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Reasearch Article

A Combination of Taxol and SC-560 Regulates Ki-67 Expression and Apoptosis in Human Ovarian Carcinoma Xenografts

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2. Keywords

Ovarian cancer; Taxol; SC-560; Ki-67; Apoptosis

1. Abstract

This study aimed to explore how SC-560, a COX-1 selective inhibitor, regulates expression of Ki-67 and apoptosis in human ovarian SKOV-3 carcinoma cells xenograft-bearing mice when combined either with taxol or cisplatin (DDP). Mice were orally administered with 3 mg/kg SC-560 two times a day, and intraperitoneal (i.p.) injections of 20 mg/kg taxol, once a week and 3 mg/kg DDP every other day, or SC-560/taxol, SC-560/DDP, for 21 days. The expression index of Ki-67 in tumor tissues was determined through immunohistochemistry. We evaluated the apoptotic index using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay. In the drug-treated group, Ki-67 expression expression in the drug-treated groups was significantly down-regulated, while the apoptotic index remarkably increased, and all reached a statistical difference (all $p < 0.05$ vs Control). Combined therapy demonstrated a synergistic effect, significantly inhibited the quantification of Ki-67 positive cells (all $p < 0.05$), and promoted cell apoptosis ($p < 0.05$) compared to when SC-560 or taxol are used independently. Conclusively, from this work, we reveal that the combination of SC-560-with taxol has a synergistic effect on inhibiting cell proliferation and inducing of cell apoptosis in human ovarian cancer xenografts.

3. Introduction

Epithelial Ovarian Cancer (EOC) is an occult disease with few distinct symptoms presenting at early stages. Earlier diagnosis is often delayed owing to the lack of effective screening mechanisms. Ovarian cancer is the leading cause of gynecologic malignancy-related mortalities. With an estimate of 22,440 new cases and 14,080 deaths in the US alone by 2017, ovarian carcinoma is regarded as the fifth most common cause of cancer deaths in the female population globally [1]. More than 70% of patients are diagnosed with peritoneal cavity metastasis and large amounts of ascites at the advanced stage (stage III / IV) [2]. The high mortality from EOC is mainly attributed to late-stage diagnosis, disease recurrence as well as resistance to standard platinum-based chemotherapy [3]. Even with the increasing use of neoadjuvant chemotherapy, surgery and combination treatment with platinum agents and taxane still is the standard care for managing, newly diagnosed patients with EOC [4]. Currently EOC treatments on trial include the incorporation of biologic agents or immune-oncology drugs to chemotherapy, the use of new combination therapy strategies such as anti-vascular drugs and PARP inhibitors [4].

Paclitaxel, a natural product isolated from the yew bark, was originally used as a microtubule-stabilizing drug. The Food and Drug Administration approved paclitaxel as suitable, for treating various tumors such as ovarian cancer, breast cancer and lung cancer. Taxol, a classic drug, used in advanced ovarian cancer, has been reported to significantly improve the survival of patients. However, taxol chemotherapy has limited success due to dose-limiting toxicity and eventual drug resistance in patients [5]. In the past decade, studies demonstrated that cyclooxygenase-1(COX-1) is over-expressed in ovarian surface epithelial cell lines at different stages of human EOC (onset and progression), [6] SC-560 is a COX-1 selective inhibitor belonging to nonsteroidal anti-inflammatory steroids (NSAIDs). Besides, Daikoku et al. using a mouse model of EOC, revealed that overexpression of COX-1 could be inhibited by SC-560 [7], a finding that is consistent with our previous study [8]. We previously revealed that the COX-1 inhibitor may improve survival time in ovarian cancer xenograft-bearing mice and inhibit tumor growth [9]. SC-560 in combination with taxol showed a satisfactory, decreasing tendency in growth-inhibitory effect in human ovarian cancer xenografts [10]. However, mechanisms by which COX-1

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inhibitors combined with chemotherapy drugs regulates ovarian tumor growth is still under research.

Therefore, in this work, we used human ovarian cancer SKOV-3 cells xenograft-bearing mice as an experimental model to evaluate the effects of SC-560 either in combination with taxol or cisplatin (DDP) on ovarian tumor growth, cell apoptosis, and Ki-67 positive cells in a human ovarian cancer xenograft.

4. Experimental Design

All chemicals used in this study were purchased from Sigma Chemical unless otherwise indicated.

The study was approved by the Ethics Committee of the Maternal and Child Health Hospital Affiliated to Nantong University in China.

4.1. Human Ovarian Tumors in Nude Mice

In the present study, we used SKOV-3 cells for *in vivo* assessment of tumor growth. The SKOV-3 ovarian cancer cell line was purchased from China Type Culture Collection (Wuhan, China) and cultured in a medium recommended under standard conditions. The SKOV-3 cells were inoculated subcutaneously into the dorsal skin (2×10^6 cells) of female athymic nude mice (nu/nu, 7-8 weeks old).The resulting tumors were visible on experiment day 7 after inoculation. At the same time, the mice were randomly divided into six groups (six mice per group): control, SC-560, taxol, DDP (as control), SC-560/taxol, and SC-560/DDP (as control).

4.2. Dose and Administration of Drugs

COX inhibitors, SC-560 (Sigma Chemical Co., St. Louis, MO, USA), a COX inhibitor, was orally administered by gavage at a dose of 3 mg/kg twice a day. Besides, Taxol (Bristol Myers Squibb SRL, Italy) was administered via intraperitoneal (i.p.) injection once a week at a dose of 20 mg/kg. The drugs were suspended in 0.5 ml solution containing 5% methylcellulose (Sigma Chemical Co.) and 0.025% Tween 20 (Sigma Chemical Co.). DDP was purchased from Haoshen Pharmaceutical Co. (Jiangsu, China), suspended in phosphate buffer saline (PBS, pH 7.2), and was administered via i.p. injection at a dose of 3 mg/kg every other day. The doses of COX inhibitors were selected for their specificity in inhibiting COX isotypes [11]. For the control group, mice were treated with physiological saline under the same conditions. Drugs or the vehicle control were administered for 21 days from one week after palpation of the tumors.

4.3. Measuring Tumor Volume

A linear caliper was used to measure tumor size twice a week. The largest diameter was recorded as "a" whereas, the smallest diameter was recorded as" b". Then, the formula V (mm^3) = a × b2 / 2 was used to calculate the tumor volume. Tumor growth was evaluated by the mean inhibition rate (IR), using the formula: IR = (C–T) / C × 100%, where "T" denotes the average tumor volume of the treatment group and "C" denotes the average tumor volume of the control group. Measurement of the weight of the animals was taken weekly throughout the experiment. On the 28th day of the experiment, all mice were sacrificed through cervical dislocation. Tumor tissue samples were collected and categorized into three groups. Group one was fixed with 10% phosphate-buffered formalin solution for pathological and immunohistochemical analysis. Group two was fixed in 4% paraformaldehyde (PFA) for 24 hours in preparation for TUNEL testing. Group three was rapidly frozen with liquid nitrogen and stored at -80℃ for subsequent experiments.

4.4. TUNEL Assay

Terminal deoxynucleoitidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay kit (Chemicon Co. China) was used in detecting apoptotic tissue sections. First, the tissue samples were fixed in 4% paraformaldehyde (PFA) for 24 hours, followed by dehydration and conventional paraffin embedding. The paraffin-embedded tissue sections were cut to 4 μm sizes and subjected to graded alcohol dealkylation treatment. They were covered with 20 μg proteinase K / mL PBS (–) for 15 minutes at room temperature, blocking the endogenous peroxidase activity. Subsequently, samples were incubated with TdT enzyme and biotin-16-dUTP in TdT buffer containing 0.01% bovine serum albumin in a humidity chamber at 37°C for 1.5h. The ABC method with DAB as a chromophore was used to detect biotin-16-dUTP nucleotides which had been incorporated into DNA fragments. In each tissue sample, we randomly selected 5 high-power fields (× 400 magnification), and calculated the apoptotic index (AI) in these fields as the percentage of positive cells as follows:

AI = (number of positive cells/total number of cells) × 100%

4.5. Immunohistochemistry of Ki-67

Here we detected the proliferation index of Ki-67. Tumors were fixed with 10% neutral formalin for 24-48 hours before paraffin embedding. After deparaffinization, the tissue sections were heated at 121°C in a pressure cooker for 15 min in 10 mM Tris HCl with 1 mM antigen unmasking EDTA (pH 9.0). Wait for the Tris HCl to cool to room temperature and take out the slice for use. Endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 10 min at room temperature, followed by incubation with the Ki-67 antibody [clone MIB-5 (M7248)] for 90 min at room temperature. Thereafter, sections were incubated with the EnVision reagent for 40 min and DAB / H_2O_2 at room temperature for 8-12 min. We selected five typical tumor areas (× 400 magnification) of the tumor and counted the number of Ki-67 positively staining nuclei as well as the total number of cancer cells to evaluate tumor proliferation. Results were expressed as the ratio of the number of positively stained cells to the total number of cells.

4.6. Statistical Analyses

SPSS software (SPSS Standard version 17.0, SPSS) was used to analyze all statistical date. Any statistical significance between the control and the treated groups was determined by Student's *t*-test. All the experimental data were expressed as means values ± SE. $P < 0.05$ was considered statistically significant.

5. Results

5.1. Inhibition of Ovarian Cancer Growth

To examine the inhibitory effect of SC-560, taxol, or DDP on the progression of ovarian cancer, we established a model using the human ovarian cancer cell line SKOV-3. The respective effect of SC-560 when used in combination with taxol or DDP on tumor growth is highlighted in (Figure 1). We observed that the tumor size in the control group increased from the date of injection throughout the examination period, while the average tumor size was significantly suppressed in all drug-treated mice. For instance, on experiment day 28, the average tumor volume of mice in the control group was 730 mm^3. Under similar conditions, the mean tumor volume of the SC-560 treated group, taxol treated group and DDP treated group was 396 mm^3, 319 mm^3 and 477 mm^3, respectively. Compared with DDP, SC-560 and taxol exhibited a higher inhibitory effect on the growth of ovarian cancer. Moreover, the inhibitory effects of SC-560, taxol, DDP and the combination groups were highly statistically significant compared to the control group ($p < 0.05$ for all).

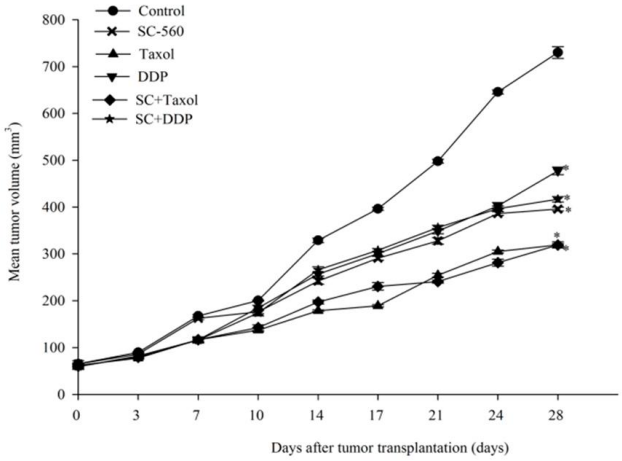

Figure 1. In vivo effects of SC-560, taxol, and DDP on tumor growth. The inhibition of drugs on tumor growth in the ovarian cancer model constructed by SKOV-3 cells was detected. After experiment day 7, the model was established successfully, mice were treated with the SC-560 (3mg / kg, twice a day), taxol (20mg / kg, once a week) and DDP (3mg / kg, once every other day) were used to treat mice for 21 days. On the 28th day, the average tumor volume of all treatment groups was significantly smaller than that of the control group. The student's t-test was used to determine statistical significance, * $p < 0.05$.

5.2. Effect on Cell Apoptosis

To evaluate the extent of apoptosis in tumor tissue, apoptotic cells were stained using the TUNEL method. The number of apoptotic-positive cells was counted under a high-power field. TUNEL assay revealed an increase in the number of apoptosis cells in mice treated with SC-560, taxol, DDP, SC-560/taxol, and SC-560/DDP. Apoptotic rates were 56.00 ± 5.23%, 53.00 ± 3.88%, 52.00 ± 2.14%, 74.00 ± 3.44%, 54.00 ± 2.32% in treatment groups SC-560, taxol, DDP, SC-560/taxol and SC-560/DDP, respectively, and were statistically significant compared to the control group (33.00 ± 8.36%, $p < 0.05$ for all). Besides, compared with the taxol group, SC-560 combined with taxol showed a synergistic effect on the apoptosis rate of tumor cells ($P < 0.05$). However, cell apoptosis in xenograft tumors of nude mice treated with SC-560 combined with DDP showed no significant difference compared with the DDP group (Figure 2). Typical tumors images of apoptotic cells immunohistochemically stained revealed the effects of SC-560, taxol, and SC-560/taxol on cell apoptosis in SKOV-3 xenograft tumors (Figure 3).

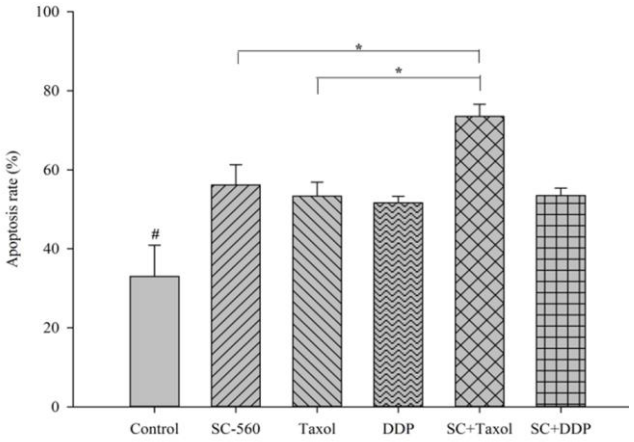

Figure 2. Cell apoptosis in the ovarian cancer model constructed by SKOV-3 cells treated with SC-560 in combination with taxol or DDP. The apoptosis index was determined by the ratio of the number of apoptosis-positive cells to the total number of nuclei. The apoptotic index illustrated the extent to which the drugs induced apoptosis on tumors. The apoptosis rate of treatment groups compared with the control group, $^{\#}p < 0.05$ for all. SC-560 combined with taxol showed a synergistic effect on the index of cell apoptosis compared to when SC-560 and taxol are used alone, $^{*}p < 0.05$; Standard errors are represented by error bars.

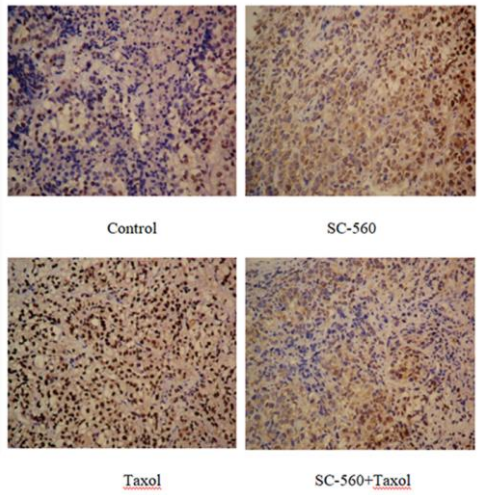

Figure 3. Immunostaining of apoptosis in ovarian tumor sections of mice in each group using the TUNEL method. Light microscopic images showed the distribution of TUNEL positive cells in the control group, SC-560, taxol and SC-560 / taxol-treated groups, respectively.

5.3. SC-560, Taxol, and DDP Inhibit Ki-67 Positive Cells

Compared to the control group, we observed a significantly lower number of Ki-67 positive cells in tumor sections in the drugs-treated group. Further in the SC-560, taxol, DDP, SC-560/taxol, and SC-560/DDP groups, the Ki-67 positive cell index were 12.00% ± 3.22%, 7.00% ± 2.27%, 17.00% ± 1.68%, 5.00 ± 1.32%, 15.00 ± 2.12%, respectively, which was statistically significant compared to the control group (29.00% ± 4.29%, $p < 0.05$ for all). We found that SC-560 combined with taxol can significantly reduce the Ki-67 positive cell index compared with when SC-560 is used alone ($P < 0.05$). This suggested that SC-560 and taxol have a synergistic effect in inhibiting cell proliferation whereas with the combination of SC-560 and DDP, no significant difference in the Ki-67 positive cell index occurs (Figure 4). Typical tumor images with immunohistochemically stained Ki-67 positive cells showed depicted the effects of SC-560, taxol and SC-560/taxol in SKOV-3 xenograft tumors (Figure 5).

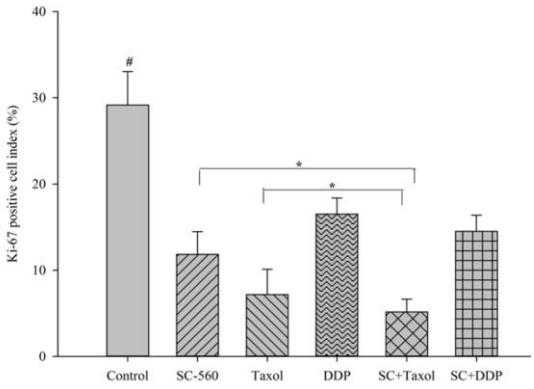

Figure 4. The Ki-67 positive cells in xenograft tumors of nude mice treated with SC-560 or/and taxol, DDP. The percent of Ki-67 positive cells was significantly decreased in the groups of SC-560, taxol, DDP and the combinations in SKOV-3 xenograft tumors compared with control, $^{\#}p < 0.05$. SC-560 combined with taxol showed a synergistic effect on Ki-67 positive cells compared with SC-560 and taxol alone, $^{*}p < 0.05$, error bars indicate standard error.

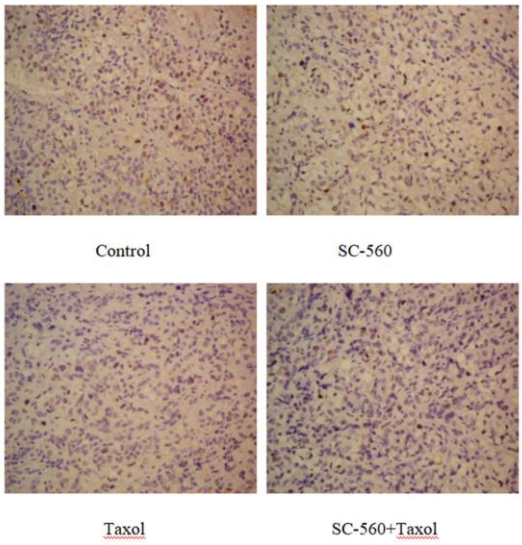

Figure 5. Immunohistochemical staining of Ki-67 in each group. The number of Ki-67 positive cells in tumor sections of SC-560, taxol, SC-560/taxol-treated groups was significantly lower than that of the control group.

6. Discussion

In this work, we mainly report that all drugs inhibited the growth of human ovarian SKOV-3 carcinoma xenografts through *in vivo* experiments. SC-560 enhanced the *in vivo* inhibitory effect of taxol on the growth of human ovarian SKOV-3 carcinoma xenografts. Also, the antitumor effect of taxol combined with SC-560 was associated with an obvious reduction in cell proliferation, this was evident by inhibition of Ki-67 expression and induction of apoptosis. Compared to SC-560 combined with DDP, the combined use of SC-560 and taxol has a preferable synergistic effect on inhibiting tumor proliferation and apoptosis induction.

The international standard of care for women with advanced or poor-prognosis early-stage ovarian cancer mainly consists of debulking surgery followed by chemotherapy with platinum and paclitaxel [12]. Taxol is accepted as a first-line drug for chemotherapy in ovarian cancer. Although between 75% and 80% of patients with ovarian cancer can respond effectively to drugs in the early chemotherapy stages, more than 80% of such patients may develop varying degrees of drug resistance, including Multi-Drug Resistance (MDR), which ultimately leads to a 5-year survival rate of only 30%. This suggests that MDR is commonly attributed to chemotherapy failure in ovarian cancer [13]. Considerable efforts have been made to overcome MDR and to define new molecular therapies, including inhibitors, modulators, and gene therapy [14]. Using a combination of more than two therapeutic drugs can effectively reduce tumor resistance, however, these drugs need to have different mechanisms of action which may lead to the blockade of irrelevant pathways that are essential for the survival of cancer cells [15].

Cyclooxygenase (COX) is a key rate-limiting enzyme that mediates a series of physiological and pathological reactions and is capable of catalyzing the synthesis of prostaglandins and thromboxane by arachidonic acid [16]. Recent assessments have described two isoforms of COX, that is COX-1 and COX-2. Using multiple approaches with mice model, a previous study revealed that human EOC manifests heightened expression of COX-1 but not COX-2 and that COX-1 plays an important role in the main pathway of PG production in EOC [17]. Epidemiological evidence shows that inhibiting COX expression potentially reduces the risk of epithelial ovarian cancer by 40% or more [18]. Subsequently, another study showed that COX-1 selective inhibitors may be a potent drug with antitumor activity in conflicting EOC [6].

Uncontrolled cell proliferation is one of the main hallmarks of cancer, and the genes directly involved in cell-cycle regulation are often damaged. Besides, Ki-67, a proliferation marker, is positively correlated with tumor grade, aiding in the identification of aggressive ovarian cancer [19]. Increased knowledge of the molecular mechanisms of cell cycle transition associated with tumor formation sug-

gests that modulators of cyclins are potential therapeutic targets in cancer therapy [20]. Taxol mainly induces G2/M cell cycle arrest via cyclin B1-associated CDK1 (cyclin-dependent kinases 1) [21] and consequently inhibits cell proliferation by blocking cell division and death through the apoptotic pathway [22]. On the other hand, SC-560 blocks the progression of the cell cycle in the G1/S phase, thus cyclin D1 is inhibited and G1 arrest occurs [10]. Previously from our investigation, we reported a significantly decreased quantification of the Ki-67 positive cells in the tumors exposed to SC-560 [23]. Similarly, in the present experiment, we found a significant decrease in Ki-67 expression in treatment groups. Furthermore, we observed that using SC-560 combined with taxol has a synergistic *in vivo* effect on a marked decrease in the Ki-67 positive cells index, highly significantly inhibiting cell proliferation in xenografts than when SC-560 or taxol is used alone. These results demonstrate that when SC-560 is used in combination with taxol, the inhibitory effect of taxol on cell proliferation is enhanced, and because SC-560 and taxol have different mechanisms of action, cell cycle arrest occurs.

The delicate balance between cell proliferation and apoptosis is favorable in controlling the periodic growth of reproductive tissues and can play an important role in preventing tumor transformation [24]. Apoptosis is a multi-step process, and increasingly several genes have been confirmed to participate in the regulation or execution of apoptosis. Taxol associated apoptosis is mainly induced by the imbalance of microtubule polymerization and depolymerization, eventually resulting in cell cycle arrest and apoptosis [25]. COX inhibitors induce apoptosis by hindering the production of COXs, reducing the level of PGE2, and cause variation in gene expression [26]. Recently, Lee et al. [27] conducted experiments on the taxane-sensitive ovarian cancer cell line, SKOV-3, and reported that the combination of taxol and SC-560 could improve the toxicity of taxane-resistant ovarian cancer cells by inhibiting the expression of MDR1 gene and ATP-Binding Cassette (ABC) transporter P-glycoprotein (P-gp). Further, in their report, they highlighted that SC-560 significantly increases the number of paclitaxel-induced deaths of drug-resistant ovarian cancer cells via a mechanism independent of prostaglandin and cyclooxygenase. Results suggested that the COX-1 inhibitor can serve as an effective therapeutic tool not only as a drug sensitizer, but also as a pro-apoptotic agent. In our experiment, we also demonstrated that SC-560 combined with taxol therapy has a synergistic effect on inducing apoptosis in xenografts than when SC-560 or taxol is applied independently. Moreover, SC-560 inhibits cell proliferation and induces apoptosis via a different mechanism of action with taxol, therefore, it has a synergistic effect on the inhibition of ovarian tumor growth when used in combination with taxol.

In conclusion, this study demonstrated that the combination of SC-560 and taxol has a synergistic effect in down-regulating of Ki-67, reflecting a decrease in proliferating cells and an increase in apoptotic cells in human ovarian SKOV-3 cancer cell xenograft mice. Therefore, our results affirm that the anti-tumor effects of taxol combined with SC-560 therapy might be partly achieved by inhibition of cell proliferation and induction of apoptosis.

7. Study Highlights

The current knowledge on the subject indicates that combination drugs can inhibit tumor growth. The research offers new directions for the treatment of ovarian cancer. It is evident from the findings that sc-560 enhances the anti-tumor activity of Taxol. Sc-560 may be incorporated in future chemotherapy for ovarian cancer.

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