

Sulfated polysaccharide-protein complex sensitizes doxorubicin-induced apoptosis of breast cancer cells *in vitro* and *in vivo*

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Abstract. The present study aimed to investigate the effect of sulfated polysaccharide-protein complex (SPPC) on the anti-tumor effect of doxorubicin (Dox) on MDA-MB-231 breast cancer cells *in vitro* and *in vivo*. MTT and Annexin V/propidium iodide staining assays demonstrated that SPPC selectively sensitized MDA-MB-231 cells to Dox-induced cytotoxicity. The half maximal inhibitory concentration of Dox against MDA-MB-231 cells was decreased from 5.3 to 1.5 μ M when it was used concomitantly with 5 μ M SPPC. SPPC potentiated Dox-induced apoptosis in breast cancer cells via the mitochondrial apoptosis signaling pathway by activating caspase-3 and caspase-9. Notably, the caspase inhibitor Z-VAD-fmk diminished the effect of SPPC on Dox-mediated apoptosis. Furthermore, combination treatment with SPPC and Dox markedly reduced the growth of breast cancer xenografts in mice. The present study demonstrated that SPPC was able to enhance the antitumor effect of Dox on breast cancer cells, thus suggesting that SPPC may be used to reduce the cumulative dose of Dox and its associated toxicities in the chemotherapy of breast cancer and other types of cancer.

Introduction

The chemotherapeutic agent doxorubicin (Dox), which is a cytotoxic anthracycline antibiotic isolated from *Streptomyces peuxetus* (1), has been widely used in the clinical treatment of a broad spectrum of cancers (2). The mechanism underlying the antitumor effect of Dox has been

associated with its ability to induce the apoptosis of cancer cells (3). In the majority of cells, caspase-dependent apoptosis is induced via the activation of either the mitochondrial (intrinsic) signaling pathway or the death receptor (extrinsic) signaling pathway (4). Unfortunately, Dox exhibits cytotoxic effects on a wide range of cells, as well as cancer cells (5). In addition, Dox may be fatal in animals as it damages several organs, including the heart, bones and kidneys (6). The clinical application of Dox has been limited due to association of cardiomyopathy and heart failure with Dox usage (7). The severity of cardiac damage is typically proportional to the cumulative dose of Dox in a patient (8). Therefore, it is not possible to increase the antitumor potency of Dox by increasing the dose of Dox due to its adverse effects.

Breast cancer is one of the most common types of cancer and is the fourth leading cause of cancer-associated mortality worldwide (9). Since the 1970s, Dox has been considered one of the most effective agents for the treatment of advanced breast cancer (10). However, recent studies have demonstrated that numerous cancer cell types, including breast cancer cells, are resistant to the apoptosis-inducing effects of Dox (11-13). Therefore, the identification of sensitizing agents that are able to increase the potency of Dox at low doses with clinically acceptable adverse effects may help to improve the treatment of breast cancer.

Laminaria japonica is a medicinal and dietary brown algae plant, whose medicinal value has been recorded in ancient Chinese books, including the Compendium of Materia Medica and Jiayou Materia Medica (14). Sulfated polysaccharide-protein complex (SPPC) is a cellular interstitial proteoglycan complex produced by *L. japonica*. Previous studies have demonstrated that SPPC has unique antitumor biological activities (15,16), and that numerous sulfated glycoproteins and sulfated polysaccharides are able to inhibit the proliferation and metastasis of tumor cells (17-19). In a study on the regulatory effect of sulfated polysaccharides on macrophage-induced tumor cell apoptosis, it was demonstrated that sulfated polysaccharides produced by *L. japonica* were able to indirectly enhance the cytotoxic effect of macrophages on HepG2 cells (16). However, the majority of previous studies have focused on the ability of SPPC to

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promote tumor cell apoptosis and inhibit tumor cell proliferation and metastasis when used alone (20,21); few studies have addressed whether SPPC has a synergistic effect when used simultaneously with other chemotherapeutic agents, such as Dox, with a view to clinically alleviate their toxicities. Therefore, the present study aimed to investigate the effect of SPPC on Dox-induced apoptosis of the MDA-MB-231 breast cancer cell line *in vitro* and *in vivo*.

Materials and methods

Reagents. Dox, Hoechst 33258, z-VAD-fmk and MTT were purchased from Sigma-Aldrich (St. Louis, MO, USA). Molecular Probe JC-1 dye was obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). SPPC of 99% purity was purchased from Shanghai Traditional Chinese Medicine Co., Ltd. (Shanghai, China) and was dissolved in phosphate-buffered saline (PBS) to 10 mM as stock solution.

Cell lines and culture. The MDA-MB-231 human breast cancer cell line and the Hs578Bst normal human mammary cell line were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured to 5×10^5 cell density in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, penicillin and streptomycin (all Gibco; Thermo Fisher Scientific, Inc.). Cells in culture flasks were incubated in 5% CO₂ at 37°C.

MTT assay. Cells were plated on 96-well plates at a density of 5×10^3 cells/well and incubated at 37°C overnight, followed by incubation at 37°C with various doses of SPPC (0–60 μ M) in complete MEM for 24 or 48 h. Following treatment, the medium was replaced and the cells were incubated at 37°C with 0.5 mg/ml MTT in complete MEM for 4 h. Hs578Bst normal human mammary cells incubated with various doses of SPPC (0–6 μ M) in complete MEM for 24 or 48 h served as a control. Dimethyl sulfoxide (DMSO; 0.1%) was used as a negative control. Viable cells converted MTT to formazan, generating a blue-purple color when dissolved in DMSO. The intensity was measured at 570 nm using a ELx800 Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). The relative percentage of viable cells was calculated by dividing the absorbance of SPPC-treated cells by that of the control in each experiment.

Annexin V/propidium iodide (PI) staining assay. Apoptosis was assessed by measuring the membrane redistribution of phosphatidylserine using an Annexin V-Fluorescein Isothiocyanate (FITC) Apoptosis Detection kit (BD Pharmingen, San Diego, CA, USA), according to the manufacturer's protocol. Briefly, cells were incubated with 1 μ M DOX, 5 μ M SPPC or both for 24 h. (22,23), and the cells were collected and washed twice with PBS followed by resuspension in 500 μ l staining solution containing FITC-conjugated Annexin V antibody (5 μ l) and PI (250 μ g/ml stock solution). Following incubation at 37°C in the dark for 15 min, the cells were analyzed by flow cytometry. Basal apoptosis and necrosis rates were identically determined for the untreated cells. The percentage of cells undergoing apoptosis was determined as an average of three independent experiments.

Hoechst 33258 staining. The MDA MB 231 and Hs578Bst cells following incubation with 1 μ M DOX, 5 μ M SPPC or both for 24 h. Following treatment, the cells were washed with isotonic PBS (pH 7.4) and then fixed in 4% paraformaldehyde solution in PBS for 1 h at 37°C. Subsequently, the nuclei were stained with 2 μ g/ml Hoechst 33258 for 10 min at room temperature and morphological changes to the nuclear chromatin were observed under a fluorescent microscope (Nikon Eclipse TE2000-U; Nikon Corporation, Tokyo, Japan).

Caspase enzyme activity. Following treatment, the cells were washed with PBS and lysed using cell lysis buffer provided in the caspase-3, -8 and -9 Assay kits (Sigma-Aldrich). Samples were incubated on ice for 10 min and centrifuged in a microcentrifuge at 12,000 \times g for 5 min at 4°C to precipitate the cellular debris. The activities of caspase-3, -8 and -9 in the supernatant were measured using the kits and a spectrophotometer at 450 nm, according to the manufacturer's protocol. Briefly, the MDA MB 231 cells were treated with 1 μ M DOX and 5 μ M SPPC, or SPPC only for 24 h before the assay. The activities of caspase 3, -8 and -9 in the MDA MB 231 cells prior to and following co-treatment with SPPC were assessed using colorimetric assay kits (K106-100, K113-100 and K119-200, BioVision, Inc., Mountain View, CA, USA) according to the manufacturer's instructions. Briefly, the cells were harvested and incubated in ice-cold cell lysis buffer for 30 min on ice. The supernatants were collected, and the proteins from each sample were incubated with the appropriate caspase substrate. After 4 h incubation at 37°C, the protease activity was determined at 405 nm using a microplate spectrophotometer (xMark Microplate Absorbance Spectrophotometer, Bio-Rad Laboratories, Inc., Hercules, CA, USA). Caspase activity was normalized to the cell lysate protein and expressed as fold activation compared with the control.

Cell observations by scanning electron microscopy. Following treatment, the cells were sequentially fixed in 2.5% glutaraldehyde solution and 1% osmium solution, dehydrated in graded alcohol, placed in tert-butanol for it to infiltrated the samples, and were then frozen at 0°C for 12 h, followed by observation under a scanning electron microscope (S-2300; Hitachi High-Technologies Corporation, Tokyo, Japan). The MDA MB 231 cells were treated with 1 μ M Dox, 5 μ M SPPC or SPPC only for 24 h. The loss of the MMP was assayed by JC 1 staining. For JC-1 staining, cells were stained with JC 1 (5 μ g/ml, T4069, Sigma-Aldrich) in the dark for 10 min at 37°C, washed with culture media and observed under a confocal laser scanning microscope.

Human tumor xenograft experiment. The present study was performed in strict accordance with the recommendations outlined in the Guide for the Care and Use of Laboratory Animals of Fudan University (Shanghai, China). The protocol was approved by the Committee on the Ethics of Animal Experiments of Fudan University. A total of 20 female nude athymic BALB/c mice aged 4–6 weeks were obtained from the Fudan University Animal Center (Shanghai, China). The mice were housed under standard and enriched conditions

for six weeks at $21\pm1^{\circ}\text{C}$ and $50\pm5\%$ humidity in controlled conventional colony rooms and under a reversed 12:12 h light:dark with water and standard rodent pellets *ad libitum*. *In vitro* cultured MDA-MB-231 cells (5×10^6 in 200 μl PBS volume) were injected subcutaneously into the right supra scapula region of the mice. On day 12 post-inoculation, when the tumors had grown to a mean volume of 150 mm^3 , the mice were equally randomized into four groups, as follows: i) Normal saline (NS) group (100 μl); ii) 40 mg/kg SPPC group; iii) 4 mg/kg Dox group; and iv) 4 mg/kg Dox plus 40 mg/kg SPPC group. SPPC was administered via intraperitoneal injection three times a week, and Dox was administered via intraperitoneal injection once a week for 3 weeks. Saline was administered to the NS group, which was considered as the control group, via intraperitoneal injection three times a week. The tumor volume was determined using a caliper twice a week and was estimated using the following formula: Tumor volume = length \times width²/2. At the end of the experiment after 24 days, the mice were sacrificed by CO_2 asphyxiation and the tumors were weighed to assess treatment efficacy. Tumor tissue samples were fixed in 10% formal saline for 24 h, paraffin embedded, sliced into 4 mm sections, stained with Harris hematoxylin and eosin and evaluated for any structural changes under a bright field microscope. Standard immunoperoxidase procedures (24) were used to visualize proliferating cell nuclear antigen (PCNA) in the tumor samples.

Statistical analysis. Data are presented as the mean \pm standard deviation. Statistical analyses were performed using SPSS 19.0 software for Windows (IBM SPSS, Armonk, NY, USA). The least significant difference test for multiple comparisons was used to detect whether there was any significant difference between the different treatment groups. Student's two-tailed t-test was used to evaluate the differences between two groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of SPPC on the growth of breast cancer cells and normal mammary cells. To determine the effects of SPPC on breast cancer cells and normal mammary cells, MDA-MB-231 breast cancer cells and Hs578Bst normal mammary cells were treated with various concentrations of SPPC (0-60 μM) for 24 or 48 h. Cell viability was assessed using MTT assays. As shown in Fig. 1, SPPC inhibited the growth of MDA-MB-231 cells in a dose- and time-dependent manner, and the half maximal inhibitory concentration (IC_{50}) was 25.4 and 14.2 μM for 24 and 48 h treatment, respectively. Conversely, SPPC did not affect the growth of Hs578Bst cells after 24 or 48 h, even at high concentrations ($>60\text{ }\mu\text{M}$). These results suggest that SPPC specifically induces cytotoxicity in breast cancer cells, without affecting normal mammary cells.

SPPC potentiates the anti-cancer effect of Dox in human breast cancer cells. To determine whether low-dose SPPC is able to enhance the anti-tumor effect of Dox, MDA-MB-231 cells were treated with 5 μM SPPC plus various doses of Dox for 24 h. Dox treatment was observed to induce breast cancer

cell death, and this effect of Dox was markedly increased by SPPC treatment. The IC_{50} of Dox against MDA-MB-231 cells was decreased from 5.3 to 1.5 μM when Dox was used concomitantly with 5 μM SPPC (Fig. 2A). In a previous study, the peak and the steady-state concentrations of Dox in human plasma were reported to be 5 μM and 25-500 nM, respectively (7); thus, 1 μM Dox was selected in the present study as representative of the plasma levels in patients treated with Dox.

Whether SPPC-mediated anti-cancer activity was associated with its ability to induce apoptosis was investigated. Flow cytometric analysis showed that 1 μM Dox increased the percentage of apoptotic MDA-MB-231 cells to 16% and co-treatment with SPPC increased it to 48%. Conversely, the percentage of apoptotic Hs578Bst cells was 11.6% following Dox treatment and 10.9% following co-treatment with SPPC (Fig. 2B).

Hoechst 33258 staining was used to assess morphological changes to the MDA-MB-231 and Hs578Bst cells following treatment. Following co-treatment with 5 μM SPPC and 1 μM Dox for 24 h, morphological changes that are characteristic of apoptosis, including chromatin condensation, nuclear fragmentation and apoptotic body formation, were observed in MDA-MB-231 cells. At the same time, some apoptotic nuclei were observed in cells treated with SPPC or Dox alone (Fig. 2C). These results suggest a preferential potentiation effect of SPPC on Dox-mediated cytotoxicity in breast cancer cells.

SPPC enhances Dox-induced caspase activation. The activities of caspase-3, caspase-8 and caspase-9 in MDA-MB-231 cells prior to and following co-treatment with SPPC were assessed using colorimetric assays. As shown in Fig. 3A-C, the activities of caspase-3/8/9 were increased in MDA-MB-231 cells exposed to 5 μM SPPC for 24 h. Dox-induced apoptosis has been associated with two distinct apoptosis pathways: The death receptor pathway via activation of caspase-8; and the mitochondrial pathway via activation of caspase-9 (25,26). Dox treatment increased the activities of caspase-3/8/9 to 7.3, 3.2 and 5.4-fold above control cells, respectively. SPPC-induced apoptosis has been associated with the mitochondrial signaling pathway via activation of caspase-9 (27,28). Co-treatment with Dox and SPPC resulted in markedly higher ratios of caspase-3 and caspase-9 activity, which were 13.1 and 8.9-fold that of controls. However, SPPC was not shown to increase Dox-induced caspase-8 activation. To further define the role of caspases in SPPC potentiated Dox-induced apoptosis, cells were treated with the broad-spectrum caspase inhibitor, z-VADfmk (100 μM), for 2 h prior to treatment with SPPC and/or Dox and apoptosis was detected 24 h following treatment. As shown in Fig. 3D, apoptosis induced by SPPC and Dox was markedly attenuated by pretreatment with z-VAD-fmk. These results suggest that SPPC sensitizes Dox-induced apoptosis via a caspase-dependent signaling pathway.

SPPC enhances the Dox-induced loss of the mitochondrial membrane potential (MMP). In the present study, Dox and SPPC co-treatment markedly enhanced caspase-9 activation but not caspase-8, thus suggesting that SPPC may sensitize Dox-induced apoptosis predominantly via the mitochondrial

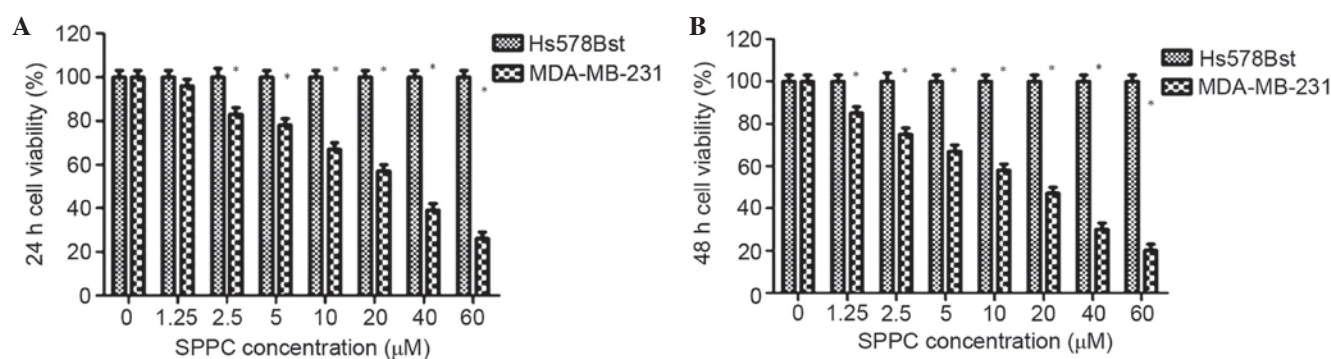


Figure 1. Effect of SPPC on the proliferation of MDA-MB-231 breast cancer cells and Hs578Bst normal human mammary cells. Cells were incubated with various concentrations of SPPC (0-60 μ M) for (A) 24 or (B) 48 h, after which cell proliferation was assessed using the MTT assay. Data are presented as the mean \pm standard deviation of three independent experiments. SPPC, sulfated polysaccharide-protein complex. * $P < 0.05$ vs. the Hs578Bst normal human mammary cell line.

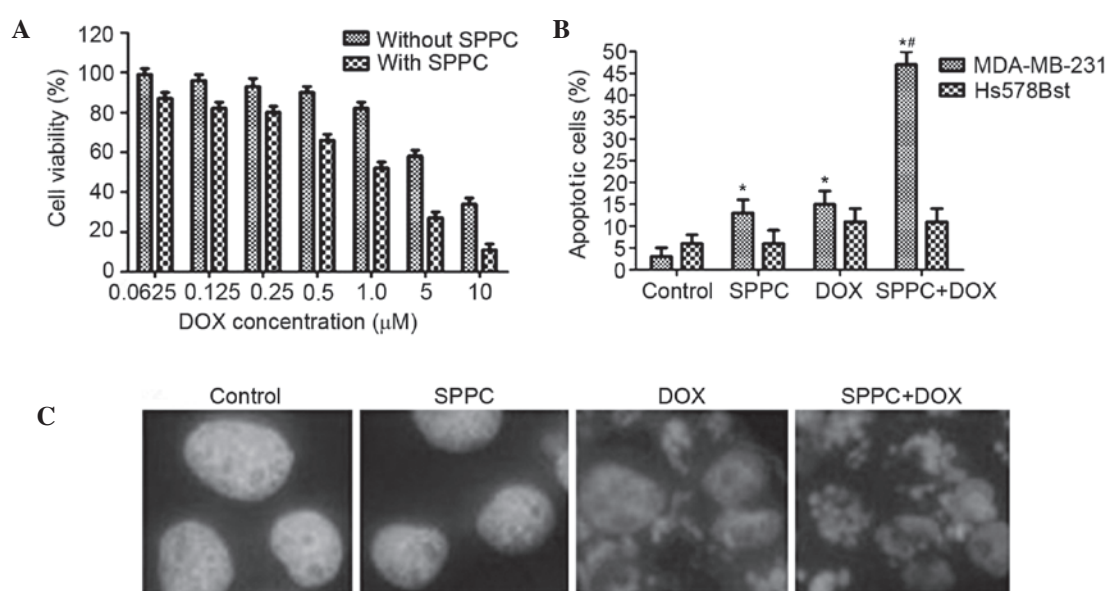


Figure 2. SPPC selectively potentiates Dox cytotoxicity in breast cancer cells. (A) MDA-MB-231 cells were incubated with various doses of DOX (0-10 μ M) with or without 5 μ M SPPC for 24 h, and then analyzed using the MTT assay to assess cell proliferation. (B) MDA-MB-231 and Hs578Bst cells were incubated with 1 μ M DOX and/or 5 μ M SPPC for 24 h, and then analyzed by flow cytometry using Annexin-V/propidium iodide double-staining to determine the percentage of apoptotic cells. (C) Apoptotic cells were photographed by scanning electron microscopy (magnification, $\times 200$). Data are presented as the mean \pm standard deviation of three independent experiments. * $P < 0.05$, vs. the control group. # $P < 0.05$, vs. the SPPC alone and DOX alone groups. SPPC, sulfated polysaccharide-protein complex; DOX, doxorubicin.

pathway. In the mitochondrial pathway, a loss of MMP precedes caspase-9 activation (29). Therefore, the present study assessed whether there was a loss of the MMP during combined treatment using the cationic lipophilic probe, JC-1. Quantitative measurement demonstrated that MDA-MB-231 cells treated with SPPC alone underwent significant dissolution, as compared with the control. Dox treatment resulted in a rapid dissipation of the MMP (Fig. 4); however, a significant decrease in the fluorescence was observed following co-treatment with SPPC. These results suggest that SPPC sensitizes breast cancer cells to Dox-induced apoptosis via the mitochondrial apoptosis pathway.

In vivo antitumor activity of Dox and SPPC. In order to confirm the anticancer activity of combined SPPC and Dox treatment on human breast cancer cells, an *in vivo* experiment was performed using an MDA-MB-231 cell-xenografted mouse model. Male nude mice inoculated with human

MDA-MB-231 cells were divided into four groups 12 days post-tumor cell inoculation. At the end of the experiment, the mean tumor volume was 1,687 mm³ for the control group and 661 mm³ for the Dox-treated group, which was significantly reduced ($P < 0.05$; Fig. 5A). Furthermore, SPPC induced regression of MDA-MB-231 tumors. The tumor growth inhibitory rate was 58.8% in the 40 mg/kg SPPC-treated mice, as compared with the control group ($P < 0.05$). Notably, our results demonstrated a significant reduction in tumor volume from day 4 post-treatment to the end of the experiment in the mice co-treated with SPPC and Dox, as compared with the control group. The tumors in the co-treatment group grew at a slower rate after co-treatment, from the mean volume of 201 mm³ in the beginning to 399 mm³ in the end. The mean tumor weight was 523 mg for the control group versus 315 mg for the Dox only group, 358 mg for the SPPC only group and 89 mg for the Dox plus SPPC group (Fig. 5B). These results

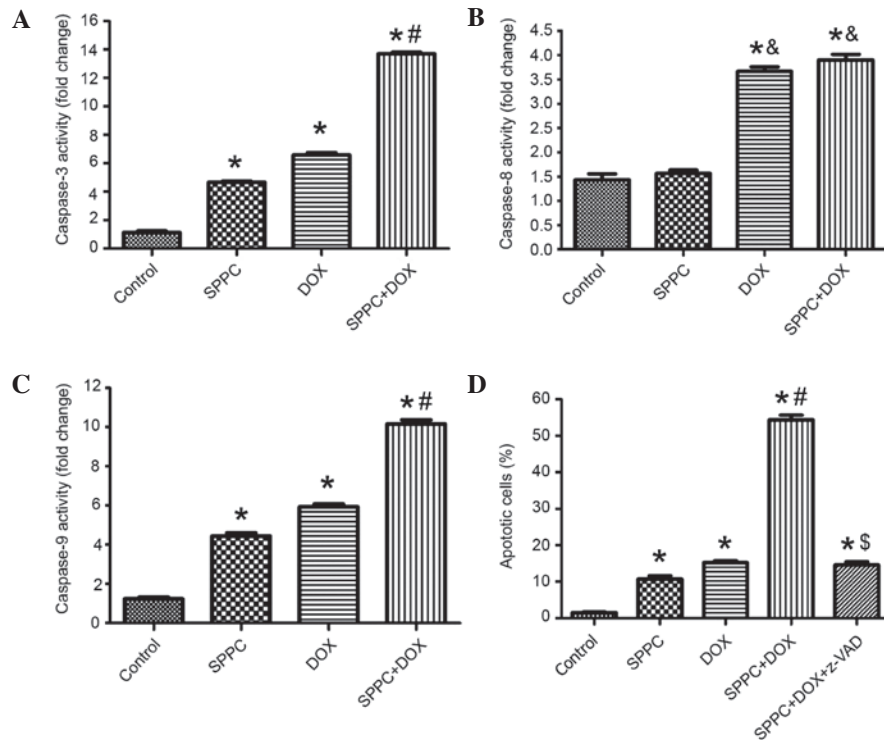


Figure 3. SPPC enhances Dox-induced activation of caspases. The activities of (A) caspase-3, (B) caspase-8 and (C) caspase-9 in MDA-MB-231 cells treated with 1 μ M DOX and/or 5 μ M SPPC for 24 h were assayed. Caspase activity was normalized to cell lysate protein and expressed as fold activation as compared with the control. (D) The caspase inhibitor, Z-VAD-fmk (100 μ M), reduced the effect of SPPC on Dox-induced apoptosis in MDA-MB-231 cells, as determined by Annexin-V/propidium iodide staining and flow cytometry. Data are presented as the mean \pm standard deviation of three independent experiments. *P<0.05, vs. the control group. #P<0.05, vs. SPPC alone group. §P<0.05, vs. the SPPC alone and DOX alone groups. P<0.05, vs. the SPPC + DOX group. SPPC, sulfated polysaccharide-protein complex; DOX, doxorubicin.

suggest that SPPC is able to augment the antitumor activity of Dox *in vivo*.

Histologically, the tumors from the mice in the SPPC plus Dox-treated group contained markedly fewer cells and were predominantly composed of acellular material. Conversely, the tumor cells in the mice administered vehicle control were arranged as nests separated by bundles of extracellular matrix (Fig. 5C). To investigate the effect of combined Dox and SPPC treatment on tumor cell proliferation, tumor sections from nude mice were assessed for PCNA expression. At the end of the experiment, the percentage of PCNA⁺ tumor cells in the combination treatment group was 34%, versus 70% in the Dox only group, 78% in the SPPC only group and 95% in the vehicle control group. These *in vivo* results suggest that SPPC is able to potentiate the Dox-mediated inhibition of tumor growth.

Discussion

Chemotherapy is the most frequently used treatment for breast cancer and other cancer types (30). Dox is an efficacious chemotherapeutic agent with a wide anti-cancer spectrum; however, it has been associated with severe dose-dependent cardiotoxicities once the cumulative dose has reached >500 mg/m² (31). Therefore, it is important to identify novel compounds that are able to enhance the anticancer effect of Dox and reduce Dox-associated side effects.

Various natural products have been shown to exhibit synergistic antitumor effects with chemotherapeutics (32).

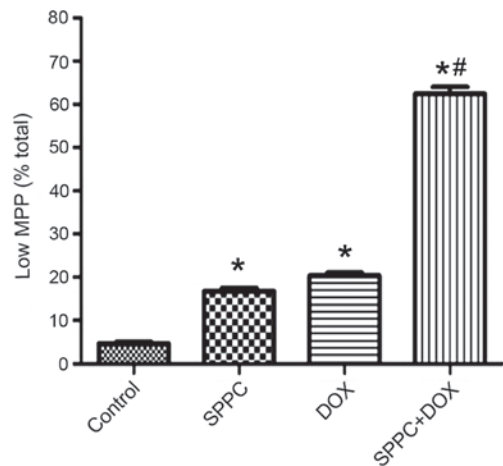


Figure 4. SPPC potentiates the Dox-induced loss of the MMP in MDA-MB-231 cells. Cells were treated with 1 μ M Dox and/or 5 μ M SPPC for 24 h. The loss of the MMP was assayed by JC-1 staining. Data are presented as the mean \pm standard deviation of three independent experiments. *P<0.05, vs. the control group. #P<0.05, vs. the SPPC alone and DOX alone groups. SPPC, sulfated polysaccharide-protein complex; DOX, doxorubicin; MMP, mitochondrial membrane potential.

In a previous study, SPPC was able to induce apoptosis and inhibit the growth of the HL-60 and K562 human leukemia cell lines without causing cytotoxicity to normal hematopoietic cells (33). Furthermore, SPPC has been shown to induce the caspase-3-dependent apoptosis of NB4 leukemia cells via the survivin-mediated apoptotic pathway (1). Previous studies

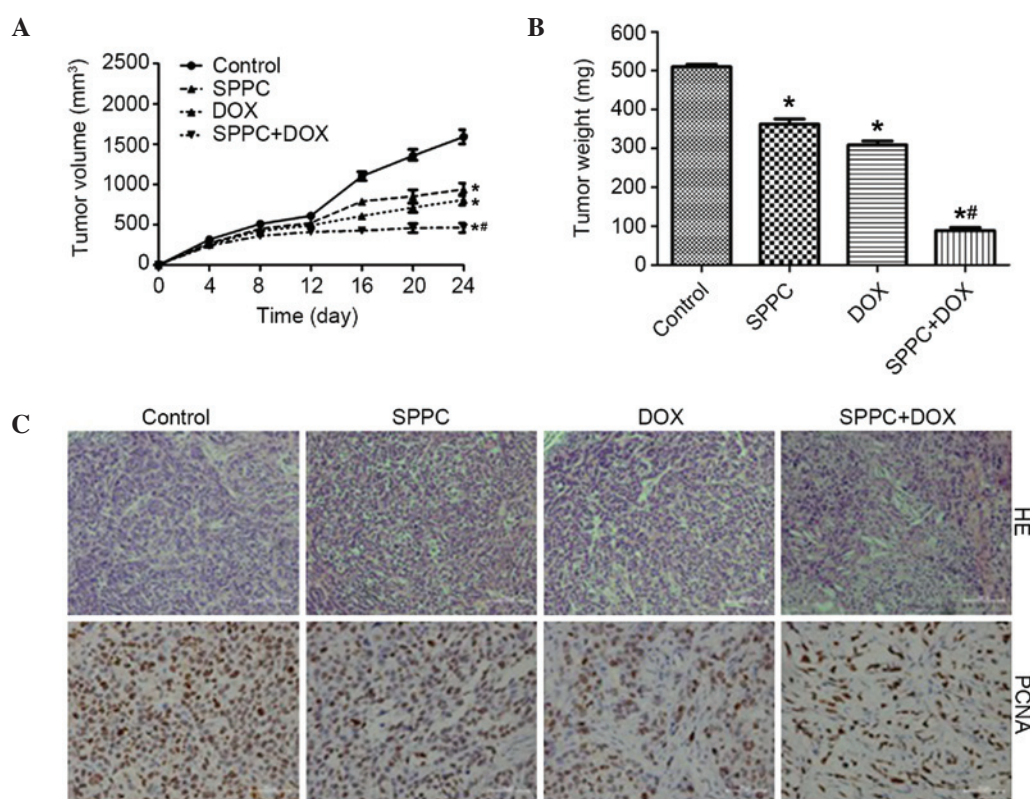


Figure 5. SPPC potentiates Dox-mediated inhibition of MDA-MB-231 cell tumor growth *in vivo*. MDA-MB-231 cells derived from tumors were injected into nude mice that were treated with saline, SPPC alone (40 mg/kg), Dox alone (4 mg/kg) or Dox (4 mg/kg) plus SPPC (40 mg/kg). (A) Tumor growth was monitored by measuring the tumor volume for 24 days. (B) At the end of the experiments, tumors were collected and weighed. (C) Tumor samples were subjected to HE staining and immunohistochemical analysis using anti-PCNA antibody (magnification, x200). Data are presented as the mean \pm standard deviation of three independent experiments. * $P < 0.05$ vs. the control group. # $P < 0.05$ vs. the SPPC alone and DOX alone groups. SPPC, sulfated polysaccharide-protein complex; DOX, doxorubicin; HE, hematoxylin & eosin; PCNA, proliferating cell nuclear antigen.

used the trypan blue exclusion assay to demonstrate that SPPC at 25 and 35 μ M was able to inhibit the proliferation of SMMC-7721 cells (15,34). Sulfated polysaccharides have previously been reported to improve antitumor activity (35). In addition, SPPC was able to promote apoptosis and cell cycle arrest in human breast cancer cells, leading to loss of the MMP and activation of caspase-3 and caspase-9 (36). However, whether or not SPPC has a synergistic effect on the activity of Dox against human breast cancer cells is currently unclear. The present study aimed to investigate the *in vitro* and *in vivo* effects of co-treatment with SPPC and DOX on the growth of the MDA-MB-231 human breast cancer cell line. It was demonstrated that SPPC significantly enhanced the anticancer activity of Dox by significantly reducing the viability of MDA-MB-231 cells. In addition, SPPC was shown to sensitize breast cancer cells to Dox-induced apoptosis. These results suggested that SPPC may be used to reduce the dose of Dox and, in turn, its associated toxicities, thereby presenting a novel therapeutic strategy for breast cancer with fewer side effects.

The present study demonstrated that z-VAD-fmk, a caspase inhibitor, was able to inhibit the apoptosis of breast cancer cells induced by treatment with Dox alone or Dox plus SPPC. In addition, it was able to reduce the difference in the apoptotic rates observed between the Dox alone and Dox plus SPPC groups, thus suggesting that the SPPC potentiation of Dox-induced apoptosis of breast cancer cells

was caspase-dependent. Since it has been demonstrated that two caspase-dependent signaling pathways are involved in Dox-induced apoptosis (37), the present study aimed to determine the apoptotic signaling pathway affected by SPPC. It was demonstrated that SPPC significantly promoted the loss of the MMP in MDA-MB-231 cells, which was consistent with the observed elevation of mitochondrial breakdown and subsequent increase in the activities of caspase-9 and caspase-3, although there was no significant difference in the activity of caspase-8 between the SPPC and control groups. These results suggested that SPPC may sensitize breast cancer cells to Dox-induced apoptosis predominantly via the mitochondrial signaling pathway.

One mechanism by which cancer cells may develop resistance to chemotherapeutic agents is by expelling intracellular anticancer drugs out of cells via IL-8 (38). Cancer cells require oxygen, food and growth proteins to grow and spread. The transport of these essential nutrients to the cancer cells is performed by blood vessels. Angiogenesis is the process of creating new blood vessels necessary to transport this food to the cancer cells, and IL-8 is involved in the process of angiogenesis. SPPC has been observed to exhibit negligible cytotoxicity against normal cells; however, it markedly inhibited the proliferation of SMMC-7721 cells and blocked the cells in the S phase in a previous study (15). In addition, SPPC was shown to inhibit human SMMC-7721 cell growth in a dose-dependent manner (20), and it induced

cell apoptosis, increased the protein expression levels of caspase-3 and the drug excretion capacity of cells, and reduced the mRNA and protein expression levels of IL-8 (16). The present study demonstrated that co-treatment with SPPC and Dox markedly potentiated the antitumor activity of DOX against MDA-MB-231 human breast cancer cells. These results suggested that the potentiation of Dox-induced apoptosis of breast cancer cells by SPPC may be due to the inhibitory effect of SPPC on IL-8 and the increased concentration of Dox within tumor cells. However, this hypothesis requires confirmation by further studies.

The adverse effects of Dox include dilated cardiomyopathy and congestive heart failure due to the induction of apoptosis in cardiomyocytes. It has previously been reported that the Dox-induced apoptotic mechanism is reactive oxygen species-dependent in normal cells and p53-dependent in cancer cells (39). In addition, SPPC, the extract of *L. japonica*, was shown to promote apoptosis by downregulating IL-10 expression (40). The present study demonstrated that SPPC reduced the viability of breast cancer cells and that, when used in combination with DOX, it was able to promote the apoptosis of breast cancer cells, while simultaneously reducing the dose and adverse effects of Dox. Whether SPPC may also reduce the toxicities associated with other anticancer agents requires investigation in further studies.

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