A Combination of Pterostilbene With Autophagy Inhibitors Exerts Efficient Apoptotic Characteristics in Both Chemosensitive and Chemoresistant Lung Cancer Cells

Ming-Ju Hsieh,*†¶ Chiao-Wen Lin,‡§ Shun-Fa Yang,¶¶ Gwo-Tarng Sheu,¶¶¶ Ya-Yen Yu,¶¶¶¶ Mu-Kuan Chen,# and Hui-Ling Chiou**††††.

*Cancer Research Center, Changhua Christian Hospital, Changhua 500, Taiwan, Republic of China; †School of Optometry and ‡Institute of Oral Sciences, Chang Shan Medical University, Taichung 40201, Taiwan, Republic of China; §Department of Dentistry, Chang Shan Medical University Hospital, Taichung 40201, Taiwan, Republic of China; ¶Institute of Medicine, Chang Shan Medical University, Taichung 40201, Taiwan, Republic of China; ¶¶Department of Medical Research, Chang Shan Medical University Hospital, Taichung 40201, Taiwan, Republic of China; ¶¶¶Institute of Medical and Molecular Toxicology, Chang Shan Medical University, Taichung City 40201, Taiwan, Republic of China; ¶¶¶¶Department of Laboratory, Department of Health, Chang-Hua Hospital, Changhua 500, Taiwan, Republic of China; **School of Medical Laboratory and Biotechnology, Chang Shan Medical University, Taichung 40201, Taiwan, Republic of China; and ††††Department of Clinical Laboratory, Chang Shan Medical University Hospital, Taichung 40201, Taiwan, Republic of China

†To whom correspondence should be addressed at School of Medical Laboratory and Biotechnology, Chang Shan Medical University, 110, Section 1, Chien-Kuo N. Road, Taichung 40201, Taiwan, ROC. Fax: +886-4-23248171. E-mail: hlchiou@csmu.edu.tw.

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The emergence of multidrug resistance (MDR), meaning that cancer cells develop simultaneous resistance to different drugs, has limited the clinical efficacy and application of chemotherapy. Pterostilbene, a naturally occurring phytoalexin exerts a variety of pharmacologic activities, including cancer prevention, cytotoxicity, and antioxidant activity. In this study, results proved the capability of pterostilbene to effectively inhibit the cell viability of docetaxel-induced MDR human lung cancer cell lines through cell cycle arrest and apoptosis. Meanwhile, the observation of LC3-II production and formation of acidic vesicular organelles revealed an induction of autophagy at an early stage by pterostilbene, which was triggered by an inhibition of the AKT and JNK pathways and activation of ERK1/2. Furthermore, pretreatment with the autophagy inhibitors 3-methyladenine and bafilomycin A1 or with beclin-1 small interfering RNA was able to enhance pterostilbene-triggered apoptosis. In conclusion, this study demonstrated that pterostilbene causes autophagy and apoptosis in lung cancer cells. Furthermore, pterostilbene in combination with autophagy inhibitors may strengthen the efficiency of chemotherapeutic strategies in both chemosensitive and chemoresistant lung cancer cells, which may be of immense value for the clinical management of lung cancer patients with MDR.

Key Words: pterostilbene; multidrug resistance; apoptosis; autophagy.

Lung cancer is the second most frequently diagnosed cancer in the United States (Jemal et al., 2009). With chemotherapy being the suggested treatment for advanced-stage cancers, the emergence of multidrug resistance (MDR), a trait referring to the ability of cancer cells to become additionally resistant to different drugs, limits the efficacy of chemotherapy (Baird and Kaye, 2003). Therefore, resensitization to chemotherapeutic agents or induction of apoptosis of MDR cancer cells may allow successful chemotherapy (Fojo and Bates, 2003).

Pterostilbene (trans-3,5-dimethoxy-4-hydroxystilbene) is a naturally occurring phytoalexin identified in the genus Pterocarpus, leaves of Vitis vinifera, and some berries and grapes (Rimando et al., 2002). It has multiple pharmacologic activities, including antioxidant and cancer prevention activity (Rimando et al., 2002) and the capability to inhibit DNA synthesis (Stivala et al., 2001). Pterostilbene is also cytotoxic to various types of cancer cells, including breast cancer, melanoma, colon cancer, liver cancer, and gastric cancer (Ferrer et al., 2005; Remsberg et al., 2008; Rimando et al., 2002). Although antiproliferative and proapoptotic activities of pterostilbene have been demonstrated in vitro, the ability of pterostilbene to induce apoptosis in drug-resistant lymphoma cell lines (Tolomeo et al., 2005). However, the impact of pterostilbene on autophagy and the underlying mechanisms remain unclear.

Autophagy is a major intracellular degradation mechanism used for responding to stress conditions to either promote survival during starvation or lead to programmed cell death type II under specific conditions such as the inhibition of apoptosis (Cohen et al., 1992; Davies et al., 2003; Green, 2002). Defective autophagy contributes to the pathogenesis of a number of diseases, including myopathies, neurodegenerative diseases, and...
some forms of cancers (Finlan et al., 2005). Recently, the role of autophagy in cancer development and therapy has been extensively studied (Cohen et al., 1992; Davies et al., 2003; Fitzpatrick and Wheeler, 2003). Studies have indicated that certain agents kill cancer cells through nonapoptotic pathways and may overcome chemoresistance (Green, 2002). Another study has revealed that tumor cells with defects in apoptosis undergo autophagy, and autophagy inhibition causes tumor cells to die through alternative mechanisms (McGrogan et al., 2008). It has also been suggested that autophagy provides a beneficial way to prevent cancer development, inhibit tumor progression, and enhance the efficacy of cancer treatments (Wong and Rabie, 2011). Therefore, the aim of this study was to characterize the effects of pterostilbene on docetaxel (DOC)-induced MDR human lung cancer cell lines and the underlying molecular mechanism of autophagy and apoptosis in pterostilbene-induced cytotoxicity.

MATERIALS AND METHODS

Cell culture. The A549 human lung cancer cell line was purchased from the American Type Culture Collection (ATCC, Manassas, Virginia) and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids, 1mM glutamine, 1% penicillin/streptomycin, 1.5 g/l sodium bicarbonate, and 1mM sodium pyruvate (Sigma, St Louis, Missouri) at 37°C in a humidified atmosphere of 5% CO2. The process for selection and establishment of drug-resistant A549 cell lines was previously described (Chiu et al., 2010). Briefly, A549 cells in low density were seeded onto 10-cm culture dish and treated with 0.5nM DOC until the surviving cells grew to an obvious colony. The selected colony was amplified in the presence of 0.5nM DOC until confluence before the drug dose was increased in multiples of 2 for the next round of selection. The DOC-resistant subline maintained at 16nM DOC is denoted as A549/D16.

Chemicals. Pterostilbene of ≥ 98% purity was purchased from Enzo Life Sciences (St Suite, San Diego, California). Stock solution was made at 50, 75, and 100nM concentration in dimethyl sulfoxide (Sigma) and stored at −20°C. Other chemicals, including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Triton X-100, bafilomycin A1 (BafA1), JNK1/2 inhibitor SP600125, 4′,6-diamidino-2-phenylindole (DAPI), and 3-methyladenine (3-MA) were obtained from Sigma Chemical Co. The ERK1/2 inhibitor U0126, phosphatidylinositol 3-kinase (PI3-kinase) inhibitor LY294002, and the general caspase inhibitor Z-VAD-FMK were purchased from Millipore Corporation, Milford, Massachusetts.

Cell cytotoxicity. The effect of pterostilbene on cell growth was assayed by the MTT method, as previously described (Hsieh et al., 2012). Briefly, 2 × 104 cells/well were cultured in 6-well plates and treated with the indicated concentrations of pterostilbene (0, 50, 75, or 100μM). After 24, 48, or 72 h, MTT was added to each well and incubated for further 4 h. The viable cell number was directly proportional to the production of formazan, reflected by the color intensity measured at 595 nm, following the solubilization with isopropanol.

DAPI staining. Cells were grown on 6-well cell culture dishes overnight. After being subjected to the indicated treatment, cells were fixed with 4% paraformaldehyde for 20 min. PBS washing was conducted between each reaction to remove any residual solvent. Cells were subjected to DAPI staining for 15 min and then observed under fluorescence microscopy equipped with UV filters.

Cell cycle analysis. Cells were first cultured for 18 h in serum-free medium for starvation and then exposed to pterostilbene. After washing, cells were fixed with 70% ethanol, incubated for 30 min in the dark at room temperature with propidium iodide (PI) buffer (4 μg/ml PI, 1% Triton X-100, and 0.5 mg/ml RNase A in PBS), and then filtered through a 40-μm nylon filter (Falcon). The cell cycle distribution was analyzed for 5000 collected cells using a FACsVantage flow cytometer that uses the Cellquest acquisition and analysis program (Becton–Dickinson FACS Calibur, San Jose, California).

Annexin V/PI double staining. To detect apoptosis in HuH7 cells after exposure to pterostilbene, an FITC Annexin V Apoptosis Detection Kit I was used to quantify cell numbers in different stages of cell death (Hsieh et al., 2012). Briefly, 1 × 106 cells were resuspended in 100 μl 1× binding buffer (0.01M Heps/NaOH (pH 7.4), 0.14M NaCl, and 2.5mM CaCl2). After addition of 5 μl FITC Annexin V and 5 μl PI, the cell suspension was gently mixed and then incubated for 15 min at room temperature in the dark. Subsequently, 400 μl of 1× binding buffer was added to each tube, followed by flow cytometry analysis within 1 h.

Cell transfection. Cells were grown on 6-well cell culture dishes overnight and then transfected with 4 μg of pEGFPC1-LC3 (Hsieh et al., 2012) for 6 h, followed by the indicated treatment. Afterward, cells were fixed with 2% paraformaldehyde for 20 min and then incubated with 0.5% Triton X-100 for 10 min. PBS washing was conducted between each reaction to remove any residual solvent. The formation of GFP-LC3 dots was detected under a fluorescence microscope after drug treatment.

Western blot analysis. Cell lysates were separated on 10% or 15% polyacrylamide gels and transferred onto a polyvinylidene difluoride membrane. The blot was subsequently incubated with 3% or 5% nonfat milk in PBS for 1 h, probed with antibody against a specific protein for 37°C at 2 h or overnight at 4°C, and then probed with an appropriate peroxidase-conjugated secondary antibody for 1 h at room temperature. Washing between incubations with wash buffer was conducted, and after the final washing, the signals were developed using the ECL detection system, and the relative photographic density was quantitated by a gel documentation and analysis system (Alpha Imager 2000, Alpha Innotech Corporation).

Detection and quantification of acidic vesicular organelle formation. The occurrence of acidic vesicular organelle (AVOs) was assessed by a previously described method (Hsieh et al., 2012). Briefly, pterostilbene-treated cells were washed with PBS, followed by staining with 1 μg/ml acridine orange (diluted in PBS containing 5% FBS; Sigma) for 30 min at 37°C. Afterward, cells were washed with PBS and then observed at 488 nm under a fluorescence microscope equipped with filters. For quantification of AVOs, acridine orange-stained cells were harvested, washed twice with PBS, resuspended in PBS containing 5% FBS, and then analyzed by flow cytometry.

RNA interference. Small interfering RNA (siRNA) targeting human beclin-1 (sc-29797) was purchased from Santa Cruz Biotechnology (Santa Cruz, California). Cells were transfected by siRNA Transfection Reagent sc-29528 according to the manufacturer’s recommendations. The Silencer negative control siRNA (sc-37007), a nonsense siRNA duplex, was used as a control.

Statistical analysis. Statistical significances of differences were analyzed by one-way ANOVA test throughout this study. A p value <.05 was considered to be statistically significant. Values represent the means ± SD, and the experiments were repeated 3 times.

RESULTS

Pterostilbene Inhibits Cellular Growth in DOC-Induced MDR Lung Cancer Cell Lines

The chemical structure of pterostilbene was shown in Figure 1A. To confirm the presence of MDR, parental A549...
A549 and A549/D16 cells were exposed to 16nM DOC or 16nM VCR (vincristine) for 24, 48, and 72 h. As shown in Figure 1B, A549/D16 cells showed resistance to the cytotoxicity of 16nM DOC and 16nM VCR, whereas the growth of parental A549 cells was effectively inhibited. To assess the effects of pterostilbene on cell viability, A549 and A549/D16 cells were treated with pterostilbene (0, 50, 75, and 100 μM) for 24, 48, and 72 h, as analyzed by the MTT assay. Cell viability rate was denoted as a percentage of untreated control (PT 0 μM) at the concurrent time point. Results are shown as mean ± SD from 3 determinations per condition repeated 3 times. *p < .05; **p < .01; ***p < .001, compared with the control (0 μM). Abbreviation: PI, propidium iodide.
with pterostilbene and then analyzed with the MTT assay. As shown in Figures 1C and 1D, pterostilbene significantly decreased the growth of A549 and A549/D16 cells in a concentration- and time-dependent manner. Results from Annexin V/PI double staining confirmed that pterostilbene induced cell death through apoptosis (Figs. 1E and F).

Pterostilbene Induces Cell Cycle Arrest and Apoptosis in DOC-Induced MDR Human Lung Cancer Cell Lines

The effects of pterostilbene on the cell cycle distribution of A549 and A549/D16 cells were examined. Results indicated that a 24-h treatment with pterostilbene induced a dose-dependent increase in the sub-G1 phase and a decrease in the G2/M phases of these cells compared with controls (Figs. 2A and B). Meanwhile, there was a time-dependent increase in the number of cells in the sub-G1 phase and a decrease of cells in the G2/M phase (Fig. 2C). The results indicated that pterostilbene delayed cell cycle progression in both A549 and A549/D16 cells. Therefore, pterostilbene-induced cell cycle arrest likely contributed to the overall growth suppression in A549 and A549/D16 cells.

To determine whether pterostilbene-induced cell death is related to apoptosis, DAPI staining was performed to analyze the changes in nuclear morphology. Results revealed the condensed and fragmented nuclei in both cell types after 48 h of pterostilbene treatment (Fig. 2D). Annexin V/PI double staining was also determined by flow cytometry, and results showed an increased percentage of cells displaying phosphatidylserine externalization in pterostilbene-treated A549 and A549/D16 cells, compared with that of untreated cells (Fig. 2E).

Pterostilbene Induces Autophagy in DOC-Induced MDR Human Lung Cancer Cell Lines

Because various numbers of vacuoles with autophagy characteristics were observed in the cytoplasm as early as at 24 h after pterostilbene treatment (Fig. 3A), whether autophagy can be attributed to the cellular response to pterostilbene was examined. As shown in Figure 3B, after transfection with pEGFP-C1-LC3 and treatment with pterostilbene for 24 and 48 h, cytoplasmic LC3 formation was observed in both A549 and A549/D16 cells treated with pterostilbene (100µM). A significant change of LC3 puncta formation was found as soon as at 24 h posttreatment, whereas increased LC3-II protein expression was also observed at 48 h in a dose-dependent manner. To further confirm the autophagy induction by pterostilbene, expression levels of LC3-II, p62, and beclin-1 were analyzed. Compared with that of control, expression levels of LC3-II and beclin-1 were increased in both A549 and A549/D16 cells after 48-hour pterostilbene treatment, whereas that of p62 was decreased (Figs. 3C and D). In the presence of pterostilbene, the formation of AVOs was induced in both A549 and A549/D16 cells in a concentration- and time-dependent manner (Figs. 3E and F). The AVO formation reached a peak at 48 h and then gradually decreased. Taken together, these results show that autophagy was induced by pterostilbene at an earlier stage and that apoptosis occurs at a later stage.

Autophagy Inhibitors Increased Pterostilbene-Induced Apoptosis in DOC-Induced MDR Human Lung Cancer Cell Lines

To clarify the interaction between pterostilbene-induced apoptosis and autophagy, 2 autophagy inhibitors were used in the following experiments. 3-MA, an autophagy inhibitor that can block autophagosome formation and prevents early stage autophagy (Zhang et al., 2009), and BafA1, an inhibitor of vacuolar H+ -ATPase that prevents late-stage autophagy by inhibiting fusion of autophagosomes and lysosomes (Shacka et al., 2006). Pterostilbene-induced autophagy activity was also confirmed by increased accumulation of LC3-II resulting from cotreatment with BafA1 (Fig. 4A). A549 and A549/D16 cells were pretreated with 3-MA or BafA1 for 1 h before pterostilbene treatment. Results, as shown in Figure 4B, indicated that the formation of AVOs was significantly reduced in the presence of 3-MA or BafA1. Meanwhile, the percentage of Annexin V-positive cells was also higher in both cells pretreated with 3-MA. BafA1-pretreated A549 and A549/D16 cells were more susceptible to pterostilbene, in addition to expressing an increased percentage of Annexin V positivity (Fig. 4C). Identical data were obtained by inhibiting autophagy with beclin-1 siRNA. Western blot analysis demonstrated that transfection with beclin-1 siRNA reduced beclin-1 expression by approximately 63% in both cells (Fig. 4D). After treatment with pterostilbene for 48 h, increased apoptosis was observed in both A549 and A549/D16 cells undergoing pretransfection with beclin-1 siRNA, compared with the same in control pterostilbene-treated groups (Fig. 4E).

Furthermore, cotreatment with pterostilbene and Z-VAD-FMK, a broad-spectrum caspase inhibitor, was conducted to show that pterostilbene-induced increase in the percentage of Annexin V positivity were abolished by Z-VAD-FMK; however, pterostilbene-induced AVO expression remained unaltered in both cells (Figs. 4B and C). Clearly, inhibition of autophagy did not hinder pterostilbene-induced cell death but further enhanced the cell toxicity of pterostilbene. Therefore, these results indicate that autophagy had a cytoprotective effect in pterostilbene-induced A549 and A549/D16 cell death.

Pterostilbene Induces Autophagy Through Inhibition of the PI3K/AKT and JNK1/2 Pathways and Upregulates ERK1/2 Activation in DOC-Induced MDR Human Lung Cancer Cell Lines

The class I phosphatidylinositol 3-phosphate kinase (PI3K)/Akt/mTOR/p70 ribosomal protein S6 kinase (p70S6K) signaling pathway and the Ras/Raf-1/mitogen-activated protein kinase 1/2 (MEK1/2)/extracellular signal-regulated kinase 1/2 (ERK1/2) pathway are involved in the regulation of autophagy (Thorburn, 2008). To investigate the effect of pterostilbene on both pathways in DOC-induced MDR cells, the expression
FIG. 2. Effect of pterostilbene on cell cycle and apoptosis in A549 and A549/D16 cells. A549 and A549/D16 cells were incubated for 18 h in the absence of serum and then treated with indicated concentrations of pterostilbene (0, 50, 75, and 100 μM) for 24 h, after which the cells were stained with PI and analyzed for DNA content by flow cytometry in a (A and B) concentration and (C) time-dependent manner. Results are shown as mean ± SD from 3 determinations per condition repeated 3 times. *p < .05; **p < .01; ***p < .001, compared with the control (0 μM) or A549. #p < .05; ##p < .01; ###p < .001, compared with the A549/D16. Cells were treated with different concentrations of pterostilbene (0, 50, 75, and 100 μM) for 48 h and then subjected to DAPI staining for DNA (blue areas) by fluorescence microscopy (D). A549 or A549/D16 cells treated with pterostilbene (0, 50, 75, and 100 μM) were harvested and then subjected to Annexin-V and PI double-stained flow cytometry in a concentration-dependent manner (E). Results are shown as mean ± SD from 3 determinations per condition repeated 3 times. *p < .05; **p < .01, compared with the A549 control (0 μM). #p < .05; ##p < .01, compared with the A549/D16 control (0 μM). Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; PI, propidium iodide.
FIG. 3. The induction of autophagy in pterostilbene-treated A549 and A549/D16 cells. A549 and A549/D16 cells were treated with 100μM pterostilbene for 24h and microscopic observation revealed the formation of vacuoles in the cytoplasm of treated cells (A). After successful transfection with pEGFP-C1-LC3, A549 and A549/D16 cells were treated with pterostilbene (100μM) for 24 and 48h, followed by an observation for LC-3 (green fluorescence) under fluorescence microscopy (B). Cells were treated with the indicated concentrations of pterostilbene (0–100μM) for 24 and 48h and then subjected to Western blotting to study the expression levels of LC3-I, LC3-II, p62, and beclin-1, with β-actin acting as the internal control (C and D), in addition to staining by acridine orange to detect AVOs. The percentage of cells showing the formation of AVOs was detected by flow cytometry in (E) concentration- and (F) time-dependent manner. Results are shown as mean ± SD. *p < .05; ***p < .001, compared with A549 control (0μM). #p < .05; ##p < .01; ###p < .001, compared with the A549/D16 control (0μM).

Abbreviation: AVOs, acidic vesicular organelle.
levels of the phosphorylated forms of PI3K, Akt, ERK1/2, and JNK1/2 were examined by Western blotting. Results showed that pterostilbene treatment led to an increased activation of ERK1/2 in a dose-dependent manner. Conversely, the levels of phosphorylated PI3K, Akt, and JNK in A549 and A549/D16 cells were decreased by pterostilbene treatment (Figs. 5A and B). To further discuss whether Akt or JNK1/2 inhibition contributes to the suppression of autophagy, the inhibitor of PI3K, LY294002, and a JNK inhibitor (SP600125) were added into this analysis system. As shown in Figures 5C and

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**Figure 4.** Effects of autophagy inhibitors on pterostilbene-induced cell death. A549 and A549/D16 cells were treated with pterostilbene (100μM) for 24h in the presence or absence of autophagy inhibitor BafA1 (10nM) and then subjected to Western blotting for LC3-II and β-actin (A). A549 and A549/D16 cells were treated with pterostilbene (100μM) in the presence or absence of autophagy inhibitor 3-MA (5mM), BafA1 (10nM), or Z-VAD-FMK (20μM) and stained with acridine orange to detect AVOs. The percentage of cells with the formation of AVOs was detected by flow cytometry (B). Subsequently, these treated cells were double stained with Annexin-V and PI and then analyzed by flow cytometry (C). Results are shown as mean ± SD. ***p < .001, compared with the pterostilbene (100μM) group. Mock: untreated cells. Furthermore, after cells were transfected with beclin-1 siRNA, the expression of LC3-II formation was investigated by Western blotting, with β-actin being an internal control (D). Beclin-1 siRNA-transfected cells were treated with pterostilbene (100μM) for 48h and then double stained with Annexin-V and PI and analyzed by flow cytometry (E). Results are shown as mean ± SD. ***p < .001, compared with the pterostilbene (100μM) group. Mock: untreated cells. Abbreviations: AVOs, acidic vesicular organelle; PI, propidium iodide; siRNA, small interfering RNA.
significant differences in the LC3-II/LC3-II ratio were observed between pterostilbene treatment in the presence or absence of LY294002 or SP600125. Again, the levels of LC3-I and LC3-II were investigated by Western blotting with β-actin being used as an internal control (C–E), in addition to detection of A VOs by flow cytometry (F and G). Results are shown as mean ± SD from 3 determinations per condition repeated 3 times. *p < .05; **p < .01, compared with the A549 control (0μM). #p < .05; ##p < .01, compared with the A549/D16 control (0μM). NS: no significance, p > .05. U0126-pretreated cells were also treated with pterostilbene (100μM) for 24h, followed by double staining with Annexin-V/PI and flow cytometry analysis (H). Abbreviation: PI, propidium iodide.

**FIG. 5.** The induction of autophagy through inhibition of the PI3K/AKT and JNK pathways and activation of the ERK1/2 by pterostilbene in A549 and A549/D16 cells. Cells were treated with different concentrations of pterostilbene (0–100μM) for 24h and then subjected to Western blotting. The levels of phosphorylation of PI3K, Akt, ERK1/2, and JNK1/2 were investigated by Western blotting, with β-actin being used as an internal control. The values indicate relative density of the band normalized to β-actin using a densitometer (A and B). Results are shown as mean ± SD. **p < .01; ***p < .001, compared with the control (0μM). Furthermore, cells were pretreated with LY294002 20μM, SP600125 20μM, or U0126 10μM for 1h, followed by treatment with pterostilbene (100μM) for 24h. The levels of LC3-I and LC3-II were investigated by Western blotting with β-actin being used as an internal control (C–E), in addition to detection of A VOs by flow cytometry (F and G). Results are shown as mean ± SD from 3 determinations per condition repeated 3 times. *p < .05; **p < .01, compared with the A549 control (0μM). #p < .05; ##p < .01, compared with the A549/D16 control (0μM). NS: no significance, p > .05. U0126-pretreated cells were also treated with pterostilbene (100μM) for 24h, followed by double staining with Annexin-V/PI and flow cytometry analysis (H). Abbreviation: PI, propidium iodide.

**FIG. 5.** The induction of autophagy through inhibition of the PI3K/AKT and JNK pathways and activation of the ERK1/2 by pterostilbene in A549 and A549/D16 cells. Cells were treated with different concentrations of pterostilbene (0–100μM) for 24h and then subjected to Western blotting. The levels of phosphorylation of PI3K, Akt, ERK1/2, and JNK1/2 were investigated by Western blotting, with β-actin being used as an internal control. The values indicate relative density of the band normalized to β-actin using a densitometer (A and B). Results are shown as mean ± SD. **p < .01; ***p < .001, compared with the control (0μM). Furthermore, cells were pretreated with LY294002 20μM, SP600125 20μM, or U0126 10μM for 1h, followed by treatment with pterostilbene (100μM) for 24h. The levels of LC3-I and LC3-II were investigated by Western blotting with β-actin being used as an internal control (C–E), in addition to detection of A VOs by flow cytometry (F and G). Results are shown as mean ± SD from 3 determinations per condition repeated 3 times. *p < .05; **p < .01, compared with the A549 control (0μM). #p < .05; ##p < .01, compared with the A549/D16 control (0μM). NS: no significance, p > .05. U0126-pretreated cells were also treated with pterostilbene (100μM) for 24h, followed by double staining with Annexin-V/PI and flow cytometry analysis (H). Abbreviation: PI, propidium iodide.

**DISCUSSION**

Extensive studies have proven that natural products may serve as ideal chemotherapeutic agents for arresting tumor promotion and progression through the control of cell proliferation, invasion, or apoptosis. Pterostilbene has been reported to be
able to inhibit herbicide-induced oxidative damage (Remsberg et al., 2008), induce apoptosis in sensitive and chemoresistant lymphoma cell lines through the caspase-independent pathway (Rimando et al., 2002), and hinder B16 melanoma growth and metastatic activity (Ferrer et al., 2005). In this study, results show that pterostilbene inhibits A549 and A549/D16 (DOC-induced MDR human lung cancer cell lines) cell growth by arresting the cell cycle (Figs. 1 and 2). Based on the significant increase in the sub-G1 phase and Annexin V positivity, we believe that pterostilbene induces apoptosis in A549 and A549/D16 cells, which is consistent with other reports showing that pterostilbene induces G0/G1 arrest in CEMC7H2 leukemia cells (Tolomeo et al., 2005) and inhibits growth by apoptosis and arrest of gastric cancer cells (Pan et al., 2007).

Autophagy is an important cellular response for various environmental stimuli, diseases, and even cancers (Høyer-Hansen and Jäättelä, 2008; Shintani and Klionsky, 2004; Yang et al., 2005). Many anticancer agents, including tamoxifen, rapamycin, and temozolomide, were reported to induce autophagy (Rosenfeldt and Ryan, 2009). Further investigation revealed that sulfaphenac causes autophagy as a defense mechanism against apoptosis in PC3 and LNCaP prostate cancer cells (Herman-Antosiewicz et al., 2006) and 7,7'-dimethoxyxagastisflavone (DMGF)-induced autophagic cell death in HepG2 cells (Longo et al., 2008). Because drug-resistant cancer cells with antiapoptotic properties may be vulnerable to death by autophagy, studies focusing on autophagy have been conducted in the cancer therapy field. For example, resveratrol, an analog of pterostilbene, induces autophagy in ovarian and drug-resistant breast cancer cells (Opipari et al., 2004; Scarlatti et al., 2008). In this study, pterostilbene resulted in apparent apoptosis at 48 hours (Fig. 2), whereas autophagy was observed as early as 24 h posttreatment together with a dose-dependent increase in LC3-II expression (Fig. 3). In addition, inhibition of autophagy by 3-MA or BafA1 may increase the sensitivity of cells to death signals. Previous studies have suggested that autophagy can be induced and that this is involved in cell death or cytoprotection in HCC cell lines (Chang et al., 2007; Longo et al., 2008). Abedin et al. (2007) found that inhibition of autophagy increases mitochondrial depolarization and apoptosis in camptothecin-treated MCF-7 cells. Our further investigation on the role of autophagy in pterostilbene-induced cell death indicated that the blockage of the class III PI3K by 3-MA, an autophagy inhibitor, inhibits the formation of autophagosomes and sensitizes cells to pterostilbene-induced apoptosis and cell death. Similarly, autophagosome accumulation can enhance cell death after BafA1 treatment alone and in combination of pterostilbene (Fig. 4C). The results suggested that the effect of pterostilbene on the autophagy inhibitors may be synergistic. The lack of a protective effect of these studied inhibitors indicated that autophagy is an important mechanism in pterostilbene-induced apoptosis in both cell lines.

This study clearly demonstrated that pterostilbene inhibits the AKT and JNK pathways and activates the ERK1/2 pathway to promote autophagy. Blocking PI3K/AKT or JNK with LY294002 or SP600125 in both cells enhances AVO induction and LC3-II production induced by pterostilbene. Ellington et al. (2006) showed that the triterpenoid B-group soy saponins induced autophagy by inhibiting AKT and activating ERK activity. These findings may highlight the common mechanisms, such as AKT inhibition and ERK1/2 activation, which regulate autophagy in cancer cells treated with anticancer agents, although our results indicated that inhibition of ERK1/2 activation by U0126 could hinder pterostilbene-induced AVO induction and LC3-II production. Nevertheless, the increased apoptosis in U0126-pretreated groups further confirmed that inhibition of autophagy sensitizes the cells to apoptosis (Fig. 5H). So far, P-glycoprotein (Pgp/ABCB1) overexpression in A549/D16 is the only observed difference between A549 and A549/D16 cell lines and this is believed to be responsible for the MDR phenotype. Therefore, the similar effects of pterostilbene in these 2 cell lines may indicate the irrelevance of P-glycoprotein to pterostilbene-induced mechanisms. Furthermore, the absence of DOC in the medium in all experiments, except that in Figure 1B, indicated that these results are unrelated to DOC.

Autophagy is suppressed by functional p53 and certain cytoplasmic p53 mutants (Baur and Sinclair, 2006). A previous study has suggested that cytoplasmic p53 suppresses autophagy and p53 inhibition induces autophagy (Cecconi and Levine, 2008). Furthermore, nuclear expression of p53 may stimulate autophagy by damage-regulated autophagy modulator (DRAM) upregulation and mammalian target of rapamycin (mTOR) inhibition (White, 1997). Therefore, pterostilbene-induced autophagy and apoptosis in A549 and A549/D16 cells may be partially attributed to functional p53, which could be verified by additional experiments exploring the role of p53 in pterostilbene-induced autophagy. Autophagic agonists have been described as anticancer drugs (Opipari et al., 2004; Scarlatti et al., 2008), with various compounds being shown to induce death in tumor cells having defective apoptotic machinery (Longo et al., 2008; Scarlatti et al., 2008). Previous studies have demonstrated that pterostilbene causes autophagy and hence this can serve as a new and promising agent for the treatment of chemoresistant bladder cancer cells (Chen et al., 2010). Chen et al. (2012) indicate that pterostilbene mediated the inhibition of epidermal growth factor receptor (EGFR), leading to apoptosis and autophagy. Thus, with the capability to induce both the apoptotic and autophagic pathways, pterostilbene may be an ideal agent targeting MDR for antitumor treatment.

In conclusion, this study is the first to demonstrate that pterostilbene suppressed cell growth and induced apoptosis in A549 and DOC-induced MDR cell lines (A549/D16) while autophagy is also induced through an inhibition of the AKT and JNK pathways and an activation of the ERK1/2 pathway. Furthermore, a combination of pterostilbene with autophagy inhibitors may strengthen the efficiency of proapoptotic chemotherapeutic strategies in both sensitive and chemoresistant lung cancer cells.
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