

Original Article

Pterostilbene simultaneously induces apoptosis, cell cycle arrest and cyto-protective autophagy in breast cancer cells

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Abstract: As a nature phytoalexin found in grapes, resveratrol has been proposed as a potential drug for cancer chemoprevention and treatment. However, its poor bioavailability limits its potential clinical application. Pterostilbene, the natural dimethylated analog of resveratrol with greater bioavailability, was confirmed to inhibit tumor growth both in vivo and in vitro, demonstrating its potential for further clinical application. In the current study, we found that pterostilbene could markedly inhibit the growth of two independent breast cancer cell lines. Both apoptosis and cell cycle arrest as well as the inhibition of wnt signaling was induced by pterostilbene. The dominant-active mutant of β -catenin could reverse the growth inhibitory effect of pterostilbene, indicating that the inhibition of wnt signaling is important to the growth inhibitory effect of pterostilbene. Interestingly, pterostilbene induced autophagy and blockage of autophagy augmented pterostilbene-induced growth inhibition, suggesting that the combination of autophagy inhibitors with pterostilbene and other therapeutics such as endocrine drugs could serve as a new and promising strategy for the treatment of breast cancer cells.

Keywords: Pterostilbene, phytoalexins, tumor growth inhibition, wnt signaling, apoptosis

Introduction

Early in 1819, an Irish doctor Samuel Black noticed the French Paradox that the French can eat so much fat but stay healthy. Only recently it turned out that part of the answer to the French paradox was resveratrol found in red wine which has been highly consumed in French. Resveratrol (trans-3,5,4V-trihydroxystilbene), a nature phytoalexin found in grapes and other food products, was believed to confer cardioprotective effects via its antioxidant and anti-inflammatory properties. Moreover, resveratrol was found to have anti-cancer activities at different stages of cancer development. It can act as antioxidant and antimutagen and inhibit the ignition and progression of inflammation, thus preventing the initiation of cancer development. In addition, it can induce cellular differentiation and inhibit the growth of cancer cells with various origin, such as leukemia, pancreatic cancer, breast cancer and prostate cancer [1-6]. Finally, resveratrol could inhibit tumor me-

tastasis by blocking angiogenesis and suppressing the expression of matrix metalloproteinases (MMPs) [7].

As a result, resveratrol has been proposed as a potential drug for cancer chemoprevention and treatment. However, the low bioavailability due to its poor absorption and rapid metabolism in mammals impeded the clinical application of resveratrol. Many approaches have been applied to enhance its bioavailability in humans, such as combinations with agents that can inhibit the in vivo metabolism of resveratrol, nanoparticle-mediated delivery and the development of nature or synthetic analogues of resveratrol [5].

As a natural dimethylated analog of resveratrol, pterostilbene has been proposed to have similar properties of resveratrol including anticancer, anti-inflammation, antioxidant, apoptosis, anti-proliferation and analgesic potential. Indeed, many studies have confirmed that pterostilbene

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could inhibit tumor growth both in vitro and in vivo. Importantly, following equimolar oral dosing in rats, plasma levels of pterostilbene and pterostilbene sulfate were markedly greater than plasma levels of resveratrol and resveratrol sulfate [8]. The greater bioavailability of pterostilbene indicated that pterostilbene could be potentially developed for clinical applications.

In the present study, we found that pterostilbene could markedly inhibit the growth of breast cancer cells by inducing apoptosis and cell cycle arrest. Interestingly, autophagy was simultaneously induced and the inhibition of autophagy significantly enhanced the cytotoxicity of pterostilbene, indicating that combination of pterostilbene with autophagy inhibitors could be a promising strategy for cancer treatment.

Materials and methods

Reagents and antibodies

Pterostilbene was bought from Beyotime (Shanghai, China). The Primary antibodies were antibodies against microtubule-associated protein 1 light chain 3 (LC3) (Cell Signaling Technology, #2775), phospho-mTOR (S2448) (Cell Signaling Technology, #2971), total mTOR (Cell Signaling Technology, #2983), phospho-P70S6K (T389) (Cell Signaling Technology, #9234), phospho-AKT (S473) (Cell Signaling Technology, #4051), total AKT (Cell Signaling Technology, #9272), GAPDH (Cell Signaling Technology, #3683). The secondary antibodies were HRP conjugated anti-rabbit (Santa Cruz Biotechnology, sc-2357) and anti-mouse IgG (Santa Cruz Biotechnology, sc-2371).

Cell cultures

Both Bcap-37 and MCF-7 cell lines were bought from cell bank (Chinese Academy of Sciences). Monolayer culture of cancer cells was maintained in RPMI 1640 supplemented with 10% FBS. Stock solution of Pterostilbene was prepared in dimethyl-sulphoxide (DMSO) (Sigma, D4540), and diluted with medium before use. Final concentration of DMSO was < 0.1%.

Cell proliferation assay

The proliferation of breast cancer cells before and after Pterostilbene treatment were deter-

mined by MTS assay (Promega, Madison, WI, USA). Cells were seeded into 96-well plates and treated with chemicals with different concentrations. After 48-h incubation, MTS was added into each well and the quantity of formazan formed was measured at 490 nm absorbance following the instructions provided.

Electron microscopy

Cancer cells were washed and fixed for 30 min in 2.5% glutaraldehyde. The samples were treated with 1.5% osmium tetroxide, dehydrated with acetone and embedded in Durcupan resin. Thin sections were then stained with lead citrate before being examined in the TECNAI 10 electron microscope (Philips, Holland) at 60 kV.

Western blot analysis

Western blotting was carried out as previously reported [9]. Proteins were resolved by SDS-polyacrylamide gel, transferred to Hybond C nitrocellulose membranes (Amersham Life Science, Buckinghamshire, UK). Membranes were blocked by 5% dry-milk containing TBS-T (Tris-Buffered Saline Tween-20) and probed with primary antibodies in blocking buffer overnight at 4°C. Finally, membranes were incubated with secondary antibodies conjugated with HRP (horse-radish peroxidase) and signals were visualized with enhanced chemiluminescence (Amersham Life Science). Membranes were re-probed with antibodies against GAPDH (Cell Signaling Technology) as the loading control.

Real-time PCR

Reverse transcription reaction was performed using 1 µg of total RNA with High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) [10]. CCND1 and c-myc expression levels were determined by quantitative real-time PCR using SYBR Green Master Mix Kit (Tiangen Biotech, Beijing, China). Human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) was used as an internal control of RNA integrity [11]. Primers used were CCND1-F: 5'-CAAATGTGTGCAGAAGGAGG, CCND1-R: 5'-AGGCCACGAACATGCAAGTG and c-myc-F, c-myc-R.

Flowcytometry analysis

For cell cycle distribution analysis, cells were

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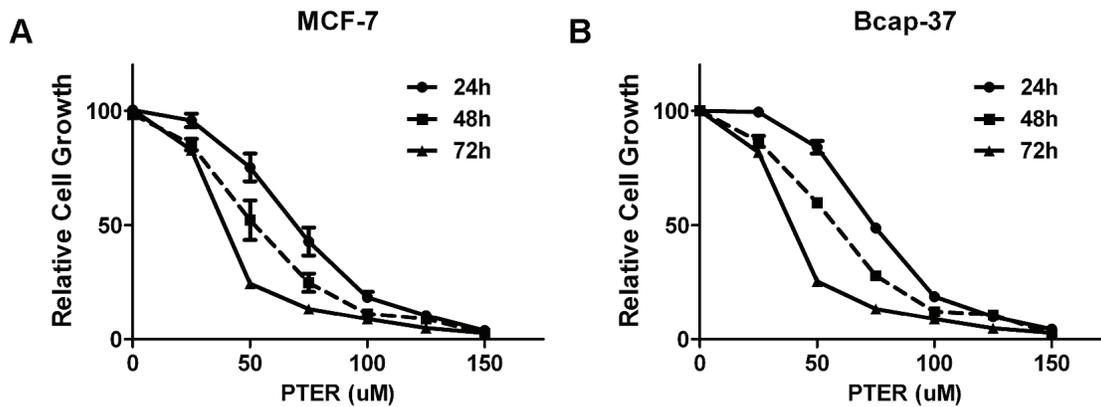


Figure 1. Pterostilbene inhibits proliferation of breast cancer cells. After the incubation with various concentrations of pterostilbene for different times, the viability of breast cancer cells (A, MCF-7 cells and B, Bcap-37 cells) was determined by MTS assay.

fixed by ice-cold ethanol and stained with RNase -containing PI (propidium iodide) solution. For apoptosis detection, cells were stained with PI and FITC labeled Annexin-V. Cells negative for PI but positive for FITC are early apoptotic cells while cells positive for both PI and FITC are cells in late apoptosis or necrosis. Stained cells were analyzed by Flow Cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical analyses

Unless otherwise stated, data were expressed as the mean \pm SD, and analyzed by Student's t test.

Results

Pterostilbene inhibited proliferation of breast cancer cells

We started to evaluate the effect of pterostilbene on the proliferation of breast cancer cells. Proliferation of breast cancer cells treated with or without pterostilbene was determined by MTS assay. In both MCF-7 and Bcap-37 cells, pterostilbene could inhibit cell proliferation in a time- and dose-dependent manner (**Figure 1A** and **1B**).

Pterostilbene induced apoptosis of breast cancer cells

Some cancer cells became floating after the exposure to pterostilbene, indicating that

pterostilbene may induce the apoptosis of breast cancer cells. Indeed, nuclear condensation was clearly observed in both cancer cell lines after pterostilbene treatment (**Figure 2A**). We further detected the expression of PARP in breast cancer cells before and after pterostilbene treatment. In both breast cancer cells, PARP was cleaved in a dose dependent manner (**Figure 2B**). Moreover, Annexin V staining revealed that phosphatidylserine (PS) was translocated from the cytoplasmic face of the plasma membrane to the extracellular face in MCF-7 and Bcap-37 cells treated with pterostilbene, confirming the induction of apoptosis by pterostilbene (**Figure 2C** and **2D**).

Pterostilbene induced cell cycle arrest in breast cancer cells

In addition, we tried to know whether there are other mechanisms responsible for the growth inhibition induced by pterostilbene. Indeed, after pterostilbene exposure more cells were arrested in G1 phase (**Figure 3A**). In consistent with this finding, cyclin D1 expression was significantly reduced in breast cancer cells with pterostilbene treatment (**Figure 3B**).

Pterostilbene suppressed oncogenic signaling pathways in breast cancer cells

To understand molecular mechanisms underlying the growth inhibition induced by pterostilbene, we compared the activation of several oncogenic signaling pathways critical to cancer

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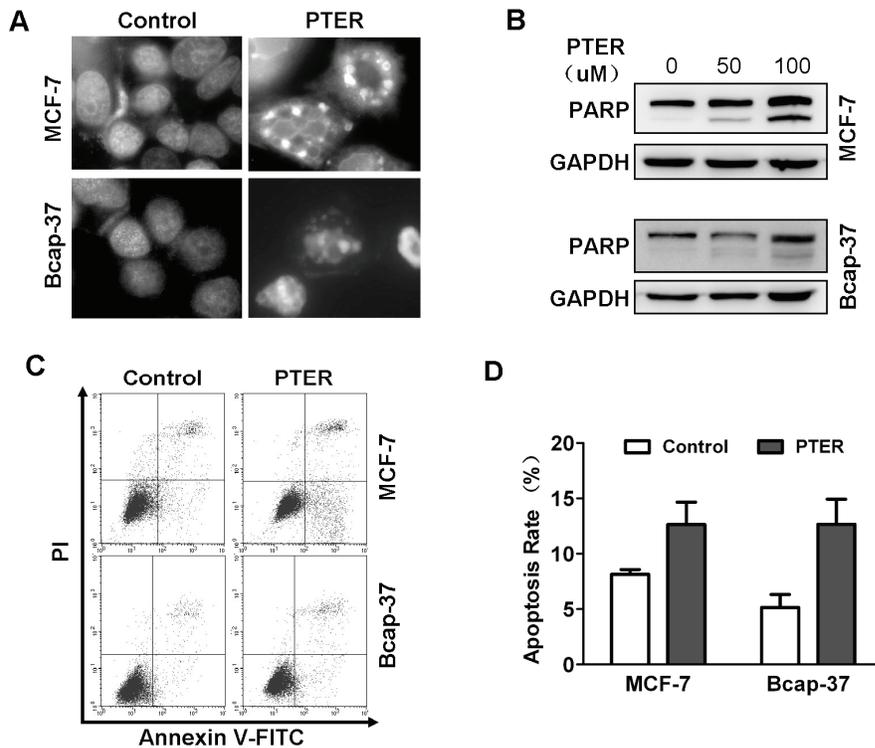


Figure 2. Pterostilbene induces apoptosis of breast cancer cells. A, After the incubation with 50 μ M of pterostilbene for 24 hours, MCF-7 cells were stained with DAPI. Photograph was taken under light microscope. B, The cleavage of PARP after the treatment of different concentrations of pterostilbene was determined by western blotting analysis. GAPDH was used as the loading control. C, Breast cancer cells were treated as in B and stained with PI and Annexin-FITC for the evaluation of apoptosis by FCM. Quantitative results were summarized in D. Asterisks indicate statistical difference ($p < 0.05$, Student's t test).

development in both cell lines using Proteome Profiler Human Phospho-Kinase Array Kit. It turned out that β -catenin level was dramatically changed before and after pterostilbene treatment (data not shown and **Figure 4A**). Consistently, the expression of CCND1 and c-myc, well-known downstream targets of wnt/ β -catenin pathway, were also significantly reduced after the exposure of pterostilbene (**Figure 4B**). Moreover, we found that upon pterostilbene treatment, GSK3 β , the upstream regulator of wnt/ β -catenin pathway, was dephosphorylated and activated (**Figure 4C**). The activity of PI3K/Akt, the upstream regulator of GSK3 β , was also reduced although PTEN expression remained unchanged (**Figure 4C**). To clarify the relevance of wnt/ β -catenin pathway to pterostilbene-induced growth inhibition, we compared the effect of pterostilbene on cancer cells in the presence or absence of a β -catenin mutant which is irresponsive to GSK3 β -mediated phosphorylation and refractory to ubiquitination-initiated degradation. As shown in **Figure 4D**, this β -catenin mutant could partly rescue the growth-inhibitory effect of pterostilbene, demonstrating the relevance of wnt/ β -catenin pathway to pterostilbene-induced growth inhibition.

Pterostilbene induced cyto-protective autophagy in breast cancer cells

Interestingly, we also observed the foamy appearance of cancer cells after pterostilbene treatment (**Figure 5A**), indicating that pterostilbene might be able to induce autophagy. We therefore further determined LC3b expression in cells with or without pterostilbene treatment. In consistent with microscopic observations, autophagy-specific LC3b-II was increased after pterostilbene treatment in a dose-dependent manner (**Figure 5B**). Furthermore, electronic microscopy analysis revealed the presence of typical autophagosomes in cancer cells treated with pterostilbene (**Figure 5C**), confirming the induction of autophagy by pterostilbene. To understand the role of autophagy in pterostilbene-induced growth inhibition, we determined the effect of pterostilbene on breast cancer cells before and after the suppression of autophagy by chloroquine (CQ). While CQ alone had no any effect on cellular proliferation, the growth-inhibitory effect of pterostilbene was significantly enhanced after CQ treatment (**Figure 5D**), indicating that autophagy in response to pterostilbene treatment played a cyto-protective

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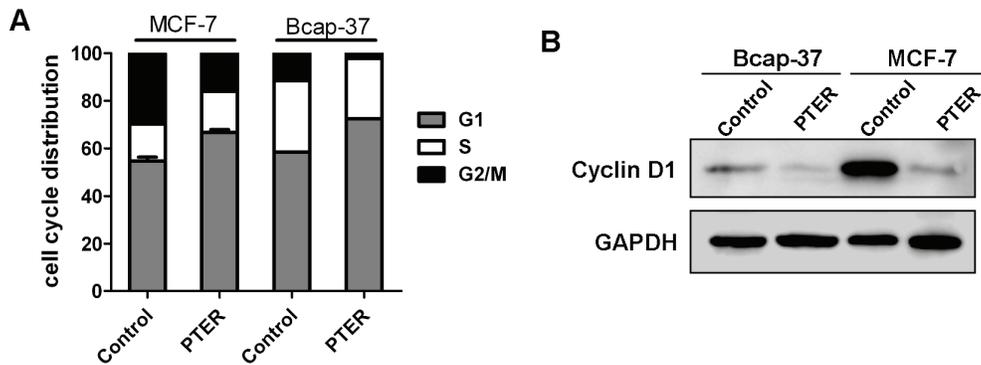


Figure 3. Pterostilbene induces cell cycle arrest in breast cancer cells. A, Breast cancer cells were treated as in Figure 2B and stained with PI for cell cycle analysis by FCM. B, The expression of cyclin D1 in breast cancer cells were treated as in Figure 2B were determined by western blotting. GAPDH was used as the loading control.

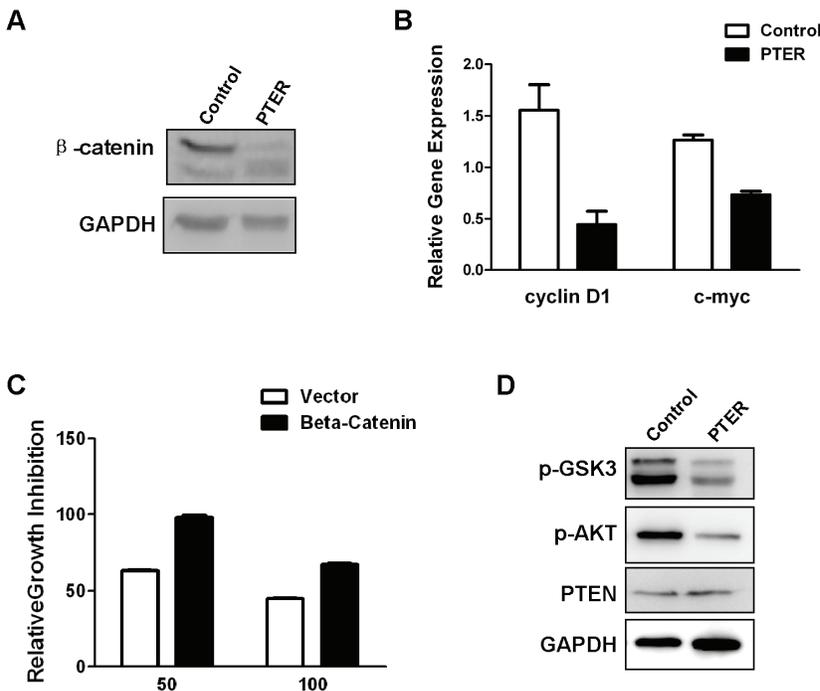


Figure 4. Pterostilbene suppresses wnt/ β -catenin signaling in breast cancer cells. A, The expression of cyclin D1 in breast cancer cells treated as in Figure 2B were determined by western blotting. GAPDH was used as the loading control. B, The expression of cyclin D1 and c-myc in breast cancer cells were treated as in Figure 2B were determined by real-time RT-PCR. GAPDH was used to control the integrity of RNA. C, The cellular level of phospho-GSK3 β , phospho-Akt and PTEN were determined by western blotting as in A. D, After the incubation with various concentrations of pterostilbene, the viability of breast cancer cells transfected with or without β -catenin mutant was determined by MTS assay, respectively.

role to help cancer cells survive under unfavorable conditions. Interestingly, cell cycle arrest induced by pterostilbene was enhanced after CQ treatment while no significant increase of apoptosis induction was observed after the inhibition of autophagy (Figure 5E).

Discussion

As the nature phytoalexin found in grapes and other foods, resveratrol was found to have anti-

cancer activities at different stages of cancer development. However, its poor absorption and rapid metabolism impeded its development for the clinical application. Pterostilbene, the natural dimethylated analog of resveratrol, has similar functions as resveratrol. For example, both stilbenes could block azoxymethane (AOM)-induced inflammation and oxidative stress, thereby preventing colon carcinogenesis. However, pterostilbene was more potent than resveratrol probably due to its possible better

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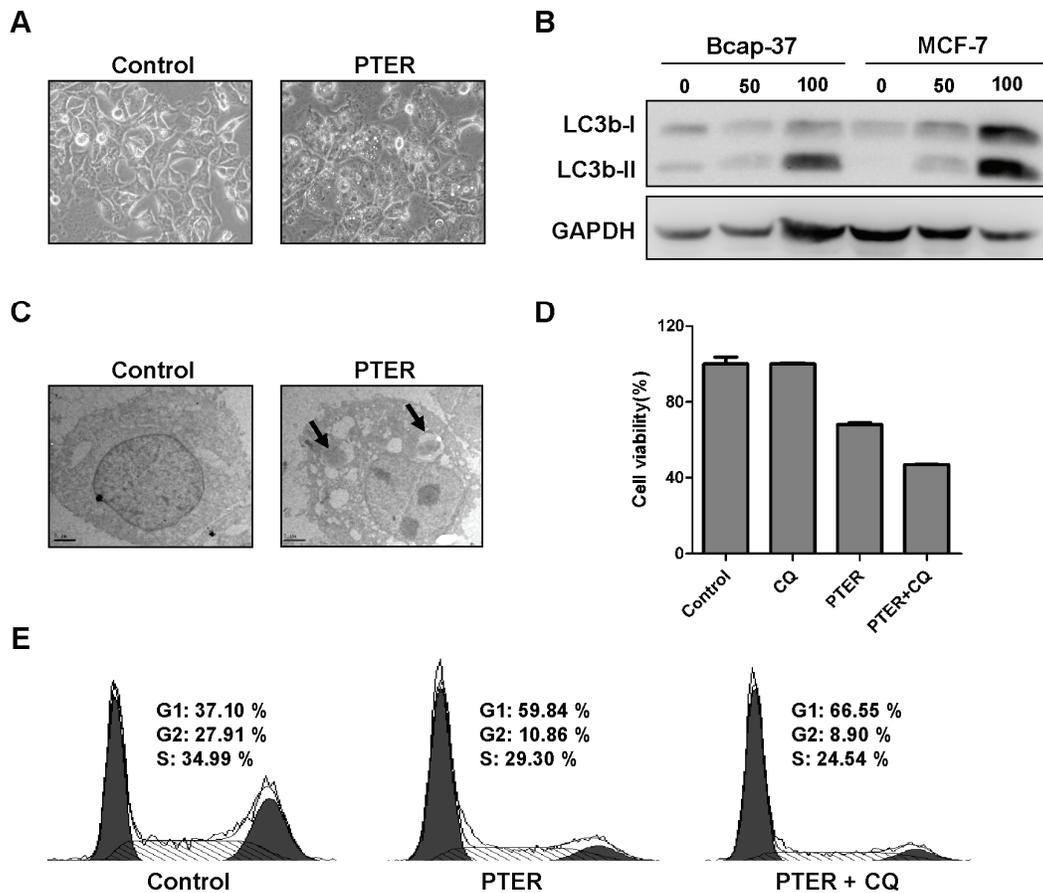


Figure 5. Pterostilbene induces cyto-protective autophagy in breast cancer cells. A, Cells treated with 50 μ M pterostilbene were photographed under light microscope. DMSO treated cells were used as the control. B, Expression of LC3 -I/II in cells treated with or without 50 μ M pterostilbene were determined by western blotting. GAPDH was used as the loading control. C, Cells treated with or without 50 μ M pterostilbene were subjected to TEM examination. Arrows indicate autophagosomes. The viability and cell cycle distribution of cells treated as indicated were analyzed by MTS assay. D, and flow cytometry analysis. E, respectively.

bioavailability since substitution of hydroxy with methoxy group increases lipophilicity, indicating that pterostilbene could be potentially developed for clinical applications [12-14].

Although the primary target of pterostilbene is still unknown, pterostilbene could influence multiple signaling pathways critical to cancer development. For example, pterostilbene strongly inhibited TPA-stimulated nuclear factor κ B (NF- κ B) and activator protein-1 (AP-1)-dependent transcriptional activity as well as TPA-induced activation of extracellular signal-regulated kinase 1/2, p38 mitogen-activated protein kinase, c-Jun N-terminal kinases 1/2 and phos-

phatidylinositol 3-kinase/Akt and protein kinase C that are upstream of NF- κ B and AP-1 [15, 16]. The expression of some oncogenic proteins including iNOS (inducible Nitric Oxide Synthase) and cyclooxygenase-2 (COX-2) were also suppressed upon pterostilbene treatment. In the current study, pterostilbene induced the reduction of β -catenin levels and caused the down-regulation of genes regulated by wnt signaling pathway such as c-myc and cyclin D1 (Figure 2-4). Although the phosphorylation of GSK-3 β (Glycogen Synthase Kinase-3 β) was indeed repressed, pterostilbene could most likely target many other signaling molecules in breast cancer cells since dominant active β -catenin failed to

completely rescue the growth inhibitory effect of pterostilbene.

It has been reported that pterostilben could induce apoptosis and cell cycle arrest in many cancer cells including gastric cancer cells, lung cancer cells and pancreatic cancer cells [17-20]. Importantly, pterostilbene had an additive inhibitory effect on breast cancer cells when combined with tamoxifen most likely through augmenting cell apoptosis [21], demonstrating the potential of pterostilben for the clinical application in the prevention and treatment of breast cancer.

Interestingly, pterostilbene could induce autophagy in addition to apoptosis and cell cycle arrest which was consistent with the finding in bladder cancer cells [22]. Moreover, the autophagy induced by pterostilbene seems to be cytoprotective since the inhibition of autophagy with chemical inhibitors could sensitize cancer cells to chemotherapeutic drugs (**Figure 5**), indicating that the combination of autophagy inhibitors and pterostilbene with classical chemotherapeutic drugs or endocrine drugs could serve as a new and promising strategy for the treatment of breast cancer cells.

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