

# Paxilitaxel induces apoptosis accompanied by protective autophagy in osteosarcoma cells through hypoxia-inducible factor-1 $\alpha$ pathway

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**Abstract.** Paxilitaxel, a drug used in cancer chemoprevention and treatment, has shown promising anti-cancer effects against a broad spectrum of tumors. However, the effect of paxilitaxel on osteoblasts has remained to be elucidated. The aim of the present study was to investigate the anti-tumor effect of paxilitaxel on human osteosarcoma cancer cells, the underlying molecular mechanism as well as drug resistance involved. The results showed that paxilitaxel not only induced apoptosis via the mitochondrial pathway but also induced autophagy, which partially inhibited cell apoptosis. The present study also demonstrated that paxilitaxel induced autophagy through the hypoxia-inducible factor (HIF)-1 $\alpha$  pathway. Moreover, paxilitaxel-induced apoptosis decreased following incubation with the autophagy inducer rapamycin. By contrast, co-treatment with the HIF-1 $\alpha$  inhibitor YC-1 or autophagy inhibitor 3-methyladenine significantly blocked autophagy and augmented the anti-tumor effects of paxilitaxel. Therefore, the results of the present study suggested that the combination of paxilitaxel with an autophagy inhibitor or a HIF-1 $\alpha$  inhibitor may be an effective and potent strategy for improved chemotherapy of osteosarcoma in the future.

## Introduction

Osteosarcoma is the most common malignant tumor of bone, which mainly affects children and adolescents and accounts for ~60% of malignant bone tumors diagnosed in the first two decades of life (1,2). Current treatment of osteosarcoma consists of multiagent chemotherapy and surgical resection. However, further therapy with additional chemotherapy is palliative and too often toxic (3,4). Thus, safe and more effective anti-cancer treatments are required for patients with osteosarcoma.

Paxilitaxel, which was isolated from the bark of the yew tree, inhibits the division of actively growing tumor cells and has become increasingly important in the treatment of a number of major cancers, including ovarian (5), pancreatic (6) and breast (7) cancer. However, the impact of paxilitaxel on osteoblasts has remained to be elucidated.

Autophagy is an evolutionarily conserved lysosomal degradation process by which cells recycle macromolecules and organelles (8). Studies have demonstrated that anti-cancer therapies (such as chemotherapy) induce autophagy in cancer cells, while the association between autophagy and therapeutic effects has remained controversial (9,10). However, an increasing number of studies have reported that autophagy is elevated in certain tumors and contributes to poor outcome by promoting resistance to chemotherapy (11). It has remained elusive whether paxilitaxel is able to induce autophagy in osteosarcoma cells and, if so, what the significance of this response is.

Hypoxia-inducible factor 1 (HIF-1) is the most critical nuclear transcription factor under hypoxic conditions in normal cells and tumor cells. Studies have reported that HIF-1 $\alpha$  protein expression is upregulated in certain tumor types and contributes to poor disease outcome by promoting tumor progression, metastasis and resistance to chemotherapy (12,13). Previous studies also showed that HIF and autophagy are closely linked (14,15). Whether paxilitaxel is able to affect the expression of HIF-1 and autophagy, as well as the complex association between them, was the subject of the present study.

The present study investigated whether paxilitaxel induces apoptosis in human osteosarcoma cells. Furthermore,

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**Abbreviations:** HIF-1, hypoxia-inducible factor 1; 3-MA, 3-methyladenine; RAPA, rapamycin; LC3I/II, microtubule-associated protein light chain 3-I/II; AIF, apoptosis-inducing factor; DMEM, Dulbecco's modified Eagle's medium; PI, propidium iodide;  $\Delta\Psi_m$ , mitochondrial membrane potential; YC-1, 3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole

**Key words:** paxilitaxel, autophagy, hypoxia-inducible factor-1 $\alpha$ , osteosarcoma

the ability of paclitaxel to induce autophagy and HIF-1 $\alpha$  expression in osteosarcoma and its possible association with resistance were investigated.

## Materials and methods

**Cell cultures.** The human osteosarcoma cell line MG-63 was obtained from the Cell Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Invitrogen Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; Gibco-BRL) at 37°C in a 5% CO<sub>2</sub> incubator. The cells were routinely sub-cultured every 2-3 days. All experiments were performed on cells harvested at the mid-logarithmic growth phase. Briefly, the cells were treated with autophagy inhibitors: 50  $\mu$ mol/l 3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole (YC-1) or 5 mM 3-methyladenine (3-MA) (both Sigma-Aldrich, St. Louis, MO, USA), or with an autophagy inducer: 2  $\mu$ M rapamycin (RAPA; Sigma-Aldrich) in DMEM with 10% FBS.

**Reagents and antibodies.** Paclitaxel was purchased from Sigma-Aldrich. Mouse monoclonal anti-B-cell lymphoma 2-associated X protein (Bax; cat. no. sc-20067), mouse monoclonal anti-apoptosis-inducing factor (AIF; cat. no. sc-13116), and mouse monoclonal anti- $\beta$ -actin (cat. no. sc-8432) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Rabbit monoclonal anti-caspase 3 (cat. no. 9664), rabbit polyclonal anti-caspase 9 (cat. no. 9502), rabbit monoclonal anti-Beclin1 (cat. no. 3495), rabbit monoclonal anti-microtubule-associated protein light chain 3 (LC3; cat. no. 12741) and rabbit monoclonal anti-HIF-1 $\alpha$  (cat. no. 14179) were purchased from Cell Signaling Technology (Danvers, MA, USA).

**Analysis of apoptosis.** Cellular apoptosis was determined using the Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit I (Clontech Laboratories Inc., Takara Bio Inc., Mountain View, CA, USA). Briefly, MG-63 cells were cultured at 4x10<sup>6</sup> cells/ml and seeded in six-well plates. Cells were harvested by trypsinization (Sigma-Aldrich), then washed twice with cold phosphate-buffered saline (PBS) and centrifuged at 700 x g. 1x10<sup>5</sup>-1x10<sup>6</sup> cells were re-suspended in 300  $\mu$ l 1X binding buffer (Clontech Laboratories Inc.), centrifuged again at 700 x g for 5 min and then the supernatant was removed. Cells were re-suspended in 300  $\mu$ l 1X binding buffer and transferred to a sterile flow cytometry glass tube. 10  $\mu$ l Annexin V-FITC was added and cells were incubated in the dark for 30 min at room temperature. Cells were then incubated in the dark with 5  $\mu$ l propidium iodide (PI) and analyzed by flow cytometry (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ, USA).

**Hoechst 33342 staining.** The cells were washed three times with PBS and fixed with 4% paraformaldehyde (Sigma-Aldrich) for 60 min at 4°C. After washing with PBS for three times, the cells were incubated with 0.2% Triton X-100 (Sigma-Aldrich) for 15 min. Cells were then blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich) for 60 min at room temperature and Hoechst 33342 (Sigma-Aldrich) was added to the cells

for 20 min. After washing three times with PBS, cells were visualized under a fluorescence microscope (SZ51; Olympus Corporation, Tokyo, Japan).

**Immunofluorescence staining.** MG-63 cells were seeded in six-well plates for 24 h. Cells were then washed once with ice-cold PBS and fixed with 4% paraformaldehyde for 30 min at 4°C. After washing with PBS three times, the cells were incubated with 1% Triton X-100 for 10 min. The cells were blocked at non-specific antibody binding sites by incubating with 10% goat serum in PBS containing 0.3% Triton X-100 and 0.5% BSA for 30 min at room temperature, followed by incubation with a mouse antibody against Beclin1 (1:400 in PBS; Cell Signaling Technology) or LC3 (1:200 in PBS; Cell Signaling Technology) overnight. The samples were then incubated with either tetramethylrhodamine (TRITC)- or FITC-conjugated goat anti-rabbit immunoglobulin (Ig)G secondary antibodies (1:100 in PBS) for 0.5 h at room temperature. Cells initially incubated with anti-Beclin1 were incubated with the TRITC-conjugated secondary antibody, whereas those initially incubated with anti-LC3 were incubated with the FITC-conjugated secondary antibody. Hoechst 33342 was added to the cells for 15 min. After washing three times with PBS, cells were visualized under a fluorescence microscope (SZ51; Olympus Corporation).

**Assessment of the mitochondrial membrane potential ( $\Delta\psi$ m).** The  $\Delta\psi$ m was analyzed using the tetraethylbenzimidazolylcarbocyanine iodide (JC-1) assay (Beyotime, Nantong, China). JC-1 is a cationic dye that indicates the  $\Delta\psi$ m by reversibly shifting its fluorescence emission between green (JC-1 monomers indicating loss of  $\Delta\psi$ m) and red (JC-1 aggregates indicating intact  $\Delta\psi$ m). In brief, a staining mixture (300 nM JC-1) was prepared according to the manufacturer's instructions of the JC-1 kit. Cells were incubated in the staining mixture for 30 min at 37°C. Thereafter, cells were washed twice with medium and re-suspended in fresh medium.  $\Delta\psi$ m was monitored using a fluorescence microscope.

**Isolation of proteins from mitochondria and cytosol and western blot analysis.** The preparation of proteins from the mitochondrial and cytosolic fractions and western blot were performed as described previously (16). The cells were washed twice in ice-cold PBS and re-suspended in five volumes of ice-cold extraction buffer [20 mM western blotting analysis (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-KOH, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid, 1 mM dithiothreitol and 0.1 mM phenylmethanesulfonyl fluoride, pH 7.5; Sigma-Aldrich]. The re-suspended cells were homogenized with ten strokes of a Teflon homogenizer (2-16P; Sigma-Aldrich). The homogenates were centrifuged twice at 750 xg for 10 min at 4°C. The supernatants were centrifuged at 10,000 xg for 15 min at 4°C to obtain the mitochondrial pellets. Cytosolic fractions were obtained after further centrifugation at 100,000 xg for 1 h at 4°C. The protein concentrations of the resulting supernatants and mitochondrial fractions were measured. The samples (10  $\mu$ g protein) were separated by 10% SDS-PAGE (Invitrogen Life Technologies). The proteins separated by SDS-PAGE were electrotransferred onto a Hybond-polyvinylidene fluoride (PVDF) membrane (Invitrogen Life Technologies). The individual SDS gels were distinguished

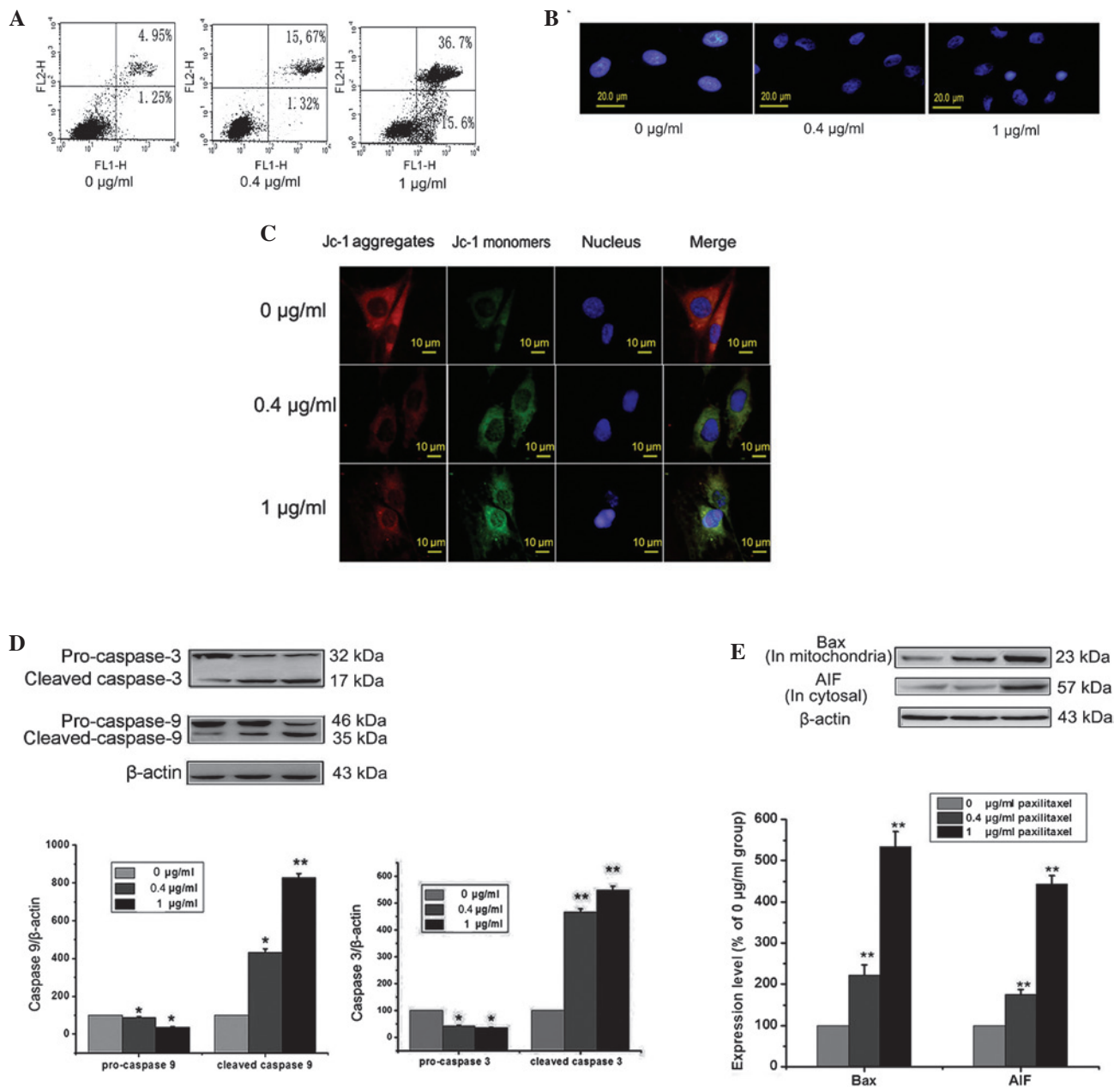


Figure 1. Effects of paclitaxel on the apoptosis of MG-63 cells. (A) Cells were treated with 0, 0.4 or 1 µg/ml paclitaxel for 24 h and apoptosis was determined by flow cytometry followed by Annexin V-propidium iodide double staining. (B) Cells were treated as above, and the nuclei were stained with Hoechst 33342 and observed under a fluorescence microscope (scale bar, 20 µm). (C) Cells were treated as above and subjected to JC-1 staining for assessment of the mitochondrial membrane potential. JC-1 monomers (green fluorescence) indicate mitochondrial membrane potential loss, while JC-1 aggregates (red fluorescence) indicate a retained mitochondrial membrane potential (scale bar, 10 µm). (D) Cells were treated as described above, and levels of caspase 3, caspase 9 were detected by western blot analysis. β-Actin was used as loading control. (E) Cells were treated as described above, and the expression of Bax (in the mitochondria) and AIF (in the cytosol) were detected by western blot analysis. β-Actin was used as loading control. Values are expressed as the mean ± standard error (n=10). (\*\*P<0.01 vs. control; \*P<0.05 vs. control). JC-1, tetraethylbenzimidazolylcarbocyanine iodide; Bax, B-cell lymphoma 2-associated X protein; AIF, apoptosis-inducing factor.

by placing the protein molecular weight marker (Invitrogen Life Technologies) in different but consistent positions. The PVDF membrane was then soaked in a blocking solution [5% nonfat milk in TBST buffer (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.1% Tween 20; Sigma-Aldrich)] for 2 h at room temperature. The soaked PVDF membrane was then incubated in TBST containing primary antibodies overnight at 4°C. The following primary antibodies were used: Anti-Bax, anti-AIF, and anti-β-actin (all Santa Cruz Biotechnology); Anti-caspase 3, anti-caspase 9, anti-LC3, anti-Becn1 and anti-HIF-1α (1:400 dilution; all Cell Signaling Technology). Following incubation,

the blot was washed with TBST buffer three times for 5 min each and incubated at room temperature for 2 h in TBST containing horseradish peroxidase (HRP)-conjugated goat anti-mouse and goat anti-rabbit IgG (Santa Cruz Biotechnology, Inc.). The membrane was washed with TBST buffer three times for 10 min each. The membranes were incubated in enhanced chemiluminescence reagent (Pierce Biotechnology, Thermo Fisher Scientific, Waltham, MA, USA) for HRP (30 sec) and exposure to autoradiography film for visualization of the bands using a LAS-1000 system (Fujifilm, Tokyo, Japan). The relative amounts of various proteins were analyzed. The results were



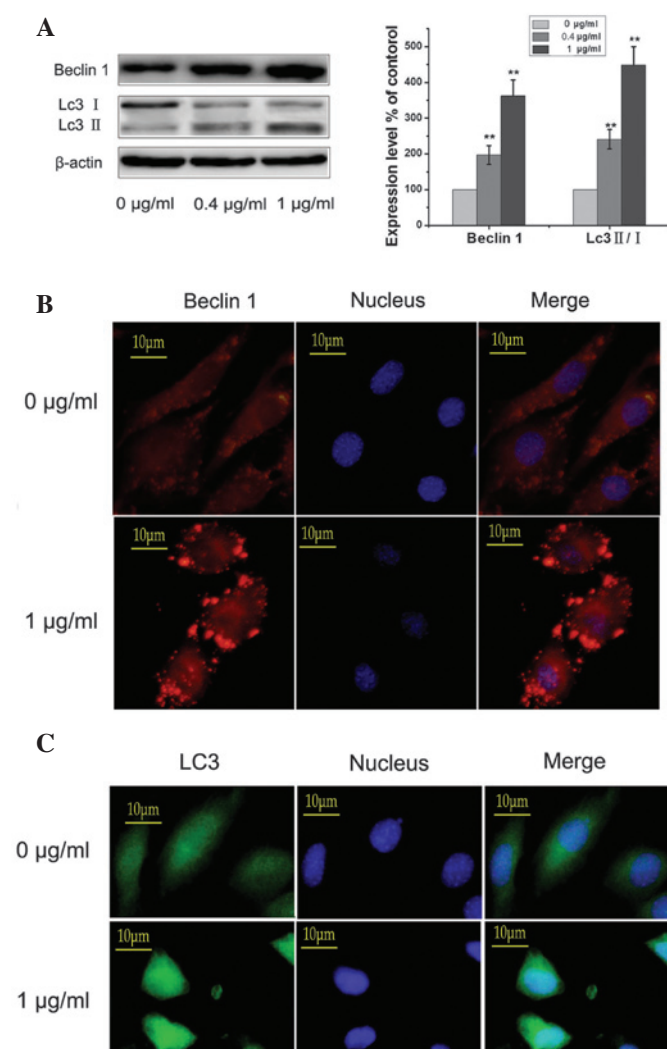


Figure 2. Effects of paclitaxel on the autophagy of MG-63 cells. (A) Cells were treated with 0, 0.4 or 1  $\mu$ g/ml paclitaxel for 24 h, and the expression of Beclin1 and LC3II/I were detected by western blot analysis.  $\beta$ -Actin was used as a loading control. Values are expressed as the mean  $\pm$  standard error (n=10). (\*\*P<0.01 vs. control). (B) MG-63 cells were treated with 0 or 1  $\mu$ g/ml paclitaxel for 24 h, Beclin1 protein was visualized by immunofluorescent staining and fluorescence microscopy. (C) MG-63 cells were treated with 0 or 1  $\mu$ g/ml paclitaxel for 24 h, and LC3 protein was visualized by immunofluorescent staining and fluorescence microscopy. LC3, microtubule-associated protein light chain 3.

quantified by Quantity One version 5.2.1 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Statistical analysis.** Values are expressed as the mean  $\pm$  standard error of the mean (n=10). Statistical analysis was performed using SPSS 18 (International Business Machines, Armonk, NY, USA). Data were analyzed using the one-way analysis of variance first. Individual comparisons were made using Tukey's multiple comparisons test. P<0.05 was considered to indicate a statistically significant difference between values.

## Results

**Paclitaxel induces apoptosis of MG-63 cells via a mitochondria-mediated pathway.** First, the effects of paclitaxel

on human osteosarcoma cancer cells were tested and the mechanisms by which paclitaxel induces MG-63 apoptosis at different concentrations were investigated. Cells were treated with 0, 0.4 and 1  $\mu$ g/ml paclitaxel for 24 h and the apoptotic rate was determined by Annexin V/PI double staining followed by flow cytometric analysis. The results showed a substantial increase in the apoptotic population among cells treated with paclitaxel (0.4 or 1  $\mu$ g/ml) at 24 h (Fig. 1A). Fluorescence microscopy (Hoechst 33342 staining) demonstrated that the paclitaxel-treated cells showed obvious nuclear damage in the form of chromatin condensation, which distinguished them from the cells in the control group (paclitaxel 0  $\mu$ g/ml) (Fig. 1B). Following treatment with paclitaxel, the  $\Delta\psi$ m in the cells was examined. Compared with control group, MG-63 cells in paclitaxel groups exhibited green JC-1 fluorescence, indicating a reduction of the  $\Delta\psi$ m (Fig. 1C). As shown in Fig. 1D and E, the expression of Bax in the mitochondria was significantly increased in the paclitaxel-treated groups compared to that in the control group (P<0.01), suggesting that the translocation of Bax into the mitochondria was involved in the induction of cell death by paclitaxel. Mitochondria-mediated apoptosis comprises caspase-dependent and caspase-independent processes, where AIF is involved in the caspase-independent response. As shown in Fig. 1E, the expression of AIF in the cytosol was significantly increased in the paclitaxel-treated groups compared to that in the control group (P<0.01). This result indicated that the release of AIF from the mitochondria to the cytosol was involved in cell death. Similarly, caspase-3 and caspase-9 were examined by western blot analysis. Levels of cleaved caspase-3 and caspase-9 in the paclitaxel-treated groups were upregulated compared with those in the control group. These results indicated that paclitaxel induced apoptosis in MG-63 cells was via a mitochondrial pathway involving the caspase-dependent pathway (caspase-3 and -9) as well as the caspase-independent pathway (AIF).

**Paclitaxel induces autophagy of MG-63 cells.** Cells were treated with 0, 0.4 and 1  $\mu$ g/ml paclitaxel for 24 h and the occurrence of autophagy was determined. As shown in Fig. 2A, the expression levels of Beclin1 and LC3 II/I were significantly increased in the paclitaxel-treated groups compared with those in the control group (paclitaxel 0  $\mu$ g/ml; P<0.01), suggesting that paclitaxel, in addition to triggering apoptosis, induced autophagy in MG-63 cells. Immunofluorescence microscopy revealed that in the control group, low levels of autophagy were sustained, while paclitaxel markedly enhanced the levels of LC3 and Beclin1 (Fig. 2B and C). These findings indicated that paclitaxel did not block autophagic flux, but induced the autophagic activity.

**Paclitaxel induces autophagy of MG-63 cells through the HIF-1 $\alpha$  pathway.** It is well known that HIF and autophagy are closely linked (13,14). Therefore, the present study investigated whether paclitaxel treatment of MG-63 cells had any effect on the expression of HIF-1 $\alpha$ . MG-63 cells were treated with 0.4 or 1  $\mu$ g/ml paclitaxel for 24 h, and the expression levels of HIF-1 $\alpha$  were assessed by western blot analysis. As shown in Fig. 3A, paclitaxel enhanced the levels of HIF-1 $\alpha$  compared to those in the control group. These

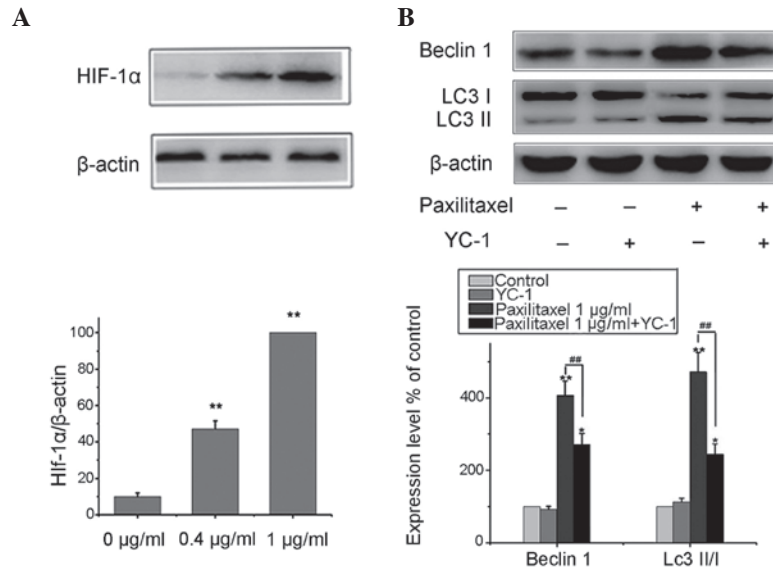


Figure 3. HIF-1α induced by paxlitaxel contributes to the autophagy of MG-63 cells. (A) MG-63 cells were treated with 0, 0.4 and 1 μg/ml paxlitaxel for 24 h, and the HIF-1α protein was detected by western blot analysis. β-actin was used as a loading control. Values are expressed as the mean ± standard error (n=10). (\*P<0.01 vs. control). (B) MG-63 cells were exposed to 1 μg/ml paxlitaxel with or without HIF-1α inhibitor YC-1 (50 μmol/l) for 24 h, and the expression of Beclin1 and LC3II/I were detected by western blot analysis. β-actin was used as loading control. Values are expressed as the mean ± standard error (n=10). (\*P<0.05 vs. control; \*\*P<0.01 vs. control; ##P<0.01 vs. 1 μg/ml paxlitaxel group). HIF, hypoxia-inducible factor; LC3, microtubule-associated protein light chain 3; YC-1, 3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole.

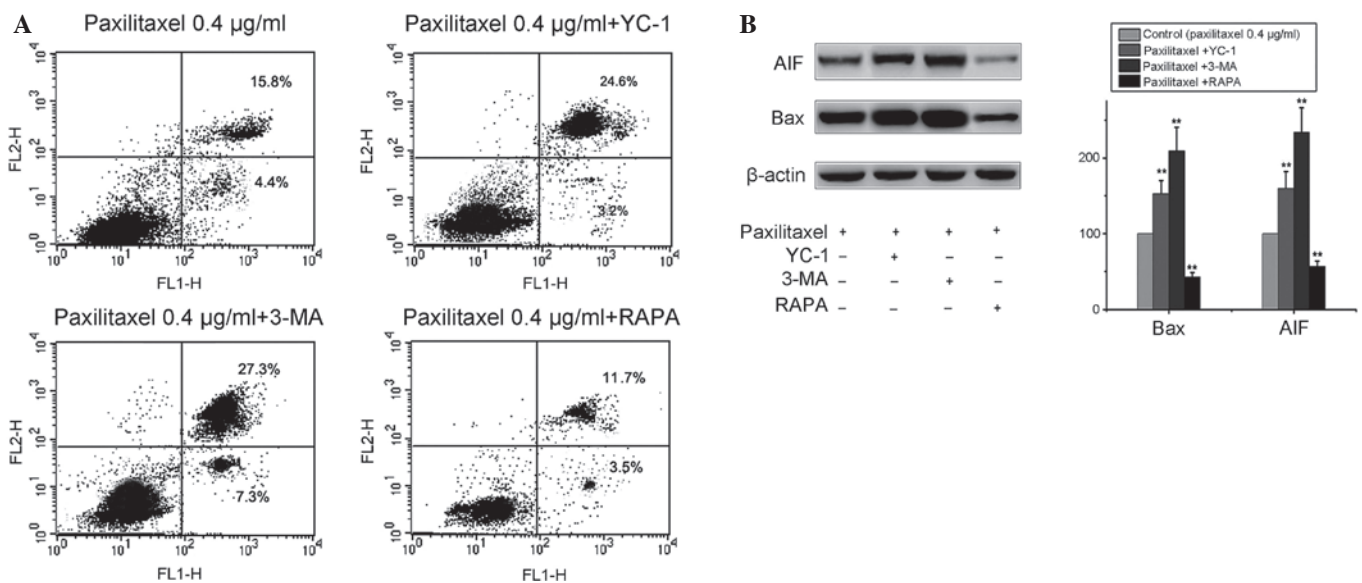


Figure 4. Autophagy is a protective mechanism in MG-63 cells. (A) Cells were treated with 0.4 μg/ml paxlitaxel with or without the autophagy inhibitors YC-1 (50 μmol/l) and 3-MA (5 mM) or the autophagy inducer RAPA (2 μM) for 24 h, and apoptosis was determined by Annexin V-propidium iodide double staining and flow cytometry. (B) Cells were treated as above, and the expression of Bax (in the mitochondria) and AIF (in the cytosol) were detected by western blot analysis. β-actin was used as a loading control. Values are expressed as the mean ± standard error (n=10). (\*P<0.01 vs. control). YC-1, 3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole; Bax, B-cell lymphoma 2-associated X protein; AIF, apoptosis-inducing factor; 3-MA, 3-methyladenine; RAPA, rapamycin.

results indicated that paxlitaxel treatment not only resulted in apoptosis, but also in the induction of autophagy and the expression of HIF-1α. Following treatment with 0.4 and 1 μg/ml paxlitaxel, autophagy-associated protein as well as and HIF-1α protein levels were increased in a dose-dependent manner (Figs. 2A and 3A). However, it appeared unlikely that paxlitaxel induced autophagy by upregulating the expression of HIF-1α alone. Therefore, MG-63 cells were exposed to 1 μg/ml paxlitaxel in the presence of the HIF-1α inhibitor

YC-1 [50 μmol/l in accordance with reference (17)] for 24 h and the expression of Beclin1 and LC3II/I was detected by western blot analysis. The results indicated that paxlitaxel-mediated upregulation of autophagy-associated protein expression was attenuated, but not fully abolished by HIF-1α inhibitor YC-1 (Fig. 3B). Therefore, these results indicated that HIF-1α generation by paxlitaxel has an important role in autophagy induction; however, there are likely to be additional signaling proteins via which autophagy is induced by paxlitaxel.

*Autophagy induced by paxilitaxel protects MG-63 cells from apoptosis.* To investigate the effect of autophagy on the apoptosis of MG-63 cells, 3-MA, a potent pharmacological inhibitor of autophagy, was used to suppress the autophagy induced by paxilitaxel. The results demonstrated that pre-treatment with 3-MA [5 mM according to reference (18)] was able to block autophagy in MG-63 cells without significant cytotoxicity. 3-MA itself scarcely induced cell apoptosis and cell death, but significantly increased apoptosis at 24 h after paxilitaxel exposure (paxilitaxel group, 20.2%; paxilitaxel plus 3-MA, 34.6%). In addition, in the group of combined treatment of 0.4  $\mu$ g/ml paxilitaxel and 50  $\mu$ mol/l YC-1 for 24 h, the apoptotic rate was higher than that in the group treated with paxilitaxel only (paxilitaxel group, 20.2%; paxilitaxel plus YC-1, 27.8%) (Fig. 4A). These results suggested that suppression of autophagy by 3-MA or YC-1 increased paxilitaxel-induced injury in MG-63 cells. By contrast, paxilitaxel-induced apoptosis decreased following co-treatment with autophagy inducer RAPA [2  $\mu$ M according to reference (19)] compared to that of cells treated with paxilitaxel alone (Fig. 4A). Treatment of MG-63 cells with RAPA or 3-MA alone did not affect cell viability (data not shown). The results were further confirmed by western blot analysis. The upregulation of the expression of apoptotic proteins AIF and Bax was more marked when cells were co-treated with 3-MA, while it was attenuated by co-treatment with RAPA (Fig. 4B). These results indicated that autophagy induced by paxilitaxel had a protective effect on MG-63 cells, and blockage of autophagy enhanced the anti-tumor effect of paxilitaxel.

## Discussion

Paxilitaxel is an active component of the herbal medicine *Curcuma wenyujin* with reported anti-tumor activity and has been approved for the treatment of malignant effusion and certain types of solid tumor in China (20,21). However, to the best of our knowledge, the effects of paxilitaxel on osteosarcoma have not been documented. In the present study, paxilitaxel was shown to inhibit the proliferation and induce apoptosis of human osteosarcoma cells. Mitochondria has an important role in the regulation of cell death. Mitochondrial dysfunction is considered an early event in apoptosis and this process accompanied with a conspicuous reduction of the mitochondrial membrane potential (22,23). The present study found that paxilitaxel-treated cells exhibited green JC-1 fluorescence, indicating a reduction of the  $\Delta\psi_m$ . Paxilitaxel treatment led to a marked upregulation of cleaved-caspase 3, cleaved-caspase 9, Bax (in the mitochondria) and AIF (in the cytosol), while significantly downregulating the levels of pro-caspase 3 and pro-caspase 9. These results suggested that paxilitaxel is capable of inducing mitochondria-mediated apoptosis involving the caspase-dependent (via caspase-3 and -9) as well as the caspase-independent pathway (via AIF).

Autophagy, an evolutionarily conserved lysosome-dependent cellular catabolic degradation process, is characterized by the formation of autophagosomes (24). Autophagy has a housekeeping role in clearing damaged organelles, including mitochondria and peroxisomes, as well as eliminating intracellular pathogens. Thus, autophagy is generally regarded as a survival mechanism (25,26). Autophagy and apoptosis are

closely associated. Apoptosis is often accompanied by the occurrence of autophagy (27,28). It was found that paxilitaxel not only induced apoptosis but also induced autophagy in MG-63 cells. The autophagy proteins Beclin1 and LC3 were upregulated by paxilitaxel. Moreover, in accordance with the results of previous studies (29,30), the present study reported that paxilitaxel activated autophagy by modulation of HIF-1 $\alpha$  signaling. Paxilitaxel induced autophagy as well as HIF-1 $\alpha$  expression in MG-63 cells. This autophagic induction by paxilitaxel was partly mediated through the activation of HIF-1 $\alpha$ , as inhibition of HIF-1 $\alpha$  expression by YC-1 reduced autophagy.

Autophagy has an important role in mediating the effects of drugs in cells. Autophagy and HIF-1 $\alpha$  are often associated with resistance to chemotherapy (31-33). The results of the present study suggested that autophagy is a pro-survival mechanism of MG-63 cells following paxilitaxel treatment and facilitates the development of acquired paxilitaxel resistance. Paxilitaxel-induced apoptosis of MG-63 cells was markedly decreased by autophagy. In addition, co-treatment with clinically applicable inhibitors of autophagy or HIF-1 $\alpha$  may be one of the important strategies for human osteosarcoma therapy. Suppression of autophagy by 3-MA or YC-1 was able to increase paxilitaxel-induced apoptosis in MG-63 cells. By contrast, paxilitaxel-induced apoptosis was decreased following co-treatment with the autophagy inducer RAPA. However, the molecular mechanisms of HIF-1 $\alpha$ -mediated activation of autophagy and paxilitaxel resistance, as well as the association between autophagy, apoptosis and other factors of paxilitaxel-resistance require further study. At present, the precise underlying mechanism of autophagy mediated by anti-apoptotic proteins remains elusive and is under investigation in our laboratory.

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