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# Effects of blocking CXCL12/CXCR4, CXCR7 and ERK signaling pathways on the growth of subcutaneous xenografts in nude mice

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**Abstract:** **To study the inhibitory effects of blocking chemokine pathways CXCL12/CXCR4, CXCR7 and intracellular ERK signaling pathway on human endometrial carcinoma in nude mice. Ishikawa cells suspension (7.5×107/ml) of endometrial cancer were used to establish animal models. 30 nude mice were randomly divided into the following five groups: AMD3100 (6 mg/kg), PD98059 (50 mg/kg), Anti-CXCR7 (1 ul/mouse), AMD3100+Anti-CXCR7 (6 mg/kg+1 ul/mouse) and Nacl (etc. volume of saline). These small molecule inhibitors were intraperitoneally injected into the body. Tumor length and short diameter to calculate tumor volume were measured every three days for three weeks. At the end of the treatment period, completely stripped the all tumors, weigh them and the expression of Survivin in subcutaneous xenografts was detected by western blotting and RT-PCR. The growth curve and weight of the tumors in the experimental groups were significantly lower than those in the control group (P<0.01). The results of western blotting and RT-PCR revealed that the expression levels of Survivin protein and mRNA in subcutaneous xenografts of experimental groups were down regulated compared with control group (P<0.01). There were no significant difference in growth curves, weights, tumor inhibition rates, Survivin protein and mRNA expression of tumors between experimental groups (P>0.05). Blocking CXCL12-specific receptors CXCR4, CXCR7 and intracellular ERK signaling pathway can inhibit the growth of endometrial carcinoma cells in vivo, this condition may be related to the down-regulation of the apoptosis-inhibiting gene Survivin.**

**Keywords:** CXCR4; CXCR7; Endometrial cancer; Survivin

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## 1. Introduction

Endometrial cancer (EC) is a common malignant tumor of the female reproductive system and it threats women's health and quality of life seriously. In recent years, the incidence of EC has been on the rise in some developed countries[1], and the age of onset has been younger[2]. The clinical treatment approach of patients with recurrent and distant metastasis is limited and its treatment is still in the bottleneck period because of unclear pathogenesis. The occurrence of tumors is closely related to the abnormal activation of multiple signaling pathways. At present, new progress in the treatment of EC is concentrated on small molecule targeting drug[3, 4].

The chemokines are known to play a key role in many physiological and pathological processes[5], including inflammatory bowel disease[6], diabetes mellitus[7], and various cancers[8-10]. To my knowledge, the current research has confirmed that CXCL12 has two receptors, CXCR4 and CXCR7. Importantly, the CXCL12/CXCR4,CXCR7 protein signaling axis are proinflammatory and further disease. In 2005, it observed that CXCL12 could bind to a second chemokine receptor previously referred to as RDC-1 or ACKR3. This receptor, now called CXCR7, has a 10-fold higher binding affinity for CXCL12 than CXCR4[5,11,12]. Research on small molecule inhibitors in the field of cancer has achieved some results, which will provides new treatment options and research direction for clinical treatment. AMD3100 (product name: plerixafor, a chemokine receptor 4 (CXCR4) Specific antagonist) can be used to block the SDF-1/CXCR4 axis (now defined as CXCL12/CXCR4) to inhibit the proliferation and invasion of EC cells[13]. Cell migration examination indicates that the neutralizing antibody of CXCR7 blocked microRNA-126 (miR-126) associated signaling pathways, which its target gene was identified as stromal cell-derived factor 1 (SDF-1), angomir-induced migration of endothelial progenitor cells[14]. PD98059 is a non-ATP-competitive MEK inhibitor that specifically inhibits MEK-1 mediated MAPK activation. The study suggested that PD98059 reduced cell proliferation in endometrial cancer via the mitogen-activated protein kinase/ERK signaling pathway[15,16].

We used small interfering RNA (siRNA) of CXCR4 and CXCR7 to silence the expression of CXCR4 and CXCR7 previously, demonstrating that CXCR4-siRNA and CXCR7-siRNA could inhibit the proliferation of endometrial cancer cells in vitro and in vivo[17-19]. Nowadays, there are a few experimental studies on the targeted treatment of endometrial cancer with specific

 

antagonists in vivo. In this paper, we used AMD3100(CXCR4 specific antagonist), anti-CXCR7 (CXCR7 neutralizing antibody) and PD98059 (ERK signaling pathway blocker) on tumor-bearing nude mice to investigate the effects of these specific antagonists on tumors by observing the growth of subcutaneous xenografts and detecting the expression of apoptosis-inhibiting gene Survivin in all groups.

## 2. Materials and methods

### *2.1. Cell culture and establishment of xenografts model*

The cell line Ishikawa of human endometrial cancer was obtained from the Central Laboratory of the Affiliated Hospital of Qingdao University (Qingdao, Shandong Province, China) and cultured in DMEM (Hyclone, USA) supplemented with 10% heat-inactivated fetal bovine serum (PAN, German) and 1% penicillin/streptomycin (Solarbio, Beijing) at 37°C in a humidified incubator with 5% $CO_2$. When the cells were in the logarithmic growth phase (cover the 75% to 85% of bottle bottom ) and 0.25% trypsin was used to digest for passage culture. All animal experiments were approved by ethical and humane committee of Qingdao University Medical College. A total of 30 female BALB/c nude mice (4-5 weeks old,14~18g) with certificate number SCXK (jing) 2016-0006 were purchased from Beijing Weitong Lihua Experimental Animal Technology Co., Ltd. (Beijing, China). They fed with special sterile feed and water and were housed in cages that were individually ventilated and had clean padding under specific pathogen-free and room temperature of 22-24°C environment with a 12-h day/night cycle. All nude mice adapted for 1 week prior to experiments.

Taking a small amount of cell suspension was counted with the cell counting plate. Next, the cells were collected by centrifugation and diluted with a mixture of Matrigel (B& D, USA) and PBS 1:1 to adjust the cell density to $7.5\times10^7$/ml. Placed the nude mouse in the clean bench and disinfected the skin, then 200μl (about 1.5 $\times10^7$cells) cell suspension was injected subcutaneously into the right shoulder of each nude mouse. The needle should be sneaked into the skin about 1cm before injecting cell suspension. Pulled out the needle after the injection and the assistant gently pressed the injection point with a sterile cotton swab to prevent the tumor cells from leaking out of the pinhole. At the injection site, a soft rounded bulge with diameter about 0.7cm was seen and the hardness gradually increased. After one week, the tumors texture were obviously hard and the diameters reached >5mm, indicating that the molds were successful. Those specimens were randomly divided into five groups (each group=6) and the experimental procedures were conducted.

30 nude mice were statistically analyzed to determine that there was no statistically difference between the groups. All animals were randomly divided into the following groups: Ⅰ: AMD3100 (6mg/kg), Ⅱ: PD98059 (50mg/kg), Ⅲ: Anti-CXCR7 (1ul/mouse), Ⅳ: AMD3100 + Anti-CXCR7 (6 mg/kg+1ul/mouse), Ⅴ: Nacl (etc. Volume of saline). The above were all injected intraperitoneally and once treatment began, observing the spirit, activity, diet, defecation and tumor growth of each mouse every day. Treated with every 3 days for 3 weeks and the long diameter (a) and short diameter (b) of the subcutaneous tumor were measured with a vernier caliper before each treatment, then calculated tumor volume according to the formula: $v= (a\times b^2) /2$ and drew tumor growth curves.

At the end of the treatment period, the nude mice were sacrificed following the institutional ethical guidelines. Measured the tumor weights after completely removing and they were frozen in a -80°C refrigerator, which were subsequently used to detect Survivin protein and mRNA in the tumors. The tumor inhibition rates were calculated according to the formula = (control group tumor weight – experimental group tumor weight) / control group tumor weight ×100%.

**Table 1. Sequences of Survivin and GAPDH**

| Gene | Primer | Sequence |
|---|---|---|
| Survivin | Forward | 5'-CTACCGCATCGCCACCTTCAAG-3' |
| | Reverse | 5'-GAAGCCAGCCTCCGCCATTC-3' |
| GAPDH | Forward | 5'-CTCAGACACCATGGGGAAGGTGA-3' |
| | Reverse | 5'- ATGATCTTGAGGCTGTTGTCATA-3' |

### *2.2. Quantitative RT-PCR for Survivin mRNA in tumor tissues*

Tumor samples were taken from -80°C and a small portion was cut out. The total RNA in tissues was extracted with trizol (Invitrogen, USA) and the concentration and purity of RNA in the sample solution were measured by an ultraviolet spectrophotometer (IMPLEN, Germany). The cDNA was synthesized by reverse transcription using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Japan), and the specific procedure was carried out in accordance with the instructions. Real Time PCR reaction was performed with SYBR® Premix Ex

Taq™ II (Takara, Japan) on a Light Cycler PCR machine (ROche, Switzerland) to express Survivin gene in tumor tissues. The primer sequences of Survivin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, as internal reference control) were shown in Table 1. The Ct value of the sample gene was calculated by using the analysis software corresponding to the PCR instrument and the method of relative expression level of the target gene was mRNA = $2^{-\Delta\Delta Ct}$. All PCR experiments were repeated 3 times.

### *2.3. Western blotting for the expression of Survivin protein*

Cut a small amount of transplanted tumor tissue and weighed it. RIPA (Boster Biological Technology co. ltd, Wuhan, China): PMSF (Boster Biological Technology co. ltd, Wuhan, China)=100:1 to prepare the lysate and added the corresponding volume of lysate to homogenate to extract tissue protein according to the net weight of tissue (g): lysate (ml)=1:10. Prepared 12% SDS-PAGE gel, after loading, electrophoresis, transfer, blocking in 5% skim milk, then adding primary antibodies Survivin (1: 1000; abcam, United Kingdom) and GAPDH (1: 5000; abcam, United Kingdom) was incubated at 4°C overnight. The next day, after washing with TBST, added HRP-labeled secondary antibody for 70min at room temperature. The target bands of protein were detected with ECL chemiluminescence solution (Millipore, USA). Used Quantity One 4.6 software for analysis, the ratio of gray values of Survivin /GAPDH bands as relative expression level of target protein. The experiment was repeated three times.

### *2.4. Statistical analysis*

The experimental data were displayed as the mean ± SD and results were analyzed by SPSS version 19.0.The comparison between groups was performed by one-way ANOVA and the two groups by independent sample test. $P<0.05$ was considered to be statistically significant.

## 3. Results

### *3.1. Outcomes of subcutaneous xenografts in each group*

Throughout the treatment cycle, the nude mice in each group had no obvious abnormalities and survived well. At the end of the treatment period, the nude mice were sacrificed and the tumors were peeled off in the ultra-clean workbench to remove fat tissue and blood stains. The tumors of each group were shown in Figure 1. The tumor growth curves were plotted with the vertical axis representing the tumor volume and the horizontal axis representing the treatment time (Figure 2). The growth rate of tumors in the four experimental groups was significantly lower than that in the control group. Comparing the body weights and tumor volumes of nude mice before and after treatment was presented in Table 2. After 3 weeks of treatment, the tumor volumes of the experimental groups were significantly smaller than that of the control group, the difference was statistically significant ($P<0.01$).

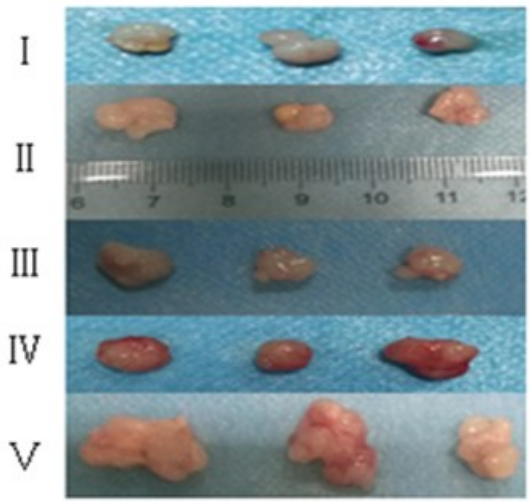


**Figure 1. Note: I: AMD3100 , II: PD98059, III: Anti-CXCR7, IV: AMD3100+ Anti-CXCR7 , V: Nacl. Tumor appearance of each group. It could be clearly seen from the figure that the tumor volumes of the AMD3100, PD98059, Anti-CXCR7, AMD3100+ Anti-CXCR7 were smaller than the Nacl group.**

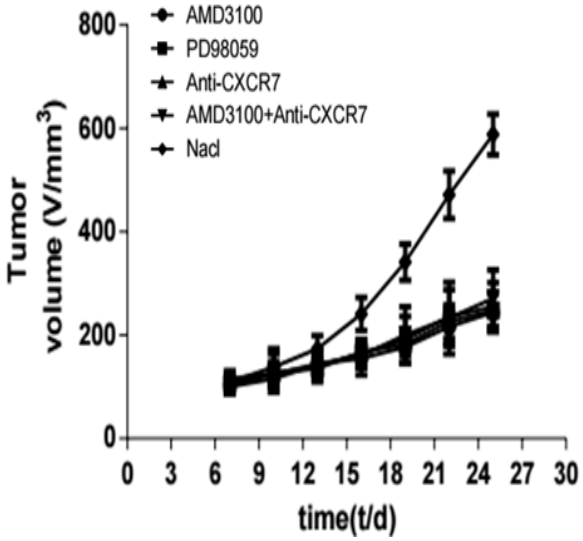


**Figure 2. Growth curve of subcutaneous xenografts in each group throughout the treatment cycle. Tumor volumes were shown with mean±SD.**

**Table 2. Comparison of experimental groups and control group before and after treatment (mean ± SD)**

| Group | Body weight before administration (m/g) | Tumor volume before administration (v/mm$^3$) | Tumor volume after administration (v/mm$^3$) | Difference in tumor volume before and after medication (v/mm$^3$) |
|---|---|---|---|---|
| AMD3100 | 19.72±0.92 | 103.90±16.82 | 244.54±38.08** | 140.65±25.64** |
| PD98059 | 19.03±0.74 | 106.26±19.32 | 247.95±32.78** | 141.69±45.43** |
| Anti-CXCR7 | 19.70±0.93 | 115.75±14.92 | 254.86±46.68** | 139.14±44.36** |
| AM+An-CX7 | 19.08±0.84 | 100.62±11.06 | 271.20±55.13** | 170.58±59.21** |
| Nacl | 19.45±0.86 | 108.86±21.93 | 588.49±39.41 | 479.63±32.37 |
| F value | 0.87 | 0.66 | 72.27 | 71.95 |

**Note: AM+An-CX7 meant AMD3100+Anti-CXCR7, **experimental groups vs control group, p<0.01.**

### *3.2. Tumor inhibition rates*

The final tumor weights of the four experimental groups were AMD3100 (0.40±0.10)g, PD98059 (0.42±0.09)g, Anti-CXCR7 (0.46±0.13)g, AMD3100+Anti-CXCR7 (0.48±0.08)g and the Nacl group was (0.84±0.11)g, respectively. The tumor inhibition rate of experimental groups were AMD3100 (53.09±6.04)%, PD98059 (50.39±4.58)%, Anti-CXCR7 (48.42±14.99)%, AMD3100+Anti-CXCR7 (45.24±8.08)%, respectively. As shown in Figures 3A and 3B, the tumor weights of the four experimental groups were significantly lower than those of the control group (F=11.35, P<0.01). There was no difference in tumor weights (F=0.44, P>0.05) and tumor inhibition rates (F=0.46, P>0.05) among the experimental groups. It showed that the combined medication did not have a stronger antitumor effect.

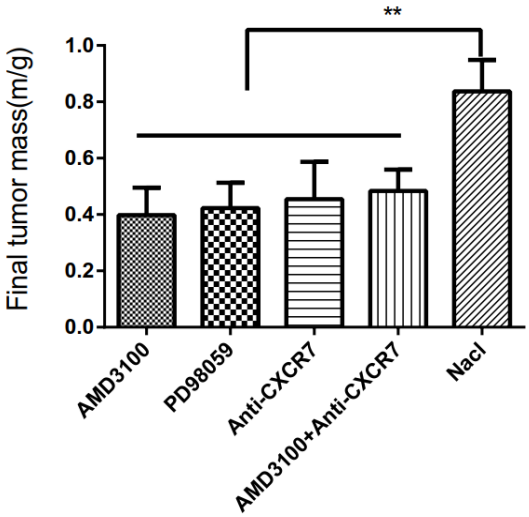


**Figure 3A. Final tumor mass in each group. It showed that the weights of tumors treated with small molecule inhibitors were significantly less than that with Nacl.** meant P<0.01 vs Nacl group.**

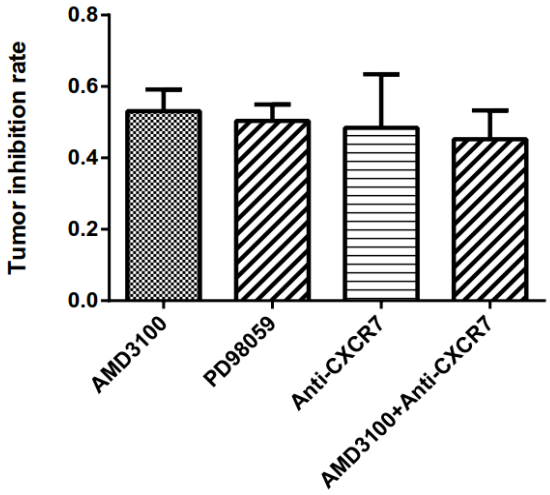


**Figure 3B. Tumor inhibition rates in the experimental groups. The four experimental groups could inhibit the growth of tumors, and there were no difference in the tumor inhibition rates between them.**

### *3.3. Down-regulation of Survivin mRNA in the experimental groups after administration*

To evaluate the inhibitory effect of the inhibitors on tumors, we examined the expression of the apoptosis-inhibiting gene Survivin in the subcutaneous xenografts by RT-PCR. We found that the relative expression levels of Survivin in AMD3100 (0.44±0.20), PD98059 (0.46±0.10), Anti-CXCR7 (0.51±0.06), AMD3100+Anti-CXCR7 (0.59±0.26) were significantly down-regulated compared with the Nacl group (F=17.16, P<0.01). However, there was no difference between the four experimental groups (F=0.50, P>0.05), as shown in Figure 4. AMD3100 and Anti-CXCR7 could inhibit tumor growth alone or combination. The combination of AMD3100+Anti-CXCR7 had no stronger inhibitory effect on the expression of the apoptosis-inhibiting gene Survivin mRNA.

 

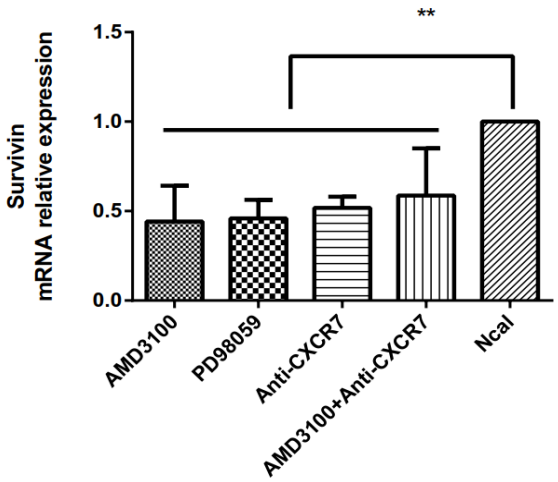


**Figure 4. Expression of apoptosis-inhibiting gene Survivin mRNA in tumor tissues of each group. **P<0.01 vs Nacl group.**

### *3.4. Expression of Survivin protein in transplanted tumors*

The expression of Survivin protein in subcutaneous xenograft of each group was detected by Western blotting. Comparing with the control group, the expression of Survivin was significantly inhibited in experimental groups (F=17.95, P<0.01). The expression of Survivin protein was not statistically significant among the experimental groups (F=1.43, P>0.05). It indicated that the combination group did not synergistically inhibit the expression of Survivin protein in tumors compared with the single-agent group, which was consistent with the mRNA results (Figure 5).

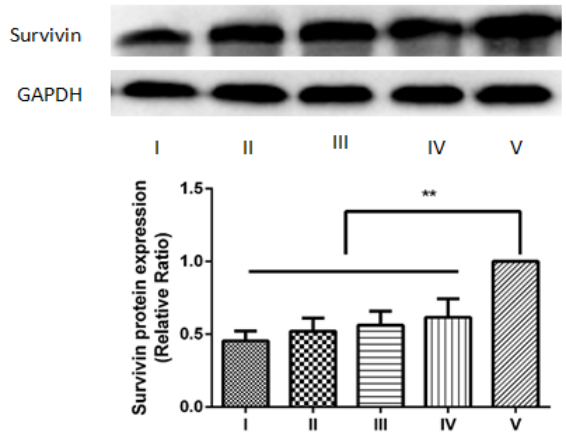


**Figure 5. Expression of Survivin protein in subcutaneous xenografts of each group. The expression of Survivin in the Nacl group was higher than that in the four experimental groups. Note: I: AMD3100, II: PD98059, III: Anti-CXCR7, IV: AMD3100+ Anti-CXCR7, V: Nacl. ** P<0.01, compared with the Nacl group.**

## 4. Discussion

Chemokines were special members of the cytokine superfamily, especially in the occurrence, development and treatment of malignant tumors, which were receiving more and more attention from researchers[20-22]. The chemokine CXCL12, also known as stromal cell-derived factor-1 (SDF-1), was a small molecule cytokine belonging to the chemokine protein family and its receptor was CXCR4. The chemokine CXCL12/CXCR4 axis played an important role in the biological behavior of many malignant tumor[23-25]. In recent years, CXCR7 as a second receptor of CXCL12 has been isolated and identified. It indicated that a series of biological effects and regulatory mechanisms triggered by CXCL12 would be more complicated. Studies have confirmed that CXCL12/CXCR4, CXCR7 chemokine axis control multiple steps for survival, adhesion and growth of tumor cells[9, 26].

In view of the close relationship between chemokines and the mechanism of tumorigenesis, researchers have now tried to use chemokine-related targeted agents for the treatment of tumors. As early as 2006, Redjal, N, et al. used CXCR4 specific blocker AMD3100 to inhibit CXCL12/CXCR4 signaling to study its inhibitory effect on glioma growth in vivo and achieved some success[27]. Regulating the mTOR signaling pathway in renal cancer cells via the CXCL12/CXCR4, CXCR7 axis, CXCR4 and CXCR7 antagonists were used in the treatment of renal cell carcinoma [28]. And related studies have confirmed that CXCR4 and CXCR7 can predict the prognosis of renal cell carcinoma[29, 30]. Moreover, studies have shown that the interaction of SDF-1α with CXCR4 on the surface of ovarian cancer cells can activate the ERK1/2 signaling pathway and this can induce proliferation and metastasis of tumor cells. However, the ERK-1/2 signaling inhibitor PD98059 could antagonize the adhesion of ovarian cancer cells treated with SDF-1α to fibronectin and type IV collagen[31]. Sun, et al.[32] found that PD98059, the ERK inhibitor was able to inhibit the effects of estrogen on the proliferation of Ishikawa cells, human endometrial cancer cell, by the interaction of estrogen-related receptor γ (ERR γ) with ERK1/2 signaling pathway. Some studies also had achieved similar results, which confirmed that the mitogen-activated protein kinase/ERK signaling pathway was crucial to estrogen-induced proliferation of endometrial cancer cells via ATX-LPA (autotaxin-lysophosphatidic acid) axis and this effect could be blocked by PD98059[15]. The application of these small molecule inhibitors in tumors allowed us to further understand the mechanism of tumor development.

Small interfering RNAs, also known as short interfering RNAs, are currently known to be primarily involved in RNA interference (RNAi) phenomena, regulating gene expression in a specific manner.

 

Therefore, this technology has been widely used in the field of exploring gene function and gene therapy of infectious diseases and malignant tumors[33, 34]. The relaxin 2 (RLN2)/relaxin receptor 1 (RXFP1) sgnaling could cause cells invasion in endometrial cancer. Fue et al. used RXFP1-siRNA to transfected into HEC-1B and Ishikawa cells, endometrial cancer cell lines and RLN2-induced invasive ability of cancer cell was significantly decreased[35]. Studies have shown that proliferation rate and invasion ability of cells were significantly reduced when the expression of protein phosphatase 2A (CIP2A) in endometrial cancer cell lines was silenced by siRNA [36]. Our previous studies also confirmed that the CXCL12/CXCR4, CXCR7 biological axis was highly transcribed in endometrial cancer tissues and cells and using siRNA-targeted silencing of CXCR4 and/or CXCR7 gene expression could inhibit the proliferation of endometrial cancer cells in vivo and vitro[17-19]. Therefore, specific targeted inhibitors may be an effective strategy for the treatment of endometrial cancer.

At present, there are a few studies on related inhibitors for endometrial cancer in vivo and the mechanism that inhibitors work in vivo is unclear. In particular, whether these inhibitors have anti-tumor effects in vivo and whether these effects are related to the apoptosis-inhibiting gene Survivin. Studies have shown that Survivin is up-regulated in 83% of endometrial cancer and down-regulation of Survivin can increase apoptosis of Ishikawa cells by curcumin micelles[37]. In this study, a variety of specific targeting small molecule inhibitors, AMD3100, Anti-CXCR7, PD98059, were injected intraperitoneally to tumor-bearing nude mouse to observe their effects on the tumors growth to determine whether they had anti-tumor effect. Importantly, our results indicated that tumor volumes, weights and growth rates with targeted inhibitors intervention were significantly lower than in the non-intervention group. We also found that the expression of the apoptosis-inhibiting gene Survivin protein and mRNA in the experimental groups were significantly reduced. This suggested that the anti-tumor effects of AMD3100, Anti-CXCR7 and PD98059 might be related to the down-regulation of Survivin in tumor tissues.

It was worth noting that there was no difference between the four groups of tumor-bearing nude mice treated with targeted inhibitors, suggesting that the combination group had no synergistic antitumor effect compared with the single-agent group. This results were consistent with our previous studies in vivo using CXCR4-siRNA and/or CXCR7-siRNA, which suggested that the signaling pathway formed with CXCL12 and its two receptors might interact[19]. Combing with previous studies, we found that CXCR4 and CXCR7 were associated with the pathological grade of endometrial cancer and could be used as indicators for predicting and evaluating disease severity[18]. Nowadays, CXCR4 inhibitors have been approved for the treatment of hematological diseases in foreign countries, but further research is needed in the clinical treatment of malignant tumors. Our research provides a new way for clinical treatment of endometrial cancer, providing theoretical support for targeted therapy that targeting is CXCR4, CXCR7 and ERK pathway.

## 5. Conclusion

Blocking CXCL12-specific receptors CXCR4, CXCR7 and intracellular ERK signaling pathway can inhibit the growth of endometrial carcinoma cells in vivo, this condition may be related to the down-regulation of the apoptosis-inhibiting gene Survivin. CXCR4 inhibitors have been approved for the treatment of hematological diseases in foreign countries, but further research is needed in the clinical treatment of malignant tumors. Our research provides a new way for clinical treatment of endometrial cancer, providing theoretical support for targeted therapy that targeting is CXCR4, CXCR7 and ERK pathway.

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## Conflict of interest

The authors declare that they have no competing interests.

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