The role of Apatinib combined with Paclitaxel (aluminum binding type) in drug-resistant ovarian cancer

CURRENT STATUS: UNDER REVIEW

Journal of Ovarian Research  ■ BMC

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DOI:
10.21203/rs.3.rs-22091/v1
SUBJECT AREAS
  Sexual & Reproductive Medicine  Cancer Biology

KEYWORDS
  Apatinib, Paclitaxel (aluminum binding type), Ovarian cancer, Combined medication
Abstract

Objective
To assess the antitumor effects and side reactions of different dosages of Paclitaxel (albumin binding) combined with Apatinib in drug-resistant ovarian cancer cell line and xenotransplantation tumor model.

Methods

Conventional cell experiments were used to evaluate the effects of Apatinib and Paclitaxel (albumin binding). SKOV-3/DDP were selected as research object and divided into 3 groups for study, a): control group, no drug intervention; b): nab-P group, Paclitaxel (albumin binding type) 40 µmol/l; c): Apatinib group, Apatinib 50 µmol/l. The IC-50 value of the drug was detected by MTT test, apoptosis related protein (Bax, bcl-2), vascular related protein (p-VEGFR-2), invasion related protein (MMP-2) expression were detected by Western blot and Cellular immunofluorescence, the invasion and migration ability of tumor cells were detected by Transwell and Cell scratch test. Based on these dates, establishing different dosages of Paclitaxel (albumin binding type) combined with Apatinib, a): Control group, no drug intervention; b): Group-1, Paclitaxel (albumin binding type) 5 µmol/l + Apatinib 10 µmol/l, c): Group-2, Paclitaxel (albumin binding type) 4.5 µmol/l + Apatinib 10 µmol/l, d): Group-3, Paclitaxel (albumin binding type) 4 µmol/l + Apatinib 10 µmol/l, the index of combined use was analyzed by Compusyn software, Western blot, Immunofluorescence, Transwell, Cell scratch test also were chose to change of inhibition effect. On the other hand, we used xenograft tumor model to verify the results in vivo. BALB/c female nude mice were randomly divided into 4 groups, a): Control group, no drug intervention; b): Paclitaxel (albumin binding type) 20 mg/kg + Apatinib 150 mg/kg, c): Paclitaxel (albumin binding type) 18 mg/kg + Apatinib 150 mg/kg, d): Paclitaxel (albumin binding type) 16 mg/kg + Apatinib 150 mg/kg. The tumor growth curve was analyzed during the test. The apoptosis related protein (Bax, bcl-2), angiogenesis related protein (CD31, p-VEGFR-2) and invasion related protein (MMP-2) were analyzed by Western blot, Immunofluorescence and Immunohistochemistry to analysis the effect of antitumor and side reactions

Result

(1) The IC-50 value of SKOV-3/DDP to paclitaxel (albumin binding type) was 45.53 ± 4.06 µmol/l, while
the role of Apatinb was 50.66 ± 4.96umol/L (48 h). (2) the expressions of bcl-2 (nab-P group, AP group), p-VEGFR-2 (AP group), MMP-2 (nab-P group, AP group) were higher than Control group, while Bax (nab-P group, AP group) lower (P < 0.01). (3) The invasion and migration of the cells decreased after the nab-P and AP treatment (P < 0.01). (4) nab-P combined with AP can increased their antitumor effect, according to Compusyn software, CL < 1. (5) After combined with AP, when nab-P were reduced dose in proper quantity, there were no obvious different in drug effect. (6) Reduced nab-P can increased nude mice’s quality of life.

Conclusion
Paclitaxel (albumin binding type), a chemotherapeutic agent, can play an anti-tumor role in drug-resistant ovarian cancer, but it can also reduce the tumor load and increase the expression of tumor vascular endothelial growth factor. When combined with Apatinib, the target drug of anti vascular endothelial growth factor, the two drugs have synergistic effect, which can improve the effect of single drug. In addition, when combined with Apatinib, the dosage of Paclitaxel (albumin binding type) can be appropriately reduced under the standard of recommended dosage to reduce the toxicity of chemotherapy drugs, without affecting the anti-tumor effect.

Introduction:
Ovarian cancer (hereinafter referred to as OC) is one of the three female reproductive system common malignant tumors, known as "silent killer". Because of its insidious onset, rapid progress, and weak awareness of cancer prevention in some women, most patients have reached the late stage of clinical diagnosis (stage III or IV) when they seek medical advice. According to the 2018 latest statistics released by the international cancer research center, the incidence and death toll of OC are 295,000 and 185,000 respectively in the world[1]. In the 2019 version, the National Comprehensive Cancer Network (hereinafter referred to as NCCN) guides the first-line chemotherapy was still represented by platinum. These drugs have obvious effect on the treatment of patients, and the 5-year survival rate can reach 47%[2], but about 60% of the patients will relapse unfortunately[3]. Moreover, among these relapsed patients, there are a group of people who belong to platinum-resistant relapse (no platinum interval ≤ 6 months). Platinum-resistance is an important factor that
makes the patient's condition difficult to control. There are studies have shown that more than 80% of patients with platinum-resistant relapse had only 12–18 months for progression-free survival (hereinafter referred to as PFS) [4], more importantly, platinum-resistant patients are increasing. Compared with 30–40% of ovarian cancer patients with platinum-resistance in 2012 [5], the data in 2019 increased to 70–80% [6]. In response to platinum-resistance, most treatments choose platinum-free drugs to increase the drugs sensitivity for patients [7], the drugs available to clinicians have doxorubicin liposome, paclitaxel (aluminum binding type) (hereinafter referred to as nab-P) and so on, they are widely used in clinical practice. nab-P is a kind of paclitaxel in the form of 130 nanoparticles which combines with human albumin, using albumin as carrier and stabilizer, adsorbing on tumor cells through SPARC protein, and then releasing toxicity to kill tumor cells, belongs to targeted chemotherapeutic drugs [8]. Compared with paclitaxel, it can improve the local concentration, enhance the effect on tumor, improve the stability and safety of drugs. In 2005, there is one phase III clinical trial of breast cancer, the response rate of nab-P was significantly higher than that of standard paclitaxel (33% and 19%, respectively, P = 0.001) [9]. Another, in a study of lung cancer, ovarian cancer, prostate cancer and colon cancer in xenograft tumor model, nab-P produced less drug toxicity than the same dose of paclitaxel, which was easily tolerated by patients [10]. In addition, nab-P also played a good synergistic effect in combination therapy, such as Volk’s research in breast cancer, combined with bevacizumab had gotten satisfactory results [11].

In recent years, the development of anti-tumor drugs has changed from traditional chemotherapy drugs to targeted drugs, promoting the precise treatment process of ovarian cancer. Tyrosine kinase (hereinafter referred to as TK) is an important target only next to G protein-coupled receptor [12], and one study has shown that tyrosine kinase inhibitors (hereinafter referred to as TKI) can increase the effect of chemotherapy drugs, mainly by inhibiting the function of ABC transporter [13]. Apatinib (hereinafter referred to as AP) is such a small molecules TKI drug, it can selectively act on the ATP binding site of vascular endothelial growth factor receptor-2 (hereinafter referred to as VEGFR-2) and block the phosphorylation of VEGFR-2 to inhibit tumor angiogenesis [14]. Many research have shown that AP has showed strong tumor inhibition and tolerance in non-small cell
l lung cancer, gastric cancer, breast cancer and other malignant solid tumors[15-17], in OC patients, many achievements have also been made, in a phase II clinical study of recurrent epithelial ovarian cancer, the objective response rate (hereinafter referred to as ORR) of patients was 41.4%, and PFS was 14.5 months[18], in 2018, an article published in *Lancet Oncol* on the treatment of advanced ovarian cancer patients with AP and Etoposide showed that up to 54% of patients achieved remission[19], and in the same year, Jiuhuan found that the combination of AP and radiation paclitaxel can significantly inhibit tumor growth[20]. Therefore, we propose whether AP combined with nab-P can improve the therapeutic effect on platinum-relapsed patients. Moreover, because the recommended dosage of nab-P in NCCN guideline is 260 mg (paclitaxel is 135–175 mg), the use of large dosage aggravates the physiological burden of patients. We assume that if we reduce the dosage of nab-P without effecting the efficacy after combination with AP. This paper preliminarily discusses the antitumor effect of the combination of nab-P and AP in the treatment of ovarian cancer, so as to provide reference for clinical medicine.

**Materials And Methods**

**Equipments and Drugs**

The equipments used in the experiment is as follows: Power Pac-TM Alkaline electrophoresis apparatus (164–5051, Bio-Rad), Gel imaging analysis system (SYNGENE G: BOXChemiXR5), Microplate Reader (Awareness Stat Fax, USA).

The drugs used in the experiment are as follows: Apatinib tablets (Jiangsu Hengrui Pharmaceutical Co., Ltd, Jiangsu, China, H20140103, 250 mg), paclitaxel (aluminum binding type) (Shiyao Holding Group Co., Ltd, Shijiazhuang, China, H20183318, 100 mg). Use after dissolving with Dimethyl Sulfoxide (DMSO, Sigma-Aldrich Co., St Louis, MO, USA).

**Cell lines and culture**

SKOV-3/DDP cell line was purchased from Procell(Procell Life Science & Technology Co., Ltd, Wuhan, China), and identified by STR analysis. The cell line was cultured in RPMI 1640 medium(Boster Biological Technology co.,Ltd, Wuhan, China), supplemented with 10% fetal bovine serum(Cellmax, Beijing, China), and incubated in 37 °C, 5% CO₂ incubator.

**Cytotoxicity assay**
The cytotoxicities of AP and nab-P for SKOV-3/DDP cell line were determined by MTT assay (Sigma-Aldrich Co., St Louis, MO, USA). Inoculate $1 \times 10^4$ cells/well in 96 well cell culture cluster. The concentration range of nab-P were $5 \times 10^{-2}$ to $5 \times 10^2 \mu$mol/l, and AP $10^{-1}$ to $10^3 \mu$mol/l. In the experimental group, 100 µl drug was added into each pore, while control group replaced serum-free medium. Add 20 µl MTT to each hole, and then 150 µl DMSO after 4 hours at 37 °C, shake for 30 minutes. Measure the optical density (OD) at 490 nm with enzyme standard meter.

**Combination index**

On the basis of the IC50 values of SKOV-3/DDP by nab-P and AP, the data map of Fa-CL plot established by compusyn (version 2.0) software was used to analyze the combination of drugs.

Synergism (Cl < 1): greater than expected additive effect. Additive effect (Cl = 1): the combined effect predicted by the mass-action law principle in the absence of synergism or antagonism. Antagonism (Cl > 1): small than expected additive effect.

**Western blot assay**

Total cellular protein was extracted with RIPA lysate buffer (Boster Biological Technology) containing protease inhibitor and phosphorylase inhibitor (Boster Biological Technology), and determined protein concentration with BCA protein detection kit (Boster Biological Technology), then separated by SDS-PAGE electrophoresis kit (cwbiotech). The amount of each group proteins was 20ug. After separation, the proteins were transferred to PVDF membrane (0.22um). When sealed by 0.5% non-fat milk powder still 1 h, the PVDF membrane was incubated with polyclonal primary antibody and secondary antibody combined with HRP. The membrane was colored by ECL enhanced chemiluminescence kit (Boster Biological Technology) and formed by Biological Spectrum Image System Scanning.

Primary antibodies used in this study include anti-β-actin (1:5000, AP0060, Bioworld Technology, Inc), anti-bcl-2 (1:1000, AF6139, Affinity), anti-Bax (1:1000, AF0120, Affinity), anti-p-VEGFR-2 (1:500, AF3279, Affinity), anti-MMP-2 (1:500, AF5330, Affinity). The secondary antibodies include HRP-conjugated affinipure goat anti-Rabbit IgG (1:5000, Boster Biological Technology, BA1054).

**Immunofluorescence**

SKOV-3/DDP different groups were inoculated in 6 well cell culture cluster. A slide was placed in each
hole for cell climbing. The inoculation amount of each hole was controlled at $10^5$ cells. After the cells adhered to the wall, PBS was soaked for 3 min × 2 times. Cells were fixed with 4% paraformaldehyde and soaked with PBS. Cells were permeable with 0.5% Triton X-100 room temperature. PBS was rinsed again. The PBS was dried by absorbent paper, and then the normal goat serum blocking solution was dripped on the slide for 30 min. the absorbent paper was dried. Each slide was dripped with a sufficient amount of diluted first antibody, and put into a wet box. After incubation at 4 °C overnight, it was soaked by PBST. After absorbing the superfluous liquid, the absorbent paper dripped with CY3 labeled Goat anti rabbit IgG, incubated in a wet box at 37 °C for 1 h, and then washed with PBS. DAPI was dripped in the dark and incubated in dark for 5 min. In the dark place, absorbent paper is used to absorb the climbing liquid, and the sealing liquid containing anti fluorescence quenching agent(Boster Biological Technology) was used for sealing. Images were collected under fluorescence microscope. Follow the same steps for frozen section

Primary antibodies used in this study include anti-bcl-2(1:200, AF6139, Affinity), anti-Bax(1:200, AF0120, Affinity), anti-p-VEGFR-2(1:200, AF3279, Affinity), anti-CD31(1:200, BS90231, Bioworld Technology), The secondary antibodies include CY3 conjugated affinipure goat anti-rabbit IgG(1:200, Boster Biological Technology, BA1032).

Transwell invasion assay
The SKOV-3/DDP cell line was treated in RPMI 1640 medium with serum free for 24 h. Then $1 \times 10^5$ cells in 100 µl medium were respectively sucked into upper chamber of transwell (8 µm pore size) with 20 µl pre-coated Matrigel(coring), 500 µl culture medium containing 20% FBS was added into the lower chamber to stimulate cell travelling. After 24 h culture at 37 °C, 5% CO$_2$ incubator, transwell chambers were fixed using 4% paraformaldehyde, and then stained with 0.1% crystal violet. Cells laid on upper surface of transwell membrane were wiped using a cotton swab, while cells traveled to the lower surface of membrane were photographed under a microscope. The average transmitted cells were counted in five random fields.

Wound healing assay
$5 \times 10^5$ cells were inoculated into 6 well cell culture cluster and maintained in incubator for 12 h-24 h
until cells reached 100% confluence. Then the cell monolayer was scratched to create a gap with 100 µl pipette tip followed by PBS washing. Finally, the culture medium was changed into serum free RPMI 1640 medium. The gaps were observed and photographed at 0 h, 12 h and 24 h after scratching, the area of scratches were calculated by Image J software.

Xenograft model
The BALB/C-nu were purchased from Charles River Laboratories China, all of these were almost 5-week-old female nude mouse. This study was accomplish with the approval of the ethical committee of Provincial Cancer Hospital, Shanxi, China, and followed the institutional guideline and ethical standard. In order to establish subcutaneous transplanted OC model, $2 \times 10^6$ SKOV-3/DDP cell line was injected into the right axilla of each mouse. When the axillary tumor volume of the nude mice was 200mm³, the nude mice were randomly divided into four groups, six in each group, and the following interventions were carried out every day. a): Control group, no drug intervention, intraperitoneal perfusion of 200 µl NS, b): Group-1, nab-P 20 mg/kg, intraperitoneal perfusion + AP 150 mg/kg, gavage administration, c): Group-2, nab-P 18 mg/kg, intraperitoneal perfusion + AP 150 mg/kg, gavage administration, d): Group-3, nab-P 16 mg/kg, intraperitoneal perfusion + AP 150 mg/kg, gavage administration. Record the diet and exercise status, and measure the tumor size axis every other day.

The xeno-grafts were measured by micrometer caliper, and the tumor volume was calculated based on length (a) and the width (b) by the following formula: tumor volume = ab².

Immunohistochemistry
The tissue was dehydrated by sucrose, then each tissue made a frozen-section, 16 µm, lined with adhesive slides. Incubated with 3% hydrogen peroxide at room temperature for 10 min, washed by PBS for 2 min × 3 times; normal goat serum was incubated at room temperature for 10 min, then sealed with appropriate proportion of diluted primary antibody, washed by PBS for 2 min × 3 times overnight at 4 °C, drip with ready to use secondary antibody, incubated at 37 °C for 30 min, washed by PBS for 2 min × 3 times, drip with fresh DAB Color developing solution: control the color developing time under the microscope, wash completely with water, re-dye by the hematoxylin solution for
several seconds, wash with water, dry the color developing tablets after alcohol, seal them with neutral gum, and observe under the microscope.

Statistical analysis
SPSS 21.0 software was used to analyze the data (Means ± SD). Paired samples were analyzed by paired sample T-test, and multiple samples were analyzed by one-way analysis of variance; P<0.05 was statistically significant.

Result
Effect of nab-P and AP on SKOV-3/DDP
Before discussing the combination of nab-P and AP, we analyzed the inhibition of SKOV-3/DDP along. Firstly, the half inhibition concentration (IC-50) values of SKOV-3/DDP for 24, 48 and 72 hours were measured by MTT test. According to the results of previous experiments, nine concentration gradients (5 × 10^{-2} to 5 × 10^{2} of nab-P and 10^{-1} to 10^{3} of AP) were established to intervene for 24, 48, and 72 hours respectively. The results showed that the drug effect on SKOV-3/DDP was obviously concentration dependent. The IC-50 values of nab-P were 45.53 ± 4.06 µmol/l (24 h), 20.88 ± 2.99 µmol/l (48 h), 8.77 ± 0.64 µmol/l (72 h), while the AP 65.74 ± 8.33 µmol/l (24 h), 50.66 ± 4.96 µmol/l (48 h), 39.547 ± 6.62 µmol/l (72 h)(Fig. 1A).

Divided SKOV-3/DDP into 3 groups and treated with 48 h IC-50 values as reference dosages, a): Control group, no drug intervention; b): nab-P group, nab-P 20 µmol/l; c): AP group, AP 50 µmol/l. Western blot(WB) and Immunofluorescence(IF) were used to detect the expression of Bax, bcl-2, p-VEGFR-2 and MMP-2, and Image J software was used to analyze (Fig. 1B, C). The average expression of the Bax were increased in nab-P and AP group, relative gray value were 0.13 ± 0.07(nab-P group) and 0.14 ± 0.09(AP group), while Control group was 0.04 ± 0.01 in Western blot, one field of cellular amount and positive rate(%) were 34 and 13.87 ± 1.91(nab-P group), 36 and 14.17 ± 1.69(AP group), Control group were 6 and 2.01 ± 0.39. On the contrary, bcl-2 protein’s relative gray value were 0.09 ± 0.01(nab-P group) and 0.09 ± 0.01(AP group), Control group was 0.26 ± 0.01. One field of cellular amount and positive rate(%) were 50 and 13.87 ± 2.45(nab-P group), 56 and 15.67 ± 1.31(AP group), while Control group were 118 and 23.9 ± 1.04. The difference in Control group and experience groups were all statistically significant(P < 0.01),while there was no obvious difference between the
experience groups. SKOV-3 is ovarian cancer cell line with high expression of VEGFR-2, and AP can inhibit tumor angiogenesis by inhibiting the formation of p-VEGFR-2. So, we detected the expression of p-VEGFR-2. The WB and IF results showed that in AP group, the expression were decreased, the relative gray value was 0.04 ± 0.01, one field of cellular amount and positive rate(%) were 13 and 7.4 ± 0.88. The difference was statistically significant compared with the other two groups (P < 0.01).

MMP-2 was closely related to the invasion of tumor cells. After the treatment of SKOV-3/DDP cells with nab-P and AP, the expression of MMP-2 were decreased, the relative gray value were 0.04 ± 0.01(nab-P group), 0.05 ± 0.01(AP group) and one field of cellular amount and positive rate(%) were 44, 10.4 ± 0.85(nab-P group) and 43, 10.83 ± 1.92(AP group), while in Control group, the relative gray value was 0.14 ± 0.01, one field of cellular amount and positive rate(%) were 108 and 19.3 ± 1.16. There had statistically significant compared with the control group(P < 0.01). Meanwhile, the inhibition effect of nab-P and AP on the invasion and migration of SKOV-3/DDP were verified by classical Transwell and Cell scratch assays (Fig. 1D, E). Through Image J software analysis, nab-P and AP inhibited the invasion and migration of SKOV-3/DDP, invasion cellular average amount were 151 and 153 respectively in nab-P and AP groups, while in Control group was 389, compared with Control group, the difference were statistically significant (P < 0.01). Area recovery(%) in nab-P and AP groups were -3.89 ± 0.51(12 h), 1.76 ± 0.12(24 h) and -9.85 ± 0.62(12 h), -5.87 ± 0.62(24 h). the difference were obvious when the two groups compared with Control group.

Effects of different doses of nab-P combined with AP on SKOV-3/DDP
After the above experiments, we can draw that nab-P and AP all can play the satisfactory anti-tumor effect when acting alone with SKOV-3/DDP cell line. Because of nab-P and AP all can play strong antitumor effects on drug-resistant cell lines, we guess whether there is a 1 + 1 > 2 effect if the drugs were combined. Therefore, we designed the following experiments.

We used nab-P: AP as 1:2 to act on SKOV-3/DDP cell line, MTT detecting the IC-50 value was nab-P 5.28 µmol/l + AP 10.56 µmol/l(48 h), the combined inhibition index(CL) was all belowed1, that men nab-P combined with AP onSKOV-3/DDP had synergistic effect. In clinical practice, the adverse reactions caused by chemotherapy drugs are positively correlated with the drug dosage. Therefore,
we assume that if the dosage of nab-P can be reduced properly after the combination of the two drugs, whether the drug effect can be unaffected. We designed four experiment groups, a): Control group, no drug intervention; b): Group-1, nab-P 5 µmol/l + AP 10 µmol/l; c): Group-2, nab-P 4.5 µmol/l + AP 10 µmol/l; d): Group-3, nab-P 4 µmol/l + AP 10 µmol/l, the expression of Bax, bcl-2, p-VEGFR-2 and MMP-2 in SKOV-3/DDP were also detected by WB and IF(Fig. 2B, C). In the Bax protein expression, the relative gray value were 0.03 ± 0.00(Control group), 0.15 ± 0.01(Group-1), 0.14 ± 0.01(Group-2), 0.14 ± 0.01(Group-3), the one field of average cellular amount and positive rate(%) were 5 and 2.7 ± 0.29(Control group), 55 and 23.07 ± 1.13(Group-1), 50 and 21.57 ± 0.67(Group-2), 51 and 21.8 ± 1.8(Group-3). In the bcl-2, the relative gray value were 0.24 ± 0.01(Control group), 0.06 ± 0.01(Group-1), 0.06 ± 0.01(Group-2), 0.06 ± 0.01(Group-3), the one field of average cellular amount and positive rate(%) were 66 and 25.7 ± 0.65(Control group), 11 and 7.57 ± 1.11(Group-1), 10 and 7.4 ± 1.1(Group-2), 14 and 8.2 ± 1.2(Group-3). In the p-VEGFR-2, the relative gray value were 0.14 ± 0.01(Control group), 0.03 ± 0.00(Group-1), 0.04 ± 0.00(Group-2), 0.04 ± 0.00(Group-3), the one field of average cellular amount and positive rate(%) were 75 and 27.8 ± 0.86(Control group), 11 and 6.8 ± 1.51(Group-1), 13 and 7.5 ± 0.94(Group-2), 16 and 8.4 ± 0.88(Group-3). In the MMP-2, the relative gray value were 0.16 ± 0.01(Control group), 0.03 ± 0.00(Group-1), 0.04 ± 0.00(Group-2), 0.04 ± 0.00(Group-3), the one field of average cellular amount and positive rate(%) were 78 and 27.1 ± 1.7(Control group), 15 and 7.97 ± 1.2(Group-1), 13 and 9.83 ± 1.01(Group-2), 17 and 11.13 ± 0.4(Group-3). The results showed that compared with Control group, Bax, p-VEGFR-2, MMP-2 expression in Group-1, 2 and 3 were higher than those in the Control group, while bcl-2 were lower(P < 0.01), and there was no significant difference between groups 1, 2 and 3 (P > 0.05). The results of Transwell and Cell scratch test were consistent with the expression of MMP-2(Fig. 2D, E), the ability of cell invasion and migration decreased after combined treatment, invasion cellular average amount and area recovery(%) were 6 and −6.56 ± 0.39(12 h), -14.8 ± 0.7(24 h)(Group-1), 12 and −5.28 ± 0.53(12 h), -14.37 ± 0.73(24 h)(Group-2), 14 and −4.5 ± 0.39(12 h), -14.15 ± 0.61(24 h)(Group-3), compared with the Control group, the difference was obviously significant (P < 0.01). And there still were no significant difference between the three medication groups. The results showed that reducing
dose of nab-P had no significant effect on the antitumor activity when combined with AP. Effects of different doses of nab-P combined with AP on xenograft tumor model Through the above experiments, nab-P combined with AP can significantly inhibit tumor growth and promote apoptosis of SKOV-3/DDP cell line. When the dosage of nab-P is properly reduced, it can still achieve satisfactory antitumor effect. Therefore, we can sum up that at the cellular level, nab-P can reduce the dosage when combined with AP. According to the conclusion, we continue to design in vivo experiments to verify whether the results of in vivo experiments are consistent with those of in vitro. After the establishment of xenograft tumor models, the tumor volumes were recorded every other day(Fig. 3A). We found that the average volume of subcutaneous tumor in the control group increased from \(234.83 \pm 28\)mm\(^3\) to \(1452.83 \pm 61.47\)mm\(^3\), the volume changes of the nude mice in the experimental groups were \(230.17 \pm 31.66\) to \(144 \pm 37.65\)mm\(^3\) (Group-1), \(224.83 \pm 28\) to \(142.33 \pm 31.4\)mm\(^3\) (Group-2), and \(33.67 \pm 29.37\) to \(138.83 \pm 31.43\)mm\(^3\) (Group-3), there was no significant difference in tumor volume between the three groups, but there was between Control group(\(P < 0.01\)). Next, we analyzed tumor mass by WB, IF and immunohistochemistry(IHC), the results were shown in Fig. 3C, D, E. In WB ’s results, the relative gray value of Bax were respectively \(0.08 \pm 0.02\) (Control group), \(0.18 \pm 0.12\) (Group-1), \(0.17 \pm 0.17\) (Group-2), \(0.17 \pm 0.02\) (Group-3), the bcl-2 were \(0.17 \pm 0.02\) (Control group), \(0.07 \pm 0.01\) (Group-1), \(0.09 \pm 0.01\) (Group-2), \(0.09 \pm 0.01\) (Group-3), the CD31 were \(0.75 \pm 0.04\) (Control group), \(0.16 \pm 0.02\) (Group-1), \(0.16 \pm 0.04\) (Group-2), \(0.2 \pm 0.01\) (Group-3), the p-VEGFR-2 were \(0.26 \pm 0.02\) (Control group), \(0.16 \pm 0.03\) (Group-1), \(0.18 \pm 0.01\) (Group-2), \(0.19 \pm 0.01\) (Group-3), and MMP-2 were \(0.71 \pm 0.02\) (Control group), \(0.29 \pm 0.03\) (Group-1), \(0.3 \pm 0.02\) (Group-2), \(0.29 \pm 0.01\) (Group-3). The three combination groups were had no difference between them, while compare with Control group, there were all significant difference(\(P < 0.01\)). Meanwhile, the IF and IHC ’s positive rate(%) as follows. The Bax were \(3.37 \pm 0.91\) and \(13.2 \pm 1.07\) (Control group), \(20.97 \pm 2.09\) and \(34.17 \pm 1.11\) (Group-1), \(19.13 \pm 2.78\) and \(32.83 \pm 0.95\) (Group-2), \(19.73 \pm 1.52\) and \(32.77 \pm 1.16\) (Group-3), the bcl-2 were \(17.17 \pm 1.69\) and \(31.73 \pm 1.67\) (Control group), \(3.23 \pm 1.35\) and \(13.33 \pm 1.59\) (Group-1), \(2.43 \pm 0.79\) and \(14.63 \pm 1.48\) (Group-2), \(2.57 \pm 0.5\) and \(13.87 \pm 1.89\) (Group-3), the CD31 were \(24.1 \pm 1.15\) and \(36.1 \pm 1.69\) (Control group), \(6.73 \pm 1.47\) and \(11.63 \pm 1.2\) (Group-1), \(9.53 \pm 0.94\) (Group-2), \(9.53 \pm 0.94\) (Group-3).
1.6 and 12.67 ± 1.52 (Group-2), 9.7 ± 1.96 and 12.33 ± 2.24 (Group-3), the p-VEGFR-2 were 16.77 ± 1.03 and 35.47 ± 2.93 (Control group), 3.0 ± 0.29 and 17.1 ± 1.31 (Group-1), 3.83 ± 0.61 and 17.53 ± 1.68 (Group-2), 4.07 ± 1.04 and 18.23 ± 1.25 (Group-3), and MMP-2 were 13.2 ± 1.53 and 34.9 ± 2.38 (Control group), 2.77 ± 0.54 and 14.53 ± 1.17 (Group-1), 2.87 ± 0.61 and 15.27 ± 1.68 (Group-2), 2.93 ± 0.69 and 15.57 ± 2.75 (Group-3).

These results had a same verdict that compared with Control group, there were significant difference in experiment groups (P < 0.01). This showed that although nab-P reduces its standard dosage, the combination of nab-P and AP still has no significant effect on the tumor reduction.

During the experiment, by observing the quality of life of the mice, we found that after the intervention of nab-P and AP, the majority side effects in the experimental groups were anorexia and decreased activity, which were enhanced along the intervention time. Therefore, we evaluated the quality of life of different groups by average daily food intake and 1 hour activity (Table 1), it can be seen from the table that there is no apparent difference in the quality of life of the 4 groups before the treatment. When the after, the side effects of different degrees were consist with unequal doses of nab-P. In the two evaluation indexes, the difference between the experimental group and the control group was statistically significant (P < 0.01), the results showed that after the treatment of nab-P combined with AP, the corresponding adverse reactions occurred in mice, and the quality of life decreased. In addition, we found that with the decrease of nab-P dose, the daily intake and activity of mice increased gradually, and the quality of life improved.

### Table 1

| Group     | Total (n) | Average daily food intake(g) | One hour activity(min) |
|-----------|-----------|------------------------------|------------------------|
|           | Before medication | After medication | P       | Before medication | After medication | P       |
| Control group | 6 | 6.79 ± 0.21 | 6.90 ± 0.19 | 0.64 | 18.63 ± 0.15 | 19.35 ± 0.14 | 0.62 |
| Group-1    | 6 | 6.78 ± 0.21 | 4.50 ± 0.17** | < 0.01 | 17.54 ± 0.15 | 6.11 ± 0.16** | < 0.01 |
| Group-2    | 6 | 6.91 ± 0.19 | 5.64 ± 0.15** | < 0.01 | 17.68 ± 0.15 | 6.29 ± 0.12** | < 0.01 |
| Group-3    | 6 | 6.62 ± 0.16 | 5.55 ± 0.13** | < 0.01 | 17.95 ± 0.14 | 6.34 ± 0.13** | < 0.01 |

Disscusion

With the continuous development of ovarian cancer technology, the prognosis of patients has been improved, while the mortality rate is still the first in gynecological cancer [21], especially for the late
recurrence of OC patients, the survival rate has not been ideal. More importantly, the widespread use of chemotherapy drugs, damaged the patients’ normal tissues, and even drug-resistance, leading to the failure of treatment[22]. The problem may be solved by finding drugs that more effective or can reverse drug-resistance, or reducing the dosage of chemotherapy drugs without affecting the therapeutic effect. In our initial experiment, SKOV-3/DDP was used as the research object to preliminarily explore the synergistic effect of targeting drug AP combined with chemotherapy drug nab-P on OC. Our results showed that AP and nab-P all have strong antitumor effects on SKOV-3/DDP, and when they are combined, their respective antitumor effects were enhanced. Furthermore, when the two drugs combined, if nab-P properly reduced, the two drugs can still achieve ideal antitumor effect. This means that if consistent with the results of clinical trials, such a drug scheme may possibly be used to reduce the adverse reactions caused by chemotherapy drugs. That’s why we designed this paper.

Firstly, we obtained the IC-50 value of SKOV-3/DDP to nab-P and AP. The results showed that IC-50 of nab-P was lower than AP, this indicated that the drug effect of nab-P on SKOV-3/DDP is slightly stronger than that of AP. Apoptosis is a form of cell death after the initiation of suicide procedure, which plays an important role in maintaining the balance between normal cell death and cell division[23]. Inhibition of apoptosis and abnormal cell proliferation and differentiation are the biological basis of tumorigenesis[24]. Promoting apoptosis of tumor cells is one of the most important methods in tumor targeted therapy[25]. The Bax and bcl-2 are the key to regulate apoptosis. So in our study, Western blot and Immunofluorescence were applied to analyze the expression of apoptosis related protein Bax, bcl-2, the results showed after nab-P and AP intervention, Bax was higher than control group, bcl-2 was contrary. That’s means nab-P and AP all can play the satisfactory anti-tumor effects on SKOV-3/DDP, which can promote tumor cell apoptosis. Tumor growth mainly depends on tumor angiogenesis[26]. Vascular endothelial growth factor (hereinafter referred to as VEGF) is an important factor in angiogenesis, it widely distribute in various normal tissues and tumor tissues, can stimulate the proliferation, survival and migration of endothelial cells through the corresponding cell surface receptors and signal pathways, which is one of the important factors for the growth and
survival of endothelial cells[27]. VEGFR is the main functional receptor of VEGF, and VEGFR-2 is considered to be the key molecule of VEGF signaling pathway to induce angiogenesis. So, it’s an effective way to treat tumor by inhibiting angiogenesis[28]. Apatinib is the ATP binding site of VEGFR-2, which can inhibit the production of p-VEGFR-2 and block the downstream signal transduction pathway. Therefore, we tested expression of p-VEGFR-2 in different groups. The results showed that in AP group, the expression of p-VEGFR-2 was reduction, while in nab-P group was slightly higher than that in Control group. In the way of MMP-2, previous studies have shown that matrix metalloproteinases plays an important role in tumor cell invasion, the invasion and migration is the main cause of tumor metastasis[29] and MMP-2 is an important part[30]. In experience groups, the expression of MMP-2 were declined when compared with Control group. We also used Transwell and Cell scratch experiments to verify the changes of invasion and migration of SKOV-3/DDP in different groups. Which was the same as the expected results, after the effects of nab-P and AP, the number of invasive cells decreased significantly, and the 24-hour cell healing rate decreased. Therefore, we can draw the first conclusion that nab-P and AP can play a good anti-tumor effect on drug-resistant SKOV-3/DDP. This results are the same with jing, Momeny, Tipton et al[31–33].

However, considering the limitation of single drug therapy, combined drugs is a routine means for clinical tumor treatment[34], for the treatment of ovarian cancer, the combination of chemotherapy targeted drugs has made a great breakthrough in the basic research and clinical cases in recent years[19, 35–36]. The nab-P is a kind of paclitaxel wrapped by human serum albumin, which can enter the tumor cells easily, increase the drug concentration in tumor cells, and achieve ideal therapeutic effect on drug-resistant ovarian cancer. However, after the therapy of nab-P, the decrease of tumor load may increase the expression of VEGF signal[37]. Therefore, if VEGFR-2 inhibitor is used at the same time, the expression of VEGFR-2 will be continuously inhibited, and with VEGF disabled, tumor angiogenesis will be reduced simultaneously, so as to achieve better antitumor effect. Fortunately, AP is a small molecule targeted drug to inhibit VEGFR-2, so we look forward to the better performance of combination of these two drugs. According to this problem, we have carried out the next cytological experiments. We also tested the inhibitory effect of nab-P combined Ap on
SKOV-3/DDP by MTT assay, and with the help of compusyn (version 2.0) software analysed the combination index of the two drugs, the results showed that nab-P combined with AP had a strong inhibitory effect on SKOV-3/DDP, and when they work together, they worked synergism effect. According to the IC-50 value, we set up the combination of nab-P with different concentration gradients combined with AP, Western blot and immunofluorescence experiments also be used to verify the expression of related proteins (Bax, bcl-2, p-VEGFR-2 and MMP-2), the results showed that when nab-P combined with AP, bcl-2, p-VEGFR-2 and MMP-2 were all expressed higher than Control group (P < 0.01), while Bax was lower, and in this three experience groups, there were no significant difference in this protein’s expression. Transwell and Cell scratch test’s results also showed the combination of drugs greatly weakened the invasion and migration of SKOV-3/DDP. Above that, we can obtain another conclusion that nab-P combined with AP can promote the apoptosis and inhibit the invasion of SKOV-3/DDP. When nab-P was reduced at a suitable concentration, the anti-tumor effect of combined AP has little effect. We can think that at the cellular level, when nab-P and AP are combined, the drug toxicity can be reduced by reducing the dosage of nab-P.

After obtaining satisfactory results at the cell level, we designed animal experiments to verify whether the results at the animal level are consistent with those at the cell. Different concentration gradients of nab-P were set up with the same combination of AP. The reduction of tumor mass were observed by measuring the volume of subcutaneous tumor in nude mice after administration, the results showed that after the treatment of nab-P combined with AP, the tumor volume decreased significantly, and the tumor inhibition rates in the experimental group were 90.08% (Group-1), 88.7% (Group-2) and 88.4% (Group-3), respectively. There was no significant difference among the three experimental groups, that is to say, after combination of AP, reduction of nab-P had little effect on anti-tumor. In addition, Western blot, immunofluorescence and immunohistochemistry were carried out to detect Bax, bcl-2, CD31, p-VEGFR-2 and MMP-2. The results showed that the expression of apoptosis related protein bcl-2, vascular related protein CD31, p-VEGFR-2 and invasion related protein MMP-2 decreased significantly and Bax increased, compared with the control group, the difference was statistically significant, but there were no obvious difference among the three groups, which was
highly consistent with the experimental results at the cell level. The side effects caused by chemotherapy drugs while killing tumor cells are important factors affecting the quality of life of patients[38]. In order to verify the effect of reducing the dosage of nab-P on the survival of nude mice, we set up two observation indexes: daily average diet and one hour exercise. We found that there was a significant negative correlation between the quality of life of mice and the intervention of nab-P. Therefore, we can draw the third conclusion, that is, in vivo experiments, after nab-p combined with AP, appropriate reduction of the recommended dosage of nab-p can achieve the purpose of reducing the adverse reactions caused by nab-P in mice.

In a word, in this experiment, we combined the targeting drug Apatinib with the chemotherapy drug paclitaxel (aluminum binding type) to act on the human ovarian cancer cell line and xenograft tumor model, and found that the combination of the two drugs can significantly inhibit the proliferation and invasion of the tumor. Secondly, after the combination, it can be properly reduced the inhibitory effect of low nab-P dose on OC cell line and xenograft tumor model. This suggests that, in the clinical treatment, the combination scheme can bring less side effects. Due to the certain toxicity of chemotherapy drugs, patients are prone to some adverse reactions such as myelosuppression, gastrointestinal reactions, etc. During the chemotherapy period, some patients are showed well intolerable, and by reducing the dose of chemotherapy drugs, it will lead to a great discount in the treatment effect. Therefore, if the combination can reduce the dosage of nab-P on the basis of not reducing the therapeutic effect, and the adverse reactions will be correspondingly reduced, which may improve the quality of life of patients.

Based on the above all, the combination of nab-P and Ap performed well in inhibiting the growth of tumor, and improving the drug efficacy and the survival rate of patients. In addition, it can also reduce the drug dose of nab-P, thereby reducing the adverse reactions brought by nab-P to improve the quality of life of patients.

Declarations
Ethics approval and consent to participate
Not applicable
Consent for publication
Not applicable

Availability of data and materials
The datasets used and analysed during the current study are available from the corresponding author (Rong Li) on reasonable request.

Competing interests
The authors declare that they have no competing interests

Funding
This study was funded by Natural science gene of Shanxi Province (Xiaoyan Wang grant number 2013011045-4).

Authors' contributions
Hong Zhao*: Put forward the whole idea of experiment design and important experiment’s design.
Hong Zhao*: Put forward the whole idea of experiment design and important experiment’s design.
Jinbin Zhang: Provide guidance for animal experiment operation.
Min Hu: Be responsible for experiment record.
Rong Li: Assist Hong Zhao to design and implement the major experiment.
Xiaoyan Wang: Be responsible for correcting errors in the process of experiment and providing technical guidance.
Xin Lu: Be responsible for part of the operation of cytology experiment and the breeding of nude mice.
Xia Zhao: Provide theoretical help for the preparation of the experiment.
Xiaoqin Song: Be responsible for conventional culture of cells.
Yangyang Liu: Be responsible for configuration of some reagents.

All authors read and approved the final manuscript

Acknowledgements
Thanks to the biochemistry and Molecular Biology Department of Shanxi Medical University for providing some necessary instruments and equipment, and thanks to the gynecologist of Shanxi cancer hospital for providing clinical guidance

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Effect of nab-P and AP on SKOV-3/DDP. A the nab-P and AP’s half inhibition concentration to SKOV-3/DDP (24, 48 and 72 hours), B the Bax, bcl-2, p-VEGFR-2 and MMP-2 protein expression of different groups by Western blot (WB), C the Bax, bcl-2, p-VEGFR-2 and MMP-2 protein expression of different groups by Immunofluorescence (IF), D the invasion in different groups by Transwell assay, E the migration in different groups by Wound healing.
assay. **P<0.01, medication group vs control group, △△P<0.01, comparison of two sets of data.

Figure 2

Effects of different doses of nab-P combined with AP on SKOV-3/DDP. A the nab-P combined with AP’s half inhibition concentration to SKOV-3/DDP(24, 48 and 72 hours) and combined inhibition index, B the Bax, bcl-2, p-VEGFR-2 and MMP-2 protein expression of different
groups by Western blot (WB), C the Bax, bcl-2, p-VEGFR-2 and MMP-2 protein expression of different groups by Immunofluorescence (IF), D the invasion in different groups by Transwell assay, E the migration in different groups by Wound healing assay. **P<0.01, medication group vs control group.
Figure 3

Effects of different doses of nab-P combined with AP on BALB/c. A Growth curve of subcutaneous tumor in four groups of mice on the first day of drug intervention, B mice and volume of tumor after exfoliation, C the Bax, bcl-2, CD31, p-VEGFR-2 and MMP-2 protein expression of different groups by Western blot, D the Bax, bcl-2, CD31, p-VEGFR-2 and MMP-2 protein expression of different groups by Immunofluorescence, E the Bax, bcl-2, CD31, p-VEGFR-2 and MMP-2 protein expression of different groups by Immunohistochemistry.