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Abstract

In search of natural bioactive microbial compounds with adjuvant properties, we have previously showed that the polysaccharopeptide (PSP), isolated from Chinese medicinal mushroom *Coriolus versicolor*, was able to enhance the cytotoxicity of certain S-phase targeted-drugs on human leukemic HL-60 cells via some cell-cycle and apoptotic-dependent pathways. The present study aimed to investigate whether the synergism of mechanisms of PSP with certain chemotherapeutic drugs also applies to human breast cancer. PSP treatment enhanced the cytotoxicity of doxorubicin (Doxo), etoposide (VP-16) but not cytarabine (Ara-C). Bivariate bromodeoxyuridine (BrdUrd)/DNA flow cytometry analysis estimated a longer DNA synthesis time (Ts) for the PSP treated cancerous cells suggesting that PSP enhanced the apoptotic effect of Doxo and VP-16 via creating an S-phase trap in the human breast cancer cell line ZR-75-30. The participation of PSP in the apoptotic machinery of the chemotherapeutic agents was further supported by a reduced ratio of protein expression of Bcl-xL/Bax of the cancer cells. This study provides further insight into the synergistic mechanisms of PSP and supports the hypothesis that the anticancer potentials of PSP is not limited to leukemia but may also be used as an adjuvant therapy for breast cancers.

Keywords

anticancer, enhances, zr, cancer, 75, 30, breast, human, etoposide, doxorubicin, cells, activity, polysaccharopeptide

Disciplines

Arts and Humanities | Life Sciences | Medicine and Health Sciences | Social and Behavioral Sciences

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Polysaccharopeptide enhances the anticancer activity of doxorubicin and etoposide on human breast cancer cells ZR-75-30

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Abstract. In search of natural bioactive microbial compounds with adjuvant properties, we have previously showed that the polysaccharopeptide (PSP), isolated from Chinese medicinal mushroom *Coriolus versicolor*, was able to enhance the cytotoxicity of certain S-phase targeted-drugs on human leukemic HL-60 cells via some cell-cycle and apoptotic-dependent pathways. The present study aimed to investigate whether the synergism of mechanisms of PSP with certain chemotherapeutic drugs also applies to human breast cancer. PSP treatment enhanced the cytotoxicity of doxorubicin (Doxo), etoposide (VP-16) but not cytarabine (Ara-C). Bivariate bromodeoxyuridine (BrdUrd)/DNA flow cytometry analysis estimated a longer DNA synthesis time (Ts) for the PSP treated cancerous cells suggesting that PSP enhanced the apoptotic effect of Doxo and VP-16 via creating an S-phase trap in the human breast cancer cell line ZR-75-30. The participation of PSP in the apoptotic machinery of the chemotherapeutic agents was further supported by a reduced ratio of protein expression of Bcl-x_L/Bax of the cancer cells. This study provides further insight into the synergistic mechanisms of PSP and supports the hypothesis that the anti-cancer potentials of PSP is not limited to leukemia but may also be used as an adjuvant therapy for breast cancers.

Introduction

Breast cancer is one of the most frequent cancers in the world (1), and it is the commonest cancer amongst women (1,2). The mortality of breast cancer is low (1), however, because of its high incidence and the increasing global trend (1,3,4), it results in medical costs worldwide estimated to be more than

US\$7 billion (3). The absence of estrogen receptor (ER) is found to be associated with higher recurrence rate (5), and patients with an ER-negative status have a significantly shorter median survival time (6). Hormonal therapy, one of the treatment used to tackle breast cancer, is reported to be completely ineffective in ER-negative breast cancers (7), therefore rendered the high importance of chemotherapy in ER-negative breast cancer patients.

However, chemotherapy comes with side effects such as alopecia (8) and leucopenia (9) because it does not differentiate between cancerous cells and normal cells (9-15). Many efforts have been put into the search for anti-tumor agents that can distinguish cancerous cells from normal cells, as well as adjuvant therapies that can enhance the cytotoxicity of the anti-tumor drugs to cancerous cells, which give potential of lowering chemotherapeutic drugs to reduce the side effects. Combined chemotherapy has also been tried in the hope of reducing the drug-induced side effects as well as increasing the anti-tumor specificity.

When searching for natural anticancer compounds for combination therapy, it is essential to identify novel compounds that are capable of potentiating the chemotherapeutic value of the anticancer drugs but at the same time exert no cytotoxic effect to normal cells. In this respect, the polysaccharopeptide (PSP), isolated from the mycelium of the medicinal Chinese mushroom of the species *Coriolus versicolor*, known as Yun Zhi, offers great potential in cancer combined treatment therapy because of its ability to distinguish cancerous cells from normal cells (16-20). PSP is currently in phase II clinical trials in China, with great success including high survival rates, improved immunological activities, appetite and comfort for patients status (19). The anticancer mechanisms of PSP include induction of apoptosis in cancer cells (21-24) and enhancement of host immune response (24-28).

The active component of Yunzhi, polysaccharopeptide (PSP), consists of 6 types of monosaccharides, namely mannose, glucose, xylose, galactose, arabinose and rhamnose, connected with a small polypeptide (17). Fig. 1 gives an illustration of the partial structure of the polysaccharide moiety of PSP. The polypeptide moieties of PSP are rich in aspartic and glutamic acids (Table I) (17-19). In a recent study, we have demonstrated that PSP was able to potentiate the chemotherapeutic activities of certain cell cycle-specific anticancer drugs. When combined with S-phase-specific

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Key words: polysaccharopeptide, bromodeoxyuridine, doxorubicin, etoposide, cytarabine, human breast cancer, Chinese medicinal mushroom

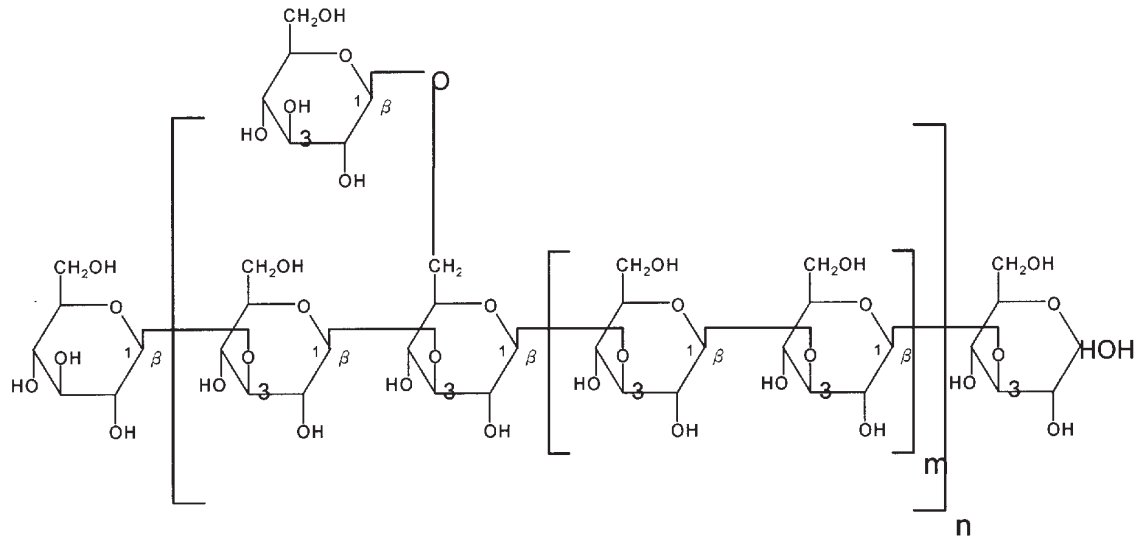


Figure 1. The partial structure of polysaccharide moiety of PSP (17).

Table I. Amino acids composition in polypeptide moiety of PSP (17).

Amino acid	Content (%)	Amino acid	Content (%)
Aspartic acid	4.0	Methionine	0.4
Glutamic acid	5.8	Isoleucine	2.2
Threonine	2.3	Leucine	2.4
Serine	3.2	Tyrosine	1.5
Proline	1.0	Phenylalanine	1.5
Glycine	2.6	Tryptophan	1.7
Alanine	2.6	Lysine	2.3
Cystine	0.9	Histidine	0.7
Valine	1.8	Arginine	1.8

chemotherapeutic drugs, we observed that PSP was effective in potentiating the cytostatic and apoptotic effects of both doxorubicin (Doxo) and etoposide (VP) on the human leukemic HL-60 cells (24). In the present study, the ZR-75-30 cancer cell line, an estrogen receptor (ER) negative human breast cancer was chosen in order to investigate whether PSP can potentiate the cytostatic and apoptotic effects of doxorubicin, etoposide and cytarabine on human breast cancer.

The 5'-Bromo-2'-deoxyuridine (BrdUrd) labeling of DNA technique with flow cytometry (29-33) was applied to investigate the cell cycle-specific actions of PSP and its interaction with Doxo, VP-16, and Ara-C. The present study showed that the combined treatment of S-phase targeting drugs with PSP also resulted in higher cytotoxicity than drugs given alone in the ZR-75-30 cancer cells. Flow cytometry analysis indicates that PSP enhanced the cytotoxicity of the tested drugs by creating an S-phase trap in ZR-75-30 cells. This study provides further insight into the synergistic mechanisms of PSP and supports the hypothesis that the anti-

cancer potentials of PSP is not limited to leukemia but may also be used as an adjuvant therapy for breast cancers.

Materials and methods

Reagents. Cytarabine (Ara-C), doxorubicin (Doxo), dimethyl sulfoxide (DMSO) and protease inhibitor cocktail were purchased from Sigma. Etoposide (VP-16) was purchased from Calbiochem. Polysaccharopeptide (PSP) was obtained from the Winsor Health Products Ltd. in form of capsules (340 mg/capsule). The crude powder of PSP was dissolved in distilled water, and the water soluble fraction was then freeze-dried. The freeze-dried powder was then dissolved in distilled water to prepare a solution at concentration of 10 mg/ml. Stock solutions of Ara-C and Doxo were prepared in distilled water, and VP-16 was dissolved in DMSO. Annexin V binding assay easy to use detection kit (Apoptosis Detection Kit) was obtained from Trevigen Inc. Monoclonal anti-human Bcl-2, anti-human Bax, anti-human Bcl-x_L and anti-human Bid antibody were supplied from Santa-Cruz Biotechnology.

Cell culture. ZR-75-30 cell line used in this study is an estrogen receptor (ER)-negative human breast cancer (34) derived from the ascite fluid of a 47-year-old post-menopausal Black women with infiltrating duct carcinoma (35) and it was a generous donation of Dr Mabel Young from the Department of Physiology, the University of Hong Kong. ZR-75-30 was routinely culture in RPMI medium supplemented with 10% fetal bovine serum. The densities of the cells were monitored to ensure they did not exceed 2x10⁷ cells per 80 cm² culture flask. Cells were cultured at 37°C in a humidified atmosphere with 5% CO₂. The cultures were supplied with fresh complete medium and the cell density was adjusted to 1x10⁶ cells per flask every three days to maintain asynchronous and exponential growth. In all experiments, they were fed with fresh complete medium a day before the experiment.

Cell proliferation analysis. The time (24, 48, 72 h) and dose-dependent (0-400 μg/ml) effect of PSP on the proliferation of

ZR-75-30 human breast cancer cells were studied by culturing the cells with or without (RPMI only) PSP as described above. Preparation of PSP has been described elsewhere by us (16,24). After trypsinization, 100 μ l cancer cell aliquots and 100 μ l of Trypan-blue solution was added to a 1.5 ml microcentrifuge tube which was then mixed thoroughly and incubated at room temperature for 5 min. Total number of cells in four 1 mm² corners of hemacytometer was counted, and the average number of cells per unit volume of medium was calculated.

Study 1: the influence of cell cycle kinetics of PSP on ZR-75-30 human breast cancer cells measured by bivariate BrdUrd/DNA flow cytometry. Previous report with human leukemic cells (16,24) indicated that one of the anticancer properties of PSP involves S-phase cell cycle arrest. This study sought to investigate the influence of cell cycle kinetics of PSP on the ZR-75-30 human breast cancer cells by using the bromodeoxyuridine labeling and bivariate BrdUrd/DNA technique analysis by flow cytometry.

To investigate the effect of PSP on tumor growth and cell cycle distribution (G_0/G_1 , S and G_2/M), ZR-75-30 cell suspensions at 2×10^6 cells were exposed with or without PSP at concentrations ranging between 5 and 400 μ g/ml. The cells were incubated for 24, 48 or 72 h, harvested and examined by various analyses. The fixed samples were stored at -20°C for further analysis by DNA/PI flow cytometry.

To study the effect of PSP on the cell cycle kinetics [labeling index (LI), relative movement (RM), DNA synthesis time (Ts) and G_0/G_1 cells returned or labeled divided cells (Lud)], ZR-75-30 cells with 48-h pretreatment of PSP (50 μ g/ml) or without (control) were pulsed with 10 μ M of BrdUrd for 20 min. After pulse-labeling with BrdUrd, cells were washed with warm cell culture medium, divided and subjected to culture incubation for 1 or 6 h. At the end of incubation, cells were harvested and fixed with ice-cold 75% ethanol. The fixed samples were stored at -20°C for further analysis by flow cytometry.

The bivariate BrdUrd/DNA technique analysis by flow cytometry is based on simultaneous measurement of the total incorporation or the rate of incorporation of the thymidine analog, BrdUrd, into newly synthesized DNA (30-32). The uptake of BrdUrd in each cell can be detected by immunocytochemical staining techniques that involve binding the BrdUrd to fluorescein isothiocyanate (FITC) labeled anti-BrdUrd monoclonal antibodies (green fluorescence). The quantity of BrdUrd (green fluorescence) and DNA (red fluorescence labels by propidium iodide) can be simultaneously measured in the same cells. This analysis estimates the relative number of cells actually involved in cell division (labeling index) and may also provide estimates of the rate of DNA synthesis.

By repeating the bivariate BrdUrd/DNA analysis of labeled cells at several time-points, it is possible to determine the rate of progression of BrdUrd-labeled cells as they accumulate more DNA. The movement of the BrdUrd-labeled cells (cycling cells) between the G_0/G_1 and G_2/M cell cycle phases can be used to calculate the 'relative movement' (RM). By using two different relative movements (RM(t)) extracted from the different BrdUrd pulse-labeling time, the DNA

synthesis time (Ts) of the cancer cells can be calculated according to the methods previously described (29-33):

$$\text{RM}(t) = (\text{F}_{\text{lud}(t)} - \text{F}_{\text{G}_0/\text{G}_1}) / (\text{F}_{\text{G}_2/\text{M}} - \text{F}_{\text{G}_0/\text{G}_1})$$

$$\text{T}_s = 0.55(t) / (\text{RM}(t) - \text{RM}(0))$$

where $\text{F}_{\text{lud}(t)}$, $\text{F}_{\text{G}_0/\text{G}_1}$ and $\text{F}_{\text{G}_2/\text{M}}$ are the mean PI fluorescence (DNA content) of the BrdUrd labelled undivided cells of the G_0/G_1 and G_2/M population at time (t) (hour) after BrdUrd pulsing. $\text{RM}(0)$ and $\text{RM}(t)$ are the relative movement values at time 0 and t (hour) after BrdUrd pulsing respectively. $\text{RM}(0)$ is estimated to be 0.55.

Immunocytochemical staining for BrdUrd/DNA flow cytometry analysis. Staining of cancer cells for bivariate analysis of BrdUrd was performed as previously described by us (29,33,36) and by other investigators (30) with some modifications. Cells were harvested into 15 ml centrifuge tubes. All samples were washed twice with 10 ml PBS and centrifuged at 400 g for 5 min. Supernatant was removed and cell pellets were re-suspended in 100 μ l ice-cooled PBS. Cells were fixed by adding ice-cold EtOH (70% w/v in Milli-Q water) dropwisely into the tubes while the cells were being vortexed. The fixed samples were stored at -20°C for further analysis.

The fixed samples were washed twice each with 10 ml PBS and the DNA of cells were partially denatured by incubation with 2 M HCl (0.5 ml) for 30 min. After denaturation, cells were washed three times with 10 ml PBS containing 0.05% Tween-20 (PBS-T). The cells were incubated with 100 μ l of anti-BrdUrd antibody (1:100 dilution) and incubated at room temperature for 1 h. After incubation, cells were washed twice with PBS-T and subsequently incubated with 100 μ l of FITC-conjugated anti-mouse IgG antibody (1:40 dilution) to label the primary antibodies in dark at room temperature for 1 h. Following the incubation, cells were washed twice with PBS and the DNA of cells was stained with propidium iodide (PI) staining solution (50 μ g/ml PI, 10 μ g/ml RNase, 0.01 M Tris-base and 10 mM NaCl in milli-Q water) for 30 min at room temperature. The stained cells were analyzed by Coulter's Epics Elite ESP flow cytometer at 525 nm and 620 nm band pass filters.

Study 2: The combined anticancer effect of PSP with doxorubicin (Doxo), etoposide (VP-16) and cytarabine (Ara-C). Based on the cell cycle kinetic study of study 1, the testing hypothesis of this study was that by creating an S-phase trap, PSP can enhance the cell killing effect of chemotherapeutic agents namely doxorubicin, etoposide and cytarabine with S-phase interference activity on the ZR-75-30 cells. The ZR-75-30 cells were treated with PSP (50 μ g/ml) or without (non PSP treated) for 48 h before adding the individual tested drugs. Both PSP pretreated and non-PSP treated cells were exposed to 5 μ M of Ara-C, VP-16 or Doxo for further 18 h. The total treatment time of PSP was 66 h. Cells were harvested for annexin V/PI flow cytometry analysis for cell death and Western blot analysis of the Bcl-2 family genes.

Quantification of cell death by annexin V/PI flow cytometry. The theoretical background of this detection method has been previously described (37). Annexin V binding assay was

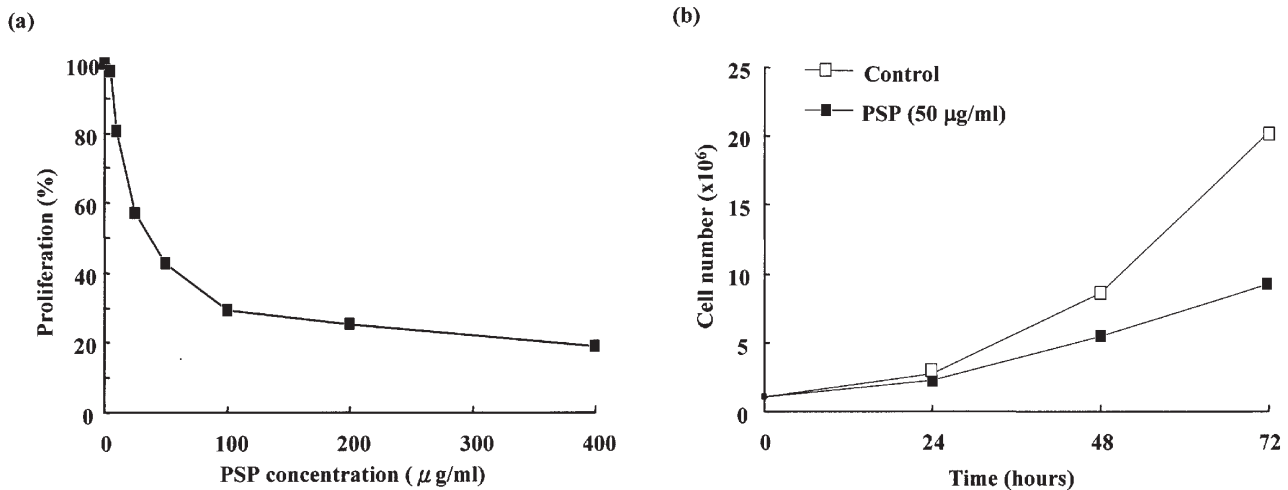


Figure 2. The time and dose effect of PSP on the proliferation of human breast cancer ZR-75-30 cell proliferation. (a) dose-dependent effect; (b) time-dependent effect.

performed by using an apoptosis detection kit. Cells (2×10^5) after incubation with or without PSP and drugs were harvested and centrifuged at $400 \times g$ for 5 min to remove culture medium. Cell pellets were washed with 3 ml phosphate buffer saline (PBS) and re-suspended in $500 \mu\text{l}$ binding buffer. After centrifugation and removal of binding buffer, $100 \mu\text{l}$ of annexin V incubation reagent ($10 \mu\text{l}$ 10X binding buffer, $10 \mu\text{l}$ propidium iodide (PI), $1 \mu\text{l}$ annexin V conjugate and $79 \mu\text{l}$ milli-Q water) was added to each sample. The samples were incubated for 15 min in the dark at room temperature. The cell suspension was then diluted with $400 \mu\text{l}$ binding buffer and was analyzed by flow cytometer.

Western blot analysis of the expression of Bcl-2 family proteins. Cells were harvested and washed twice with ice-cold PBS (45 ml) followed by centrifugation at $400 \times g$ for 5 min. Cell pellet was re-suspended in lysis buffer (5×10^6 cells/ $100 \mu\text{l}$ lysis buffer) with HEPES (25 mM; pH 7.5), NaCl (150 mM), EDTANa₂ (1 mM), DTT (1 mM), Triton X-100 (1%) and protease inhibitor cocktail. The suspension was then frozen and thawed three times by cold methanol at -80°C . The iced suspension was sonicated to melting point. Cell suspension was then placed in ice for further 30 min and then centrifuged. After centrifugation at $14,000 \times g$ for 30 min at 4°C , the suspension was collected and stored at -80°C . Protein quantity was determined by Bradford assay. Protein extracts were mixed with equal volume of 2X sample buffer (0.125 M Tris-HCl, 4% SDS, 20% v/v glycerol, 0.2 M DTT, 0.02% bromophenol blue, pH 6.8) and the mixture was boiled in water for 3 min.

Equal amounts of total protein ($20 \mu\text{g}$) were subjected to 12.5% SDS-PAGE followed by Western blotting onto a PVDF membrane. Membranes were incubated with anti-human Bcl-2, anti-human Bax, anti-human Bid and anti-human Bcl-x_L antibodies, and detected with the matching species-specific secondary HRP-conjugated antibodies. Proteins were detected using the ECL system (GE Healthcare) and the band intensity was measured by Quantity One software (Bio-Rad).

Statistical analysis. All data are presented as mean \pm standard error of the mean (SEM). Statistical significance was calculated using two-tail Student's t-test for two groups and One-way ANOVA analysis for multi-group comparison. $p < 0.05$ was considered as statistically significant.

Results

The anti-proliferation effect of PSP on ZR-75-30 cells. Treatment with PSP delayed the proliferation of ZR-75-30 cells in a dose-dependent (Fig. 2a) and time-dependent (Fig. 2b) manner. At $50 \mu\text{g/ml}$ of PSP, proliferation was decreased by 36.8% after 48-h treatment. Measured by annexin V/PI flow cytometry, at 72-h treatment, PSP induced significant cell death ($p < 0.001$) by 23.9% (Fig. 3).

Effect of PSP on the human breast cancer ZR-75-30 cell cycle distribution. The cell cycle distribution of ZR-75-30 cells, measured by DNA/PI flow cytometry, shows that PSP was capable of inhibiting the cell proliferation via alteration of cell cycle. At 48 h, PSP induced cell arrest in the S-phase by 34% ($p < 0.01$) with a corresponding decrease of cell proportions in G₀/G₁ and G₂/M phases (Fig. 4) compared to control.

Effect of PSP on labeling index (LI), relative movement (RM), and DNA synthesis time (Ts) of human breast cancer ZR-75-30 cells. Fig. 5 presents the contour plot of the bivariate BrdUrd/DNA flow cytometry analysis from the 1- and 6-h BrdUrd pulse labeling of the PSP treated and control (non-PSP treated) cells. The S-phase BrdUrd labeled cell population of the 1-h BrdUrd contour plot is used to estimate the labeling index (LI) of the cancer cells as described (29-33). The 6-h contour plots show that the BrdUrd labeled cells have moved through the S-phase in the cell cycle and some had not yet divided (Lud) whereas others had done so. The newly divided daughter cells population detected by anti-BrdUrd-mono-clonal antibodies of the G₀/G₁ phase was used to

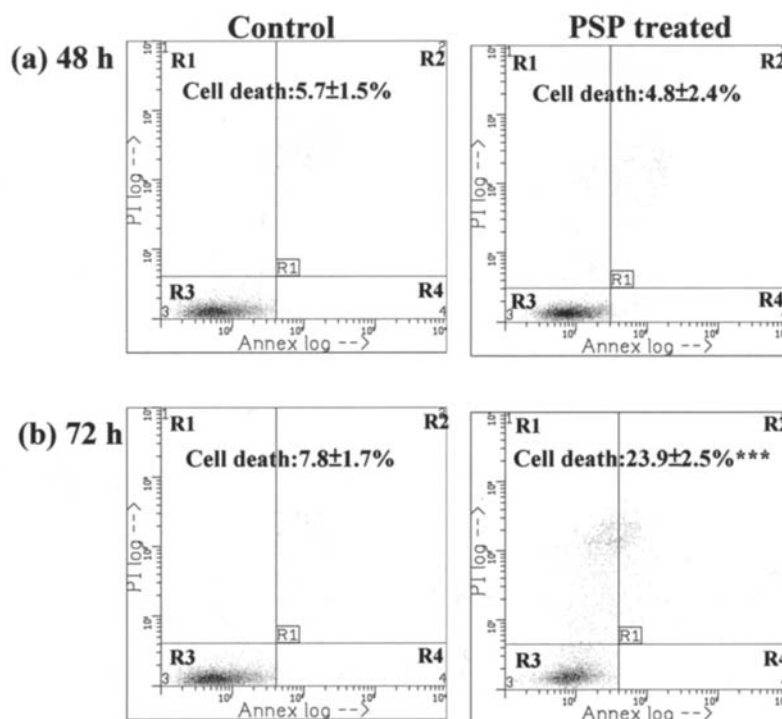


Figure 3. The effect of PSP on cell death of ZR-75-30 cells. The cells without (control) and with PSP (50 $\mu\text{g/ml}$) treatment for 48 and 72 h were subjected to annexin V/PI flow cytometry analysis. The dot plots representing data on viable cells (R3) and dead cells (R1, R2, R4) at 48 and 72 h, respectively. Values are mean \pm SEM (n=4). ***p<0.001 compared with control.

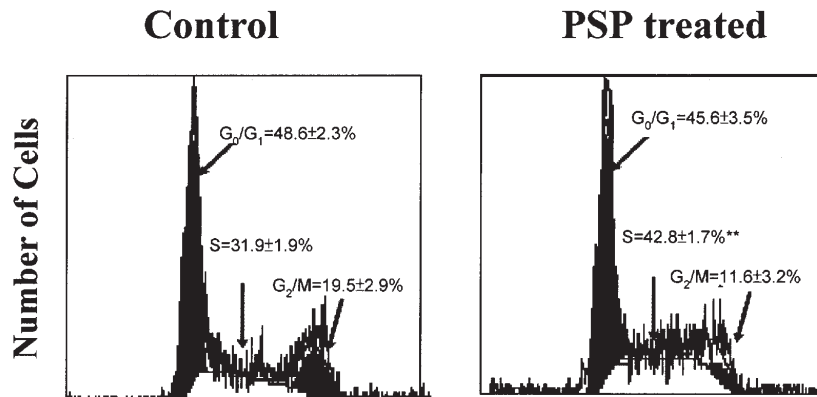


Figure 4. The effect of PSP on the cell cycle distribution. Cancer cells without (control) or with 50 $\mu\text{g/ml}$ PSP incubation for 48 h were fixed with PI for staining of DNA. The cell population (%) distribution among the G₀/G₁, S and G₂/M phase was analyzed by DNA/PI flow cytometry. Values are mean \pm SEM (n=4). **p<0.01 compared with control.

calculate the labeled divided cells (Ld). The relative movement calculated from the 6-h BrdUrd/DNA flow cytometry (RM 6) (Fig. 5) was used to calculate the DNA synthesis time (Ts) of the cancer cells. Table II summarized the effect of PSP on LI, RM, Ld and Ts of the cancer cells with and without PSP treatment. The data show that PSP arrested cells in S-phase and resulted in significant higher calculated labeling index. It is noteworthy that PSP treatment significantly (p<0.01) retarded the relative movement (1 and 6 h) of the cancer cells. PSP arrested cells in S-phase resulted in a hyper-prolongation from 12.51 to 18.31 h for the DNA synthesis of the cancer cells. The DNA

synthesis time of ZR-75-30 cells was extended by 46.4% (p<0.01). Calculation of the labeled divided cells also dropped from 8.58 to 3.67% suggesting some interference might have occurred at the G₂/M phase.

The effect of anti-tumor drugs alone and PSP pretreatment with anti-tumor drug on cell death and cell viability in ZR-75-30. To investigate the interaction of PSP with the chemotherapeutic drugs, cell death was performed with annexin V/PI flow cytometry. Fig. 6 indicates that comparing to the cancer cells administered only the corresponding individual chemotherapeutic agent treatment, the apoptotic effect of Ara-C,

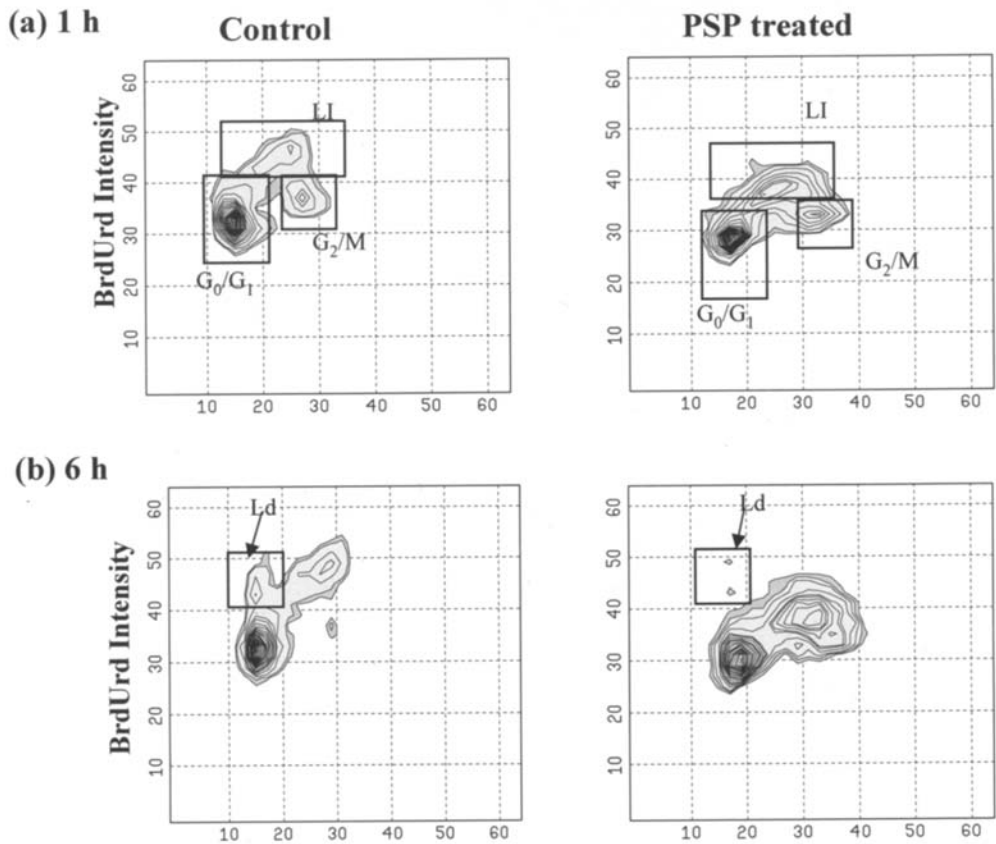


Figure 5. The effect of PSP on the labeling index and labeled divided cells: measured by bivariate BrdUrd/DNA flow cytometry. Contour plots of bivariate BrdUrd/DNA flow cytometry distribution of the breast cancer cells without or with PSP (50 $\mu\text{g/ml}$) treatment for 48 h were generated from the 1 and 6 h BrdUrd pulse labels, respectively. The S-phase cells situated in between 2N (G_0/G_1) and 4N (G_2/M) labeled positively with BrdUrd, and this population of cells were used to estimate the labeling index (LI, %). The 6-h BrdUrd/DNA contour plot shows that some S-phase cells incorporated BrdUrd and had progressed through the G_2/M phase; further divided and appeared in G_0/G_1 as daughter cells or labeled divided cells (Ld). Some cells remained undivided and expressed as labeled undivided cells (Lud). Values are mean \pm SEM (n=4). **p<0.01 compared with control.

Table II. The effect of PSP on labeling index, labeled divided cells, relative movement and DNA synthesis time of the ZR-75-30 cells.

	Time (h)	LI (%)	Ld (%)	RM	Ts
Control	1	31.9 \pm 0.4		0.66 \pm 0.01	
PSP	1	42.8 \pm 1.3 ^a		0.59 \pm 0.01 ^a	
Control	6		8.58 \pm 0.52	0.82 \pm 0.01	12.51 \pm 0.56
PSP	6		3.67 \pm 0.24 ^a	0.73 \pm 0.01 ^a	18.31 \pm 0.63 ^a

All data are expressed as mean \pm SEM (n=4). ^ap<0.01 vs control; PSP (50 $\mu\text{g/ml}$). LI, labeling index, extracted from the 1-h bivariate BrdUrd/DNA flow cytometric contour plot; Ld, labeled divided, extracted from the 6-h bivariate BrdUrd/DNA flow cytometric contour plot; RM, relative movement; extracted from both 1- and 6-h bivariate BrdUrd/DNA flow cytometric contour plot; Ts, DNA synthesis time; $T_s = 0.55(t)/(RM(t) - RM(0))$, where $Flud(t)$, FG_0/G_1 and FG_2/M are the mean PI fluorescence (DNA content) of the BrdUrd labelled undivided cells of the G_0/G_1 and G_2/M population at time (t) (hour) after BrdUrd pulsing; $RM(0)$ and $RM(t)$ are the relative movement values at time 0 and t (hour) after BrdUrd pulsing, respectively. $RM(0)$ is estimated to be 0.55.

Doxo and VP-16 on the ZR-75-30 cells was further enhanced in cells with PSP pretreatment by 18.8%, 55.4% (p<0.001) and 161% (p<0.001), respectively. The viability of these cells dropped by 8.8, 33.2 (p<0.001) and 48.8% (p<0.001) with Ara-C, Doxo, and VP-16, respectively.

Effect of PSP with and without Ara-C, Doxo and VP-16 on apoptotic protein expression in human breast cancer ZR-75-30 cells. Western blot analysis of protein level presented in Fig. 7 shows that among the apoptotic genes measured, Bax gene expression was the strongest in the ZR-75-30 cells. Fig. 8

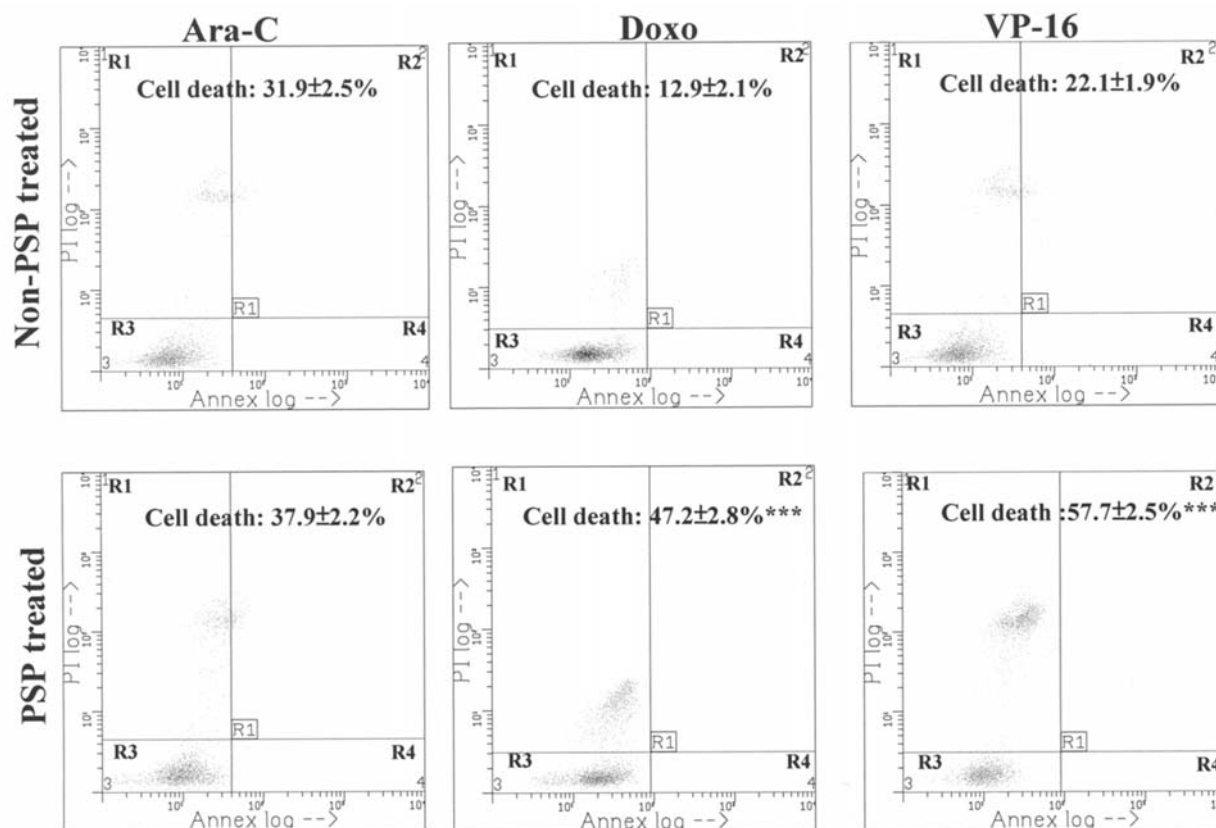


Figure 6. The combined effect of PSP with Ara-C, Doxo or VP-16 on the cell death of ZR-75-30 cells. The cancer cells either without (non-PSP) or with PSP (50 $\mu\text{g}/\text{ml}$) pretreatment for 48 h all received 5 μM of Ara-C, Doxo or VP-16 for 18 h. The total treatment time of PSP was 66 h. The population of dead cells was determined by Annexin V/PI flow cytometry. The R3 represents viable cells and the R1, R4 and R2 areas represent dead cells (PI⁺, Annexin⁺ or both). Values are mean \pm SEM (n=4). ***p<0.001 compared with non-PSP treated.

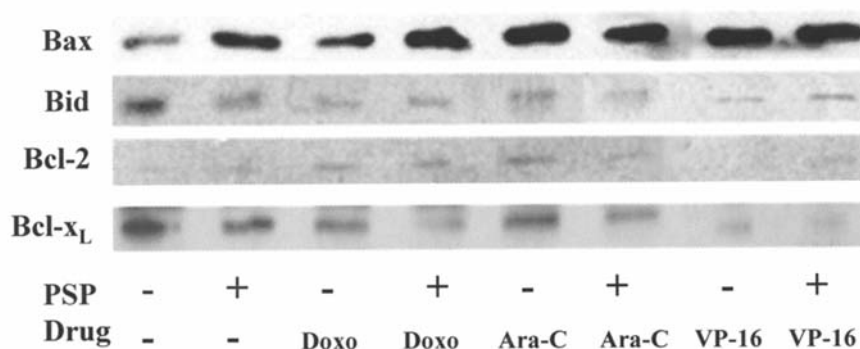


Figure 7. The combined effect of PSP with Ara-C, Doxo, VP-16 on expression of the cell death proteins Bcl-x_L, Bid and Bax. The cancer cells either with or without PSP pretreatment for 48 h all received 5 μM of Ara-C, Doxo or VP-16 for 18 h. The total treatment time of PSP was 66 h.

summarizes the relative expression of the Western plotted proteins and shows that comparing to the control, PSP treatment alone increased the pro-apoptotic Bax expression by 107% while that of Bid was reduced by 45%. Expression of the anti-apoptotic protein Bcl-x_L was reduced by 51%. When comparing the chemotherapeutic agents, they produced similar effects, i.e. increasing Bax expression and decreasing Bcl-x_L and Bid expression. Doxo treatment increased Bax expression by 39% and reduced Bcl-x_L and Bid expression by 39 and 62%, respectively. Ara-C treatment

increased expression of Bax by 209% and decreased Bcl-x_L by 45%. Expression of Bid was decreased by 70% as well. VP-16 treatment increased Bax expression by 220%, while Bcl-x_L and Bid expression were decreased by 52 and 90%, respectively.

Compared with the corresponding non-PSP treated groups, combination treatment of PSP with Doxo increased the expression of Bax by 92.1% and slightly decreased the expression of Bcl-x_L by 24.6%. The change of bid expression in Doxo with PSP is insignificant. In the combination treatment of

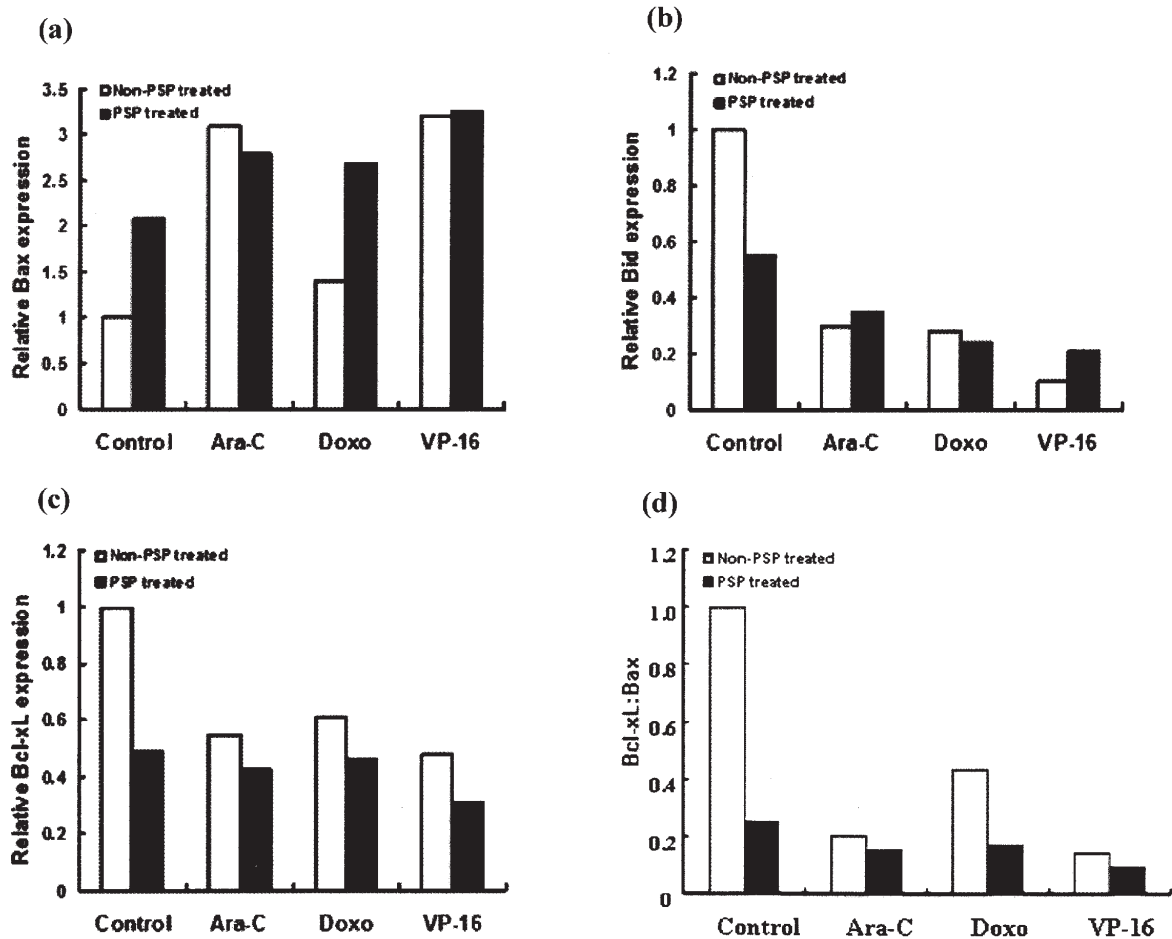


Figure 8. The combined effect of PSP with Ara-C, Doxo or VP-16 on the relative expression of the cell death proteins Bcl-x_L, Bid, Bax and Bcl-x_L/Bax ratio. The cancer cells either with or without PSP pretreatment for 48 h all received 5 μ M of Ara-C, Doxo or VP-16 for 18 h. The total treatment time of PSP was 66 h. The relative expression of (a) Bax; (b) Bid; (c) Bcl-x_L and (d) Bcl-x_L/Bax ratio.

PSP with VP-16, the change in Bax expression is insignificant while Bid expression was doubled. Bcl-x_L expression was decreased by 35.4%. Combined treatment of PSP with Ara-C, to our surprise, reduced the expression of Bax by 9.4%, while Bcl-x_L expression was decreased by 21.8%. Change in Bid expression is insignificant.

Fig. 8d shows that all treatment groups decreased the Bcl-x_L/Bax ratio. PSP alone decreased the ratio by 76.45% while Ara-C, Doxo and VP-16 given alone decreased the ratio by 82.4, 56.0 and 84.9%, respectively. Compared with the corresponding non-PSP treated groups, PSP further decreased the Bcl-x_L/Bax ratio induced by Doxo by 60.6%, Ara-C by 13.0% and VP-16 by 37.5%.

Discussion

Breast cancer is one of the most frequent cancers in the world (1), and it is the commonest cancer amongst women (1,2). Because of the advancement in breast cancer therapy, the mortality is low compared to other types of cancers (1). Therapies used for breast cancer include hormonal therapy (7,38), chemotherapy (39), a combination of both (40), and surgical removal of the malignant tissue. Surgical removal results in psychological issues such as low self-esteem (41),

which is highly undesirable. Hormonal therapy is reported to be ineffective in ER-negative breast cancers (7), therefore chemotherapy in ER-negative breast cancers is very important. Many chemotherapeutic agents, however, are also toxic to normal cells (10-15), leading to side effects like loss of hair (8), impaired immune function (9), etc. which limited their efficacy.

Combined chemotherapy offers hope and the success depends on the interaction between the candidate drugs (42-44). Several drugs with similar cell cycle-dependent properties can be scheduled properly to produce a maximal killing effect in combined chemotherapy. In this regard, PSP is likely a potential candidate as an adjuvant therapy for cancer treatment via its cell cycle interaction with the cytotoxic action of several S-phase targeting chemotherapeutic agents. The superior property of PSP over other chemotherapeutic drugs can be its ability to distinguish cancerous cells from normal cells (16,18-20).

Generally speaking, a phase-specific anticancer agent will produce a maximal cell killing effect only if it allows cycling cells to enter the cytotoxic phase, i.e. the S-phase (42-46). In the present study, we have demonstrated that by encouraging the cycling cells to enter the cytotoxic S-phase, PSP potentiated the cell killing effect of the two S-phase targeting drugs

Doxo and VP-16 (10,47-52) but not Ara-C on the human breast cancer ZR-75-30 cells (Fig. 6). The flow cytometric method used in the current study is based on simultaneous measurements of BrdUrd incorporation into cellular DNA (S-phase) and total cellular DNA content. This technique potentially overcomes many of the problems associated with the use of radioactive labels and autoradioactivity required to measure cell kinetics. By use of a single 'pulse' labeling of BrdUrd and harvesting and staining of the cancer cells for BrdUrd and DNA at two time points, it was possible to obtain detailed estimates of DNA synthesis time T_s . The extended T_s (from 12 to 18 h) of the PSP-pretreated ZR-75-30 cells indicated that DNA synthesis of the cycling cells in the cytotoxic phase had been affected to a certain extent. With the interference of DNA synthesis process, PSP provoked the cell death of the ZR-75-30 cells induced by cell cycle specific cytotoxic anticancer agents Doxo and VP-16 by 55 and 161%, respectively (Fig. 6).

The ability of PSP to arrest the cancer cells in the DNA synthesis S-phase (led to a higher labeling index as time of measurement) is thus our proposed mechanism on how PSP sensitized the human breast cancer ZR-75-30 cells to undergo cell death induced by the two S-phase targeting drugs Doxo and VP-16 in the present study (Fig. 6). The 'S-phase trap theory' of PSP was first observed in human leukemic HL-60 cells reported by us (16). The elevation of the DNA enzyme topoisomerase II α and the G₁-S-phase checkpoint cyclin, cyclin E (24) measured in the PSP-treated HL-60 cells supported the interference of DNA synthesis as part of the mechanistic anticancer action of PSP.

Recent evidence indicates that PSP induces cell death of cancer cells via well defined apoptotic program (16,22-24,47). When combined with the anticancer agents, the annexin V/PI flow cytometric data indicated that PSP was only able to enhance the apoptotic induction effect of Doxo and VP-16, but not Ara-C on the ZR-75-30 cells. Another interesting observation drawn from this study is that Ara-C was in fact found to be the most potent anticancer agent among the three anti-tumor agents tested alone, while Doxo was found to be the least effective in ZR-75-30 breast cancer.

This is unclear as to why PSP hinders the cytotoxic effect of Ara-C, but our data indeed reflects the importance of proper drugs selection for cancer treatment. Such antagonistic result from the combined treatment of Ara-C and PSP may be caused by the removal of the ZR-75-30 cells by PSP in the G₁-S boundary, which was reported as the target phase of Ara-C (46,48). Ara-C, as a pyrimidine analogue, exerts its cytotoxic action essentially by incorporation into the genome of target cells (48). Whether PSP was able to delay the cytotoxic action of Ara-C by preventing its incorporation into the DNA of the cancer cells DNA has yet to be determined.

Western blot analysis of the Bcl-2 family proteins, which are essential in modulating apoptosis (49-53), was performed to investigate the cell death mechanism of action of PSP on the ZR-75-30 cells at protein level. It has been shown that some of the breast cancer cell lines do not express Bcl-2 (54), e.g. ZR-75-1 which is a close clone of ZR-75-30. Bcl-x_L has been shown to confer multi-drug resistance in several squamous cell carcinomas (52), as well as to delay apoptosis in breast cancer (53). Our results showed that the expression

of Bcl-2 in ZR-75-30 is too low to be detected (Fig. 7), while Bcl-x_L was expressed, suggesting Bcl-x_L may have taken the anti-apoptotic role of Bcl-2. Bax, on the other hand, was shown to be pro-apoptotic in various cancer cell lines (50-55), including breast cancer (56-58), and is expressed in the ZR-75-30 cells. Bid, another pro-apoptotic protein (59,60), was also expressed in ZR-75-30 cells.

It is found that Bax expression (Fig. 8a) was increased to different extents under all treatments. PSP alone increased Bax protein level. Among the three chemotherapeutic drugs tested, both Ara-C and VP-16 given alone caused a significant increase in Bax expression while Doxo caused only a slight increase. However, with PSP pretreatment, the expression of Bax was further increased with Doxo and slightly increased with VP-16, while that of Ara-C was decreased. The Western blot result coincides with the cell death data analyzed by DNA/PI flow cytometry (Fig. 6), which showed that the synergistic effect was not observed in Ara-C enhanced the cell death with PSP pre-treatment. On the other hand, PSP alone and all drugs on their own decreased Bcl-x_L expression (Fig. 8c). With PSP pretreatment, the Bcl-x_L expression was further decreased to different extent. Compared with the single drug treatment groups, the Bcl-x_L/Bax ratio was decreased in the combined treatment group (Fig. 8d). This may explain the discordance of Bax expression with cell death data, because it has been suggested that Bcl-x_L/Bax ratio is a more important indicator of cell death (61,62), and we have previously reported that the apoptotic machinery induced by PSP involves a decrease in the Bcl/Bax ratio (16). Expression of Bid, surprisingly, was suppressed in all kinds of treatments. The change of Bid expression in drugs alone comparing to drugs with PSP pretreatment is insignificant, suggesting PSP may not potentiate the cytotoxicity of Doxo, Ara-C and VP-16 in ZR-75-30 cells via modulation of Bid expression, which is one of the common ways to induce apoptosis (60).

In conclusion, the present study indicates that PSP can activate the cellular apoptotic program of Doxo and VP-16 on human breast cancer cells by creation of an S-phase trap. The data provide support for some early reports (16,20-24) that PSP derived from Cov-1 strain promotes cancer cell suicide through a firmly controlled program (apoptosis). Up-regulation of pro-apoptotic Bax and down-regulation of anti-apoptotic Bcl-x_L, and therefore, a decrease in Bcl-x_L/Bax ratio was found to be associated with cell death induction synergism of mechanisms of PSP with certain chemotherapeutic drugs. Activation of the cellular apoptotic program is a current strategy for the treatment of cancers. The present study provides the first evidence to support the development of PSP as an adjuvant therapy in human breast cancer because of its ability to activate the cytotoxicity of chemotherapeutic drugs. However, the data also imply the importance of understanding its interaction with certain individual anticancer agents.

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