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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.

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