

Effects of a cyclooxygenase-1-selective inhibitor in combination with taxol or cisplatin on cyclin D1, apoptosis, and vascular endothelial growth factor in a xenograft model of ovarian cancer

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Summary

This study evaluated the effectiveness of SC-560 (a cyclooxygenase-1-selective inhibitor) in combination with taxol or cisplatin (DDP) in mice bearing SKOV-3 human ovarian carcinoma xenograft. Cyclin D1 expression, apoptotic index, and vascular endothelial growth factor (VEGF) mRNA level in tumor tissues were determined by immunohistochemistry, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling assay, and reverse transcription-polymerase chain reaction, respectively. Cyclin D1 and VEGF mRNA expression were inhibited, whereas the apoptotic index was markedly increased in the therapeutic group (all $p < 0.05$ in comparison with the control). The group of SC-560 + taxol had synergistic effects on suppressing cyclin D1 and VEGF expression and promoting apoptosis in comparison to SC-560 or taxol alone ($p < 0.05$). Compared with SC-560 or DDP group, SC-560 + DDP treatment made a greater reduction on level of VEGF mRNA ($p < 0.05$). The results of this study revealed that the combined therapy of SC-560 and taxol had synergistic effects on suppression of cyclin D1 and VEGF expression, and apoptosis-promoting in mice bearing SKOV-3 human ovarian carcinoma xenografts.

Key words: Epithelial ovarian cancer; SC-560; Cisplatin; Taxol; Cyclin D1; Apoptosis; Vascular endothelial growth factor.

Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecologic malignancy. With an estimated 22,240 new cases in the United States alone, ovarian carcinoma was the fifth most common cause of cancer-related deaths (14,070 cases) in females in 2018 [1]. More than 70% cases had been diagnosed in the advanced stage of EOC (stage III/IV) with intraperitoneal metastasis and massive malignant ascites [2]. The high mortality rate of EOC is related to late-stage diagnosis, recurrence, and drug resistance. At present, optimal primary cytoreductive surgery is carried out mainly in patients with early-stage of ovarian malignant tumor (stage I/II), this surgery for the patients with later period tumor (stage III/IV) is not always possible [3]. The most important adjuvant therapy for preventing recurrence of later period ovarian cancer is chemotherapy. The backbone of first-line chemotherapy in ovarian cancer is a platinum agent and a taxane, has never been changed in the past ten years. Platinum-based drugs such as cisplatin (DDP) are the most active agents for the treatment of ovarian cancer, with their effects mediated through the formation of intrastrand crosslinks with DNA [4], which primarily lead to cellular apoptosis. Platinum-based chemotherapy plays a central role in the treatment of ovarian cancer and has improved outcome [5]. However, it is well known that resistance to

DDP greatly reduces the effectiveness of chemotherapy, results in recurrence, metastasis and poor prognosis of ovarian cancer [6]. Paclitaxel, a natural product originally isolated from the bark of *taxus brevifolia*, has been approved by the U.S. Food and Drug Administration for the treatment of ovarian cancer. In randomized controlled trials of stage III/IV ovarian cancer, overall and progression-free survival of patients with incomplete cytoreductive surgery have been prolonged by the combination therapy of cisplatin and paclitaxel [7]. However, taxol chemotherapy has limited success because of its dose-limiting toxicity and eventual drug resistance in patients. Multi-drug resistance (MDR) is a primary reason for the failure of chemotherapy in ovarian malignant carcinoma [8]. However, considerable efforts have been made to overcome MDR and to define new molecular therapies including inhibitors, modulators, and gene therapy [9]. Concurrent use of combination chemotherapy can block the development of multiple intracellular escape pathways by the different mechanisms of action and differing modes of drug resistance [10].

In the past decade, studies have demonstrated that cyclooxygenase-1 (COX-1) is overexpressed in ovarian epithelial cell lines [11], especially in high-grade serous and endometrioid ovarian cancers [12]. A previous study showed that daily intake of non-steroidal anti-inflammatory

steroids (NSAIDs) reduced the risk of ovarian cancer by 47% [13]. SC-560 is a COX-1-selective inhibitor that belongs to NSAIDs. It has been shown to markedly reduce the growth of ovarian tumors by suppressing proliferation or inducing apoptosis [14]. Co-treatment of SC-560 and taxol has been proven to be a powerful therapeutic tool to promote the sensitization of paclitaxel-resistant ovarian cancer cells [11]. In this study, the authors investigated ovarian tumor growth in mice with transplanted human ovarian cancer SKOV-3 cells, evaluated the effectiveness of SC-560 in combination with taxol or DDP on cell apoptosis, expression of cyclin D1 protein and vascular endothelial growth factor (VEGF).

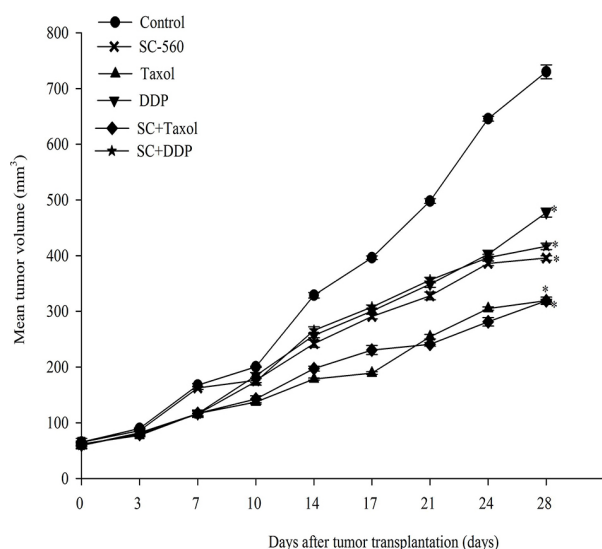


Figure 1. — The therapeutic efficacy of SC-560, taxol, DDP, combination and NS on the growth of tumor of SKOV-3 cells xenografts. SC-560 (3 mg/kg, bid, oral gavage), taxol (20 mg/kg, weekly, i.p.), and DDP (3 mg/kg, qod, i.p.) were administered at 1 week after the tumors became visible to the naked eye. The duration of administration was 21 days. Mean tumor volume in the control group was significantly larger than the therapeutic groups on day 28. Statistical significance was analyzed by the Student's *t*-test with SPSS software (version 17.0), **p* < 0.05.

Materials and Methods

The SKOV-3 cell line was cultured in the recommendatory culture medium under normative conditions. Nu/nu female athymic mice (7–8 weeks old) were transplanted with 2×10^6 SKOV-3 cells in the dorsal skin. 36 mice were divided into six groups by stochastic method in the 7th day of inoculation: control, SC-560, taxol, DDP, SC-560 + taxol, and SC-560 + DDP. All experimental procedures were performed in conformity to the guidelines for laboratory animals' care and use, and the present ethics of animal experiments were approved by the Ethics Committee of Zhejiang University School of Medicine (Hangzhou, China).

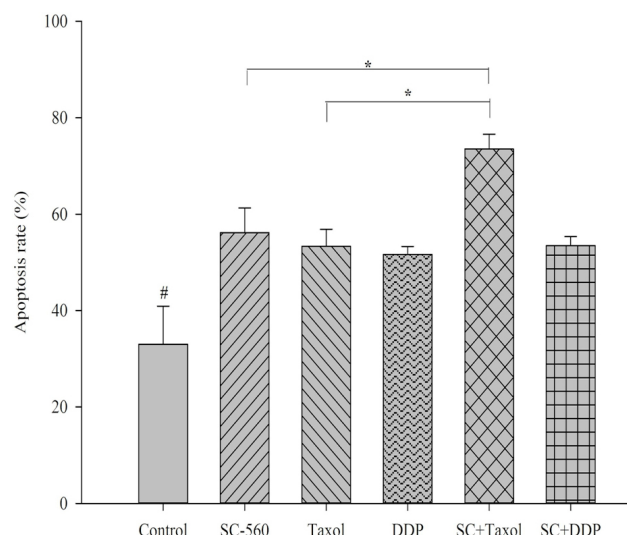


Figure 2. — Apoptosis rate of the SC-560, taxol, DDP, combination and control groups. The apoptotic index was applied to assess the percentage of apoptotic cells in the specimens of xenografts, and was calculated by the ratio of number of positive cells/total number of cells. Apoptosis rate in the control group was significantly lower than the treatment groups (#*p* < 0.05 for all). In comparison with SC-560 or taxol group, synergistic effects on apoptotic index were found in the group of SC-560 + taxol, **p* < 0.05; error bars represent standard error.

Medicines were administered as follows: SC-560 (3 mg/kg, bid, oral gavage), Taxol (20 mg/kg, weekly, intraperitoneal injection (i.p.)), were suspended in a 0.5 mL suspension of 5% methylcellulose and 0.025% Tween 20. DDP (3 mg/kg, qod, i.p.) was suspended in phosphate-buffered saline (PBS, pH 7.2). Mice were injected with normal saline (NS) under similar conditions as the control. Medicines or NS were administered at 1 week after the tumors became visible to the naked eye, and lasted for a duration of 21 days. The researchers weighed the mice every week. On day 28, samples of xenograft tissue were gathered after sacrificing mice, and were preserved in solution of 10% phosphate-buffered formalin for immunohistochemistry or immediately snap-frozen in liquid nitrogen (-80 °C) until further analysis.

Apoptosis of tumor cell was evaluated by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay, which can be applied to identify cell apoptosis. The samples of tumor tissue were treated as follows: fixed with 4% paraformaldehyde (24 h), dehydrated, and embedded with paraffin, sliced into 4 μ m-thick sections. The sections were immersed in 20 μ g proteinase K/mL PBS (-) (15 min, 25 °C) after deparaffinization in a graded series of ethanol, then the activity of endogenous peroxidase was blocked. The samples were cultivated with TdT buffer (contain TdT enzyme, biotin-16-dUTP and 0.01% bovine serum albumin) in a humidity chamber (1.5 h, 37 °C). The avidin-biotin complex method

(DAB as the chromogen) was applied to detect Biotin-16-dUTP nucleotides, which had been incorporated into DNA fragments. The apoptotic index (AI) was calculated in five randomly captured high-power fields ($400\times$ magnification) by the following equation:

$$\text{AI} = (\text{number of positive cells} / \text{total number of cells}) \times 100\%$$

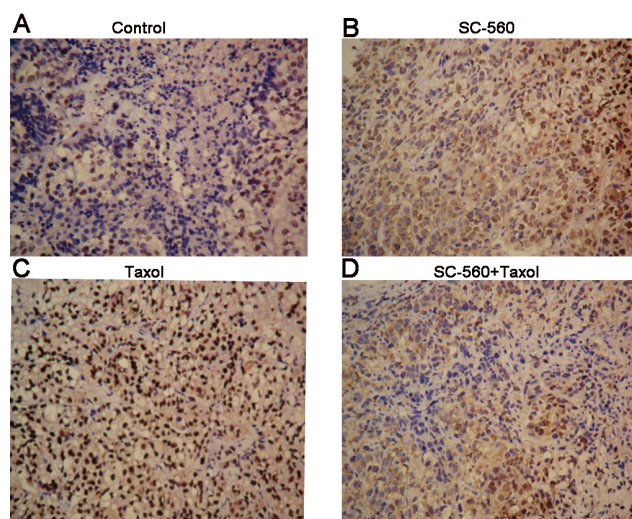


Figure 3. — Apoptotic cells in the specimens of xenografts were labeled by the TUNEL method. Cell apoptosis of control, SC-560, taxol and SC-560 + taxol were displayed by TUNEL-positive cells in the optical microscope representative pictures.

Immunohistochemistry was applied to detect Cyclin D1 protein. The tumor tissues were treated as follows: fixed with 10% neutral buffered formalin (24–48 h), embedded in paraffin, deparaffinization, heated in 10 mM Tris-HCl with 1 mM EDTA (121 °C, 15 min, pH 9.0), blocked endogenous peroxidase by 3% H_2O_2 in methanol (10 min, 25 °C). At room temperature, the samples were successively incubated with cyclin D1 antibody (90 min), EnVision reagent (40 min) and DAB/hydrogen peroxide (8–12 min). The nuclear expression of cyclin D1 was evaluated in each section by determination of the percentage of staining positive cells in 5 randomly captured fields ($400\times$ magnification).

Statistical significance between the control and the treatment group was analyzed by the Student's *t*-test with SPSS software (version 17.0). Data from all experimental groups were expressed as the mean \pm standard error of the mean. Results were considered statistically significant for $p < 0.05$.

Result

The SKOV-3 cell line was used to evaluate the inhibitory effect of medicines and NS on the growth of ovarian tumor. Figure 1 showed the therapeutic efficacy of six experimental groups on the tumor growth. There was a consistent growth of the neoplasm dimension in the control group,

whereas the average neoplasm dimension of the other five groups were markedly suppressed (all $p < 0.05$). The mean tumor volumes of the SC-560, taxol, DDP and control groups were 396 mm^3 , 319 mm^3 , 477 mm^3 and 730 mm^3 on the 28th day, respectively. The inhibitory effect of ovarian cancer growth in SC-560 and taxol group were better than DDP group. In the tumor sections, apoptotic cells were detected by the TUNEL method and AI was calculated in five high-power fields. The TUNEL assay showed the up-regulation of AI in all treatment groups. The apoptotic rates were $56.00 \pm 5.23\%$, $53.00 \pm 3.88\%$, $52.00 \pm 2.14\%$, $74.00 \pm 3.44\%$, and $54.00 \pm 2.32\%$ in the SC-560, taxol, DDP, SC-560 + taxol, and SC-560 + DDP group, respectively. There were statistically significant in comparison with the NS group ($33.00 \pm 8.36\%$, all $p < 0.05$). The group of SC-560 + taxol showed synergistic effects on the apoptotic rate of tumor cells in comparison with the taxol group ($p < 0.05$). However, there was no difference in AI between SC-560 and SC-560 + DDP (Figure 2). Representative immunohistochemistry images of tumor specimens showing the effects of control, SC-560, taxol, and the combination of SC-560 + taxol on cell apoptosis were shown in Figure 3.

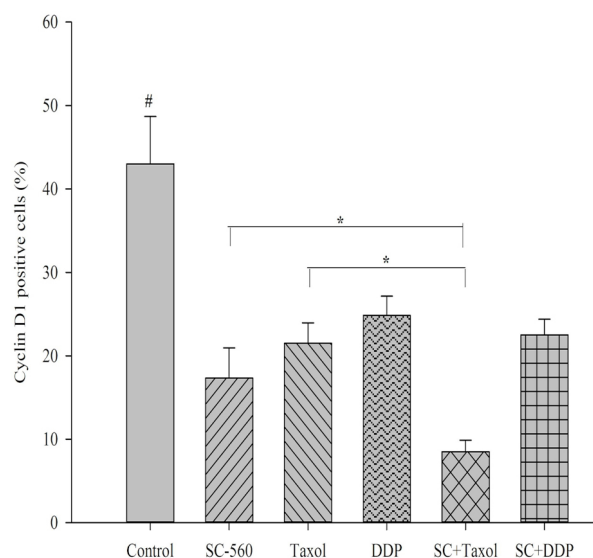


Figure 4. — SC-560 and/or taxol or DDP had profound inhibitory effects on the positive rates of cyclin D1. Cel-cyclD1+ (%) in the treatment groups compared with the control group, $\#p < 0.05$ for all. In comparison with SC-560 or taxol alone, the group of SC-560 + taxol had a synergistic inhibitory effect on cel-cyclD1+, $*p < 0.05$; error bars represent standard error.

The protein changes of cyclin D1 in ovarian cancer cells were detected by immunohistochemistry. Quantification analysis showed that the proportion of cyclin D1-positive cells (cel-cyclD1+) in the control, SC-560, taxol, DDP, SC-560 + taxol, and SC-560 + DDP group was $43.00 \pm 6.12\%$, $17.00 \pm 4.23\%$, $22.00 \pm 1.94\%$, $25.00 \pm 2.13\%$,

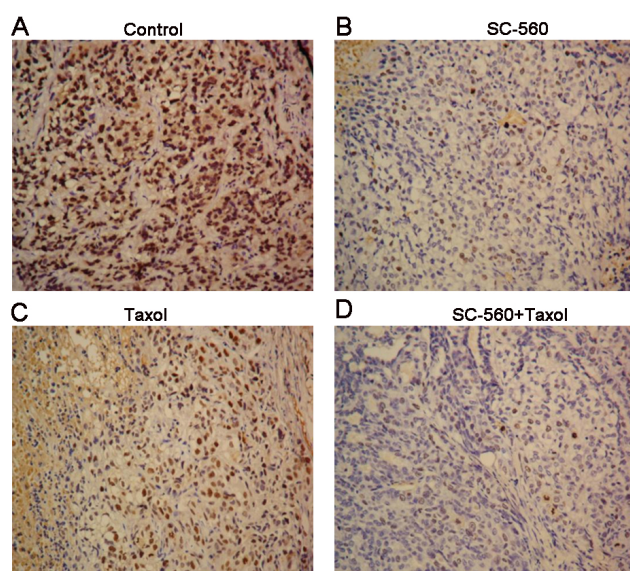


Figure 5. — Representative images of cyclin D1 immunohistochemical staining in the control, SC-560, taxol, SC-560 + taxol. Cel-cyclinD1+ (%) in xenografts sections of the therapeutic groups were significantly lower than the control.

9.00 ± 1.44%, and 23.00 ± 1.87%, respectively. There were significant inhibitory effects in the groups treated with chemotherapy (all $p < 0.05$ in comparison with the NS group). The group of SC-560 + taxol had a synergistic effect on the proportion of cel-cyclinD1+ in comparison to the taxol group ($p < 0.05$), whereas synergistic effect wasn't found in the SC-560 + DDP group (Figure 4). The cel-cyclinD1+ of control, SC-560, taxol, and SC-560 + taxol were showed in representative images (Figure 5).

VEGF levels were measured in xenograft tumors by quantitative PCR (qPCR). Three different molecular isoforms of VEGF (containing 189-, 165-, and 121-amino acid residues) were generated by alternative mRNA splicing. The qPCR analysis indicated the ΔC_t ($\Delta C_t = C_{t,selectedgene} - C_{t,\beta-actin}$) of VEGF in the six groups (Table 1). The expression levels of VEGF mRNA were observably repressed in the therapeutic groups (Figure 6, all $p < 0.05$). In addition, SC-560 + DDP or SC-560 + taxol showed more synergistic repressions on VEGF mRNA expression than SC-560 or DDP or taxol alone ($p < 0.05$).

Discussion

The major discovery of this research was that the inhibitory effect of taxol on the growth of SKOV-3 xenografts was enhanced by SC-560 in female nu/nu athymic mice. Anti-tumor efficacy of SC-560 + taxol was concerned with remarkable decline of cyclin D1, VEGF mRNA expression and induction of apoptosis, whereas SC-560 alone enhanced the inhibitory effect of DDP on VEGF mRNA expression.

The 3-weekly regimen of intravenous carboplatin and paclitaxel remains the international standard chemotherapy

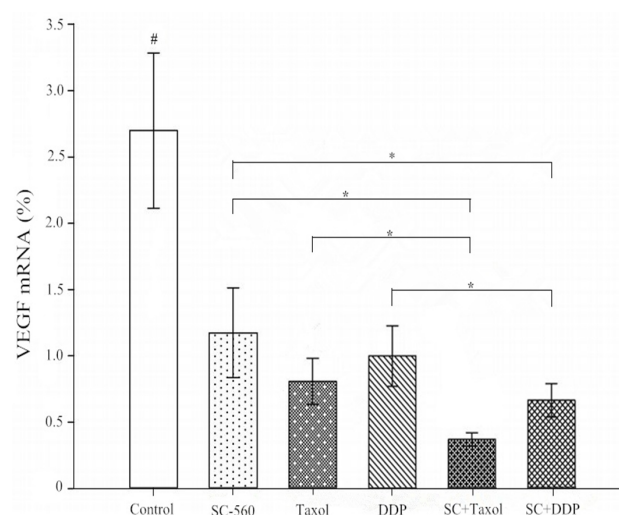


Figure 6. — Effects of the six groups on the expression of VEGF mRNA. In comparison with the control, the expressions of VEGF mRNA were observably repressed in the therapeutic groups (# $p < 0.05$); * $p < 0.05$; error bars represent standard error.

Table 1. — ΔC_t of VEGF in the six groups (control, SC-560, taxol, DDP, SC-560 + taxol combination group and SC-560 + DDP combination group)^a.

Group	VEGF 121	VEGF 165	VEGF 189
Control	6.94 ± 0.23	4.58 ± 0.26	6.34 ± 0.23
SC-560	7.13 ± 0.30	5.34 ± 0.30	6.54 ± 0.21
Taxol	7.87 ± 0.32	6.28 ± 0.49	7.79 ± 0.29
DDP	7.71 ± 0.38	5.63 ± 0.51	7.14 ± 0.28
SC-560 + Taxol	9.14 ± 0.33	7.00 ± 0.48	8.99 ± 0.23
SC-560 + DDP	8.16 ± 0.36	6.83 ± 0.37	8.27 ± 0.42

^aMolecular isoforms of VEGF were generated by alternative splicing, rendering proteins containing 189-, 165- and 121-amino acid residues. VEGF 189, 165 and 121 were routinely detected in this series of ovarian cancer.

regimen for first-line therapy in advanced-stage epithelial ovarian cancer [15]. Clinical efficacy of DDP is limited by drug resistance, which is the consequence of a wide variety of modulatory mechanisms. MDR is contributed to inefficiency of chemotherapy in epithelial ovarian tumor [16], and multidrug combination therapy was utilized to overcome drug resistance.

The expression of COX-1 (one isoform of COX) occurs constitutively in most tissues and COX-1 maintains homeostasis, whereas the expression of COX-2 (another isoform of COX) is induced by multiple stimuli including mitogens, cytokines and hypoxia. Our previous study showed that the ovarian epithelial cancer growth was inhibited by celecoxib (COX-2-selective inhibitor) in a COX-2 dependent mechanism [18]. However, a subsequent study showed that COX-1-selective inhibitors might also have potent anti-tumor activity in EOC. SC-560 suppresses ovar-

ian epithelial cancer growth in vivo and also attenuates cell proliferation and promotes apoptosis [19]. Previous studies have shown that anti-angiogenic and apoptosis-promoting effects of taxol were enhanced by SC-560, better than celecoxib [20]. Therefore, the efficacy of COX-1 inhibitor on ovarian malignancy was evaluated in present study.

Anti-angiogenic therapies were tested in ovarian cancer currently for its high vascular, which is essential for tumor growth and the development of metastases. A previous research demonstrated that anti-angiogenic effect of taxol was enhanced by SC-560 [20]. COX-1 is involved in the regulation of angiogenesis, positively correlated with the expression of VEGF, while inhibition of COX-1 can reverse this relevance [21]. SC-560 can inhibit tumor angiogenesis by inhibiting COX-1 expression [11], the pivotal mechanism is related to decrease tumor-associated VEGF expression [20, 22] and the expression of MDR1 P-glycoprotein (P-gp). The VEGF mRNA expression level in SC-560 group was significantly suppressed, which was different from the control group in present study. There were synergistic repressions on expression of VEGF mRNA in SC-560 + DDP and SC-560 + taxol group. Chang *et al.* [22] found that SC-560 attenuated angiogenesis by downregulating COX-, nuclear factor kappa B- and VEGF-mediated pathways, and improved hepatopulmonary syndrome in cirrhotic rats. A previous report [23] also suggested that ximenynic acid (COX-1-selective inhibitor) reduced the expression level of VEGF-B and VEGF-C. Recently, Lee *et al.* [24] reported that paclitaxel combined with SC-560 can suppress the expression of MDR1 gene and P-gp (an ATP-binding cassette transporter), further facilitate cytotoxicity of paclitaxel in the treatment of taxane-resistant ovarian cancer. Their findings suggested that a COX-1 inhibitor might be a powerful medicine as chemosensitizer, anti-angiogenic and apoptosis-promoting agent. The data from the current study was consistent with this previous study, which was performed in taxane-sensitive ovarian cell lines. Therefore, the powerful antiangiogenesis of SC-560 + DDP or SC-560 + taxol may be the central mechanism of action in SKOV-3 xenografts.

This study also determined whether there is another function of SC-560 in epithelial ovarian cancer, which remains unknown. Uncontrolled proliferation is another feature of malignant tumor. The molecular mechanisms of the cell cycle transition involved in tumor formation have been getting more attention, modulators of cyclins may be a potential target in the treatment of malignant tumor [25]. Taxol mainly induces G2/M cell cycle arrest by cyclin B1-associated cell division cycle 2 kinase, ultimately leads to cessation of cell division and suppression of cell proliferation [26]. Similarly, DDP induces the G0/G1 cell cycle arrest and apoptosis of tumor cells, represses expression of cyclin D1 [27]. Cyclin D1 plays an important role in the transition to the S phase (DNA synthesis), and its overexpression contributes to transformation of malignant tumor. Our previous study has shown that cyclin D1 expression

was positively correlated with tumor cell proliferation [19]. Degradation of cyclin D1 might be a potential chemotherapeutic target for inducing G1 cell cycle arrest of ovarian epithelial cancer [28]. The percentage of cel-cyclD1+ in the SC-560 group was significantly suppressed in comparison with the control group, indicated that there might be a relevance between COX-1 and cyclin D1. Cai *et al.* [23] found that ximenynic acid (COX-1-selective inhibitor) suppressed the mRNA expression of cyclin D3 and cyclin E1, resulted in arrest of G1/S phase transition. This finding raised the possibility that SC-560 can attenuate the growth of human ovarian cancer xenografts by a COX-1-dependent manner. More researches are needed to explore this possibility. In this study, the combination of SC-560 and taxol had significant synergistic effects on cel-cyclD1+ compared with SC-560 or taxol alone. Therefore, SC-560 might enhance the inhibitory effect of taxol on cyclin D1 expression.

The unbalance of apoptosis and cell proliferation is indispensable for neoplastic transformation. Apoptosis is a multi-step process, and the rapidly accumulating data of genes allows systematic studies on the control or execution of apoptosis. Administration of taxol can trigger cell cycle arrest and apoptosis by inducing imbalance polymerization and depolymerization of microtubule [29]. This study showed that combination of SC-560 and taxol made synergistic effects on inducing apoptosis compared to SC-560 or taxol alone in xenografts, this result was similar with the research of Lee *et al.* [24]. Therefore, induction of apoptosis and suppression of proliferation by the combination of SC-560 and taxol might be another mechanism of suppressive effect in the ovarian cancer xenografts.

Significant inhibitory effects on the positive rates of cyclin D1 were observed when nude mice were administered taxol or DDP in present study. In addition, SC-560 + taxol had significant synergistic effects on cel-cyclD1+ and the apoptotic rate compared with SC-560 or taxol alone, whereas there was no synergistic effect on cel-cyclD1+ and apoptotic rate in the SC-560 + DDP group. The possible reason might be the ovarian carcinoma cell line employed (SKOV-3 is a platinum-resistant cell line). Ishiguro *et al.* [30] found that paclitaxel was promising for its effectiveness regardless of the platinum-sensitivity status. More researches are needed to clarify its molecular mechanism.

In conclusion, the synergistic effects of SC-560 combined with taxol on the downregulation of cyclin D1 and VEGF mRNA, reflecting apoptosis-promoting and proliferation suppression in SKOV-3 xenografts. However, SC-560 combined with DDP did not have synergistic effects on cyclin D1 and apoptosis.

Acknowledgments

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Conflict of interest

The authors declared that they have no conflict of interest.

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