Piperlongumine induces apoptosis and autophagy in leukemic cells through targeting the PI3K/Akt/mTOR and p38 signaling pathways

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Abstract. Piperlongumine is an alkaloid compound extracted from *Piper longum* L. It is a chemical substance with various pharmacological effects and medicinal value, including anti-tumor, lipid metabolism regulatory, antiplatelet aggregation and analgesic properties. The present study aimed to understand whether piperlongumine induces the apoptosis and autophagy of leukemic cells, and to identify the mechanism involved. Cell viability and autophagy were detected using MTT, phenazine methyl sulfate and trypan blue exclusion assays. The apoptosis rate was calculated using flow cytometry. The protein expression levels of microtubule-associated protein 1A/1B-light chain 3, Akt and mechanistic target of rapamycin (mTOR) were measured using western blotting. The cell growth of leukemic cells was completely inhibited following treatment with piperlongumine, and marked apoptosis was also induced. Dead cells as a result of autophagy were stained using immunofluorescence and observed under a light microscope. Phosphoinositide 3-kinase (PI3K)/Akt/mTOR signaling was suppressed by treatment with piperlongumine, while p38 signaling and caspase-3 activity were induced by treatment with piperlongumine. It was concluded that piperlongumine induces apoptosis and autophagy in leukemic cells through targeting the PI3K/Akt/mTOR and p38 signaling pathways.

Introduction

Leukemia is a kind of malignant tumor of the hemopoietic system posing a great threat to human health (1). The mortality rate ranks sixth in males and eighth in females among all malignant tumors in China in all age groups, and first among children and adults under 35 years of age (2). There is a higher number of patients with acute myeloid leukemia (AML) than with acute lymphoblastic leukemia (ALL) (AML:ALL=2.67:1.00); AML is mostly reported in young adults (76.2%) and has a negative effect on health (3). In contrast to solid tumors, leukemia is a kind of 'liquid tumor' that cannot be removed by surgery (4). At present, chemotherapy is still the most widely used method in the treatment of patients with leukemia due to the difficulty in matching bone marrow for transplantation and the high price of molecular targeted therapy (5). However, the current research focus is to identify novel chemotherapy drugs with strong activity and low toxicity from natural drugs that are able to overcome chemotherapeutic resistance and adverse side reactions (6).

Following cell apoptosis, autophagy has become the hot research topic as type-II programmed death in recent years (5). Autophagy involves the formation of autophagic vacuoles, their integration with lysosomes and the degradation of the contents of these autophagosomes (7). In contrast to cell apoptosis, autophagy has a dual regulatory function in cell survival, which serves an important role in the occurrence and development of tumors (8). Previous studies have shown that intracellular autophagy in certain tumor cells can kill these cells and maintain the normal internal body environment in the early stages of cancer (9,10). However, upon the formation of tumors, autophagy promotes apoptosis by removing damaged organelles, so as to reduce the growth of tumor cells (11,12). Therefore, researching the mechanism of autophagy development has a good prospect in clinical application for the prevention and treatment of cancer (13).

Piperlongumine (Fig. 1) belongs to the family of alkaloids, and exhibits a variety of pharmacological effects and pharmacological activities, including antiplatelet aggregation, analgesia and antifungal properties (14). In addition, piperlongumine has a marked cytotoxic effect on tumor cells and can regulate blood lipid metabolism in hyperlipidemic rats (14). Previous studies have shown that piperlongumine has a specific cytotoxic effect on a variety of tumor cells, but exhibits low toxicity towards normal cells (14,15). Therefore, piperlongumine is an extract of a herb used in traditional Chinese medicine, which has the potential to selectively kill tumors (14). The pharmacological effects of piperlongumine mainly include antitumor, antiplatelet aggregation, analgesic, antifungal, anti-schistosomiasis, anti-anxiety and

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anti-depression effects (16). The aim of the present study was to investigate whether piperlongumine induces apoptosis and autophagy in leukemic cells, and to explore the underlying molecular mechanisms.

Materials and methods

Cell lines and mice. Leukemic monocytic lymphoma U937 cells were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium (pH 6.8) supplemented with 10% fetal bovine serum (both from Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and antibiotics (50 IU/ml penicillin G and 50 μg/ml streptomycin), and were incubated at 37˚C in a humidified atmosphere containing 5% CO_2.

Cell viability assay and immunofluorescence. The effect of piperlongumine on the viability of U937 cells was evaluated. U937 cells (2.5-5.0x10^4 cells/200 μl of RPMI-1640 medium/well) were seeded in 96-well tissue culture plates and incubated with piperlongumine (0-20 μM) for 48 h at 37˚C in the presence of 5% CO_2. MTT (2.0 mg/ml) and phenazine methyl sulfate (0.92 mg/ml) were added into every well and incubated at 37˚C for 3 h. The absorbance was measured at 490 nm in an ELISA reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cells (at 60-70% confluence) were seeded in 6-well tissue culture plates and treated with piperlongumine (0-20 μM) for 48 h at 37˚C in the presence of 5% CO_2.

U937 cells treated with piperlongumine (0, 5, 10 and 20 μM) were washed with PBS and fixed using 4% paraformaldehyde for 15 min at room temperature. Cells were incubated with Triton X-100 (0.1%) for 10 min at room temperature and then incubated with anti-LC3 antibodies (cat. no. 3868; 1:3,000; Cell Signaling Technology, Inc., Beverly, MA, USA) at 4˚C overnight. Cells were incubated with Alexa Fluor 488-labeled goat anti-rabbit secondary antibody (Thermo Fisher Scientific, Inc.) for 1 h at room temperature in darkness. Cells were observed using a Nikon Eclipse E800 fluorescence microscope and NIS-Elements 4.0 software (all from Nikon; Tokyo, Japan).

Apoptosis assay. U937 cells (2.5x10^5 cells/ml) were incubated with piperlongumine (0-20 μM) for 48 h at 37˚C in the presence of 5% CO_2. Cells were washed twice with PBS and resuspended in 100 μl of Annexin V binding buffer (pH 7.4) (BD Biosciences, Franklin Lakes, NJ, USA). Then, annexin V-fluorescein isothiocyanate (BD Biosciences) was added and incubated for 15 min under dark conditions. Propidium iodide (0.1 μg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added just prior to signal acquisition. The apoptosis rate was acquired using a FACSAria flow cytometer (BD Biosciences) and analyzed with FACSDiva 7.6.1 software (BD Biosciences).

Western blotting. Total cellular proteins were isolated from U937 cells (2.5x10^5 cells/ml) incubated with piperlongumine (0-20 μM) for 48 h at 37˚C in the presence of 5% CO_2. Cells were lysed using radioimmunoprecipitation assay (RIPA) buffer (Beyotime Institute of Biotechnology, Jiangsu, China) and centrifuged for 10 min at 4˚C at 10,000 x g. Protein concentration was estimated using the bicinchoninic acid method (Beyotime Institute of Biotechnology). Electrophoretic separations were carried out on 10% SDS-PAGE, and the proteins were then electrotransferred onto a polyvinylidene fluoride membrane. Blots were blocked for 1 h at 37˚C in TBS containing 0.01% Tween-20 (TBST) and 5% skimmed milk, and probed overnight at 4˚C with appropriate primary antibodies: Anti-microtubule-associated protein 1A/1B-light chain 3 (LC3-I; cat. no. 3868; 1:3,000), anti-phosphorylated (p)-Akt (cat. no. 4228; 1:3,000), anti-Akt (cat. no. 6211; 1:3,000), anti-p-mechanistic target of rapamycin (mTOR; cat. no. 2974; 1:3,000), anti-p-p38 (cat. no. 4511, 1:2,000) and anti-β-actin (cat. no. 4970, 1:2,000) (all from Cell Signaling Technology, Inc., Beverly, MA, USA) antibodies. Next, the membranes were washed with TBST and incubated with anti-rabbit IgG, HRP-linked antibody (cat. no. 7074, 1:5,000, Cell Signaling Technology, Inc.) at 37˚C for 1 h. Proteins were visualized using BeyoECL Plus (Beyotime Institute of Biotechnology) and analyzed uisng Bio-Rad Laboratories Quantity One software 3.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

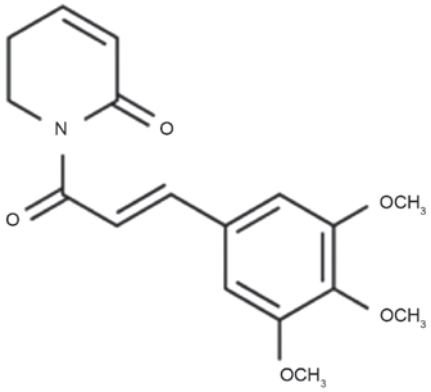

Figure 1. Chemical structure of piperlongumine.

Measurement of caspase activity. U937 cells (2.5-5.0x10^4 cells/200 μl of RPMI-1640 medium/well) were seeded in 96-well tissue culture plates and incubated with piperlongumine (0-20 μM) for 48 h at 37˚C in the presence of 5% CO_2. Cells were lysed using RIPA buffer (Beyotime Institute of Biotechnology) and centrifuged for 10 min at 4˚C at 10,000 x g. Protein concentration was estimated using the BCA Protein Assay kit (Beyotime Institute of Biotechnology). U937 cell lysates (5 μg) were incubated in 50 μl of reaction buffer and acetyl-Asp-Glu-Val-Asp p-nitroanilide (p-NA) (Beyotime Institute of Biotechnology) at 37˚C for 5 h for determination of caspase-3 activity. The emission of pNA was measured at 405 nm in an ELISA reader (Bio-Rad Laboratories, Inc.).

Statistical analysis. All experiments were repeated independently at least three times using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). The values were expressed as mean ± standard error of the mean, and statistical analyses were performed with a two-way analysis of variance followed by the Student-Newman-Keuls test.

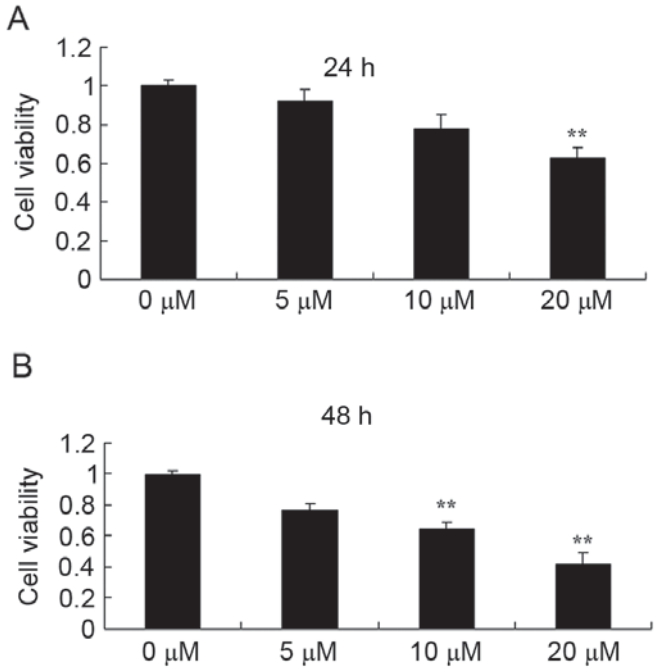

Figure 2. Piperlongumine suppresses the proliferation of leukemic cells at (A) 24 and (B) 48 h. **P<0.01 vs. U937 cells incubated with 0 μM of piperlongumine.

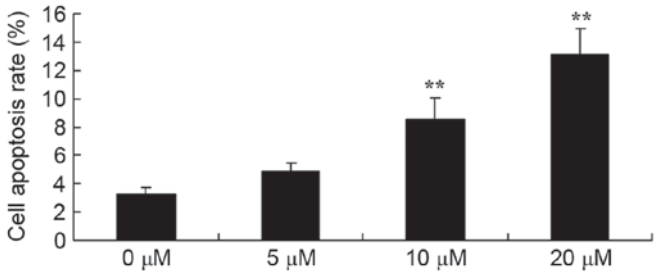

Figure 3. Piperlongumine induces the apoptosis of leukemic cells. **P<0.01 vs. U937 cells incubated with 0 μM of piperlongumine.

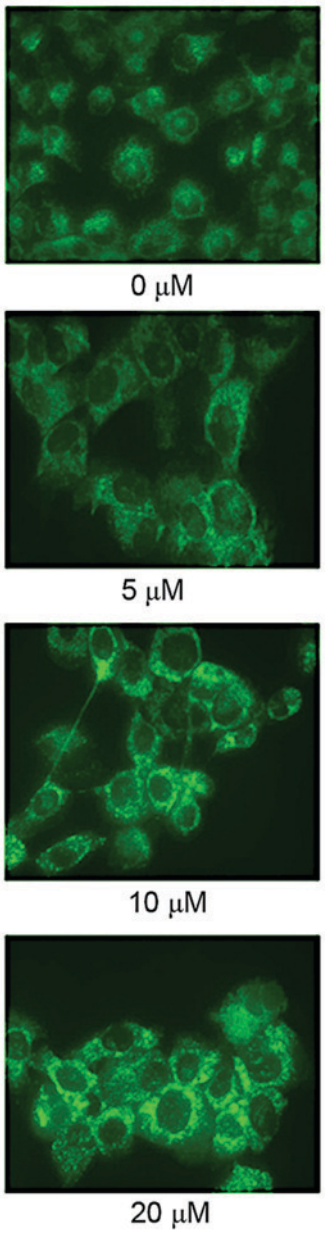

Figure 4. Piperlongumine induces the autophagy of leukemic cells. Measured via immunofluorescence targeted at microtubule-associated protein 1A/1B-light chain 3.

Results

Piperlongumine suppresses cell proliferation in leukemic cells. The anticancer effect of piperlongumine in terms of suppressing the proliferation of leukemic cells was evaluated. An MTT assay was used to analyze the change in cell proliferation in U937 cells. As shown in Fig. 2, 0-20 μM of piperlongumine could suppress the proliferation of U937 cells in a time- and dose-dependent manner. At 20 μM, piperlongumine significantly suppressed the proliferation of U937 cells at 24 h, while 10 or 20 μM of piperlongumine significantly suppressed the proliferation of U937 cells at 48 h (Fig. 2).

Piperlongumine induces apoptosis in leukemic cells. To further confirm the role of apoptosis in the effect of piperlongumine on leukemic cells, the apoptosis rate was evaluated using flow cytometry. The results revealed that 0-20 μM of piperlongumine induced the apoptosis of U937 cells in a dose-dependent manner (Fig. 3). Treatment with 10 or 20 μM of piperlongumine significantly induced the apoptosis of U937 cells, compared with that of U937 cells incubated with 0 μM of piperlongumine (Fig. 3).

Piperlongumine induces autophagy in leukemic cells. The autophagy of U937 cells was measured by fluorescence microscopy targeted at LC3. An increase in the autophagy of U937 cells incubated with 0-20 μM of piperlongumine was observed (Fig. 4). Treatment with 10 or 20 μM of piperlongumine activated the autophagy of U937 cells, compared with that of U937 cells incubated with 0 μM of piperlongumine (Fig. 4).

Piperlongumine induces LC3-I expression in leukemic cells. Upon co-incubation of U937 cells with 0-20 μM of piperlongumine for 48 h, the protein expression of LC3-I was explored using western blotting. The protein expression of LC3-I was induced by 0-20 μM of piperlongumine in a dose-dependent

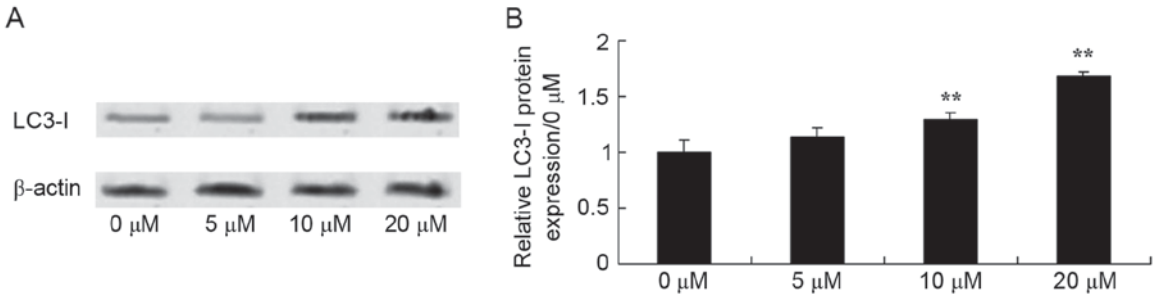

Figure 5. Piperlongumine induces LC3-I expression in leukemic cells. (A) Western blot analysis of LC3-I protein expression induced by piperlongumine. (B) Statistical analysis of LC3-I protein expression in leukemic cells. **P<0.01 vs. U937 cells incubated with 0 μM of piperlongumine. LC3-I, microtubule-associated protein 1A/1B-light chain 3.

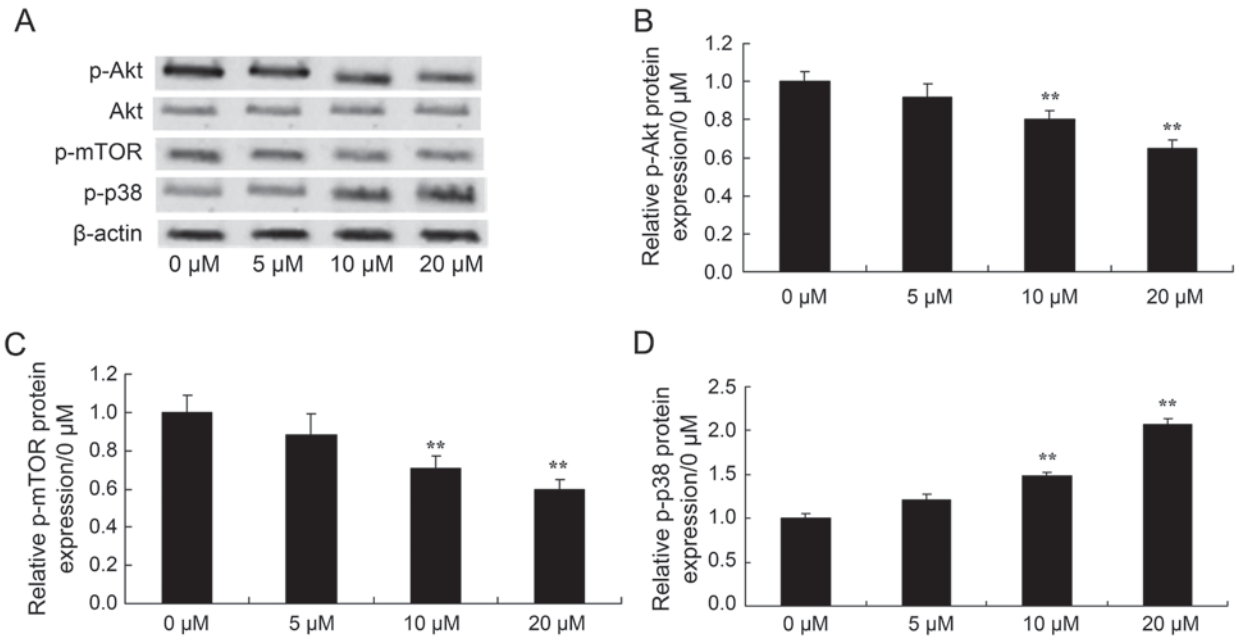

Figure 6. Piperlongumine reduces p-Akt and p-mTOR, and induces p-p38 protein expression in leukemic cells. (A) Piperlongumine affects p-Akt, Akt, p-mTOR and p-p38 protein expression, according to western blot analysis. Statistical analysis of (B) p-Akt/Akt, (C) p-mTOR and (D) p-p38 protein expression levels in leukemic cells. **P<0.01 vs. U937 cells incubated with 0 μM of piperlongumine. p, phosphorylation; mTOR, mechanistic target of rapamycin.

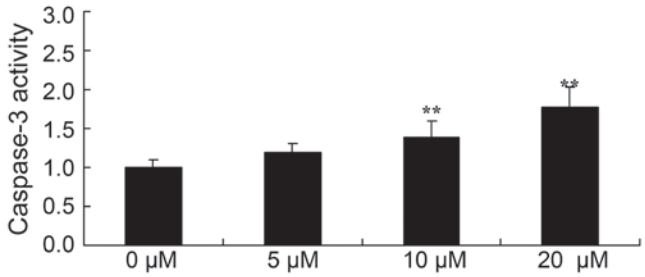

Figure 7. Piperlongumine induces caspase-3 activity in leukemic cells. **P<0.01 vs. U937 cells incubated with 0 μM of piperlongumine.

manner (Fig. 5). In particular, 10 or 20 μM of piperlongumine significantly increased LC3-I protein expression in U937 cells, compared with that of U937 cells incubated with 0 μM of piperlongumine (Fig. 5).

Piperlongumine reduces Akt levels in leukemic cells. To analyze the mechanism of piperlongumine on autophagy, the phosphorylation of Akt in U937 cells was determined by western blotting. As shown in Fig. 6, a dose-dependent decrease in the ratio of p-Akt/Akt protein expression was detected in U937 cells following treatment with 0-20 μM of piperlongumine. Particularly, 10 or 20 μM of piperlongumine significantly reduced the p-Akt/Akt ratio in U937 cells, compared with that of U937 cells incubated with 0 μM of piperlongumine (Fig. 6A and B).

Piperlongumine reduces mTOR levels in leukemic cells. To further analyze the mechanism of piperlongumine on autophagy, the protein expression of p-mTOR was measured. Fig. 6 shows that 0-20 μM of piperlongumine inhibited p-mTOR protein expression in U937 cells in a dose-dependent manner. Treatment with 10 or 20 μM of piperlongumine significantly inhibited p-mTOR protein expression in U937 cells, compared with that of the 0 μM of piperlongumine group (Fig. 6A and C).

Piperlongumine induces the phosphorylation of p38 protein in leukemic cells. To investigate the protein expression of p38,

U937 cells were incubated with piperlongumine (0-20 μM) for 48 h. Treatment with 0-20 μM of piperlongumine induced the protein expression of p-p38 in U937 cells (Fig. 6A and D). Treatment with 10 or 20 μM of piperlongumine significantly induced p-p38 protein expression in U937 cells, compared with that of U937 cells incubated with 0 μM of piperlongumine (Fig. 6A and D).

Piperlongumine induces caspase-3 activity in leukemic cells. In order to assess the role of caspase-3 activity in U937 cells, the cells were co-incubated with 0-20 μM of piperlongumine for 48 h. As shown in Fig. 7, 0-20 μM of piperlongumine increased the caspase-3 activity of U937 cells in a dose-dependent manner. Particularly, treatment with 10 or 20 μM of piperlongumine significantly increased the caspase-3 activity of U937 cells, compared with that of U937 cells incubated with 0 μM of piperlongumine (Fig. 7).

Discussion

Leukemia is a type of malignant tumor of the hemopoietic system posing a severe threat to human health, and its mortality rate ranks first among all malignant tumors among children and adults under 35 years of age (17). Its morbidity and mortality are increasing, and it is urgently required to identify novel therapeutics against leukemic cells (18). Dhillon *et al* reported that piperlongumine induces pancreatic cancer cell death (19). The present data clearly demonstrated that piperlongumine significantly suppresses cell proliferation and induces apoptosis in U937 cells.

Autophagy is a conserved self-degradation system in eukaryotic cells, which is involved in numerous physiological and pathological processes (13). The autophagosome is the typical characteristic of autophagy, and the regulation of the formation and degradation of the autophagosome is the main regulation of autophagy (8). Due to the dual characteristics of autophagy in promoting both cell growth and death, autophagy determines cell survival to certain extent (20). Autophagy is closely associated with tumors, and is involved in tumor development, metastasis and drug resistance (12). Targeted autophagy may become a new strategy for the treatment of cancer and drug resistance (21). Further study of autophagy in leukemic cells will clarify the mechanism of induction and regulation of autophagy in leukemic cells, and may provide a new treatment target and strategy for leukemic cells (22). In the present study, it was demonstrated that piperlongumine induces autophagy and induces expression of LC3-I protein in leukemic cells. Makhov *et al* demonstrated that piperlongumine promotes autophagy in a xenograft mouse model through inhibition of Akt/mTOR signaling, and mediates cancer cell death (23). Wang *et al* (24) showed that Piperlongumine induces autophagy of cancer cell by targeting p38 signaling.

The main function of mTOR is to inhibit the occurrence of self-autophagy via two mechanisms: i) mTOR regulates the transcription and translation of autophagy-associated genes through the activation of downstream effectors, which affects the signal transduction pathway; and ii) inhibition of mTOR can induce the occurrence of autophagy (25). PI3K/Akt is the upstream signaling pathway that activates mTOR (26). PI3K is important in cancer development, while the serine-threonine protein kinase Akt is the downstream effector of PI3K and is involved in the regulation of various biological processes, including cell metastasis, growth, development, apoptosis, and regulation of gene transcription, protein synthesis and nutritional metabolism (27). Akt may be key to the inhibition and survival signal pathway and inhibit autophagy by phosphorylating mTOR and contacting PI3K/Akt (27). The activated mTOR signal transduction pathway can inhibit the apoptosis and autophagy induced by various factors, which leads to cell cycle progression, cell growth and proliferation. It is also associated with angiogenesis, thus serving an important role in the formation, invasion and metastasis of tumors (28,29). Numerous tumors exhibit mutated genes coding for proteins involved in mTOR signaling, and the resulting over-activated mTOR signaling pathway is mainly caused by abnormal expression of these proteins (28,29). Previous studies have demonstrated that breast cancer, leukemia, small cell lung cancer, urinary system tumors and other diseases progress through PI3K/Akt/mTOR signaling (22,27). The present study has demonstrated that piperlongumine significantly reduces Akt/mTOR signaling in U937 cells. Wang *et al* previously demonstrated that piperlongumine induces apoptosis and autophagy through inhibition of the PI3K/Akt/mTOR signaling pathway in human lung cancer cells (16).

The p38/mitogen-activated protein kinase (MAPK) signaling pathway is involved in the activation of autophagy in macrophages (30). MAPK p38 mainly inhibits autophagy, and the effect of the p38/MAPK signaling pathway is markedly complex in the development of cells (31). The activation of this pathway leads to the inhibition of cell proliferation. p38 can also induce the arrest of the cell cycle into the stationary phase and promote DNA repair against the DNA damage induced by chemotherapy (32). In particular, p38 has been reported to exhibit anti-apoptotic properties in various cell lines, and may have a direct effect on tumor infiltration and metastasis (33). The present study noticed that piperlongumine significantly induced p-p38 protein expression and increased caspase-3 activity in U937 cells. Xiong *et al* reported that piperlongumine induces the autophagic death of primary myeloid leukemia cells through the p38/c-Jun N-terminal kinase signaling pathway (34).

In summary, the present study has demonstrated that piperlongumine significantly suppresses cell proliferation and induces apoptosis in U937 cells. Preferentially, piperlongumine significantly induced the autophagy of U937 cells through targeting the PI3K/Akt/mTOR and p38 signaling pathways. The present data suggest that piperlongumine could be applied in the treatment of leukemic cells.

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