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Picropodophyllin inhibits epithelial ovarian cancer cells *in vitro* and *in vivo*

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ABSTRACT

Epithelial ovarian cancer (EOC) is one of the leading causes of gynecological cancer death. Approximately 70% of the patients experience recurrence accompanied by the development of drug resistance 2–3 years after chemotherapy. Picropodophyllin (PPP) is a newly identified insulin-like growth factor-1 receptor (IGF-1R) inhibitor that has been shown to have anticancer properties. In this study, we investigated the effect of PPP on EOC growth *in vitro* and *in vivo*. The EOC cell line SKOV-3 was treated with increasing concentrations of PPP or cisplatin, and cell viability and apoptosis were evaluated. To study the effects of PPP on EOC growth, apoptosis, and toxicity *in vivo*, a BALB/c nude mouse xenograft model was established. Mice were treated with normal saline (controls), PPP, cisplatin, or PPP in combination with cisplatin. In addition, the expression of phosphorylated IGF-1R (pIGF-1R) was examined *in vitro* and *in vivo*. PPP induced a dose-dependent decrease in SKOV-3 cell viability *in vitro* and reduced tumor volume and weight in the *in vivo* xenograft model. Furthermore, PPP in combination with cisplatin was more effective in inhibiting the growth of SKOV-3 cells and xenografts than either drug alone. PPP-mediated growth inhibition was associated with apoptosis induction *in vitro* and *in vivo*. PPP was well tolerated *in vivo* and exerted its effects with minimal hepatotoxicity and renal toxicity. PPP downregulated the expression of pIGF-1R *in vitro* and *in vivo*, an effect that appeared to be associated with its growth inhibitory properties. Our results indicate that PPP may have therapeutic application in the treatment of EOC.
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1. Introduction

Epithelial ovarian cancer (EOC) accounts for 90% of ovarian cancers [1]. The majority of EOC patients present with advanced-stage disease at the time of initial diagnosis and experience recurrence despite the excellent response rates observed with platinum-based chemotherapy (e.g., cisplatin) [2]. Disease recurrence typically occurs within 2–3 years after first-line chemotherapy and is accompanied by the development of cisplatin resistance [3]. Therefore, there is an urgent need to develop effective pharmacologic agents for the treatment of EOC.

Picropodophyllin (PPP), a stereoisomer of podophyllotoxin, is a recently identified antitumor agent that inhibits cell proliferation by inducing mitotic arrest [4]. PPP has also been shown to induce tumor regression and substantially prolong survival *in vivo* [5], such as human adrenocortical carcinoma [4], hepatocellular carcinoma [6], and uveal melanoma [7]. Studies have demonstrated that the anticancer properties of PPP appear to be associated with its effects on insulin-like growth factor-1 receptor (IGF-1R). In recent years, IGF-1R has become a promising therapeutic target for EOC because of its key role in regulating the normal biology of ovarian surface epithelial cells [8]. Inhibition of IGF-1R with small molecule IGF-1R kinase inhibitors such as NVP-AEW541 [9] and BMS molecules [1] has been shown to decrease the proliferation of EOC cells. To date, the therapeutic effects of PPP on EOC have not been reported. Therefore, the present study investigated the effects of PPP on EOC growth *in vitro* and *in vivo*.

2. Materials and methods

2.1. Drugs

PPP (99.86% purity, extracted from podophyllin) was provided by Yabang Pharmaceutical and Development Co. Ltd. (Changzhou, China). Cisplatin was provided by Qilu Pharmaceutical Co. Ltd. (Jinan, China).

2.2. Cell line

The EOC cell line SKOV-3 was provided by the Cell Bank of Chinese Academy of Sciences (Beijing, China). The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37 °C under 5% CO_2.

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2.3. In vitro clonogenic survival and growth assay

2.3.1. Cell viability assay

The 3-(4,5-dimethylthiazoyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the viability of SKOV-3 cells. SKOV-3 cells were plated at a density of 1×10^4 cells/well in a 96-well microplate. Cells were treated with PPP (0.01, 0.10, 0.25, 0.50, 1.50, and 2.50 μmol/L), cisplatin (0.1, 0.5, 2.5, 5.0, 10.0, and 40.0 μmol/L), or PPP plus cisplatin (PPP, 0.01, 0.10, 0.25, 0.50, 1.50, and 2.50 μmol/L; cisplatin, 5.0 μmol/L). Untreated cells served as controls. After 48 h, 20 μl MTT was added to each well, and cells were incubated for an additional 4 h at 37 °C. The culture medium was carefully aspirated, and 200 μl dimethyl sulfoxide was added to each well and mixed by pipetting. Cell viability was obtained by measuring the absorbance (A) at a wavelength of 570 nm. Cell viability rate was calculated using the following formula: $A/A_{control} \times 100\%$. The assay was performed in triplicate.

2.3.2. Flow cytometry analysis

SKOV-3 cells were treated with PPP (0.25 μmol/L), cisplatin (5.0 μmol/L), or PPP plus cisplatin (5.0 μmol/L). Untreated cells served as controls. The percentage of apoptotic cells was determined by flow cytometry using an Annexin V-FITC kit (BD Biosciences, Franklin Lakes, USA) according to the manufacturer's instructions. Cells that are in early apoptosis are FITC Annexin V positive and PI negative, while cells that are in late apoptosis or already dead are both FITC Annexin V and PI positive. The assay was repeated 3 times.

2.3.3. Western blot analysis

Western blot was used to determine the effect of PPP on IGF-1R expression in EOC cells. SKOV-3 cells were treated with PPP (0.1, 0.25, 0.5, 1.5, and 2.5 μmol/L) or cisplatin (0.1, 0.5, 2.5, 10.0, and 40.0 μmol/L) for 1 h. Untreated cells served as controls. Protein lysates (20 μg) were electrophoresed on a 10% sodium dodecyl sulfate–polyacrylamide gel, electroblotted onto a polyvinylidene difluoride membrane, and blocked in 5% nonfat milk in phosphate-buffered saline containing 0.05% Tween 20 (PBS-T) for 1 h. The blots were incubated with primary antibody diluted in 5% bovine serum albumin in 1× PBS-T overnight at 4 °C. The following primary antibodies were used: IGF-1Rβ (1:500; Abcam, Cambridge, UK), phosphorylated IGF-1R (pIGF-1R) antibody (1:500; Abcam, Cambridge, UK), and β-actin (1:2000; Sigma, St. Louis, USA). β-Actin was used as a protein loading control. The blots were incubated with a horseradish peroxidase-conjugated secondary antibody (1:1000; Amersham-GE Healthcare, Little Chalfont, UK) diluted in 5% nonfat milk in 1× PBS-T for 1 h at room temperature. Western-Bright ECL Plus detection reagent (Advansta, Menlo Park, USA) was used to visualize protein bands. The experiments were repeated 3 times.

2.4. In vivo inhibition observation

2.4.1. Animals and xenografts model

Female BALB/c (nu/nu) nude mice (180–240 g) were provided by the Department of Laboratory Animal Science of the Peking University Health Science Center (Beijing, China). Mice were housed in a temperature-conditioned room (22–24 °C) with an alternating 12-h light/dark cycle and allowed free access to food and water. A single cell suspension of SKOV-3 cells (1×10^7 cells/ml) was prepared in RPMI-1640 and 200 μl was injected subcutaneously into the right side of the back of each mouse. Tumors developed after 35 days (mean tumor volume = 300 ± 18.5 mm^3), and 32 SKOV-3 xenograft models were successfully established. The 32 mice were randomly assigned to 4 groups ($n = 8$): (1) PPP group (40 mg/kg), (2) cisplatin group (5 mg/kg), (3) PPP plus cisplatin group (40 mg/kg PPP+ 5 mg/kg cisplatin), and (4) control group (normal saline). Drugs were administered by intraperitoneal injection every 3 days over the treatment period (18 days) for a total of 6 doses. All procedures were approved by the Ethics Committee of Experimental Animals of the Wenzhou Medical College (Wenzhou, China) and performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH, Bethesda, MD, USA).

2.4.2. Tumor growth

The mean tumor volume (V) was calculated daily during the treatment period using the following formula: $V = a \times b \times b/2$ (a, maximum tumor diameter; b, vertical diameter of the maximum diameter). Tumor growth curves were constructed based on tumor volume. The antitumor efficacy of the treatments was measured as tumor growth inhibition (TGI) using a modified method described previously by Liu et al. [6]. TGI was calculated using the following formula

$$[(1 - T/C) \times 100]\%$$

where T represents the final mean tumor weight in the treatment group and C represents the final mean tumor weight in control groups.

2.4.3. Immunohistochemistry analysis

Immunohistochemistry was used to assess the effect of PPP on the *in vivo* expression of pIGF-1R in EOC. Tumor grafts were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 4-μm-thick sections. pIGF-1R-positive cells were stained brown in the cell membrane. The most intensely labeled regions under low power (100×) were selected for observation. The number of pIGF-1R-positive cells in 10 randomly selected areas in an objective grid was counted under a high-power field (400×) in a blinded manner. The labeling index was used to quantify the extent of pIGF-1R expression and was calculated as follows: number of positive-stained cells/500 × 100%.

2.4.4. TUNEL assay

Apoptosis in the SKOV-3 xenograft mouse model was determined using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. The TUNEL assay was performed using an In Situ Cell Death Detection kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. Briefly, paraffin-embedded sections (4 μm) were deparaffinized, permeabilized with 20 μg/ml proteinase K, and incubated with 3% hydrogen peroxide. Sections were incubated with a nucleotide and terminal transferase (TdT) mixture for 1 h and then incubated with horseradish peroxidase for 30 min at 37 °C. Staining was developed using the 3, 3′-diaminobenzidine tetrahydrochloride, and the nuclei were lightly counterstained with hematoxylin. Positive controls were pretreated with 1 U/ml deoxyribonuclease, whereas negative controls were incubated without TdT. The cells that had brownish-stained-nuclei and morphological characteristics of apoptosis, such as nuclear fragmentation and condensation, were considered TUNEL positive. The number of TUNEL-positive cells was counted in 10 randomly selected fields (400×) in a blinded manner. Apoptotic index was calculated using the following formula: TUNEL-positive cells/500 × 100%.

2.4.5. Hepatotoxicity and renal toxicity assessment

Bodyweight was used to assess toxicity among the treatment groups. The bodyweight of each animal was recorded before and at the end of the experiment. In addition, hepatotoxicity and renal toxicity were evaluated by measuring serum alanine transaminase (ALT), blood urea nitrogen (BUN), and creatinine (Cr) levels at the end of the experiment. Liver and kidney tissues were collected

and stained with hematoxylin-eosin (HE) for histological evaluation.

2.5. Statistical analysis

Results are expressed as the means ± standard deviation (SD). Statistical analyses were performed based on the normal distribution and homogeneity of the data. Differences between 2 or multiple groups were analyzed using one-way analysis of variance, Fisher's least significant difference test, and Student's *t*-test. All statistical analyses were performed using SPSS 19.0 software (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. PPP treatment inhibits the viability of SKOV-3 cells

As shown in Fig. 1A, PPP inhibited the viability of SKOV-3 cells in a dose-dependent manner (IC50 = 0.18 μmol/L, 95% intervals from 0.11 μmol/L to 0.28 μmol/L). In addition, the combination of PPP and cisplatin was more effective in inhibiting the viability of SKOV-3 cells than either drug given alone ($P < 0.05$).

3.2. PPP treatment decreases tumor volume and weight in an SKOV-3 xenograft model

Growth curves based on the average tumor volume are shown in Fig. 1B. In the control group, tumors grew rapidly in size. In the cisplatin group, tumor volume almost remained unchanged during the first 12 days and increased slowly thereafter. The PPP and the PPP plus cisplatin treatments decreased tumor volume and exhibited similar growth curves over treatment time. However, the PPP plus cisplatin group has the lowest average tumor volume among all the groups at the end of the experiment ($P < 0.01$).

Tumor weight was decreased in the 3 treatment groups compared to the control group (Fig. 1C). Cisplatin, PPP, and PPP plus cisplatin produced TGIs of 26.53%, 39.18%, and 72.17%, respectively. Tumor weights were significantly lower in the PPP plus cisplatin group than in the cisplatin and PPP group ($P < 0.01$).

3.3. PPP treatment induces SKOV-3 cells apoptosis

When compared to untreated control cells, PPP promoted both early (FITC Annexin V+/PI−) and late phase (FITC Annexin V+/PI+) apoptosis in SKOV-3 cells *in vitro* ($P < 0.01$, Fig. 2A and B). In addition, the percentage of apoptotic cells was significantly higher in PPP plus cisplatin group than in the cisplatin and PPP group ($P < 0.05$). Mice in the PPP group showed a significantly higher apoptotic index than mice in the control group ($P < 0.01$). The apoptotic index was higher in the PPP plus cisplatin group than in the cisplatin and PPP group ($P < 0.01$, Fig. 2C–G).

3.4. PPP treatment downregulates IGF-1R phosphorylation in SKOV-3 cells

In the *in vitro* studies, IGF-1R expression was unaffected by PPP or cisplatin treatment at the doses studied. However, pIGF-1R expression was inhibited by PPP in a dose-dependent manner. In contrast, cisplatin did not have any effect on the pIGF-1R expression (Fig. 3A). Similar treatment effects on pIGF-1R expression were observed in the *in vivo* studies (Fig. 3B–F). Both the PPP and the PPP plus cisplatin groups showed significant downregulation of pIGF-1R expression in comparison to the control group ($P < 0.01$); however, pIGF-1R expression was not statistically different between these 2 groups. Cisplatin did not affect pIGF-1R expression in SKOV-3 xenografts ($P > 0.05$).

3.5. PPP exhibits low hepatotoxicity and renal toxicity

Body weight was not significantly different among the treatment groups ($P > 0.05$, Fig. 4A). Renal toxicity and hepatotoxicity are the most common side effects of antitumor agents. Therefore, serum ALT, BUN and Cr levels, which are important indices of liver and kidney function, were evaluated at the end of therapy. Serum ALT, BUN and Cr levels were not significantly different between the PPP group and the control group ($P > 0.05$, Fig. 4B–D). In contrast, BUN and Cr levels in the cisplatin group and PPP plus cisplatin group were increased markedly in comparison to the control group ($P < 0.05$). ALT level in the cisplatin group was not higher than that of the control ($P > 0.05$). BUN and Cr levels were similar in the cisplatin and PPP plus cisplatin groups ($P > 0.05$). These results indicate that the toxicity of cisplatin was not increased by PPP. However, there were no significant histological changes in the HE-stained sections of the liver and kidney.

4. Discussion

Approximately 70% of EOC patients experience recurrence and ultimately die of the disease despite favorable initial responses to platinum-based chemotherapy [1,2]. Development of resistance to platinum drugs, such as cisplatin, has hindered the effective treatment of recurrent EOC. To date, few treatments are capable of curing recurrent EOC. Therefore, developing new chemotherapeutics

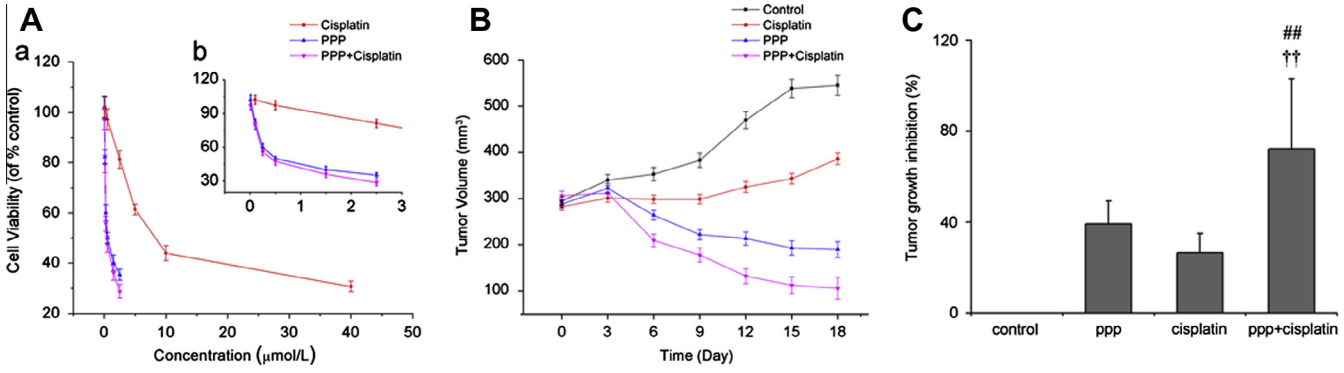

Fig. 1. PPP treatment inhibits the growth of SKOV-3 cell line *in vitro* and *in vivo*. (A) PPP treatment inhibits the viability of SKOV-3 cells. (A) The cell viability of concentration between 0 and 50 μmol/L. (B) The cell viability of concentration between 0 and 3 μmol/L. (B) PPP treatment decreases tumor volume in an SKOV-3 xenograft model. (C) PPP treatment decreases tumor weight in an SKOV-3 xenograft model. $^{\#\#}P < 0.01$ versus the PPP group, $^{\dagger\dagger}P < 0.01$ versus the cisplatin group.

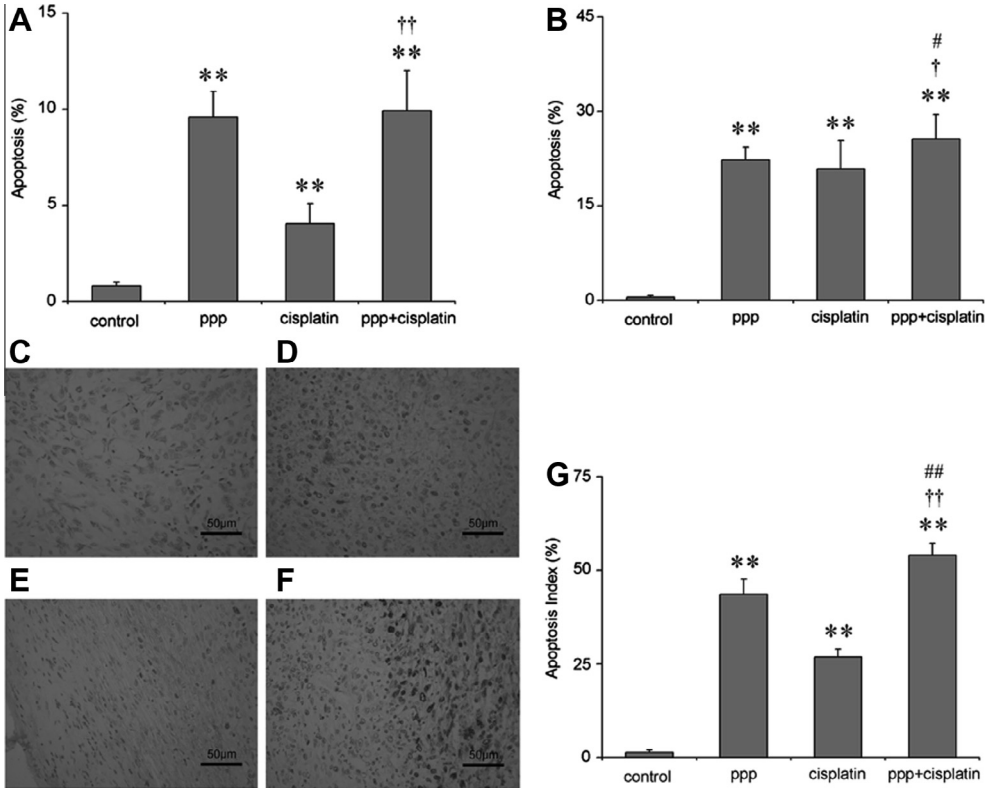

Fig. 2. PPP treatment induces SKOV-3 cell apoptosis. (A and B) PPP treatment induces the apoptosis of SKOV-3 cell line *in vitro*. (A) The early phase (FITC Annexin V +/PI−) and (B) late phase (FITC Annexin V +/PI+) were detected by using the flow cytometry. Data demonstrate that PPP treatment promoted both the early phase and late phase apoptosis of SKOV-3 in comparison with those of the control group. (C–G) PPP treatment induces the apoptosis of SKOV-3 cell line *in vivo*. (C–F) Photomicrographs of representative TUNEL assay of the control group (C), the PPP group (D), the cisplatin group (E), and the PPP+ cisplatin group (F) (400×). (G) Quantitative analyses of apoptosis indices. $^{**}P < 0.01$ versus the control group; $^{\#}P < 0.05$ versus the PPP group, $^{\#\#}P < 0.01$ versus the PPP group; $^{\dagger}P < 0.05$ versus the cisplatin group, $^{\dagger\dagger}P < 0.01$ versus the cisplatin group.

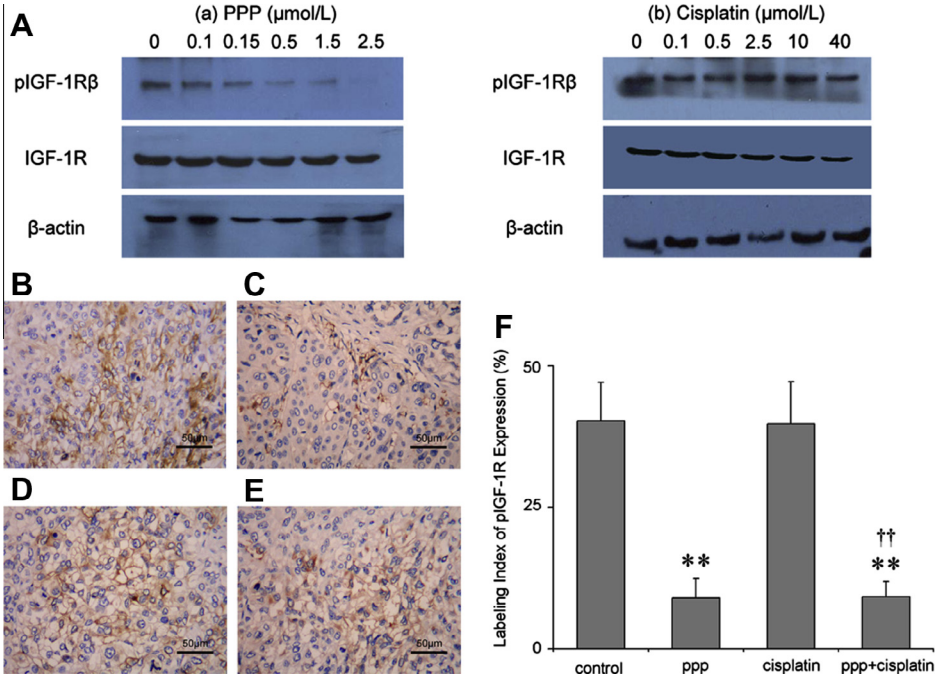

Fig. 3. PPP treatment downregulates IGF-1R phosphorylation in SKOV-3 cells *in vitro* and *in vivo*. The IGF-1R phosphorylation levels in SKOV-3 cells were detected by western blot assay. (A) Representative immunoblots of IGF-1R and pIGF-1Rβ. (A) The cells were incubated in PPP with the concentration of 0, 0.1, 0.25, 0.5, 1.5, and 2.5 μmol/L. (B) The cells were incubated in cisplatin with the concentration of 0, 0.1, 0.5, 2.5, 10, and 40 μmol/L. '0' represents the cells incubated with culture medium only. The IGF-1R phosphorylation levels in SKOV-3 xenografts were detected by immunohistochemistry assay. (B–E) Representative sections of immunohistochemical staining for pIGF-1R of the control group (B), the PPP group (C), the cisplatin group (D), and the PPP+ cisplatin group (E) (400×). (F) Labeling indices of pIGF-1R expression. $^{**}P < 0.01$ versus the control group; $^{\dagger\dagger}P < 0.01$ versus the cisplatin group.

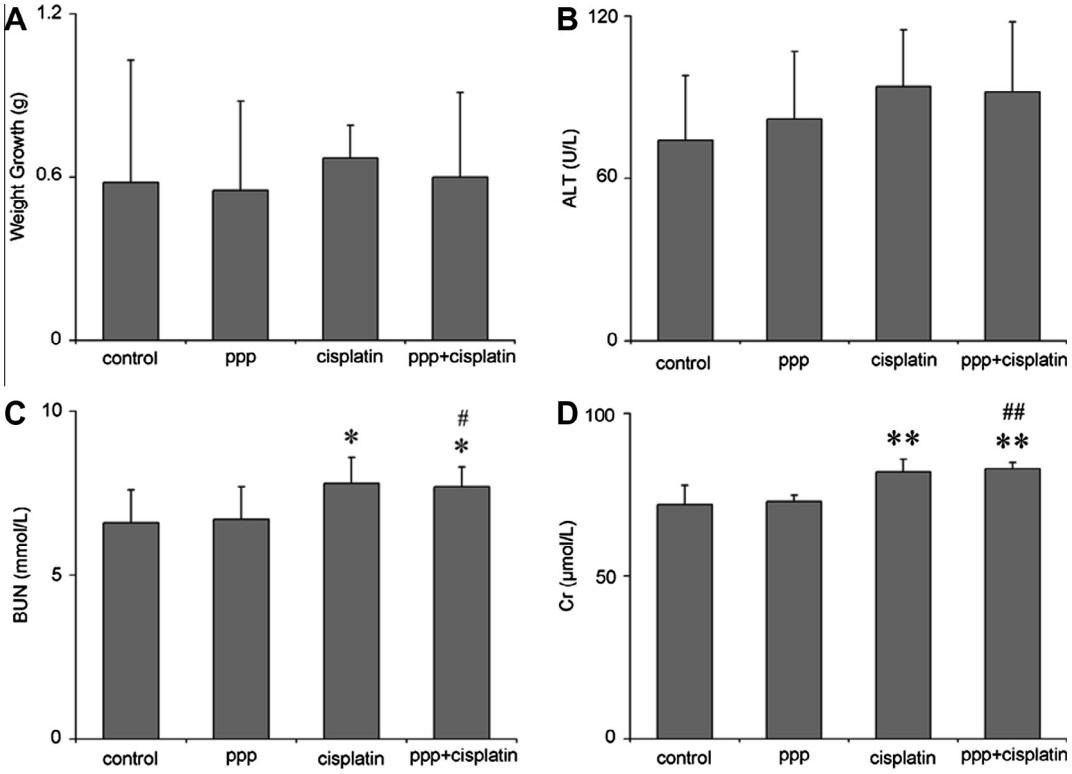

Fig. 4. PPP exhibits low hepatotoxicity and renal toxicity. (A) The weight growth of mice. (B) The ALT levels of mice. (C) The BUN levels of mice. (D) The Cr levels of mice. $^{*}P < 0.05$ versus the control group, $^{**}P < 0.01$ versus the control group; $^{\#}P < 0.05$ versus the PPP group, $^{\#\#}P < 0.01$ versus the PPP group.

for EOC patients with unfavorable responses to first-line cisplatin-based chemotherapy or recurrence has received increasing attention in the gynecological oncology field. IGF-1R, a multifunctional membrane-associated tyrosine kinase, is involved in the regulation of transformation, proliferation, differentiation and apoptosis. Recent studies have implicated IGF-1R in EOC tumorigenesis and progression [1–4]. The anticancer effects of IGF-1R inhibitors have identified IGF-1R as a valuable therapeutic target for EOC [1,9].

PPP, a newly identified IGF-1R inhibitor, has been shown to reduce the viability of human basal-like breast cancer cells *in vitro* and *in vivo* [10]. The anticancer effects of most chemotherapeutics are associated with the induction of apoptosis. PPP inhibited cell growth, induced apoptotic cell death, and downregulated the IGF pathway in primary medulloblastoma cells [11]. Previous studies also reported that PPP treatment leads to apoptosis in myocytes and lung adenocarcinoma cells [12]. Our study showed that PPP inhibited EOC growth by inducing apoptosis *in vitro* and in an *in vivo* xenograft mouse model. PPP treatment at a concentration of 1 μmol/L activated Caspase-3, a marker of early apoptosis in medulloblastoma cells [11]. In the present study, PPP induced both early and late apoptosis, especially the late phase. Together our results indicate that PPP may represent an effective therapeutic agent for EOC.

Although PPP and cisplatin exhibited similar antitumor efficacy, their mechanisms of action were different. We found that PPP decreased pIGF-1R expression in a dose-dependent manner without affecting total IGF-1R expression, indicating that PPP targets IGF-1R activation. Downregulation of pIGF-1R was also observed in the *in vivo* xenograft mouse model. In contrast, pIGF-1R expression was unaffected by cisplatin treatment. Our results are consistent with previous reports demonstrating PPP-mediated reduction in pIGF-1R expression in medulloblastoma [11], melanoma [7], adrenocortical carcinoma [4], and glioblastoma [5]. IGF-1R consists of two α chains and two β chains. The extracellular α-subunits form the ligand-binding domain, whereas the intracellular β-subunits are critical for receptor activation and subsequent initiation of downstream signaling pathways [11]. In the present study, phosphorylation of the β-chain of IGF-1R was significantly decreased in a dose-dependent manner by PPP treatment. Our results are consistent with a previous study that demonstrated the selective inhibition of IGF-1Rβ activity by PPP [13]. The cytoplasmic tyrosine kinase domain of IGF-1R activates the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) and the mitogen-activated protein kinase (MAPK) signaling pathways [14]. These downstream signaling pathways would be primed to accommodate the expression of some related genes, which leads to the development of malignant tumors including ovarian cancer [15,16]. In contrast, downregulation of IGF-1R leads to massive apoptosis of cancer cells [17]. Therefore, the growth inhibitory effects of PPP in EOC may be associated with the downregulation of IGF-1R activation. IGF-1R expression has been confirmed in 100% of the ovarian carcinoma samples by immunohistochemistry [18]. Accumulating data suggest that several IGF-1R kinase inhibitors like BMS molecules and NVP-AEW541 inhibit the growth of various ovarian cancer cell lines, including SKOV-4, OVCAR-3 and OVCAR-4 [9,19,20]. Whether PPP acts on other ovarian cancer cell lines than SKOV-3, and the precise mechanisms underlying PPP-induced growth inhibitory effects in EOC need to be investigated in future studies.

IGF-1R has been shown to enhance resistance to conventional chemotherapeutic drugs [21]. In contrast, IGF-1R inhibitors have been shown to sensitize many cancer cell types to cisplatin [22,23]. Previous studies have demonstrated that PPP exhibits additive, but not necessarily synergistic, effects when administered in combination with cytotoxic chemotherapy [24]. In our study, the PPP and cisplatin combination therapy was more effective in inhibiting EOC growth *in vitro* and *in vivo* than either drug alone, which indicates that PPP may exert additive effects in combination with cisplatin in EOC.

Cisplatin is a first-line antineoplastic agent used in the treatment of many solid tumor types and hematological malignancies [25]. Because of the non-specific targeting of cisplatin, patients commonly suffer adverse effects and toxicities including renal insufficiency, electrolyte disturbance, ototoxicity, myelosuppression, peripheral neuropathy, hepatotoxicity, and retinopathy [26]. PPP inhibits malignant cell growth by downregulating pIGF-1R expression without affecting the highly homologous insulin receptor and other growth factor receptors including fibroblast growth factor receptor, platelet-derived growth factor receptor, and epidermal growth factor receptor. PPP has also been shown to be non-toxic in rodents (LD50 $\geqslant$ 500 mg/kg) [27,28]. In our xenograft mouse model, PPP was well tolerated and had little effect on BUN or Cr levels. In contrast, cisplatin and PPP plus cisplatin treatments increased serum BUN and Cr levels. Although renal function was impaired in the cisplatin and PPP plus cisplatin groups, histological damage was not detected. Lack of histological alterations may be related to the dose and duration of cisplatin treatment and will need to be investigated in future studies. Importantly, BUN and Cr levels were similar in the cisplatin and the PPP plus cisplatin groups. Therefore, PPP may exert additive effects in combination with cisplatin, without increasing cisplatin cytotoxicity. PPP in combination with cisplatin may be useful in treating EOC patients with renal insufficiency and those requiring high-dose cisplatin therapy.

In conclusion, PPP was found to be a highly effective antitumor agent for EOC, with low cytotoxicity. Furthermore, PPP may exert additive effects in combination with cisplatin in EOC. PPP represents a promising therapeutic agent for the treatment of EOC.

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