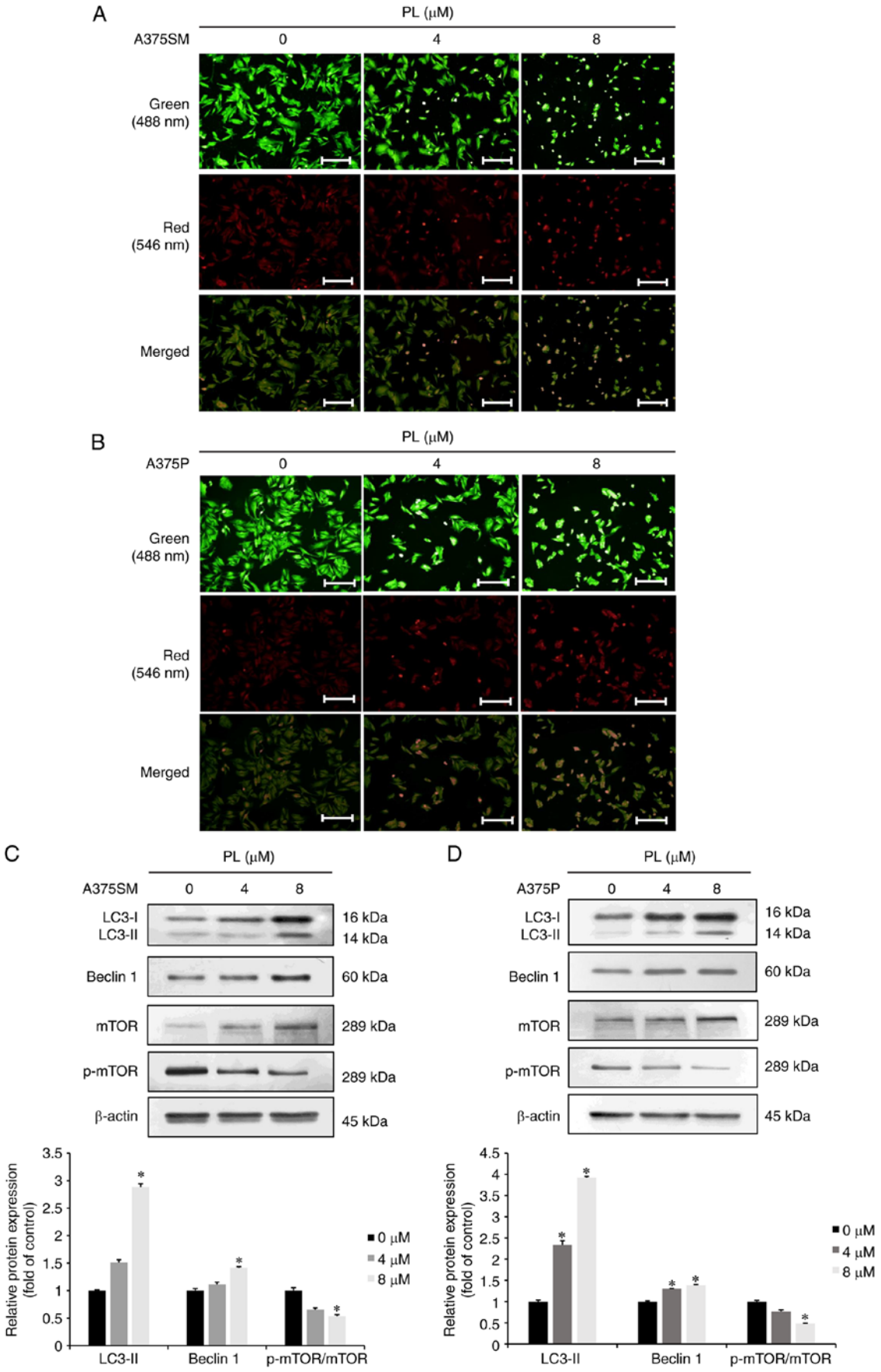

Figure 5. Effect of PL on AVO formation and autophagy proteins in human melanoma cells. (A) A375SM and (B) A375P cells were treated with PL and stained with 5 μg/ml acridine orange to detect AVOs (red). Green indicates cytoplasm and nucleus. Scale bar, 200 μm. Levels of the autophagy-related proteins LC3, Beclin 1 and p-mTOR in (C) A375SM and (D) A375P cells were observed using western blotting. β-actin was used as loading control. Data are presented as the mean and SD for three samples. Data are presented as mean and SD of three samples. *P<0.05 vs. 0 μM. AVO, acid vesicular organelle; PL, piperlongumine; p, phosphorylated.

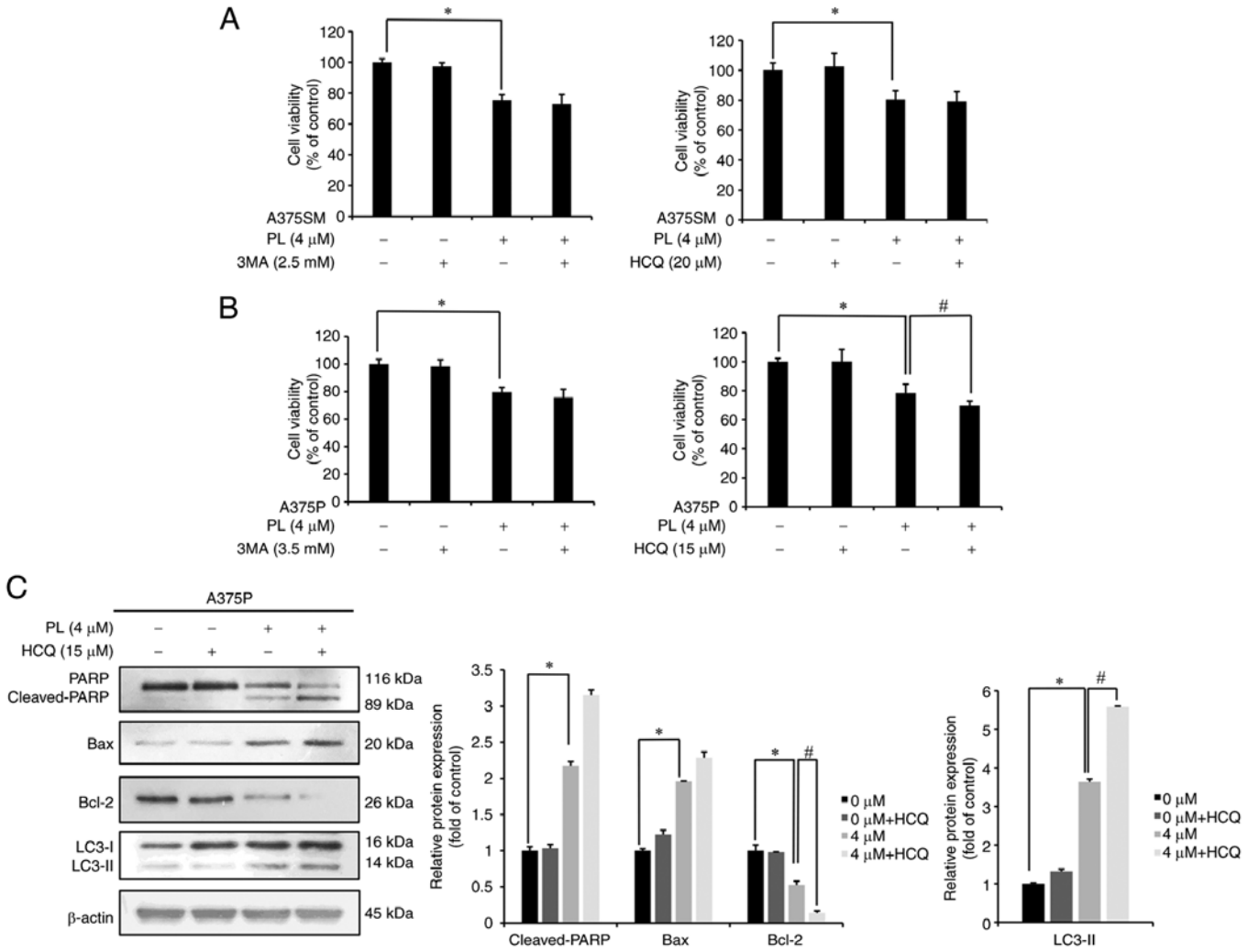

Figure 6. Effect of PL on induction of cell-protective autophagy in human melanoma cells. (A) A375SM and (B) A375P cells were pre-treated with 3-MA or HCQ and subsequently treated with indicated concentrations of PL. Cell viability was measured by MTT assay. (C) Protein levels of PARP, Bax, Bcl-2 and LC3 were measured by western blotting in A375P. β-actin was used as the loading control. Data are presented as the mean and SD of three samples. *P<0.05 vs. 0 μM; #P<0.05 vs. 0 μM + HCQ. PL, piperlongumine; 3-MA, 3-methyladenine; HCQ, hydroxychloroquine.

autophagy early-stage inhibitor 3-MA or the autophagy late-stage inhibitor HCQ for 3 h before incubation with PL for 24 h and MTT assay. The survival rate of A375SM cells treated with PL was 75.53%, whereas that of PL + 3-MA (2.5 mM)-treated cells was 72.92%. In another set of experiments the survival rate of PL-treated A375SM cells was 80.54%, whereas that of PL + HCQ (20 μM)-treated cells was 79.26%, showing an insignificant decrease (Fig. 6A). For A375P cells, the survival rate following PL treatment was 78.34%, whereas that after PL + 3-MA (3.5 mM) treatment was 75.80%. In another experiment set, the survival rate of PL-treated cells was 79.60%, whereas that of PL + HCQ (15 μM)-treated cells was 69.66%, demonstrating a significant decrease (Fig. 6B). Based on these results, A375P cells were selected for further experiments to investigate the association between apoptosis and autophagy as these cells showed a significant decrease in survival. Western blotting was performed to investigate apoptosis-related protein expression in A375P cells pretreated with HCQ for 3 h followed by PL for 24 h. Compared with cells treated with PL alone, those treated with PL + HCQ showed increased Bax expression and significantly decreased Bcl-2 expression. The expression of cleaved-PARP and LC3-II was also significantly increased in PL + HCQ-treated cells compared with that in PL-treated cells (Fig. 6C). These results suggested that apoptosis increased when autophagy was suppressed during PL treatment.

PL induces ERK-mediated apoptosis and autophagy in melanoma cells. A375P cells were pretreated with 25 μM PD98059 (ERK inhibitor) or SP600125 (JNK inhibitor) for 3 h, followed by 0 or 4 μM PL for 24 h and MTT assay. The survival rate of cells treated only with PL was 75.02% whereas that of cells treated with PL + PD98059 was 84.53%. The survival rate of cells treated only with PL (4 μM) was 78.58% whereas that of cells treated with PL + SP600125 was 82.72% (Fig. 7A). This indicated that, within the MAPK pathway, ERK was involved in the PL-induced decrease in the survival of melanoma cells. As ERK had a significant effect on survival of A375P cells, western blotting was performed after treatment with PD98059 to investigate the effects of ERK on apoptosis- and autophagy-related protein expression. The pro-apoptotic

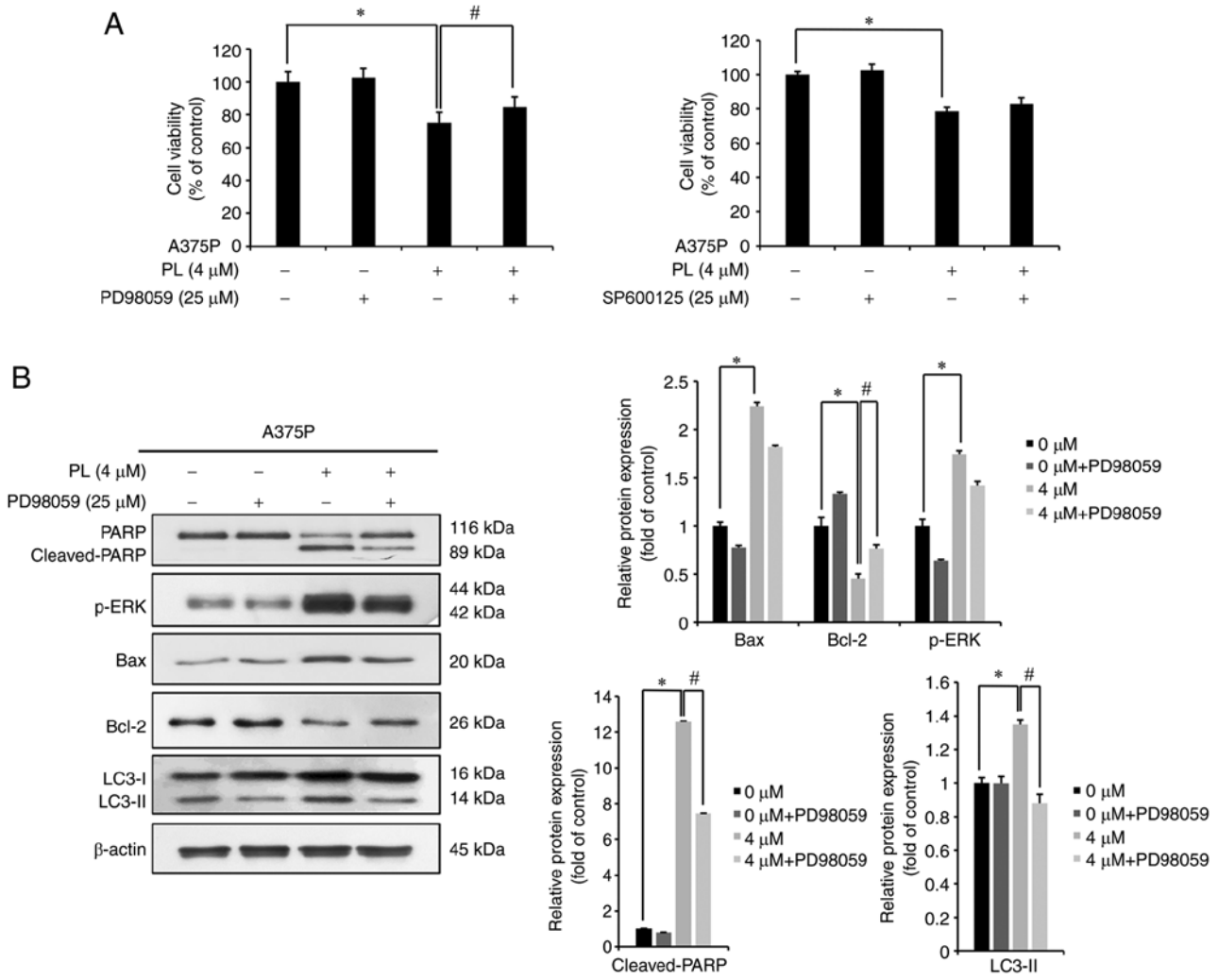

Figure 7. Effect of PL on ERK-mediated apoptosis and autophagy in A375P human melanoma cells. (A) Viability of cells pre-treated with PD98059 and SP600125 and treated with PL. (B) Levels of the apoptosis- and autophagy-related proteins PARP, p-ERK, Bax, Bcl-2 and LC3 in A375P cells pre-treated with PD98059 and treated with PL. β-actin was used as the loading control. Data are presented as the mean and SD of three samples. *P<0.05 vs. 0 μM; #P<0.05 vs. 0 μM + PD98059. PL, piperlongumine; p, phosphorylated.

proteins cleaved-PARP and Bax showed significantly increased expression in PL-treated cells compared with that in the control cells, whereas both showed insignificantly decreased expression in PL + PD98059-compared with PL-treated cells. By contrast, expression of the anti-apoptotic protein Bcl-2 was significantly decreased in PL-treated cells but increased significantly in PL + PD98059-treated cells. The representative autophagy protein LC3-II showed significantly decreased expression in PL + PD98059-treated cells compared with that in PL-treated cells (Fig. 7B). These findings suggested that ERK was involved in induction of apoptosis and autophagy in A375P cells.

Discussion

PL is an amide alkaloid primarily found in *Piper longum* and its anticancer effects have been investigated in several types of cancer (15-19). To the best of our knowledge, however, studies on the effect of PL on human melanoma cells are lacking. A375SM has a higher metastatic ability than A375P (31) and A375SM cells are more invasive than other melanoma cells (32). Therefore, the anticancer capability of PL against human melanoma was investigated in A375SM and A375P. It was hypothesized that A375P cells with lower metastatic properties would have higher sensitivity to PL than A375SM cells with higher metastatic properties. The present study investigated whether the PL-induced reduction in the survival of A375SM and A375P melanoma cells was due to apoptosis, analyzed the cytoprotective role of PL-induced autophagy and verified whether apoptosis and autophagy were affected by the MAPK/ERK pathway.

PL induced a dose- and time-dependent decrease in the survival of melanoma cells. PL has been shown to exert anticancer effects against human intestinal cancer cells: In human intestinal cancer cells INT-407 and HCT-116 treated with PL for 24 and 48 h, the IC_{50} of INT-407 was 13 at 24 and 9 μm at 48 h and IC_{50} of HCT-116 was 8 at 24 and 6 μm at 48 h, with the survival rates showing a dose-dependent decrease (35). Similarly, PL-treated WRO thyroid cancer cells show a time- and dose-dependent decrease in cell survival; the IC_{50} for 24 and 48 h of treatment with PL is 10.24 and 5.68 μm, respectively (36). Standard of concentration was

set to medium concentration as 4 μm, when cell survival rate was 80±5%. High concentration was set at 8 μm when cell survival rate was 50±5% based on previous studies (35,36). The aforementioned reports are similar to the present findings and support the conclusion that PL decreases melanoma cell survival in a dose-dependent manner. PL does not cause toxicity in normal human cells. There is no significant change in cell survival following PL treatment of human kidney cell line HK-2, pancreatic duct epithelial cell line HPDE, hepatic cell line LO-2 and breast epithelial cell line MCF10A (37). In addition, a previous study showed that PL does not decrease in survival rate in normal cells compared with cancer cells (38) DAPI staining is used to detect apoptosis as it shows nuclear changes such as condensation by strongly binding to adenine- and thymine-rich regions. Annexin-V staining targets areas with loss of membrane phospholipids, enabling the detection of early apoptosis. PI, which binds to DNA, cannot penetrate intact cell membranes and indicates cell damage, representing late apoptosis (39). DAPI and Annexin V-PI staining were used to verify if the decreased survival rate of melanoma cells determined by MTT assay was due to apoptosis. In PL-treated human melanoma cells, DAPI staining revealed a dose-dependent increase in DNA condensation, which is characteristic of apoptosis. FACS with Annexin V-PI staining found that early and late apoptosis was increased in human melanoma cells treated with PL compared with that in control cells. In a previous study on lung cancer cells (A549 and NCI-H460) treated with 20 μm PL for 24 h, DAPI staining revealed an increase in apoptotic bodies, demonstrating that PL induces apoptosis in lung cancer cells (40). Similarly, in PL (1, 2, and 4 μm)-treated DU145 prostate cancer cells, Annexin V-PI staining reveals a dose-dependent increase in apoptosis (41). The aforementioned studies support the present findings that PL induced apoptosis in melanoma cells. In summary, the decrease in melanoma cell survival induced by PL was due to apoptosis.

The first stage of apoptosis involves deactivation of PARP, which in turn prevents DNA repair. PARP cleavage prevents necrosis and enables caspase-mediated apoptosis (24). Other molecules that regulate apoptosis include Bcl-2, a protein important for tumor development, and pro-apoptotic protein Bax (42). The present study observed an increase in cleaved-PARP and Bax expression and decrease in Bcl-2 expression in PL-treated human melanoma cells. In a previous study, A546 lung cancer cells treated with PL (6 or 10 μm) for 48 h showed apoptosis due to increased cleaved-PARP and Bax and decreased Bcl-2 expression (16). The present findings showed trends similar to those of previous studies (42,16), providing evidence that PL decreases melanoma cell survival by inducing apoptosis.

The MAPK pathway serves an important role in cell proliferation, differentiation, apoptosis, angiogenesis and tumor metastasis. JNK and p38 are primarily involved in cell stress and apoptosis, while ERK is associated with cell proliferation and differentiation (43). ERK also serves an important role in halting the cell cycle and inducing apoptosis after DNA injury (44). Therefore, the present study investigated the role of the MAPK pathway in apoptosis induction using western blotting and found that PL-treated melanoma cells showed dose-dependent increases in expression of p-ERK, p-JNK, and p-p38. In a previous study, a natural compound (shikonin) with anticancer properties increased the expression of p-ERK, p-JNK, and p-p38 in A375SM melanoma cells, resulting in MAPK pathway-mediated apoptosis (45). Another natural compound (cudraflavone c) with anticancer properties was found to increase expression of p-ERK, p-JNK, and p-p38 in A375S2 melanoma cells (46). Collectively, these findings indicate that PL induced apoptosis in melanoma cells by increasing p-ERK, p-JNK and p-p38 expression and activating the MAPK pathway.

Autophagy serves various physiological roles, including anti-aging, apoptosis and tumor suppression (39). During autophagy, AVOs are produced by cells. AVOs protect cells by preventing acidification of the cytoplasm and providing necessary catabolites for recovery. However, excess production of AVOs leads to necrosis or apoptosis (47). The present study used AO staining to determine whether PL induced autophagy and observed a dose-dependent increase in AVOs when melanoma cells were treated with PL. Western blotting to analyze the levels of autophagy-associated proteins demonstrated PL induced autophagy in melanoma cells by decreasing p-mTOR and increasing Beclin 1 and LC3-II expression. Previously, PL was found to induce autophagy by decreasing p-mTOR and increasing LC3-II expression in thyroid cancer cells and increasing LC3-II expression in gallbladder cancer cells (35,48). These previous results are consistent with the present study, which strengthens the evidence that PL induces autophagy in melanoma cells. The present study used the autophagy inhibitors 3-MA (early-stage inhibitor) and HCQ (late-stage inhibitor) to investigate the role of PL-induced autophagy in melanoma cells. Combined treatment with PL + 3-MA induced no significant change in survival of A375P melanoma cells. However, PL + HCQ resulted in a significant decrease in cell survival. Western blot analysis of apoptosis-associated proteins revealed that PL + HCQ combined treatment increased cleaved-PARP and Bax expression and decreased Bcl-2 expression. Previously, endometrioma cells treated with HCQ and a natural anticancer agent (resveratrol) showed reduced survival and increased cleaved-PARP expression compared with those treated with only resveratrol, which indicates that using HCQ with resveratrol) induces apoptosis to a greater extent than only resveratrol), suggesting that autophagy has a protective effect against apoptosis (48). These findings are consistent with those of the present study. The present study showed that PL-induced autophagy had a cytoprotective effect in A375P cells and that co-treatment with PL + HCQ enhanced apoptosis to inhibit late autophagy for a stronger anticancer effect.

The present study investigated the roles of ERK and JNK by pretreating A375P cells with p-ERK inhibitor PD98059 or p-JNK inhibitor SP600125, followed by treatment with PL and cell survival measurement using MTT assay. There was a significant increase in the survival rate of PD98059-treated cells, suggesting involvement of the ERK pathway in the reduced survival rate of PL-treated melanoma cells. In a previous study, 1 h pretreatment with p-ERK inhibitor UO216 increased the survival rate of PL-treated colon cancer cells, suggesting that increased p-ERK levels affect cell survival (49). Here, western blotting revealed decreased cleaved-PARP and

Bax and increased Bcl-2 expression in melanoma cells treated with PL after 3 h PD98059 pretreatment compared with that in PL-treated cells. This suggested that when A375P melanoma cells were treated with PL, inhibition of ERK activity prevented apoptosis induction. To determine whether autophagy induction was associated with ERK protein expression, western blotting was performed on cells pretreated with PD98059 for 3 h and then treated with PL. There was decreased expression of LC3-II, indicating that autophagy occurred downstream of the MAPK/ERK pathway. However, ERK may not act independently in PL-induced apoptosis and autophagy in A375P cells. Further studies are needed to explore more detailed molecular signaling pathways.

In conclusion, there was no difference between A375SM and A375P in sensitivity to the concentration of PL. PL significantly decreased survival of melanoma cells due to apoptosis resulting from increased Bax and cleaved-PARP and decreased Bcl-2 expression. PL-induced apoptosis was associated with induction of the MAPK/ERK pathway. Moreover, PL-induced autophagy was identified based on an increase in AVOs and expression levels of autophagy-related proteins. Experiments using HCQ revealed the cytoprotective role of autophagy and the involvement of the MAPK/ERK pathway. In summary, when PL-induced late autophagy was inhibited in melanoma cells, the anticancer effects increased and PL induced apoptosis and autophagy via the MAPK/ERK pathway. The present study suggested that PL has potential as an anticancer agent and a greater anticancer effect could be achieved by regulating ERK expression. However, the present study did not confirm toxicity in normal cells, assuming that there was no toxicity in normal cells based on previous studies (37,38). In addition, experiments were only conducted on melanoma cells *in vitro*. Therefore, *in vivo* research is needed to assess anticancer effects of PL and confirm its potential clinical application.

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Availability of data and materials

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

Authors' contributions

JSJ, EYC and EJH designed the experiments, curated data and reviewed manuscript. JSJ wrote the manuscript. MJM and SWL analyzed and interpreted data. SHJ and JYJ analyzed the results and reviewed the manuscript. JYJ edited the manuscript and acquired funding. All the authors have read and approved the final manuscript. All authors confirmed the authenticity of all the raw data.

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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