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Styrylpyrone Derivative Induces Apoptosis through the Up-Regulation of Bax in the Human Breast Cancer Cell Line MCF-7

Alvin Lee Teck Chien* and Azimahtol Hawariah Lope Pihie
School of Biosciences & Biotechnology, Faculty of Science & Technology, National University of Malaysia, 43600 Bangi, Selangor, Malaysia

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In the fight against cancer, novel chemotherapeutic agents are constantly being sought to complement existing drugs. Various studies have presented evidence that the apoptosis that is induced by these anticancer agents is implicated in tumor regression, and Bcl-2 family genes play a part in apoptosis following treatment with various stimuli. Here, we present data that a styrylpyrone derivative (SPD) that is extracted from the plant *Goniothalamus sp.* showed cytotoxic effects on the human breast cancer cell line MCF-7. SPD significantly increased apoptosis in MCF-7 cells, as visualized by phase contrast microscopy and evaluated by the Tdt-mediated dUTP nick end-labeling assay and nuclear morphology. Western blotting and immunostaining revealed up-regulation of the proapoptotic Bax protein expression. SPD, however, did not affect the expression of the anti-apoptotic protein, Bcl-2. These results, therefore, suggest SPD as a potent cytotoxic agent on MCF-7 cells by inducing apoptosis through the modulation of Bax levels.

Keywords: Apoptosis, Bax, Bcl-2, *Goniothalamus*, Styrylpyrone derivative

Introduction

Negative cell growth is an important aspect of maintaining normal tissue homeostasis. This regulation involves the suppression of cell proliferation, as well as the induction of cell death (Symmonds *et al*., 1994). In cancer therapy, one approach that suppresses the tumor growth is by activating the apoptotic machinery in the cell (Fan *et al*., 1998). Apoptosis is the ability of a cell to self-destruct by the activation of an intrinsic cellular suicide program when the cells are no longer needed or when they are seriously damaged. Evidence that was obtained during the last few years is beginning to establish that a large majority of cancer chemotherapy agents effect tumor cell killing *in vivo* and *in vitro* through launching the mechanisms of apoptosis (Hannun, 1997). Morphologically, apoptosis is characterized by the appearance of membrane blebbing, cell shrinkage, chromatin condensation, DNA cleavage, and the fragmentation of the cell into membrane-bound apoptotic bodies (Kerr *et al*., 1972).

Bax is a member of the Bcl-2 family of proteins that has been associated with apoptotic cell death both *in vitro* and *in vivo*. Apoptosis is controlled by the ratio of various Bcl-2 family members (Reed *et al*., 1996). When the levels of apoptosis promoters (Bax and Bcl-X_S) increase, then apoptosis is accelerated in response to external stimuli; whereas, when the inhibitors of apoptosis (Bcl-2 and Bcl-X_L) increase, then the cells are predisposed to be resistant to apoptosis (Cohen, 1997; Pastorino *et al*., 1998; Srinivasan *et al*., 1998).

In this study, we tested the styrylpyrone derivative (SPD), a novel compound that is extracted from the plant *Goniothalamus sp*. of the Annonaceae family. Among the species of *Goniothalamus* are *G. umbrosus*, *G. andersonii*, *G. macrophyllus*, and *G. malayanus* (Jewers *et al*., 1972). In some parts of Southeast Asia, the water extract from this plant is a part of the diverse traditional medication that is used by indigenous folk. *G. macrophyllus* and *G. uvaroides* have been widely used as treatments for malaria, cholera, fever, as well as a tonic with antifertility or abortive properties (Ahmad *et al*., 1991).

Previous studies on SPD suggest this bioactive compound as an antitumor and anti-implantation agent. The antifertility effect of SPD on mice was caused by the inhibition of DNA synthesis, which resulted in the antiimplantation on the endometrium (Azimahtol Hawariah *et al*., 1994). *In vitro*, SPD was antiproliferative towards a panel of cancer cell lines [*i.e*., ovarian carcinoma (Caov-3), breast carcinoma (MCF-7,

*To whom correspondence should be addressed.
Tel: 603-8921 5993; Fax: 603-8925 2698
E-mail: alvinlee@email.com

T47D, MDA-MB-231), and cervical carcinoma (Hela)]. SPD, however, was not significantly cytotoxic towards normal cells (BHK, VERO, BGM and MDBK) (Azimahtol Hawariah *et al*., 1998). On the *in vivo* models, SPD was reportedly capable of tumoricidal and tumoristatic effects on Sprague-Dawley rats with 7, 12-dimethylbenzanthracene (DMBA)-induced mammary tumors (Meenakshii *et al*., 2000). The mechanism of action for SPD, however, remains to be clarified.

Here, we studied the antiproliferative effects of SPD on the MCF-7 human breast carcinoma. We found that SPD induced a high percentage of cells to undergo apoptosis. In these cells, we observed that SPD was able to modulate the Bax expression by increasing the level of this proapoptotic protein while having little effect on the expression of the anti-apoptotic Bcl-2. The SPD treatment did not effect the proliferation of the normal cell lines MDBK and Changs Liver, which further supports its selective antitumor property.

Materials and Methods

Cell culture MCF-7, Changs Liver, and MDBK cells were obtained from the American Type Culture Collection (ATCC) and maintained in DMEM that was supplemented with 10% fetal bovine serum and 2 mM glutamine. Styrylpyrone derivative (SPD) was isolated from the bark of *Goniothalamus umbrosus*, as previously described (Azimahtol Hawariah *et al*., 1994).

Cell viability assay The cells were treated with SPD at increasing concentrations to evaluate its *in vitro* antitumor activity. The IC_{50} values were obtained for these cell lines, as previously described (Lin and Hwang 1991; Teoh and Azimahtol Hawariah, 1999).

Apoptotic Index Staining with Hoechst 33258 was performed, as described elsewhere (Hishikawa *et al*., 1999). Briefly, the floating and trypsinized-adherent SPD-treated cells were collected and washed with PBS. The cells were then fixed with 4% paraformaldehyde for 30 min. After washing, the cells were incubated in Hoechst 33258 (Sigma) at a final concentration of 30 μg/ml at room temperature for 30 min. Nuclear morphology was then examined with a Zeiss fluorescent microscope. DNA fragmentation that was characteristic of the apoptotic cells was quantified by Tdt-mediated dUTP nick end labeling (TUNEL) with the Apoptosis Detection Kit, Fluorescein (Promega) according to the manufacturer's instructions. To calculate the percentage of TUNEL positive cells, we counted all of the cells from four random microscopic fields at 100× and 400× magnifications.

Western blotting Protein aliquots of 20 μg from both the treated and untreated cells were separated on 12% SDS-polyacrylamide gels. After electrophoresis, the proteins were blotted onto polivynyl-difluoride membranes (PolyScreen, Nen Life Science). The membranes were dried, preblocked with 5% non-fat milk in phosphate-buffered saline and 0.1% Tween-20, then incubated with a primary antibody for Bax or Bcl-2 (Pharmingen) diluted 1 : 2000, and detected with horseradish peroxidase-labeled antibodies to rabbit or mouse IgG. Following exposure on a Kodak OMAT x-ray film, a densitometry analysis was performed with a GS 670 Imaging Densitometer with the software Molecular Analyst (Bio Rad, Hercules, USA). The membranes were reprobed with β-actin (Sigma) antibodies as an internal control.

Immunostaining of bax The cells that were fixed on the slides were permeabilized with 0.2% Triton-X100 for 20 min on ice and blocked with 2% fetal calf serum in PBS for 2 h at 37^0C. After washing, the cells were incubated overnight with anti-Bax antibodies (Pharmingen) at a 1 : 200 dilution at 4^0C. Next, the slides were incubated with secondary antibodies that were conjugated with FITC. Following washes, the slides were visualized with a fluorescence microscope, and a densitometry analysis was performed, as described in Western blotting.

Results

Effect of SPD on cell viability We treated the MCF-7 cells with 10^{-5} to 10^{-8} M SPD. As shown in Fig. 1, SPD significantly reduced MCF-7 cell viability in a dose-dependent manner. At 10^{-6} M SPD, cell viability decreased by 50%. We then treated the non-malignant cell lines, MDBK and Changs Liver, with similar concentrations of SPD. At 10^{-6} M, the cell viability for MDBK and Changs Liver did not decrease significantly when compared to the untreated cells; therefore, SPD inhibits MCF-7 breast cancer cells without being significantly toxic to normal cells. Previously, SPD was also reported to be non-cytotoxic towards a panel of normal cell lines, but killed various breast, ovarian, and cervical carcinoma (Azimahtol Hawariah *et al*., 1998; Teoh and Azimahtol Hawariah, 1999).

SPD-induced apoptotic cell death When viewed with a phase-contrast microscope, the untreated MCF-7 cells

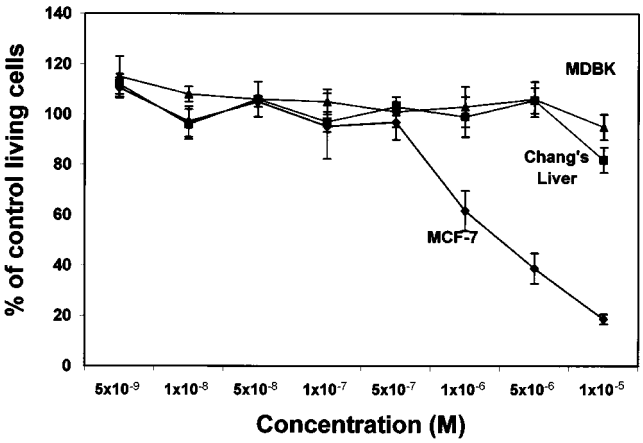

Fig. 1. Effect of SPD on cell viability. Treatment of SPD on MCF-7 cells significantly decreased the number of viable cells in a dose-dependent manner. The IC_{50} obtained was 3.0×10^{-6} M. Non-malignant Changs Liver and MDBK cells were not significantly affected by SPD and no IC_{50} was evident.

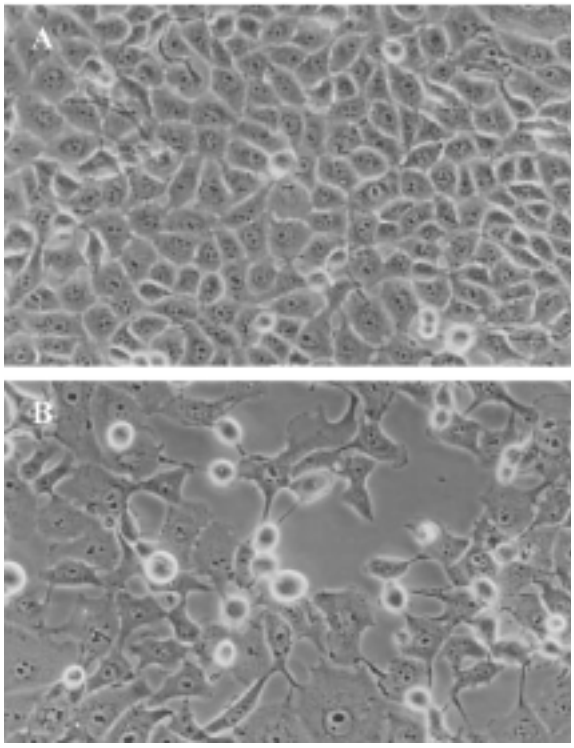

Fig. 2. Morphological characteristics of MCF-7 cells visualized with a phase-contrast microscope. (A) Confluent untreated cells at 48 h showed normal morphology. Cells remained flat with a uniform polygonal shape. (B) SPD-treated cells (10^{-6} M) at 48 h showed apoptotic morphology. Treated MCF-7 cells became irregularly shaped with elongated lamellipodia, and exhibited condensed chromatin in nuclei with occasional enucleation. There was a dramatic increase in the number of apoptotic cells rounding-up and detaching from the substrate.

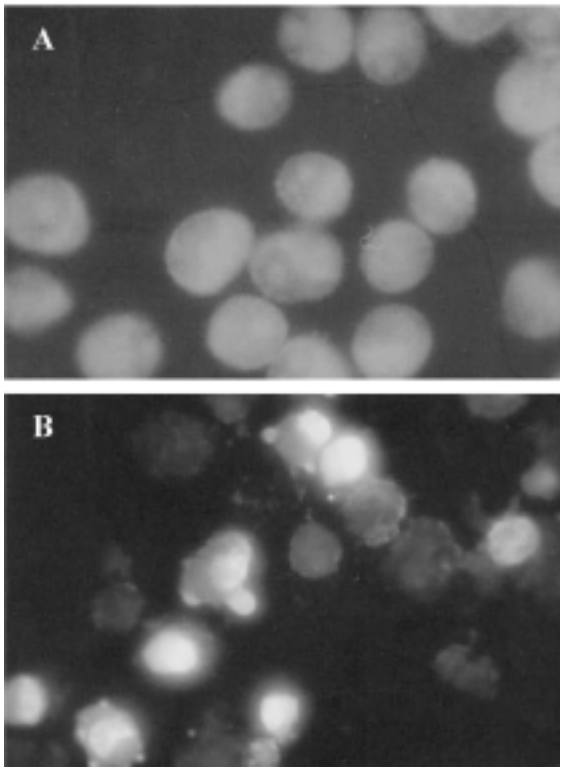

Fig. 3. Nuclear staining of MCF-7 cells with Hoechst 33258. Cells were treated with SPD (10^{-6} M) and compared to untreated controls. Nuclear morphology was observed by staining with the nuclear fluorochrome Hoechst 33258 as described in Materials and Methods. (A) Untreated control MCF-7 cells remained uniformly stained with round and unpunctuated nucleus. (B) SPD-treated MCF-7 cells showed apoptotic morphology; cell shrinkage, DNA condensation, and nuclear fragmentation.

exhibited typical growth patterns and a smooth, flattened morphology with normal nuclei (Fig. 2A). When treated with SPD, the MCF-7 cells exhibited condensed chromatin, elongated lamellipodia, and many detached cells (Fig. 2B). Lamellipodia in adherent epithelial cells contain many actin, and cross-linking, severing, and bundling proteins that are responsible for cell attachment (Majumdar *et al*., 2001). The apparent elongated lamellipodia of the SPD-treated cells may have led to the disruption of cell adhesion, thus causing the detachment of cells from the substratum and their neighbors.

Also, apparent with the SPD-treatment was a significant increase in the number of floating dead apoptotic cells, identified morphologically as round and condensed phase-bright cells. Previous studies on apoptotic morphology also report these morphological characteristics, including shrinkage of cells, condensation of nuclear material, cell membrane blebbing, apoptotic bodies, and narrowing of lamellipodia, similar to the MCF-7 cells that were treated to anti-Fas and TNF (Srinivasan *et al*., 1998). These observations provide evidence that an apoptotic pathway is occurring with the SPD treatment.

The mode of killing that is induced by most anticancer agents is by apoptotic cell death; DNA fragmentation is a hallmark of apoptotic cells (Kerr *et al*., 1972). When stained with a nuclear fluorochrome, the chromatin of the SPD-treated MCF-7 cells can be seen as condensed into lumps, thus exhibiting the punctuated morphology typical of apoptotic cells (Fig. 3). The fragmented DNA generates 3-OH DNA ends, which can be labeled with fluorescein-12-dUTP using the principle of TUNEL assay. Here, we labeled the SPD-treated cells to visualize the extent of DNA fragmentation in a time-course manner. The labeled DNA was then visualized directly by fluorescence microscopy (Fig. 4); the percentage of apoptotic cells was quantitated from the average of at least six experiments (Fig. 5). SPD treatment demonstrated an increase of the number of apoptotic cells to >75% by 24 h when compared to less than 5% in untreated cells. Previously, SPD treatment in DMBA-induced mammary tumors in Sprague-Dawley rats also exhibited similarly high levels of apoptosis in treated rats (Meenakshii *et al*., 2000).

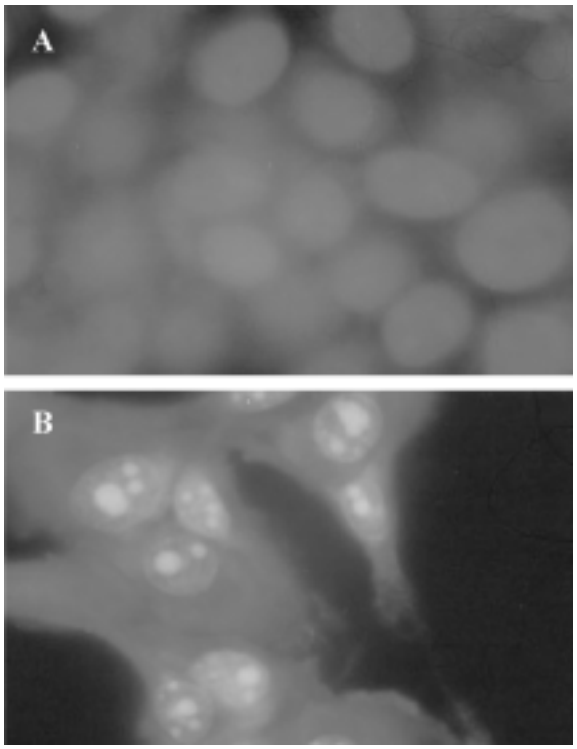

Fig. 4. TUNEL labeling of MCF-7 cells. SPD-treated and control cells were identified for DNA fragmentation by TUNEL assay as described in Materials and Methods. (A) Untreated cells. No fluorescence was detected in the nucleus, as the cells were not apoptotic and did not exhibit DNA fragmentation. (B) In cells treated with SPD at 10^{-6} M, fluorescence was detected in the nuclear region of the MCF-7 cells indicating DNA fragmentation and nuclear condensation, characteristic of apoptosis.

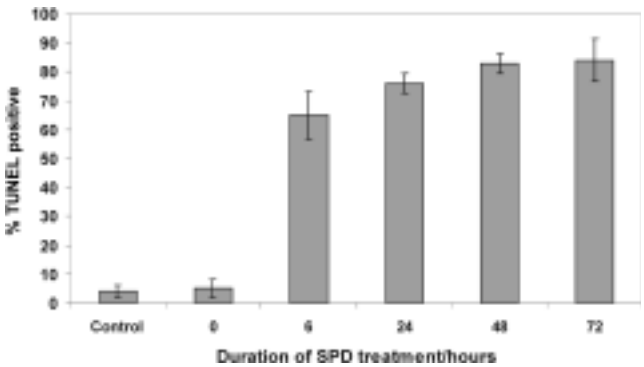

Fig. 5. Apoptosis levels as judged by TUNEL assay. SPD treatment (10^{-6} M) significantly increased the level of apoptosis in MCF-7 cells as compared to untreated controls. Augmented levels were observed till 72 h of SPD treatment as judged by Tdt-mediated dUTP nick end-labeling assay. Results are presented as the means ± SD of 6 independent experiments.

Bax protein expression was up-regulated in SPD-treated MCF-7 cells An early event in the cell that sensitizes it to apoptosis is the expression of the proapoptotic protein Bax. In some models, Bax up-regulation alone can induce the commitment of a cell to apoptosis (Xiang *et al*., 1996). Here, we detected a significant increase in the Bax expression following treatment with SPD at 10^{-6} M by immunoblotting, while the anti-apoptotic Bcl-2 levels were low throughout the treatment period (Fig. 6A). When stained with a specific Bax antibody, the fluorescence intensity in the treated cells was compared to the controls. We found that SPD dose-

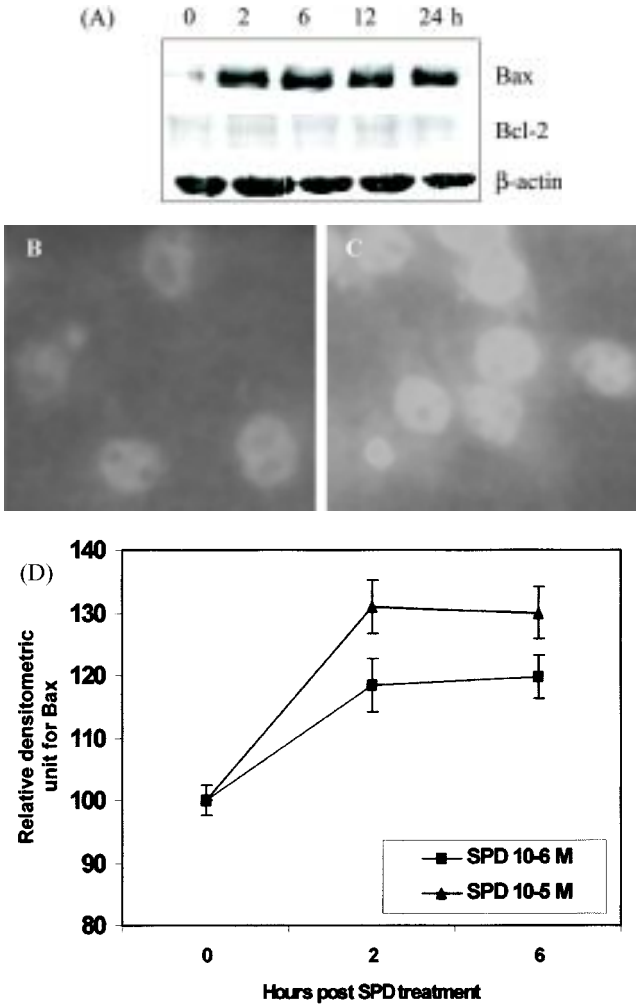

Fig. 6. Western analysis of Bax and Bcl-2 and immunostaining of Bax protein in MCF-7 cells. (A) MCF-7 cells treated with 10^{-6} M SPD for the indicated times were resolved on a 12% PAGE and submitted to Western blotting. Bax protein expression increased as early as 2 h following SPD treatment, while Bcl-2 levels were not altered and remained low throughout the experiment. (B) Bax protein was then visualized with a fluorescent microscope after immunostaining. Untreated MCF-7 cells at 0 h of SPD treatment showed a low level of Bax. (C) MCF-7 cells after 2 h treatment of SPD at 10^{-6} M exhibited a marked increase in immunofluorescence for Bax protein. (D) SPD-induced Bax protein expression increased in a dose-dependent manner as seen in immunostaining. Results are presented as the means ± SD of 3 independent experiments.

dependency increased the Bax expression, which was evident at 10^{-5} M (Fig. 6). The observed, reduced cell viability and increased levels of apoptosis, together with this marked increase in the levels of the proapoptotic Bax protein, suggested a Bax-dependent apoptotic mechanism by SPD.

Discussion

There is an increasing realization that chemotherapeutic agents act primarily by inducing cancer cell death through the mechanisms of apoptosis (Lowe and Lin, 2000). However, there are many cancers that are intrinsically resistant to apoptosis, making it vital to develop novel drugs for combination chemotherapy. In the present study, we provide evidence that a styrylpyrone derivative (SPD) compound of plant-origin induces apoptosis in the human breast cancer cell line, MCF-7. Breast cancer is the most common type of cancer effecting women. It is the number 2 killer (after lung cancer) of women aged 35-54. Approximately 20% of these cases occur in women under 30 years of age, and 70% in women over 50 years of age (Holmes *et al*., 2001).

In a large percentage of human cancers, the anti-apoptotic Bcl-2 proteins were over-expressed or pro-apoptotic Bcl-2 proteins like Bax appeared to be reduced (Constantini *et al*., 2000). These alterations in the expression of the Bcl-2 family members can render tumor cells more resistant to a wide variety of cell death stimuli including chemotherapeutic drugs (Reed *et al*., 1996). All anti-apoptotic Bcl-2-like genes are potentially oncogenic. Bcl-2 protects against diverse cytotoxic insults, including γ and UV-irradiation, cytokine withdrawal, dexamethasone, staurosporine, and cytotoxic drugs, while pro-apoptotic family members like Bax may act as tumor suppressors (Adams and Cory, 1998). An important goal in chemotherapy is, therefore, to find new cytotoxic agents that are able to increase or restore the ability of tumor cells to undergo apoptosis.

From the results in this study, we observed that the Bcl-2 protein levels in the SPD-treated cells were at a basal level and maintained a low level throughout the experiment. The Bax expression, however, increased as early as 2 h after the SPD treatment and was maintained at a markedly higher level than the controls throughout the experiment. Before the SPD treatment, the MCF-7 cells showed a low Bax protein expression. This is consistent with previous reports that breast cancer-derived cell lines show a low Bax expression (Bargou *et al*., 1996). However, when the Bax expression was up-regulated in these cells with transfected Bax cDNA, sensitivity towards apoptosis strongly increased. These researchers also found that the non-transfected MCF-7 cells, which expressed a low level of Bax, exhibited only a weak apoptotic effect, amounting to an apoptotic index of ~30%, as compared to the ~85% apoptotic cells in the MCF-7 cells that expressed high levels of Bax. They also show that in breast cancer tissue samples, no Bax signal was detected. This low Bax expression in breast cancer cell lines correlated with the resistance towards apoptosis. In contrast, the non-malignant cell lines showed a strong expression of Bax and were highly sensitive to the induction of apoptosis (Bargou *et al*., 1995).

Therefore, in the SPD-treated MCF-7 cells, the increased level of the Bax expression may play a positive role in increasing the susceptibility of these cells to apoptosis. SPD treatment resulted in massive cell death by apoptosis, which is possibly explained by the high level of the Bax protein in these cells, while Bcl-2 remains essentially unchanged. Bax is a dominant negative inhibitor of Bcl-2, and the over-expression of Bax sensitizes the MCF-7 cells to apoptosis (Sakakura *et al*., 1996). Previous studies also found that the overexpression of Bax sensitizes other non-breast-derived cells to apoptosis (Oltvai *et al*., 1993; Xiang *et al*., 1996; Zha *et al*., 1996).

Interestingly, the breast cancer patient survival rate and response to chemotherapy correlate with Bax immunostaining (Krajewski *et al*., 1995). Loss (<10%) of the Bax expression is correlated with poor chemotherapy response rates and shorter survival in women with metastatic breast adenocarcinoma. The study of Bax in tumor growth *in vivo* also found that the Bax expression led to a significant reduction in tumor growth in SCID mice that were transplanted with a Bax-expressing breast cancer cell line (Bargou *et al*., 1996). Since anticancer agents can kill tumor cells via apoptosis; therefore, the increase in the Bax protein expression may restore sensitivity to apoptotic stimuli in breast cancer cells. Here, our results provided evidence that the plant-derived SPD was able to inhibit the proliferation of MCF-7 cells, a breast cancer cell line, by inducing apoptotic cell death. SPDs ability to modulate the Bax expression by increasing the level of this proapoptotic protein further purports it as a potential anti-cancer agent in breast carcinomas, thus making it a promising agent for chemotherapy, which merits further study.

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