Primary research

Styrylpyrone Derivative (SPD) induces apoptosis in a caspase-7-dependent manner in the human breast cancer cell line MCF-7

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Abstract

Background: Styrylpyrone derivative (SPD) is a plant-derived pharmacologically active compound extracted from *Goniothalamus sp*. Previously, we have reported that SPD inhibited the proliferation of MCF-7 human breast cancer cells by inducing apoptotic cell death, while having minimal effects on non-malignant cells. Here, we attempt to further elucidate the mode of action of SPD.

Results: We found that the intrinsic apoptotic pathway was invoked, with the accumulation of cytosolic cytochrome *c* and processing of the initiator caspase-9. Cleaved products of procaspase-8 were not detected. Next, the executioner caspase-7 was cleaved and activated in response to SPD treatment. To confirm that apoptosis was induced following caspase-7 activation, the caspase inhibitor Ac-DEVD-CHO was used. Pre-incubation of cells with this inhibitor reversed apoptosis levels and caspase-7 activity in SPD-treated cells to untreated levels.

Conclusions: Taken together, these results suggest SPD as a potent antiproliferative agent on MCF-7 cells by inducing apoptosis in a caspase-7-dependent manner.

Background

Cancer is an aberrant net accumulation of atypical cells, which can arise from an excess of proliferation, an insufficiency of apoptosis, or a combination of the two [1]. The frequency of apoptosis could contribute to cell loss in tumours and promote tumour regression. Thus, in cancer therapy, the focus is on strategies that suppress tumour growth by activating the apoptotic program in the cell [2]. Evidence accumulated to this date has established that many agents of cancer chemotherapy affect tumour cell killing through launching the mechanisms of apoptosis [3]. Manifestations of apoptosis are easily discernible by the appearance of cell shrinkage, membrane blebbing, chromatin condensation, DNA cleavage, and finally, fragmentation of the cell into membrane-bound apoptotic bodies [4].

Expressed as inactive proenzymes, caspases are members of a family of cysteine proteases that play a central role in the apoptotic pathway [5]. Two major mechanisms exist that initiate the caspase cascade: the extrinsic, involving caspase-8; and the intrinsic pathway, involving caspase-9 as the apical caspase. Observations from several studies have suggested that a caspase-8 pathway can be up-regulated after drug treatment, and these include the drugs cisplatin [6], etoposide [7], doxorubicin and methothrexate

[8]. Once activated, caspase-8 is thought to activate the downstream caspases by proteolytic cleavage of their zymogen forms [9,10], thus amplifying the caspase signal. The other initiator caspase, caspase-9, controls the apoptotic response to lethal cellular insults such as ionizing radiation or certain chemotherapeutic drugs [11]. In many systems, release of cytochrome *c* from the mitochondria to cytosol has been demonstrated to be a crucial step in the activation of apoptosis [12–14]. Once released from mitochondria, cytochrome *c* acts as a co-factor and interacts with Apaf-1 and procaspase-9, which in turn activates caspase-9 [15].

The role of active caspase-8 and -9 is to generate the active forms of downstream executioner caspases, including caspase-3 and -7, by limited proteolysis, and thereby transmit the apoptotic signal to the execution phase. Activation of these executioner caspases during apoptosis results in the cleavage of critical cellular substrates, thus disabling critical homeostatic and repair enzymes as well as key

structural components that culminate in cell death [16,17].

Styrylpyrone derivative (SPD) is a pharmacologically active compound extracted from the plant *Goniothalamus sp.* of the Annonaceae family [18]. Among the species of *Goniothalamus* are *G. umbrosus*, *G. andersonii*, *G. macrophyllus* and *G. malayanus*. Previous studies on SPD suggest this bioactive compound as an antiproliferative and selective cytotoxic agent. *In vitro*, SPD was found to selectively inhibit the proliferation of several cancer cell lines without being significantly cytotoxic towards non-malignant cells [19–21]. On *in vivo* models, SPD is reported to be capable of tumoricidal and tumoristatic effects on experimental rats with mammary tumours [22]. Recent work done to elucidate SPD's mechanism of action found evidence that SPD modulates the gene expression of *Bcl-2* and *Bax* in ovarian carcinoma [20]. In breast cancer cells, SPD induces an increase of the proapoptotic Bax protein, culminating in cell death by apoptosis [21].
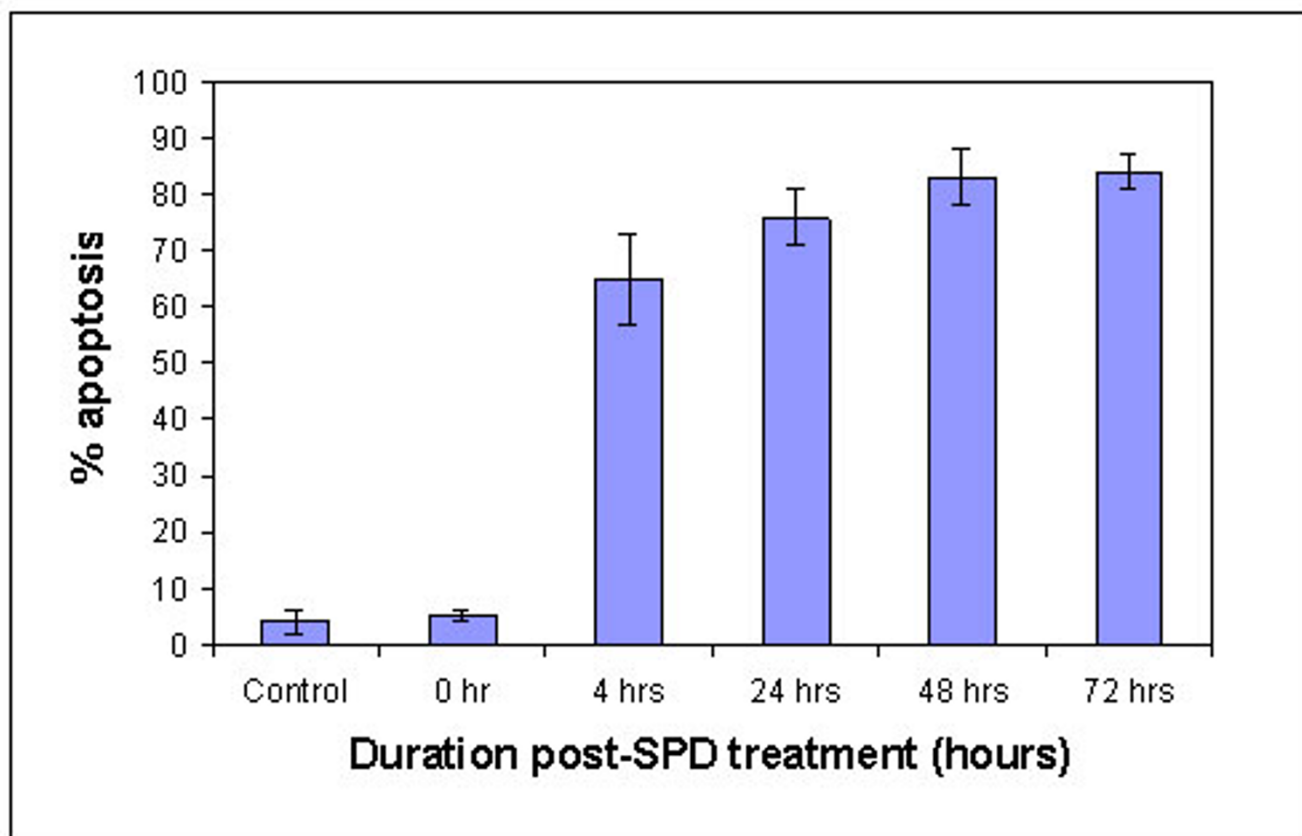

Figure 1
Apoptotic levels in SPD-treated cells. SPD treatment (10^{-6} M) significantly increased the level of apoptosis in MCF-7 cells when compared to untreated controls, as judged by apoptotic morphology by nuclear staining and DNA fragmentation by TUNEL assay described in the Experimental Procedures. Increased levels were observed till 72 hours of SPD treatment. Results were presented as the means ± SD of 6 independent experiments.

In this study, we further demonstrate the mechanism of apoptosis induced by SPD. We show that procaspase-8 was not activated in MCF-7 cells but caspase-9 activation was detected in response to SPD treatment, with the release of cytochrome *c* into the cytosol. This was followed by the activation of the executioner caspase-7. To further examine the involvement of this executioner caspase, we found that caspase-7 activity decreased and apoptosis was abrogated when SPD-treated cells were pre-incubated with the caspase-7 inhibitor, Ac-DEVD-CHO, suggesting a caspase-7-dependent apoptotic pathway induced by SPD.

Results

SPD induced apoptotic cell death
DNA condensation and fragmentation characteristic of apoptotic cells were examined and quantitated by the TUNEL assay and nuclear fluorochrome Hoechst 33258. As reported previously [21], SPD induced apoptosis in MCF-7 cells at 10^{-6} M in a time-dependent manner (Figure 1).

Procaspase-8 is not processed in SPD-treated cells
During apoptosis, initiator caspases are activated in response to proapoptotic signals [23]. By SDS-PAGE and subsequent Western blot analysis with a caspase-8 specific antibody, it was found that SPD treatment did not lead to the activation of the initiator caspase-8. Procaspase-8, expressed in two functionally active isoforms, caspase-8a and caspase-8b [24] was not processed. From immunoblotting, the two bands observed were the 55/50-kDa procaspase-8 isoforms (Figure 2), similarly reported by Sun *et al.*, [25] and Dirsch *et al.*, [26] and the active p18 subunit could not be detected. As processing of this caspase did not occur, it is possible that the other initiator caspase, caspase-9 may be involved in SPD-induced apoptosis.

Involvement of caspase-9 and cytochrome c
From immunoblot analysis, untreated MCF-7 cells exhibited the ~48-kDa proform of caspase-9. When MCF-7 cells were treated with 10^{-6} M SPD, the observed zymogen of caspase-9 slowly diminished in the course of the experiment (Figure 2). The disappearance of the procaspase-9 band reflects the processing of the zymogen to generate the active form of caspase-9, as has been interpreted in previous reports [27,28].

For activation of caspase-9, a multimeric structure termed the apoptosome is involved, consisting of cytochrome *c*, the apoptotic protease activating factor-1 (Apaf-1), and ATP or dATP [29]. Cytochrome *c* seems to be a major trigger for the assembly of this complex, and various studies have found that cytochrome *c* is released from the mitochondria into the cytosol during cell death [29–31]. When cytochrome *c* levels in the cytosol were examined, we
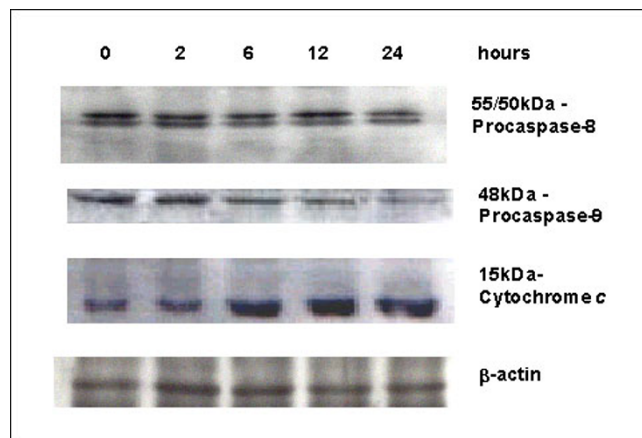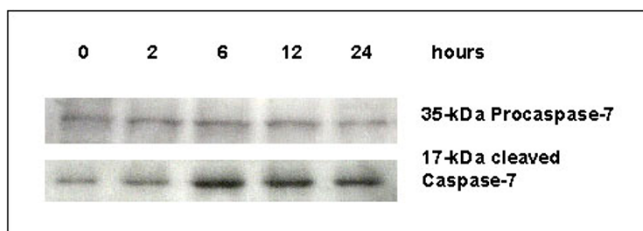
Figure 2
Western Blot analysis of apoptotic proteins in SPD-treated cells. Proteins from MCF-7 cells treated with 10^{-6} M SPD for the indicated times were resolved on 12% SDS-PAGE and submitted to Western Blotting with an anti-pro-caspase-8 antibody. Two bands were observed, corresponding to the uncleaved 55/50-kDa procaspase-8 isoforms. The active p18 subunit was not detected. Samples were also detected for procaspase-9 with an anti-procaspase-9 antibody (Clone B40). The anti-caspase-9 antibodies recognized the proenzyme; and the decrease of this band indicated activation of caspase-9. When proteins from cytosolic fractions of MCF-7 cells treated with 10^{-6} M SPD were resolved on 15% SDS-PAGE and submitted to immunoblotting with the cytochrome *c* antibody (Clone 7H8.2C12), increasing amounts of cytochrome *c* were detected in the cytosol in a time-dependent manner. All blots were then washed and reprobed with β-actin to confirm equal loading.

detected increasing levels in the SPD-treated MCF-7 cells (Figure 2). Untreated control cells did not exhibit similar high levels of cytochrome *c*, indicating that the release of cytochrome *c* from the mitochondria into the cytosol was an effect of SPD treatment.

The "executioner", caspase-7, is activated in SPD-induced apoptosis
The role of the initiator caspase-9 is to generate the active forms of executioner caspase-3 and -7 by limited proteolysis, and thereby transmit the apoptotic signal to the execution phase. Here, we used the caspase-3-deficient MCF-7 cell line. As with previous reports [32,33], caspase-3 activity was not detected (data not shown). Immunoblot analyses of lysates obtained from MCF-7 cells treated with SPD at 10^{-6} M found that caspase-7 was cleaved to the 17-kDa fragment required for its activation (Figure 3). When the activity of caspase-7 was assayed, SPD-treated cells showed increase in activity compared to untreated controls (Figure 4). To confirm that the SPD-induced
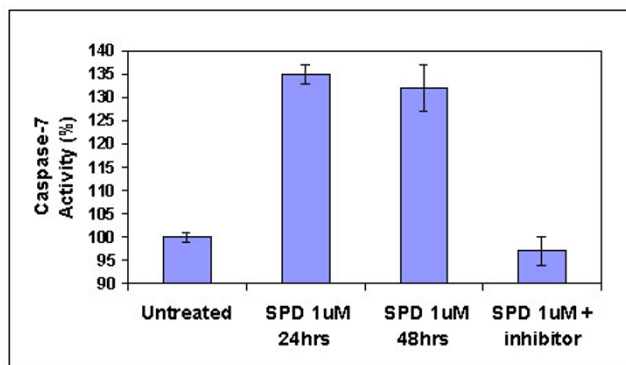
Figure 3
Cleavage of procaspase-7 after SPD treatment. Proteins from MCF-7 cells treated with 10^{-6} M SPD for the indicated times were resolved on a 15% PAGE and submitted to Western Blotting using a monoclonal antibody against caspase-7 (B94-1), which recognizes the pro- (35-kDa) and the active (17-kDa) forms of caspase-7. Following SPD treatment, the caspase-7 proenzyme was cleaved, generating the catalytically-active 17-kDa fragment.

Figure 4
Activation of caspase-7 after SPD-treatment. The activity of caspase-7 was measured with a Colorimetric Assay Kit (Chemicon) that recognizes cleavage of the sequence DEVD by active caspase-7. Caspase-7 activity increased when cells were treated with SPD, indicating the catalytic activation of this executioner caspase. When MCF-7 cells were incubated with the caspase-7 inhibitor, DEVD-CHO, prior to SPD treatment, caspase-7 DEVDase activity diminished.

apoptotic cell death was due to the involvement of caspase-7, cells were also treated with SPD in the presence of the specific inhibitor of caspase-7, Ac-DEVD-CHO [34]. SPD-treated cells preincubated with the inhibitor exhibited repressed caspase-7 DEVDase activity. Also, preincubation of MCF-7 cells with this inhibitor at 50 μM to 100 μM inhibited apoptosis and brought apoptotic levels down to the level similar to controls (Figure 5), thus purporting an apoptotic pathway dependent on caspase-7.

Discussion

There is an increasing realization that chemotherapeutic agents act primarily by inducing cancer cell death through the mechanisms of apoptosis [35]. 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 compound of plant-origin, SPD, may be a promising new anticancer agent for human breast cancers.

Previously, we have shown that MCF-7 cells treated with SPD displayed elevated levels of apoptosis and a marked increase in the expression of the proapoptotic Bax protein [21]. In addition to the loss of viability, Bax expression produces other typical manifestations leading to apoptosis, namely caspase activation [5]. Here, we found that caspase-9 was activated, together with accumulation of cytochrome *c* in the cytosol. Caspase-9 can activate downstream executioner caspases including caspase-7, which is termed caspase-3-like due to its similarity in specificity with caspase-3. Caspase-3-like activity has been detected in the apoptosis induced by various chemotherapeutic drugs [36]. Caspase-3 deficiency in MCF-7 is due to a deletion mutation in exon 3 of the gene [32,33]. Previous studies on MCF-7 with exogenously-expressed cas-

pase-3 indicates that caspase-3 plays an important role in apoptotic pathways [25,32,33,37]. Studies using etoposide and doxorubicin, active chemotherapeutic agents and key adjuvant drugs for breast cancer treatment, concluded that MCF-7 cells were sensitized to apoptosis only when these cells were reconstituted with caspase-3 [38]. Chemoresistance is often caused by aberrant apoptosis that in some instances has been related to defects in caspase activation [39,40]. Given the importance of caspase-3 in apoptotic execution, it is then postulated that caspase-3 deficiency might significantly contribute to chemotherapeutic resistance.

In our studies, we observed manifestations of apoptosis in SPD-treated MCF-7 cells. Previous reports by Hishikawa and colleagues [41] and Heerdt *et al.*, [42] have also demonstrated similar apoptotic hallmarks in MCF-7 cells when induced with connective tissue growth factor (CTGF) and tributyrin, respectively. These suggest that the mechanism for induction of apoptosis is present and functional in MCF-7 cells, but is dependent on the external stimuli. Caspase-7 is highly related to caspase-3 and shows the same synthetic substrate specificity *in vitro* [43] suggesting that caspase-3 and -7 have possibly overlapping roles in apoptosis [44]. Without caspase-3, SPD-treated MCF-7 cells may utilize an alternate caspase pathway to affect cell death [45]. Here, we demonstrated that caspase-7 was activated in SPD-induced apoptosis. In MCF-7 cells treated with SPD, the 35-kDa proenzyme was
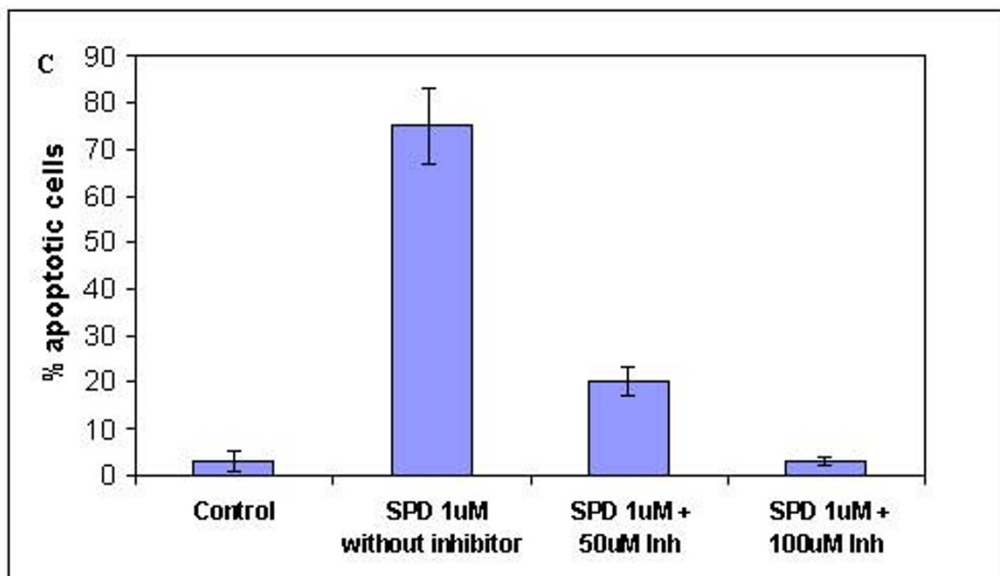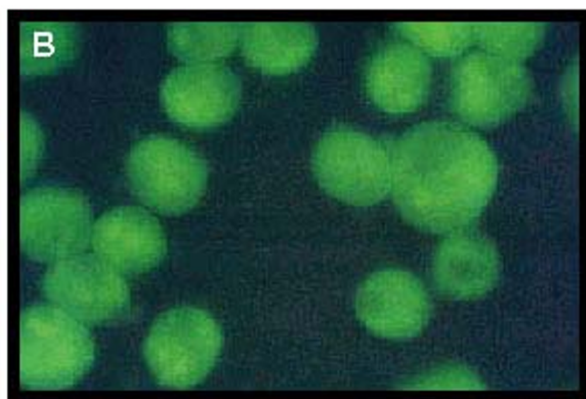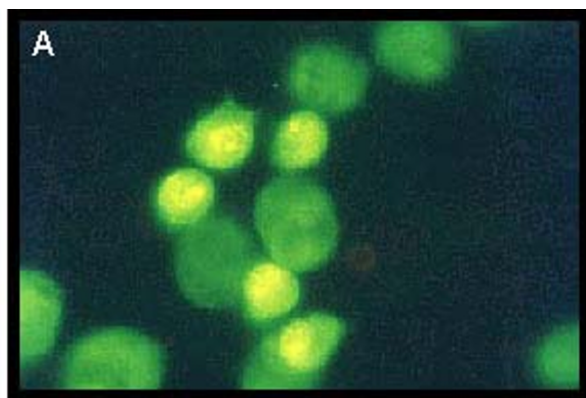
Figure 5
Apoptosis inhibited by DEVD-CHO. When MCF-7 cells were incubated with the caspase-7 inhibitor, DEVD-CHO at (A) 50 μM and (B) 100 μM, prior to SPD treatment, apoptosis levels decreased to control untreated levels as detected by nuclear staining, suggesting the important role played by this executioner caspase in SPD-induced apoptosis. (C) Results were presented as the means ± SD of 3 independent experiments.

cleaved into its active 17-kDa subunit. Synthesized as inactive precursors, caspases must be proteolytically cleaved to become active enzymes [16]. Overexpression of full-length caspase-7 in the MCF-7 does not induce apoptosis, whereas the activated 17-kDa subunit induces apoptotic cell death [46].

Activation of executioner caspases, caspase-3 or -7 results in the cleavage of critical cellular substrates and homeostatic enzymes, bringing about the manifestations of apoptosis [16,23]. Experimental inhibition of apoptosis by peptide caspase inhibitors presents the opportunity to investigate the importance of this protease family. When MCF-7 cells were preincubated with the caspase-7 inhibitor Ac-DEVD-CHO before treatment with SPD, apoptosis levels decreased to a level similar to controls. Cell death was thus inhibited and treated cells had morphology similar to controls, further supporting the involvement of caspase-7 in SPD-induced apoptosis.

Previous reports have found that MCF-7 cells are relatively insensitive to many chemotherapeutic agents due to the absence of caspase-3 [38]. Our studies here have shown that the mechanism for apoptosis is functional in MCF-7 and SPD is able to induce an alternate caspase pathway, possibly via caspase-7. Tumours accumulate mutations that increase their resistance to apoptotic inducers; *e.g.* abrogation of caspase-3 has been associated with acquired multidrug resistance [47]. Therefore, finding new therapeutic agents that induce tumour cell apoptosis in a manner independent of caspase-3 may have important clinical implications. By not requiring caspase-3, SPD may evoke an apoptotic pathway different from clinical oncology drugs such as doxorubicin and etoposide [38], thus making it a promising agent for combination chemotherapy that merits further study.

Methods
Cell culture
MCF-7 human mammary carcinoma cells were obtained from the American Type Culture Collection (ATCC) and maintained in DMEM supplemented with 10% fetal bovine serum and 2 mM glutamine. Styrylpyrone derivative (SPD) was isolated from the bark of *Goniothalamus umbrosus* as described previously [48].

Apoptotic index and nuclear morphology
Staining with Hoechst 33258 was done as described previously [41]. Briefly, cells were treated with SPD at 10^{-6} M for various incubation times. For inhibitor studies, cells were incubated with the caspase-7 inhibitor, Ac-DEVD-CHO (N-Acetyl-Asp-Glu-Val-Asp-al) (Sigma-Aldrich) 1 h prior to SPD treatment. After treatment periods, floating and trypsinized adherent cells were collected and washed with phosphate-buffered saline (PBS). Cells were then

fixed with 4% paraformaldehyde for 30 min. After washing, cells were incubated in the nuclear fluorochrome 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 and apoptotic cells were counted. DNA fragmentation characteristic of 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, four random microscopic fields at 100× and 400× magnifications were taken, and calculations were based on at least 6 independent experiments.

Isolation of cytosolic fractions
Cytosolic extracts were prepared as previously described [13]. Briefly, treated cells were harvested by centrifugation and washed with ice-cold phosphate-buffered saline and resuspended in 5 volumes of extraction buffer containing 250 mM sucrose. Cells were homogenized and the homogenates were centrifuged twice at 750 × g for 10 min at 4°C. The supernatant was then centrifuged at 10,000 × g for 15 min at 4°C, and the resulting mitochondrial pellets were discarded. The supernatant was then dissolved in electrophoresis sample buffer and used for Western blotting.

Protein extraction for caspases
As previously described [49], cells were scraped with a rubber policeman after incubation in extraction buffer and put on ice. Cells were then submitted to 3 freeze-thaw cycles and centrifuged at 10,000 rpm for 20 min at -4°C. Supernatant was collected and added with 1:1 sample buffer and boiled at 95°C for 5 min. After protein concentration was determined by standard procedures, protein aliquots of 20 μg were applied to 12% or 15% SDS-polyacrylamide gels for separation.

Western Blotting
After electrophoresis, proteins were blotted onto polivynyl-difluoride membranes (PolyScreen, Nen Life Science). 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 caspase-8, caspase-9 (Clone B40), caspase-7 (Clone B94-1) or cytochrome *c* (Clone 7H8.2C12) (all from Pharmingen), and detected with horseradish peroxidase-labeled antibodies to rabbit or mouse IgG. Following exposure on a Kodak BIOMAX x-ray film, densitometry analysis was done with a GS 670 Imaging Densitometer with the software Molecular Analyst (Bio Rad). Blots were stripped with Re-Blot Plus (Chemicon) before reprobing with β-actin antibody to determine equal loading.

Caspase-7 activity assay

Caspase-7 activity in the caspase-3-deficient MCF-7 cells [32,33] was assayed with the Colorimetric Assay Kit (Chemicon International, USA) that provides a means to assay the activity of caspases that recognize the sequence DEVD. The tetrapeptide DEXD is the optimal recognition motif for caspase-3 and caspase-7 [23]. The assay was done according to the manufacturer's instructions. Briefly, MCF-7 cells were treated with SPD at 10^{-6} M for 24 and 48 h. After the treatment period, cells were counted and then pelleted at 1,500 rpm for 10 min. Cells were then resuspended in chilled Cell Lysis Buffer and incubated on ice before centrifugation at 10,000 × g for 5 min. The supernatant (cytosolic extract) was then transferred to a fresh microcentrifuge tube and put on ice. The protein concentration for each sample set was then assayed using standard protocols. Assay mixture was prepared in a 96-well plate and mixed with Assay Buffer, dH_2O and the caspase substrate, Ac-DEVD-pNA, and incubated at 37°C for 1.5 h. For inhibitor studies, the sample was pre-incubated with the caspase-7 inhibitor, Ac-DEVD-CHO, for 10 min at room temperature before adding the substrate solution. After incubation, samples were read with a Dynex MRX microtiter plate reader at 405 nm. Increase in caspase-7 activity was then determined by comparing the OD reading from the SPD-treated samples with the level of the untreated control.

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