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Pterostilbene Inhibits Breast Cancer *In Vitro* Through Mitochondrial Depolarization and Induction of Caspase-Dependent Apoptosis[1]

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Background. **Epidemiologic studies suggest that diets high in fruits and vegetables reduce cancer risk. Resveratrol, a compound present in grapes, has been shown to inhibit a variety of primary tumors. Pterostilbene, an analogue of resveratrol found in blueberries, has both antioxidant and antiproliferative properties. We hypothesized that pterostilbene would induce apoptosis and inhibit breast cancer cell growth *in vitro*.**

Methods. **Breast cancer cells were treated with graduated doses of pterostilbene. Cell viability was measured by MTT assay. Apoptosis was evaluated *via* DNA fragmentation assay and TUNEL assay. Apo-ONE caspase-3/7 assay was used to evaluate caspase activity. Flow cytometry was used to evaluate mitochondrial depolarization, superoxide formation, and cell cycle. Student's *t*-test and two-way ANOVA with Bonferroni posttests were utilized for statistical analysis.**

Results. **Pterostilbene decreased breast cancer cell viability in a concentration- and time-dependent manner. Pterostilbene treatment increased caspase-3/7 activity and apoptosis in both cell lines. Caspase-3/7 inhibitors completely reversed pterostilbene's effects on cell viability. Pterostilbene treatment triggered mitochondrial depolarization, increased superoxide anion, and caused alteration in cell cycle.**

Conclusions. **Pterostilbene treatment inhibits the growth of breast cancer *in vitro* through caspase-dependent apoptosis. Mitochondrial membrane depolarization and increased superoxide anion may contribute to the activation downstream effector caspases. Caspase inhibition leads to complete reversal of pterostilbene's effect on cell viability. Further *in vitro* mechanistic studies and *in vivo* experiments are warranted to determine its potential for the treatment of breast cancer.**

Key Words: **pterostilbene; apoptosis; phytochemicals; breast cancer.**

INTRODUCTION

Breast cancer is the most common malignancy and second leading cause of cancer-related death in women. While 5-y survival rates for localized disease are over 90%, outcomes for treatment of regional and systemic disease are not as successful. One reason successful therapy is difficult to achieve is that tumors are often resistant to apoptosis. This allows neoplastic cells to evade the body's natural surveillance mechanisms and initiate tumor growth. Furthermore, unregulated cellular survival mechanisms result in the promotion of tumor growth and metastasis. These alterations in intracellular signaling cascades can render cells resistant to standard therapies. In fact, many therapeutic modalities act principally through the promotion of apoptosis. Interventions designed to attenuate resistance to apoptosis may sensitize tumors to conventional modalities of cancer therapy. To this end, development of novel chemopreventive and/or chemotherapeutic agents and adjuncts may improve the treatment of breast cancer.

Epidemiologic studies have suggested that diets rich in fruits and vegetables are associated with risk reduction for a number of common cancers. Literature reviews on this subject advocate for increased intake of fruits and vegetables [1]. However, randomized trials have yet to show the association of a high fruit, vegetable, and fiber diet with a reduction in breast cancer [2].

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Phytochemicals are non-nutritive chemicals found in plants that have protective or disease preventive properties. Chemopreventive phytochemicals including curcumin (a spice in curry), catechin (found in green tea), and resveratrol (found in the skin of grapes) have been shown to prevent tumor promotion or progression through a variety of mechanisms, such as the amelioration of oxidative DNA damage and ability to alter abnormal cellular signaling [3]. Resveratrol, a stilbene with antioxidant and antiproliferative properties, has been shown to inhibit a variety of primary tumors [4–11]. Pterostilbene, an analogue of resveratrol found in blueberries, has been shown to suppress carcinogenesis in animal models of gastric cancer, colon cancer, leukemia, and melanoma [12–15]. Antioxidant capacity of pterostilbene has been shown through its ability to scavenge ABAP [2,2′-azo-bis(2-amidinopropane)] and inhibit oxidation of ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]) [16, 17]. *In vitro* studies using a variety of cancer cell lines, including breast cancer, demonstrate a concentration-dependent antiproliferative effect when treated with pterostilbene [16]. Similar results were found in an organ culture model where pterostilbene inhibited carcinogen-induced preneoplastic lesions in murine mammary tissue [17]. The goal of this study was to examine the effects of pterostilbene treatment on breast cancer *in vitro* and to identify intracellular mechanisms by which pterostilbene exerts its effects.

MATERIALS AND METHODS

Chemicals

Pterostilbene was purchased from Sigma-Aldrich (St. Louis, MO). 5-[(S)-(+)-2-(methoxymethyl)pyrrolidino]sulfonylisatin, a reversible inhibitor of caspase-3 and caspase-7, was obtained from Calbiochem (La Jolla, CA). Compounds were dissolved in dimethyl sulfoxide (DMSO) and further diluted in sterile culture medium immediately prior to use.

Cell Lines and Culture

Two breast cancer cell lines, MCF-7 and MDA-MB-231, were purchased from the American Type Culture Collection (ATCC: Manassas, VA). MCF-7 cells express estrogen receptor (ER) and are a common example of estrogen-responsive breast cancer cells. MDA-MD-231 cells are ER negative with a highly invasive *in vitro* assay profile. Cells were maintained as monolayers in T-25 flasks. MCF-7 cells were maintained in minimal essential medium with Earle's salts and L-glutamine (MEM; Mediatech, Inc., Herndon, VA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT), 1% penicillin streptomycin (Mediatech, Inc.), and 0.01 mg/mL insulin. MDA-MB-231 cells were maintained in Leibovitz's modified medium with L-glutamine (L-15: Mediatech, Inc.) supplemented with 10% fetal bovine serum and 1% penicillin streptomycin. Flasks were kept at 37°C in a water-jacketed 5% CO_2 incubator (Fischer Scientific, Houston, TX). For experiments, cells were harvested from culture monolayers at 80% to 90% confluence. Cells were rinsed with sterile phosphate buffered saline (PBS: Mediatech, Inc.), and live cells were detached using 0.25% trypsin in 0.1% EDTA (Mediatech, Inc.). Cells were then centrifuged at 1000 rpm for 5 min and resuspended in growth medium. Cells were seeded at 10^4 cells per well in 96-well plates and allowed to attach overnight. Cells were then exposed to various doses of pterostilbene (10–100 μM).

Growth Inhibition

Cells were added to 24-well plates at 10^4 cells/well and incubated to allow for adherence. After 24 h, half of the media was changed and replaced with pterostilbene at 20–100 μM for 24, 48, and 72 h. Cells were then harvested and counted by hemocytometer. The growth of treated cells was expressed as a percentage of untreated control cells. The concentration of pterostilbene that decreased cell count by 50% (IC_{50}) was calculated by nonlinear least-squares curve fitting of experimental data utilizing Graphpad Software (San Diego, CA).

Cell Viability Assay

The MTT colorimetric assay was performed to detect cell viability after 24, 48, and 72 h of exposure to pterostilbene (10–100 μM). Culture media was removed and MTT, a tetrazolium dye (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide thiazolyl blue; Sigma, St. Louis, MO) diluted in culture media was added to each well. Plates were incubated at 37°C in the CO_2 incubator for 1 h. Mitochondrial dehydrogenase activity reduced the yellow MTT dye to a purple formazan, which was solubilized in DMSO (Sigma) and absorbance was read at 540 nm on an ELISA plate reader.

DNA Fragmentation Assay

The Cell Death Detection ELISA kit (Roche, Mannheim, Germany), a sandwich-enzyme-immunoassay-based method, was used to detect the occurrence of nuclear DNA fragmentation. This kit employs mouse monoclonal antibodies directed against DNA and histones to recognize released nucleosomes after DNA nucleosomal fragmentation. Cells were plated 10^4 cells per well into 96-well plates and allowed to adhere for 24 h. Cells were then exposed to various doses of pterostilbene for 18 h. Adherent cells were lysed and centrifuged to produce a nucleosome-containing supernatant. Samples were transferred to a streptavidin-coated microplate and incubated with anti-histone and anti-DNA antibodies followed by a peroxidase substrate resulting in color change. Color development was proportional to the amount of nucleosomes captured in the antibody sandwich, and was measured spectrophotometrically at 405 nm.

TUNEL (Terminal Deoxynucleotide Transferase dUTP Nick End Labeling) Assay

Cells were seeded at 5×10^5 cells per well into 6-well plates and allowed 24 h to adhere. Cells were then exposed to control vehicle (DMSO) or pterostilbene (25 or 75 μM) for 24 h. Cells were fixed in 1% (wt/vol) paraformaldehyde in PBS and the APO-BRDU (Phoenix Flow Systems, Inc., San Diego, CA) TUNEL kit was utilized for measuring apoptosis by flow cytometry. Apoptotic cells with exposed 3'-hydroxyl DNA ends were labeled with bromolated deoxyuridine triphosphate nucleotides (BR-dUTP). Fluorescein labeled antiBrdU monoclonal antibodies were then used to stain apoptotic cells. Propidium iodide was used to label total cellular DNA. Samples were analyzed with flow cytometry per kit instructions.

Caspase Activity Assay

Cells were seeded at 10^4 cells per well into 96-well plates with opaque sidewalls. After an allotted 24 h for cell adherence, half of the media was replaced and cells were exposed to 75 μM pterostilbene for 0, 12, 24, and 36 h. The Apo-ONE homogeneous caspase 3/7 assay

substrate (Promega, Madison, WI) was utilized to evaluate the activities of caspase-3 and -7. The caspase-3/7 substrate rhodamine 110,bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide; Z-DEVD-R110), is acted upon by caspase-3 and-7 resulting in a fluorescent leaving group. The amount of fluorescent product generated is proportional to the amount of caspase-3/7 cleavage activity present in the sample. Caspase-3/7 substrate was added to each well and incubated at room temperature for 1–2 h. A spectrofluorometer was used to measure fluorescence (excitation wavelength 485±20 nm, emission wavelength 528 ± 20 nm).

Caspase Inhibition

Cells were plated 10^4 cells per well into 96-well plates and allowed to adhere for 24 h. Cells were then pretreated for 30 min with 50 μM of caspase-3/7 inhibitor. Cells were then treated with 50 or 75 μM pterostilbene for 48 h. Cell viability was then assayed using MTT as described above.

Mitochondrial Depolarization

Cells were seeded at 3×10^5 cells per well into a 6-well plate. After 24 h for cell adherence, 2 μM of JC-1 (Molecular Probes, Eugene, OR) was added to each well for 20 min at 37°C. Cells were then washed with PBS and treated with DMSO control or pterostilbene (25, 50, 70, 100 μM) for an additional 30 min. Cells were then trypsinized, resuspended in PBS and run on a Coulter Elite flow cytometer. The excitation peak of JC-1 is 488 nm. The approximate emission peaks of monomeric and J-aggregates forms are 529 nm and 590 nm, respectively.

Cell Cycle

Cells were seeded at 3×10^5 cells per well into a 6-well plate. The next day, cells were treated with DMSO or pterostilbene for 24 h, then washed in PBS, trypsinized, and fixed in cold ethanol for 2 h. Cells were then washed and resuspended in PBS + 0.1% Triton X-100 + 100 ug/mL RNase A (Sigma, St. Louis, MO) + 40 ug/mL propidium iodide (MP Biomedicals Solon, OH) for 30 min at 37 in the dark. Cells were run on a Coulter Elite flow cytometer. Propidium iodide, when bound to nucleic acids, has an excitation maximum at 535 nm and emission maximum at 617 nm. Cell populations were analyzed and categorized into cell cycle phases using Modfit LT 3.0 software.

Intracellular Superoxide Anion

Cells were seeded at 3×10^5 cells per well into a 6-well plate and allowed 24 h to adhere prior to the addition of 5 μM Mitosox Red (Molecular Probes, Eugene, OR). Cells incubated with Mitosox for 20 min at 37°C. Cells were then washed with PBS then treated with either DMSO control or pterostilbene (25, 50, 70, 100 μM) for 20 min. Cells were then washed with PBS, trypsinized and run on the Coulter Elite flow cytometer. Mitosox Red excites at 510 nm and emits at 580 nm.

Statistical Analysis

Data were presented as mean values ± standard error. Statistical comparisons among groups were performed by Student's *t*-test or analysis of variance (ANOVA) followed by Bonferroni post-tests for multiple comparisons.

RESULTS

In Vitro Antitumor Activity of Pterostilbene

Pterostilbene induced a significant concentration- and time-dependent decrease in MDA-MB-231 and MCF-7 cell viability (Fig. 1). The values of the inhibitory concentration at 50% effect level (IC_{50}), shown in Table 1, indicate the antitumor potency of this agent in both breast cancer cell lines. Potency was similar in both cell lines. Pterostilbene treatment for 24 h, at concentrations of 56 and 59 μM, inhibited cancer cell

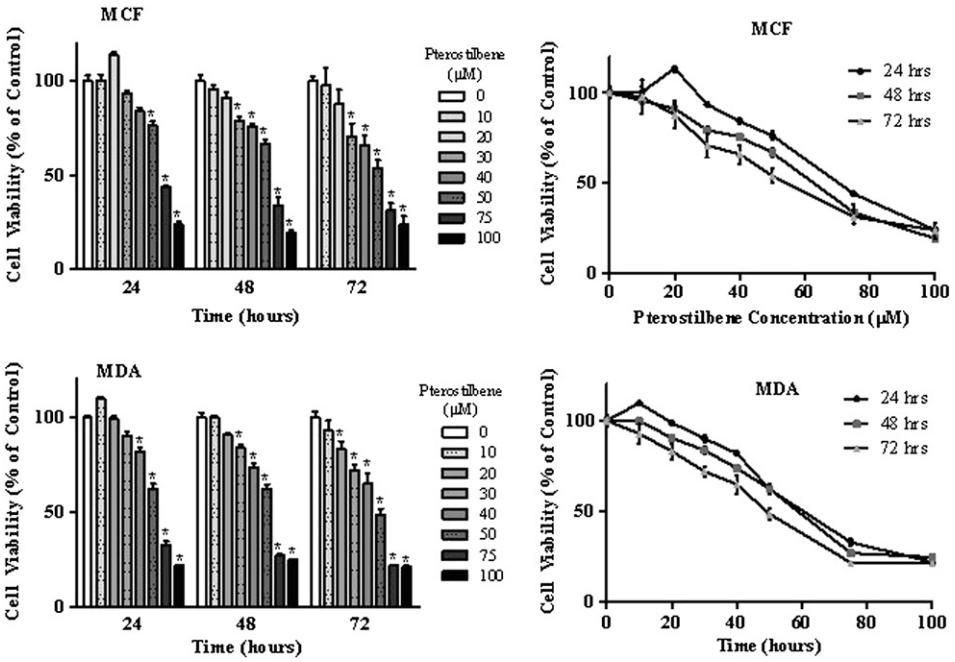

FIG. 1. Cell proliferation inhibition by pterostilbene.

TABLE 1

Pterostilbene-Mediated Cell Growth Inhibition After 24, 48, and 72 h

	IC50 (μM), mean±SEM	
	MDA-MB-231	MCF-7
24 h	56.37±17.56	59.42±7.89
48 h	29.6±4.77	40.51±10.72
72 h	20.21±2.88	26.42±10.84

Experiments were performed in triplicate and values expressed as means±SEM.

growth by 50% of control values in MDA-MB-231 and MCF-7 cells, respectively.

Pterostilbene Induces Apoptosis in Breast Cancer Cells

Programmed cell death is characterized by chromatin condensation, membrane blebbing, inter-nucleosomal degradation of DNA, and apoptotic body formation. To investigate whether cytotoxic effects of pterostilbene were due to necrosis or apoptosis an assay looking at released nucleosomes was performed (Fig. 2). There was a statistically significant fold increase in released nucleosomes in MDA-MB-231 (2.15 ± 0.42, $P = 0.011$) and MCF-7 (6.18 ± 2.33, $P = 0.018$) cells exposed to pterostilbene compared with vehicle-only treated controls. Using a TUNNEL assay modified for use with flow cytometry, percentages of apoptotic cells were analyzed. MCF cells treated with 25 μM pterostilbene had a 2.5-fold increase in apoptosis, and 75 μM pterostilbene resulted in a 4-fold increase in apoptotic cells. Similarly, with MDA there was a 2.17- and 4-fold increase in apoptotic cells when treated with 25 and 75 μM pterostilbene.

Caspase-3 and Caspase-7 Upregulation with Pterostilbene

To ascertain whether the biologic activity of pterostilbene could involve effector caspases, an assay was performed looking at the activity of two enzymes involved in the effector phase of apoptosis: caspase-3 and caspase-7. Pterostilbene treatment resulted in a significant fold-increase in caspase-3/7 activity compared with vehicle-alone treated controls in both MCF-7 (6.16 ± 1.92, $P < 0.01$) and MDA-MB-231 (3.86 ± 0.25, $P < 0.5$) cells (Fig. 3). Caspase 3/7 activity was slightly greater in MCF-7 cells compared to MDA-MB-231 cells. Of note, the MCF-7 cells had a quicker cell turnover compared to the slower growing MDA-MB-231 cells.

Caspase-3/7 Inhibition Restores Cell Viability in Pterostilbene Treated Cells

The role of effector caspase-3 and caspase-7 is essential for pterostilbene to inhibit the growth of breast cancer cells *in vitro*. Inhibition of caspase-3/7 reversed the effect of pterostilbene on both MCF and MDA cell viability (Fig. 4). Pterostilbene (75 μM) inhibited MCF cell viability to $13.33\% \pm 6.42\%$ of control, and caspase inhibition restored viability to $88.63\% \pm 4.58\%$ of control ($P < 0.001$ *versus* pterostilbene treatment). Pterostilbene (75 μM) inhibited MDA cell viability to $42.30\% \pm 22.67\%$ of control, and caspase inhibition restored viability to $106.02\% \pm 8.90\%$ of control ($P < 0.001$ *versus* pterostilbene treatment).Viability for cells exposed to pterostilbene with caspase 3/7 inhibition was not statistically different from vehicle-only treated controls.

Pterostilbene Increases Superoxide Anion

Hydroethidine (HE) has been widely used to detect intracellular superoxide anion. Mitosox Red is a derivative of HE. The oxidation of HE by superoxide leads to the fluorescent product 2-hydroxy-ethidium. This oxidation product becomes highly fluorescent upon binding to nucleic acids. The flow cytometry results show increased fluorescence of pterostilbene treated cells compared with vehicle (DMSO) treated cells (Fig. 5). There is a concentration dependent increase in superoxide anion in pterostilbene treated MDA and MCF breast cancer cells.

Pterostilbene Modification of Cell Cycle in MCF-7 Cells

Flow cytometric analysis revealed that after 24 h of pterostilbene treatment normal cell cycle progression was disrupted in the insulin dependent MCF7 cell

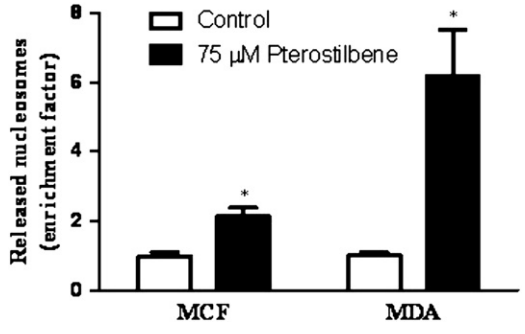

FIG. 2. Nucleosome release by pterostilbene.

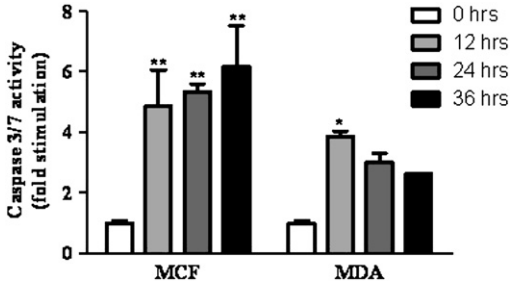

FIG. 3. Caspase 3/7 activity by pterostilbene.

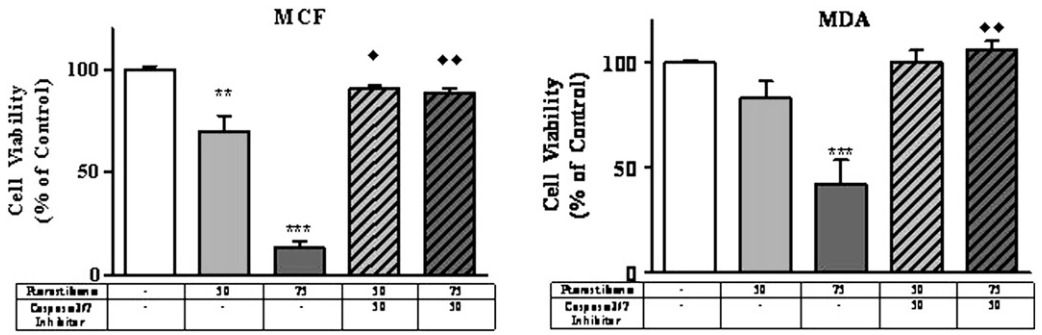

FIG. 4. Caspase 3/7 inhibition and pterostilbene treatment.

line but not MDA. The MCF7 breast cancer cell line showed an increase in cells in S phase, particularly at lower concentrations of pterostilbene. MDA-MB-231 showed no differences in cell cycle after 24 h.

Mitochondrial Depolarization with Pterostilbene

JC-1 is a cationic dye that exhibits a potential-dependent accumulation in the mitochondria of cells with healthy mitochondria. This accumulation leads to the formation of red fluorescent aggregates and a fluorescence emission shift from green to red. Mitochondrial depolarization causes JC-1 leakage and less aggregation of the dye in the mitochondria. Mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. MCF-7 and MDA-MB-231 showed a concentration-dependent increase in mitochondrial depolarization with pterostilbene treatment (Table 2).

DISCUSSION

The present study demonstrates *in vitro* anticancer activity of pterostilbene on two different breast cancer

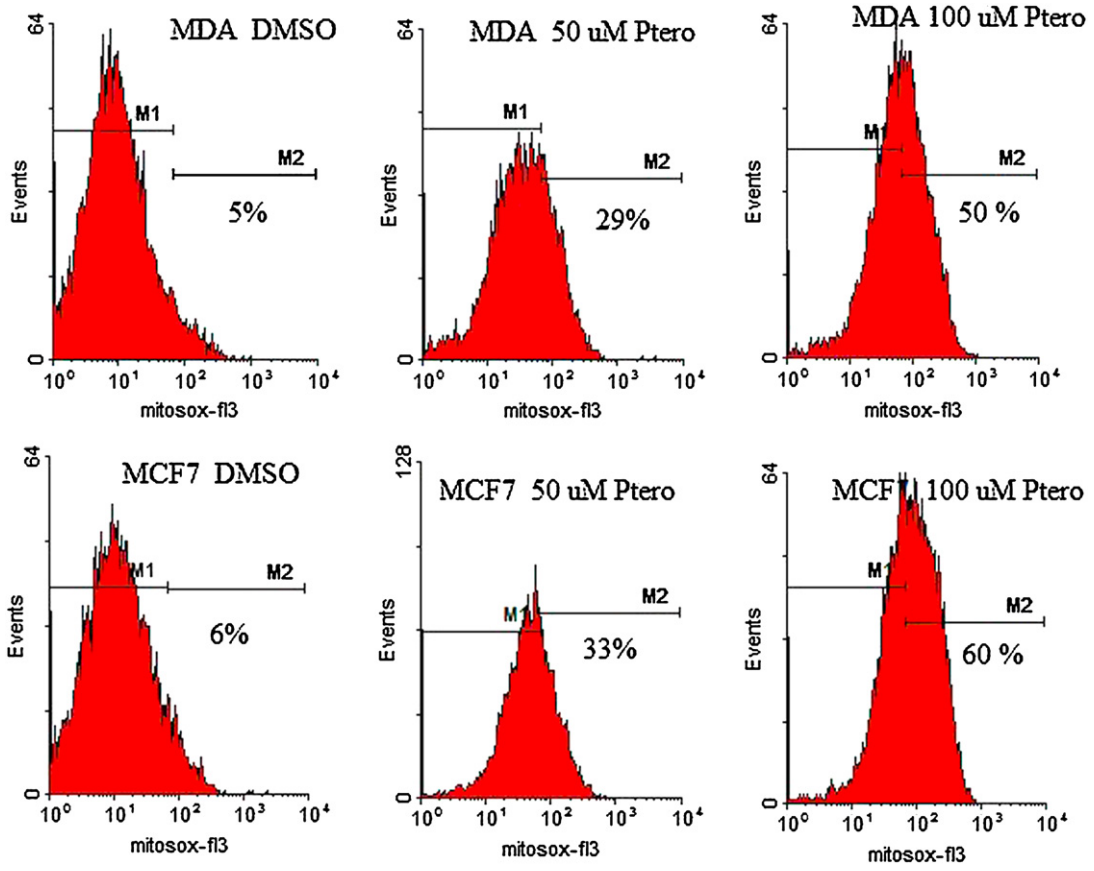

FIG. 5. JC-1 mitochondrial depolarization by pterostilbene. (Color version of figure is available online.)

cell lines. We have shown that pterostilbene treatment leads to caspase-dependent apoptosis. Anticancer effects of resveratrol, an analogue of pterostilbene, have been well established in breast cancer (Alkhala *et al.* 2008 [18]; Pozo-Guisado *et al.* 2005 [10]). Our hypothesis was that pterostilbene would act in a manner similar to resveratrol. Our data with pterostilbene show inhibition of breast cancer cells through the induction of apoptosis as evidenced by increased internucleosomal fragments and increased apoptotic cells identified with TUNEL labeling. Further examination of the mechanisms leading to apoptosis, we show increased caspase 3/7 with pterostilbene treatment. Furthermore, our results demonstrate that caspase 3/7 inhibition reverses the toxicity of pterostilbene on both breast cancer cell lines. Resveratrol has been shown to induce caspase-dependent apoptosis in MDA-MB-231 breast cancer cells, and similarly to our studies, inhibition of caspase-3 blocked apoptosis [18]. Our data are also congruent with the work by Mohan *et al.* on colon cancer, in which inhibition of caspase-3 prevents resveratrol-induced cell death [19]. Of note, the MCF-7 breast cancer cell line lacks a functional caspase-3 gene [20]. Caspase-3 deficiency may account for the insensitivity of MCF-7 cells to many chemotherapeutic agents. We chose to look at caspase-3 and caspase-7 combined and used a caspase inhibitor that targeted both of these caspases. Whether attributable to caspase-3 or caspase-7, our results indicate that effector caspases are crucial for the progression of apoptosis seen when breast cancer cells are exposed to pterostilbene.

Sareen *et al.* demonstrate activation of the mitochondrial (intrinsic) apoptotic pathway and caspase-3 in human retinoblastoma cells treated with resveratrol [21].

Pterostilbene has been shown to up-regulate the expression of genes involved in mitochondrial functions [22]. Pan *et al.* studied the effects of pterostilbene on human gastric carcinoma cells [13]. They proposed a pathway by which pterostilbene causes increased reactive oxygen species (ROS), which induces altered mitochondrial transmembrane potential, causing release of cytochrome-c, followed by activation of the caspase cascade triggering programmed cell death. In our study, we show both an increase in superoxide anion and mitochondrial depolarization in breast cancer cells treated with pterostilbene. However, we have not shown a temporal or causal relationship between these 2 findings. Pterostilbene may cause increased ROS which in turn activates the intrinsic (mitochondrial) apoptotic pathway. Alternately, Pterostilbene may act directly on the mitochondrial membrane.

There is evidence that fluctuations of the oxidative states of intracellular proteins may be important in regulating the cell cycle [23]. ROS act as potent second messengers that trigger signal transduction mechanisms involved in regulation of cell growth, transformation, aging and apoptosis. ROS increase cell cycle transition from G1 to S phase. Resveratrol has been shown to induce S-phase arrest for different cancer cell types [24]. Our data show both an increase in ROS and a disruption of normal cell cycle progression in MCF-7 cells treated with pterostilbene. MCF-7 breast cancer cells showed an increase in S phase. This finding was not replicated with MDA-MB-231 cells, which in culture grew more aggressively compared to the MCF-7 cells. The discrepancy between cell lines is similar to work by Tolomeo *et al.* on leukemia using two myeloid leukemia cell lines (HL60 and K562), where pterostilbene caused an increase in S phase in HL60 cells, but no modification of cell cycle with K562 cells [15].

Although our study did not look at the cytotoxic profile of pterostilbene on noncancerous cells, studies have shown it to be nontoxic to normal PMN's and hematopoietic stem cells at concentrations similar to those used in our studies (25–100 μM) [15, 22].

TABLE 2

Mitochondrial Depolarization With Pterostilbene Treatment

	Red/green ratio	
	MCF	MDA
DMSO	4.556	11.500
25 μM pterostilbene	4.000	9.000
50 μM pterostilbene	3.000	2.704
75 μM pterostilbene	2.571	0.299
100 μM pterostilbene	0.710	0.031

Mitochondrial depolarization is indicated by a decrease in the red/green ratio.

CONCLUSIONS

In conclusion, our data indicate that pterostilbene induces apoptosis in breast cancer cells. Apoptosis, however, is a complex signaling cascade with multiple triggers and many levels of checks and balance when determining cell fate. The exact mechanism by which apoptosis is triggered remains to be determined. We have shown that pterostilbene treatment leads to increased ROS, mitochondrial membrane depolarization, and activation of effector caspases. It remains unclear whether our data represent one pathway leading to apoptosis, or if these findings correlate with multiple apoptotic triggers and pathways. Further research to determine the molecular mechanism of action is warranted. This information will clarify whether

pterostilbene will serve as a chemopreventative agent or as an adjunct, allowing practitioners to decrease doses of chemotherapy and radiation, while achieving similar clinical results.

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