

Full Length Research Paper

Selenium-enriched polysaccharides from *pyracantha fortuneana* (Se-PFPs) augment docetaxel-induced tumor suppression in human ovarian cancer

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Pyracantha fortuneana (Maxim.) contains many healthy ingredients, including selenium-enriched polysaccharides. Selenium-enriched polysaccharides from *P. fortuneana* (Se-PFPs) have anti-mutation and anti-oxidation activities, along with anti-tumor activity in ovarian cancer and breast cancer cells. Docetaxel (DTX) is widely applied in therapeutic treatment for ovarian cancer. However, its use in clinics is restricted due to its unfavorable side-effects. Thus there is a need to improve the therapeutic efficacy of Docetaxel (DTX). This study aimed to determine whether Se-PFPs and DTX can be used together to generate additive and/or synergistic effect in therapeutic treatment for ovarian cancer. *In vitro* and *in vivo* experiments were conducted to compare the anti-proliferative and apoptosis-inducing effects of the combined use of both drugs with that of each one used alone. Two ovarian carcinoma cell lines, SKOV3 and A2780 cells, were used and divided into four groups: 1) Saline control (vehicle); 2) Se-PFPs only; 3) DTX only; and 4) Se-PFPs+DTX. The apoptotic rate of group treated with a combination of Se-PFPs and DTX was found to be significantly higher than those of groups treated with either agent alone. This finding was confirmed by increased TdT-mediated dUTP nick end labeling (TUNEL)-positive rate, rate of apoptotic cells, enhanced cleavage of caspase-3 and PARP, and reduced cell viability and Ki-67 expression. The combined drugs did not significantly increase the toxicity HOSE cells, the human ovarian surface epithelial cells immortalized with hTERT. Similar results were obtained in the *in vivo* experiments by measuring tumor weight and volume. Thus, Se-PFPs can augment DTX tumor inhibitory effect. The combination of Se-PFPs and DTX can be a promising therapeutic strategy for ovarian cancer.

Key words: Selenium-enriched *Pyracantha fortuneana* (Maxim.) Li polysaccharides, docetaxel, tumor suppression, ovarian cancer.

INTRODUCTION

Ovarian cancer is one of the most aggressive diseases characterized with widespread invasion and metastasis.

Its mortality is extremely high among gynecologic cancers. Docetaxel, a chemotherapy drug, has been

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applied to treat previously incurable ovarian cancer in recent years (Torre et al., 2018; Hou et al., 2018). However, more than 80% of patients with oophoroma at the stage III or IV succumb in the short term owing to relapse (Testa et al., 2018).

Compared with surgical treatment, natural plant extracts can have better effectiveness to fight disease and enhance life quality of patients (Al Sawah et al., 2015). *Pyracantha fortuneana* (Maxim.) is a shrub found in China, Vietnam, and Europe. Its fruits are of significant medicinal value. Selenium-enriched *P. fortuneana* Li polysaccharides (Se-PFPs), derived from *P. fortuneana* have been demonstrated to have an anti-mutation ability, is able protect immunologic function (Peng et al., 2016), and have a hepatoprotective effect (Yuan et al., 2015, 2010). Furthermore, it is also capable of inhibiting the growth of MDA-MB-231 breast cancer cells (Yuan et al., 2016). Moreover, Se-PFPs could inhibit the invasion and migration of ovarian cancer cells (Sun et al., 2016). Thus, treatment of ovarian cancer with Se-PFPs as an adjuvant in a short term deserves further exploration.

Docetaxel, derived from the Taxodaceae family, is one of the most studied taxanes of natural products (Kenmotsu and Tanigawara, 2015). It induces cell apoptosis through a mechanism involving suppressing microtubule dynamics (Azarenko et al., 2014) and arresting cell-cycle at the G2/M phase (Han et al., 2016). However, its clinical application is still limited due to its severe side-effects, such as nausea and fatigue (Sohail et al., 2018). Reduction dosage is an effective way to reduce side effects. Combination chemotherapy is brought into sharp focus on account of its lower toxicity and higher sensitivity. Researchers have already studied the effects of combination of taxanes with alkylating agents, topoisomerase inhibitors and anthracyclines (Joensuu et al., 2017). This study aimed to enhance the sensitivity of ovarian cancer cells to lower dosage of DTX by combining it together with Se-PFPs by analyzing the effects of the combination on induction of apoptosis and synergistic function.

MATERIALS AND METHODS

Reagents and cell lines

Selenium-enriched *P. fortuneana* fruiting bodies were obtained from Enshi mountain area in Hubei province, China. Se-PFPs were extracted from Se-enriched *P. fortuneana* as described by Yuan et al. (2015, 2010, 2016). Briefly, fruit were dried in an infrared dryer, and crushed into fine powder by a multifunctional disintegrator. The resulting products were refluxed and degreased twice with 5 L of petroleum ether at 75°C for 5 h each time. After cooling, the extract obtained was placed into hot water, concentrated under a vacuum at 55°C and precipitated with 4-fold volume of ethanol at 4°C overnight. The precipitate was dissolved in Sevag reagent to remove protein, followed by dialysis against distilled water for 48 h. After centrifugation at 2000×g for 15 min, the supernatant was pooled, condensed and lyophilized successively to obtain crude Se-PFPs.

Docetaxel purchased from Sigma-Aldrich (St. Louis, MO, USA)

was dissolved in dimethylsulfoxide (DMSO). Primary antibodies against cleaved caspase-3 and poly ADP ribose polymerase (PARP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). SKOV3 and A2780 cells were initially obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured at 37°C under 5% CO₂ in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin.

MTT assay

Cell viability was determined as described by Yuan et al. (2012). Briefly, SKOV3 and A2780 cells were seeded at a density of 3000 cells per well in triplicate into 96-well plates, and exposed to 200 µg/mL Se-PFPs, 2 nM DTX, and 200 µg/mL Se-PFPs + 2 nM DTX for 24 and 48 h, respectively. Cells without exposure to these agents were taken as the negative control. Cell viability was determined using an MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) assay, and the absorbance was measured at 570 nm.

Apoptosis assay

SKOV3 and A2780 cells were treated with 200 µg/ml Se-PFPs, 2 nM docetaxel (DTX) or 200 µg/mL Se-PFPs + 2 nM DTX for 48 h. Thereafter, the apoptosis was measured by two ways: part of the cells was measured by flow cytometry with Annexin V-fluorescein isothiocyanate (FITC) and 50 µg/mL propidium iodide (PI) staining, the remaining cells were fixed in 4% paraformaldehyde for 10 min and stained with 10 µg/mL Hoechst 33342 for 15 min, and apoptotic cells were counted under light microscopy.

Western blot

The procedures of protein extraction from SKOV3 and A2780 cells or animal tissues and the protein concentration measurement were performed as described in our previous study (Yuan et al., 2015). The proteins (50 µg per well) were separated by SDS-PAGE (8 - 15%) and transferred onto polyvinylidene fluoride (PVDF) membrane. After being blocked with 5% defatted milk for 1 h, the membrane was incubated with the indicated primary antibodies overnight at 1:500 or 1:1000 dilution, and then incubated with corresponding secondary antibodies for 1 h. β-actin was used as the internal loading control. The membrane was developed and detected using enhanced Chemiluminescence Reagent (Pierce, Rockford, IL, USA) and exposed to X-ray film (Koda, Japan).

Animal experiments

SKOV3 cells (4×10⁶) were subcutaneously injected into the flank of BALB/C female mice (30-day-old) following a protocol approved by the Ethics Committee Guide of China Three Gorges University. After ten days of injection, the mice were randomly assigned into four groups (10 mice in each group): 1) saline control (vehicle); 2) Se-PFPs only; 3) DTX only; and 4) Se-PFPs+DTX. The control group received saline intraperitoneal (i.p.) injections twice a week, as well as saline through oral gavage once a day. The mice treated with DTX only received DTX (5 mg/kg) injection i. p. twice a week, as well as saline through oral gavage once a day. The Se-PFPs+DTX treatment mice received Se-PFPs (250 mg/kg/day body weight) through DTX (5 mg/kg) i.p. injections twice a week and oral gavage once a day. The mice were weighed every other day, and the sizes of tumors were measured using a vernier caliper every three days. The mice were treated for 24 days. After the last

gavage, the mice in four groups were fasted for 16 h, weighted and euthanized through CO₂ inhalation. The tumors were then excised, weighted, and their volumes were calculated with the formula: $\pi/6 \times \text{length} \times \text{width}$. Specific organs, such as spleens, were excised and used for determining the activity and proportions of different immune cells. Blood was collected and used to determine the counts of white blood cells and measure the hepatic and renal function. The excised tumors were used to detect the proliferation and apoptosis rates.

Experimental design and calculation for CI

We performed the pretreatment and based on the results, the baseline data of the untreated control was used for normalization. We determined the dosage range for the possible coordination of drugs A and B and determined the fixed ratio of two drugs. At least three dosages and maximal six measures of drug A were selected. Given the fixed ratio of Se-PFPs: DTX = 1:2, then,

Se-PFPs (nM):	100	200	300	400	500
DTX (nM):	200	400	600	800	1,000
Combination:	A100+B200	A200+B400	A300+B600	A400+B800	A500+B1,000

The correspondingly matched dosages for drug DTX were calculated using software CalcuSyn. The experiments were repeated three times.

Immunohistochemical detection of Ki-67 and TUNEL assay for apoptotic cells

The partial tumor tissues were fixed in 4% formaldehyde and embedded in paraffin, and then cut into 4-micron sections. The commercial TM SP Kit (Zhongshan Corp., Beijing, China) was used to perform the immunohistochemical examination according to the manufacturer's instructions. The sections were exposed to 1:500 dilution of Ki-67 antibody (Santa Cruz Biotechnology) to evaluate the cell proliferation. The negative control was performed by using phosphate buffered saline; i.p., intraperitoneal (PBS) to replace Ki-67 antibody. For TUNEL assay, the other nearly frozen sections were used to detect the apoptotic cells using the TUNEL assay kit (Boehringer Mannheim, Indianapolis, IN, USA) following the manufacturer's instruction. Briefly, sections were treated with protease K at 37°C for 30 min, then incubated with a terminal deoxynucleotidyl transferase end labeling cocktail at 37°C for 2 h, and finally incubated with 50 μ L of FIFC in the dark for 1 h. The staining was scored as per Yuan et al. (2012).

Statistical analysis

For cell viability, Annexin V-FITC and morphologic analysis of apoptotic cells, values were presented as the means \pm standard deviation (SD) for at least three independent experiments done in triplicate. Statistical analyses were performed using one-way analysis of variance (ANOVA) or Student's t-test using Prism Graphpad 5 software. $P < 0.05$ was set for statistical significance. Analysis of synergism was done using a commercial CalcuSyn software program (Biosoft, Ferguson, MO, USA). A synergistic, additive, or antagonistic interaction were defined as $CI < 1$, $CI = 1$, $CI > 1$, respectively.

Ethical considerations

The animal experiments were approved by the Ethics Committee

Guide of China Three Gorges University.

RESULTS AND DISCUSSION

Se-PFPs and DTX interacted synergistically to inhibit cell proliferation and induce apoptosis in ovarian cancer cells

Figure-1A indicates that Se-PFPs or DTX alone significantly suppressed the proliferation of SKOV3 and A2780 cells at 24 and 48 h, respectively ($P < 0.05$). However, a combination of Se-PFPs and DTX showed a significant and synergistically inhibitory effect on the proliferation of both SKOV3 and A2780 cells ($P < 0.05$, Figure 1A-C). To clarify whether reduced cell proliferation after Se-PFPs and DTX treatment was caused by induction of cell death, this study carried out cell apoptosis assays using multiple methods. Annexin V and propidium iodide (PI) staining showed that treatment of 200 μ g/ml Se-PFPs or 2 nM docetaxel individually for 48 h caused cell apoptosis by 14.9 and 15.8% in SKOV3 cells, and by 14.3% and 15.4% in A2780 cells, respectively. However, the treatment of cells with a combination of Se-PFPs and DTX resulted in a significant increase in the percentage of apoptotic cells to 67.5 and 68.8% in SKOV3 and A2780 cells, respectively ($P < 0.01$, Figure 2, panels A and B). It is noteworthy that, Se-PFPs and DTX did not show significantly synergistic effect on induction of apoptosis of HOSE cells, an immortalized non-tumorigenic human ovarian surface epithelial cell line (Figure 2 panel C). The synergistic apoptosis-inducing effect was further validated by elevated PARP cleavage in combined treatment of SKOV3 and A2780 cells with Se-PFPs and DTX (Figure 2B). We also discovered that combination index (CI) values of Se-PFPs and DTX were less than 1.0 for both SKOV3 and A2780 cells, indicating a highly synergistic interaction between Se-PFPs and DTX administration in inducing apoptosis in both SKOV3 and A2780 cells (Figure 2C-D).

P. fortuneana has been used as a traditional Chinese medicine for thousands of years because of its wide range of pharmacological activities. It has been demonstrated by previous researches that Se-enriched polysaccharides possess the antiproliferation and antitumor activities in multiple cancer lines (Mao et al., 2016), especially in breast cancer (He et al., 2013) and osteosarcoma (Wang et al., 2013). Polysaccharides induce apoptosis through many signaling pathways, such as mitogen activated protein kinase (MAPK) (Zhang et al., 2013) and NF- κ B signaling pathway (Lee et al., 2014). It has been known that the NF- κ B can control many cellular processes, including inhibition of apoptosis, induction of proliferation and cell invasion. Kinoshita et al. (2010) and Zhang et al. (2003) have shown that some drugs can lead to a high level of DTX-induced activation of caspase-3 in cancer cells through inhibiting NF- κ B expressions. In addition, another kind of paclitaxel in the

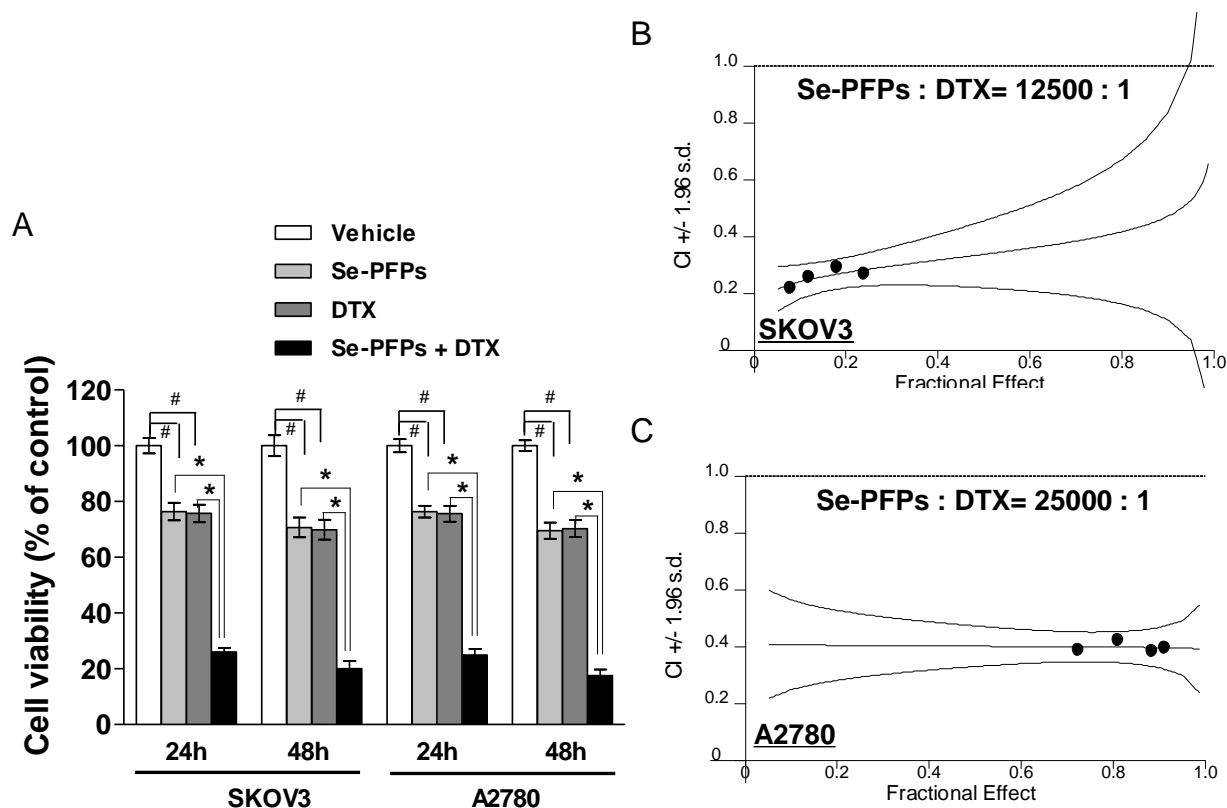


Figure 1. Synergistic interaction of Se-PFPs with DTX to inhibit proliferation in ovarian cancer cells. (A) SKOV3 cells and A2780 cells were exposed to 200 µg/ml Se-PFPs, 2 nM DTX or 200 µg/ml Se-PFPs + 2 nM DTX for 24 and 48 h, respectively. Cell viability was measured using MTT method. The data are expressed as the ratio to the control cells. (B) SKOV3 and (C) A2780 cells were incubated with a range of Se-PFPs (200 to 500 µg/ml) and DTX (2 to 5 nM) alone and in combination at a fixed ratio (e.g. SKOV3, 12500:1; A2780, 25000:1) for 48 h. At the end of the exposure, the cell viability was measured as mentioned above. Fractional effect values were analyzed by CalcuSyn software. CI values <1.0 indicate a synergistic interaction. All the experiments were repeated thrice independently, and the data were expressed as mean ± SD. #*p* < 0.05; **p* < 0.01, comparing with the indicated group.

taxane family has been proved to have inhibitory effects on NF-κB family (Kinoshita et al., 2016; Zhang et al., 2003). Based on these observations, the increased caspase-3 activation in our study suggests that the combination therapy might also function through inhibition of the NF-κB activation. Thus, Se-PFPs and DTX may induce apoptosis of ovarian cancer cells through a common signaling pathway, which may be one of the cellular and molecular bases underlying their synergistic effect. However, further study is needed to confirm this hypothesis.

The combination therapy was initially used in treatment of lymphomas and leukemia (Andrew and Brown, 2017). Nowadays, it is widely used for treatment of malignant tumors. Without a single established criterion, it is difficult to clearly examine whether the combination is of antagonism or synergy. Some effective methods have been applied, such as the CI-isobologram (Liu et al., 2017) and the CI method. To assess the effects of Se-PFPs and DTX combination treatments, this study conducted a statistical analysis by determining CI values

in this research. The CI values have shown that the interaction between Se-PFPs and DTX has moderately synergistic effects against A2780 and SKOV3 cell lines. Similar results have been revealed by cell viability analysis, Annexin V and PI staining. Moreover, the similar synergistic effects demonstrated in our animal experiment suggest that the combined treatment has great potential in the real clinical application.

Se-PFPs enhanced DTX-induced inhibition on tumor growth and induction of apoptosis *in vivo*

To verify whether the *in vitro* findings in cultured cells could be repeated *in vivo*, nude mice xenografted model carrying SKOV3 cells were divided into four groups and treated with saline control; 5 mg/kg DTX (i.p. twice a week); 250 mg/kg Se-PFPs (oral gavage, once a day) or a combination of 5 mg/kg DTX + 250 mg/kg Se-PFPs, respectively. The effects of combination group were then compared with those of the other three groups. The mice

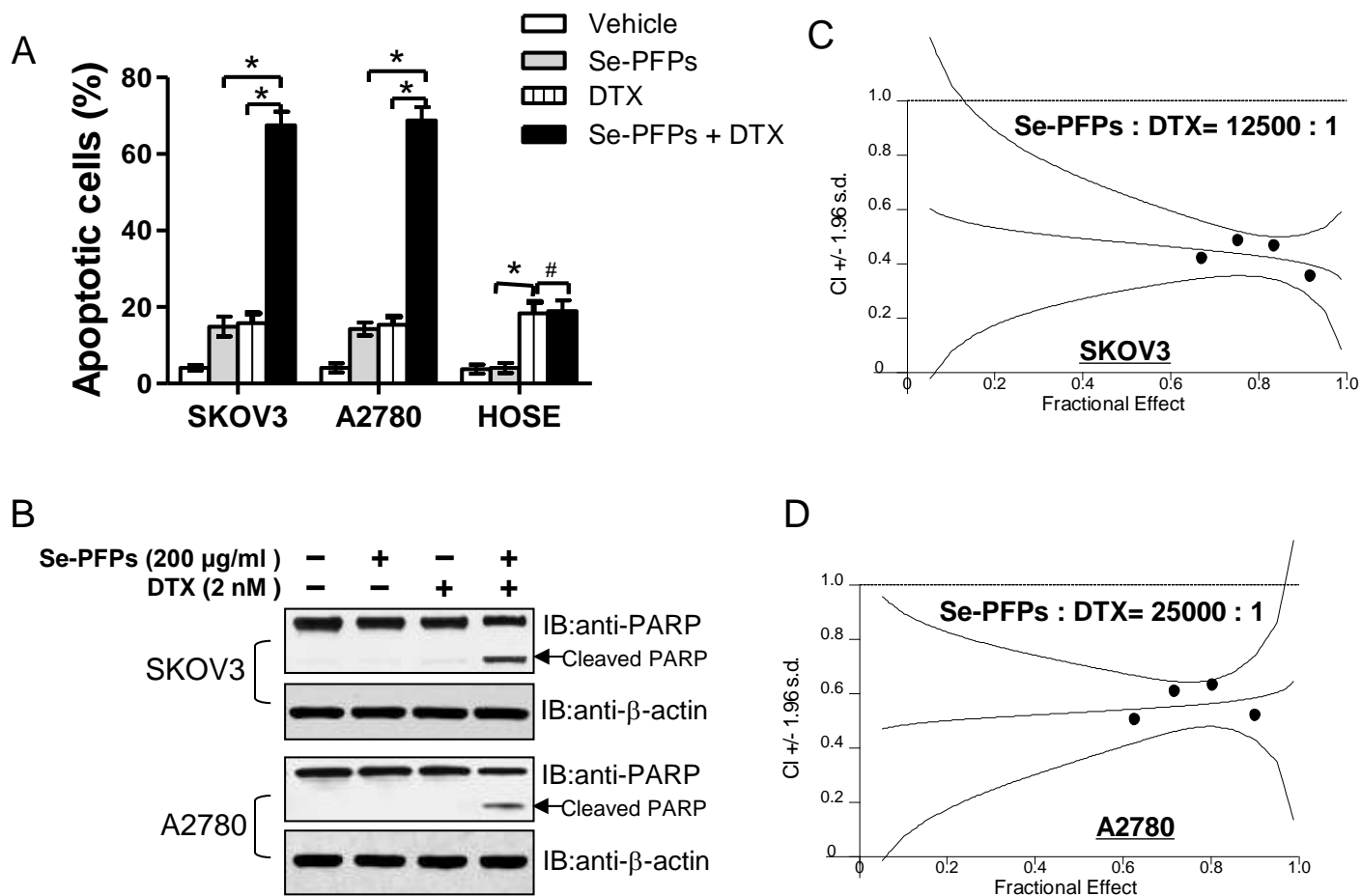


Figure 2. Co-administration of Se-PFPs and DTX induced apoptosis in ovarian cancer cells. (A) SKOV3, A2780 cells and (B) immortalized, non-tumorigenic HOSE cells were treated with 200 µg/ml Se-PFPs, 2 nM DTX or 200 µg/ml Se-PFPs + 2 nM DTX for 48 h, respectively. Apoptosis was determined by flow cytometry as described in this study. (C) SKOV3 and A2780 cells were exposed to 200 µg/ml Se-PFPs ± 2 nM DTX for 48 h, the cells were lysed and proteins separated by SDS-PAGE, and Western blot analysis was performed to monitor the degradation of PARP. (D) SKOV3 and A2780 cells were incubated with a range of Se-PFPs (200 to 500 µg/ml) and DTX (2 to 5 nM) alone and in combination at a fixed ratio (e.g. SKOV3, 12500:1; A2780, 25000:1) for 48 h. At the end of the exposure, apoptosis was measured by flow cytometry. Fractional effect values were analyzed by CalcuSyn software. CI values <1.0 indicate a synergistic interaction. All the experiments were repeated three times independently, and the data were expressed as mean ± SD. * $p < 0.01$, comparing with the indicated group, whereas # $p > 0.05$, comparing with DTX group.

treated with either Se-PFPs or DTX exhibited reduced tumor volume to a certain extent while the mice treated with combination of Se-PFPs and DTX displayed decreased tumor weight and tumor volume after 18 days compared with those in groups of mice treated with vehicle control, DTX or Se-PFPs alone, respectively. At 24 days, the difference in the reduction ranges between the treatment with combined DTX or Se-PFPs and those treated with either DTX or Se-PFPs became much larger ($P < 0.05$, Figure 3A and B). However, there were no differences in body weight of mice among these groups (data not shown).

In order to evaluate the potential mechanisms by which Se-PFPs augment DTX-induced tumor-inhibition effect, the sections from the treated mice were stained antibody against Ki-67 (a marker for proliferation), or subjected to

TUNEL assay (for apoptotic cells). Figure 3C and D indicate that the immunohistochemical staining of Ki-67 and TUNEL assay revealed that proportion of Ki-67-positive cells was significantly decreased while the proportion of TUNEL positive cells was significantly increased in xenografted tumor tissues of mice treated with combination of DTX or Se-PFPs, as compared with those of mice treated with vehicle control, DTX or Se-PFPs alone, respectively ($P < 0.05$). Consistent with our findings of *in vitro* experiments, these results obtained from the *in vivo* experiments support the conclusion that Se-PFPs can augment DTX-induced tumor inhibition in ovarian cancer.

This study clearly demonstrated that Se-PFPs could augment synergistically the anti-cancer function of DTX in ovarian cancer. However, the underlying molecular

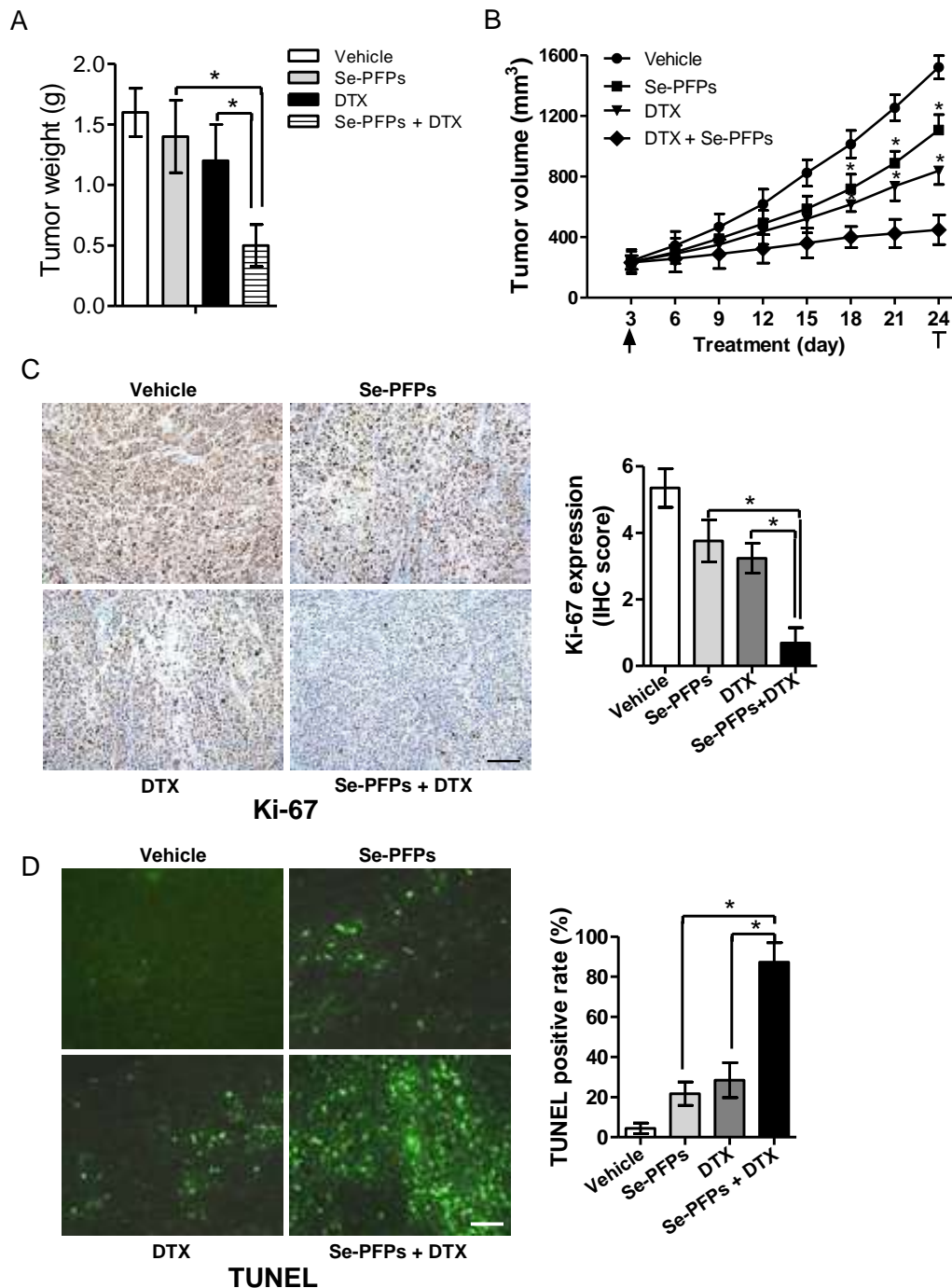


Figure 3. Augmentation of DTX-induced tumor inhibition *in vivo* using Se-PFPs. The (A) weight and (B) volume of tumors from SKOV3-xenografted mice were shown. SKOV3 cells (4×10^6) were injected subcutaneously into mice. After ten days of injection, the mice were treated as described in this study. The tumor size was measured every 3 days. After 24 days of treatment, the mice were euthanized by CO₂ inhalation. The tumors were separated and weighed. C) IHC staining for Ki-67 (200x) and (D) TUNEL (40x). * $p < 0.05$ (n=10), compared with the combined treatment group.

mechanisms are not clearly understood and still needs to be further elucidated. It has been reported that DTX can induce suppression of peripheral blood mononuclear cells (PBMC) without decreasing expression levels of Th1-

derived cytokines. Whether or not the combination of DTX and Se-PFPs are involved in immunomodulation of different types of immune cells and in regulation of proinflammatory cytokines requires to be further

investigated. Furthermore, based on the results of the experiments with HOSE, it can be concluded that the combination of DTX and Se-PFPs has relatively slight impact on normal ovarian cells (Figure 2C-D). However, it remains to be confirmed with other types of normal cells and to be determined whether inhibitory effects of their combination are tumor-specific; if it is proved to be the case, the combination therapy of DTX and Se-PFPs will be of therapeutic and clinical significance.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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