

Picropodophyllin inhibits the growth of Ewing's sarcoma cells through the insulin-like growth factor-1 receptor/Akt signaling pathway

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Abstract. Ewing's sarcoma (ES) is the second most common type of pediatric bone tumor, and is associated with a poor prognosis. Picropodophyllin (PPP), a novel selective inhibitor of insulin-like growth factor-1 receptor (IGF-1R), is able to strongly inhibit various types of cancers. However, the effect of IGF-1R on ES remains unclear. Following treatment with various concentrations of PPP for various times, cell viability was determined using an MTT assay. In addition, cell proliferation and apoptosis was investigated separately by bromodeoxyuridine staining and flow cytometry, respectively. The PPP-associated signaling pathway was also investigated. The results of the present study suggested that PPP inhibited cell proliferation and viability of A673 and SK-ES-1 human Ewing's sarcoma cells in a dose- and time-dependent manner. In addition, cell apoptosis rates were increased following treatment with PPP. Further investigation of the underlying mechanism revealed that PPP inhibited Akt phosphorylation. Fumonisin B1, an Akt-specific activator, reversed the inhibitory effects of PPP on cell growth. Furthermore, the results suggested that PPP decreased the expression levels of IGF-1R, a common activator of Akt signaling. PPP inhibited the growth of human Ewing's sarcoma cells by targeting the IGF-1R/Akt signaling pathway. Therefore, PPP may prove useful in the development of an effective strategy for the treatment of Ewing's sarcoma.

Introduction

Ewing's sarcoma (ES) is the second most common type of primary bone and soft malignant tumor, often occurs in children and adolescents, and is characterized by a pathognomonic chromosomal translocation known as t(11;22) (q24;q12) or t(21;22) (q22;q12) (1,2). Despite rapid advances in modern biomedical therapy, the 5-year survival rate of patients with ES has only reached 50-60% (3,4). Numerous studies have focused on investigating the underlying mechanism of ES, as well as therapeutic targets for patients with ES; however, the process of ES carcinogenesis remains largely unknown (5-9). Therefore, developing an effective therapeutic strategy for the treatment of ES is critical for young patients.

Insulin-like growth factor 1 receptor (IGF-1R) represents an important therapeutic target in the pathogenesis of ES cells, and is regarded to be an effective biological therapy for ES (10-12). A previous study reported that mutations in IGF-1R may induce apoptosis and inhibit tumorigenesis, as well as enhance chemosensitivity in ES cells (13). Previous studies involving the inhibition of IGF-1R to regulate cell proliferation and apoptosis were also conducted (14,15). Increasing evidence suggests that the IGF-1R inhibitor NVP-AEW541 may possess the ability to enhance cell apoptosis, inhibit proliferation or arrest the cell cycle in ES (16,17). Therefore, identifying more effective inhibitors of IGF-1R may prove to be advantageous in the prevention of ES.

Picropodophyllin (PPP), an epimer of podophyllotoxin, may be a novel selective inhibitor of IGF-1R. PPP strongly inhibits cell growth in various types of cancer, including lymphoma, asopharyngeal carcinoma and colorectal carcinoma (18-21). Although PPP induces apoptosis in cultured IGF-1R-positive tumor cells, the mechanism underlying these effects remains to be elucidated (22). In addition, its effect on ES and the underlying mechanism also remain to be clarified. In the present study, the effects of PPP on the proliferation and apoptosis of ES cell lines was investigated, along with the associated signaling pathway.

Materials and methods

Cells and culture. A673 and SK-ES-1 human ES cell lines were obtained from the American Type Culture Collection

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Abbreviations: ES, Ewing's sarcoma; PPP, picropodophyllin; IGF-1R, insulin-like growth factor I receptor; FB1, fumonisin B1

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(Manassas, VA, USA). The A673 cells (5×10^5) were maintained in RPMI-1640 medium (Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen Life Technologies, Carlsbad, CA, USA), streptomycin (100 mg/ml; Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., Shanghai, China), and penicillin (100 U/ml; Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.) in a 5% (v/v) CO₂ incubator at 37°C. The SK-ES-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies) supplemented with 15% FBS. The cells were also incubated in a humidified atmosphere containing 5% (v/v) CO₂ at 37°C.

Cell viability assay. An MTT assay was used to determine the effects of PPP or fumonisin B1 (FB1) on the viability of the ES cell lines. Briefly, the cells were seeded in 96-well plates (3×10^3 cells/200 μ l) for 24 h. The cells were treated with various concentrations (0.05, 0.1, 0.2, 0.4 and 0.8 μ M) of PPP (cat. no. UNO-000037; 99% pure; UNO, Zhongshan, China) or 50 μ M FB1 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 48 h. MTT solution (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 0.5 mg/ml was subsequently added and the samples were incubated for a further 4 h at 37°C. The medium (RPMI-1640 or DMEM) was then discarded, and 200 μ l dimethyl sulfoxide (Gibco Life Technologies) was added to dissolve the formazan dye crystals for 15 min. Absorbance was finally measured at 570 nm using a microplate reader (Molecular Devices, LLC, Sunny Vale, CA, USA) with a reference wavelength of 630 nm. The results were expressed as a percentage of the MTT reduction, and assumed that the absorbance of the control cells was 100%. Each experiment was performed in triplicate.

Cell proliferation analysis. A bromodeoxyuridine (BrdU) Cell Staining kit (Invitrogen Life Technologies) was used to investigate the effects of PPP on ES cell proliferation, according to the manufacturer's instructions. Briefly, the cells were seeded in 96-well plates (5×10^3 cells/well) for 24 h, and exposed to various concentrations of PPP for a further 48 h. The cells were then fixed with 10 μ l BrdU for 5 h, and the medium (RPMI-1640 or DMEM) was discarded prior to the addition of 100 μ l/well fixing/denaturing solution (Beyotime Institute of Biotechnology, Nantong, China), incubated at room temperature for 15 min. The solution was then removed and 100 μ l/well prepared detection antibody solution (mouse anti-human BrdU monoclonal antibody) was added and incubated for 1 h at room temperature. The plates were then washed three times with phosphate-buffered saline (PBS), followed by the addition of 100 μ l/well horseradish peroxidase (HRP)-conjugated secondary antibody solution, incubated for 30 min at room temperature. The plates were further washed three times with washing buffer, and 100 μ l 3,3',5,5'-tetramethylbenzidine substrate was added, and incubated for 30 min at room temperature. The quantity of BrdU incorporated into the cells was determined at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Apoptosis analysis. The cell lines cultured in RPMI-1640 were seeded in 96-well plates (2×10^4 cells/well), treated with

PPP or FB1 for 24 h, and harvested with trypsin (Gibco Life Technologies). Following two washes with PBS on ice, the cells were incubated with fluorescein isothiocyanate-conjugated Annexin V (Sigma-Aldrich) in binding buffer (50 mM HEPES, 700 mM NaCl, 12.5 mM CaCl₂, pH 7.4) for 20 min at 37°C in the dark. The cells were then washed with PBS, and incubated with 10 ml phosphatidylinositol (PI) solution (1 mg/ml; Sigma-Aldrich) for 20 min at room temperature in the dark. The cells were then analyzed at 488 nm and 633 nm using a FACScan Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA) to determine the relative apoptosis levels.

Western blot analysis. For western blot analysis, the cells were lysed with 2X lysis buffer containing 250 mM Tris-HCl (pH 6.5), 2% SDS, 4% β -mercaptoethanol, 0.02% bromophenol blue and 10% glycerol. Protein concentration was determined using a Bicinchoninic Acid Protein Assay kit (Bio-Rad Laboratories, Inc.) and equal quantities of protein were analyzed by SDS-PAGE (20 mg/lane) on a 5% stacking gel and a 10% separating gel (Beyotime Institute of Biotechnology), prior to being transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA) at 10 V for 30 min. The membranes were blocked for 2 h with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (Beyotime Institute of Biotechnology) (TBST), and incubated at 4°C overnight with the following primary antibodies: Rabbit anti-human Akt (cat. no. 9272; Cell Signaling Technology, Inc., Danvers, MA, USA; 1:1,000), rabbit anti-human phosphorylated (p)-Akt (cat. no. SAB4301497; Sigma-Aldrich; 1:800), rabbit anti-human p-IGF-1R (cat. no. I2033; Sigma-Aldrich; 1:500), rabbit anti-human IGF-1R (cat. no. 3027; Cell Signaling Technology, Inc.; 1:800) and mouse anti-human β -actin (cat. no. 3700; Cell Signaling Technology, Inc.; 1:1,000). Following washing with TBST, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (cat. no. A0545; Sigma-Aldrich; 1:80,000) or HRP-conjugated horse anti-mouse IgG (cat. no. 7076; Cell Signaling Technology, Inc.; 1:20,000) secondary antibodies targeting rabbit or mouse in TBST for 45 min at room temperature. Following three washes with TBST, the proteins were developed using an Enhanced Chemiluminescence kit (GE Healthcare Life Sciences, Chalfont, UK). Detection was performed using an Enhanced Chemiluminescence system (EMD Millipore).

Statistical analysis. The results are expressed as the mean \pm standard deviation of at least three independent experiments. Statistical analysis was conducted using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). The statistical significance of the differences between the control and drug-treated groups were evaluated using an unpaired Student's t-test. $P < 0.05$ was considered to indicate a statistically significant result.

Results

PPP inhibits cell viability in a dose- and time-dependent manner. Cell viability evaluation is one of the most important steps in the quality control process for therapeutic drug use (23). To investigate the effects of PPP on ES, an MTT assay was first used to assess the influence of PPP on the cell growth of

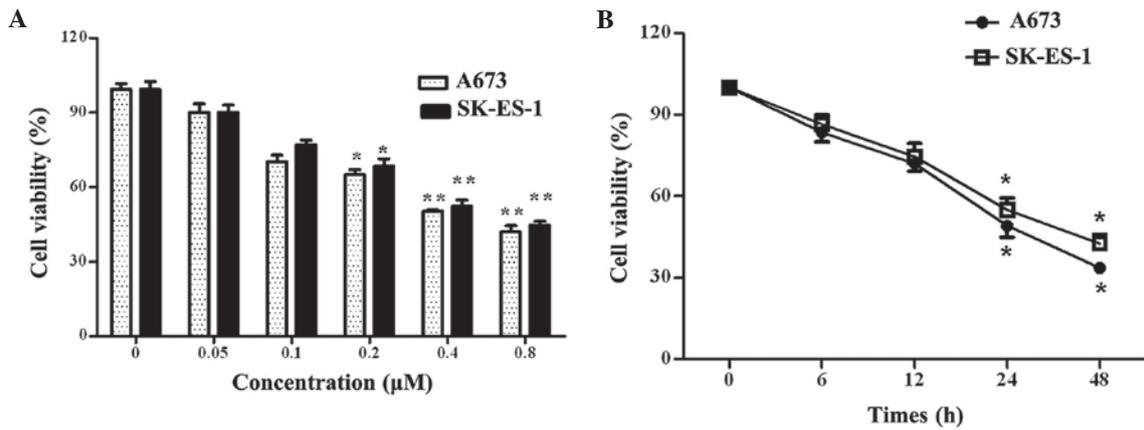


Figure 1. Effects of PPP on Ewing's sarcoma cell viability *in vitro*. (A) A673 and SK-ES-1 cell lines were treated with various concentrations of PPP (0, 0.05, 0.1, 0.2, 0.4 and 0.8 μM). A total of 48 h later, an MTT assay was performed to analyze cell viability. The error bars represent the mean \pm standard deviation. (B) Following treatment with 0.4 μM PPP for 6, 12, 24 or 48 h, cell viability was detected using an MTT assay. * $P < 0.05$ and ** $P < 0.01$, vs. the control group. PPP, picropodophyllin.

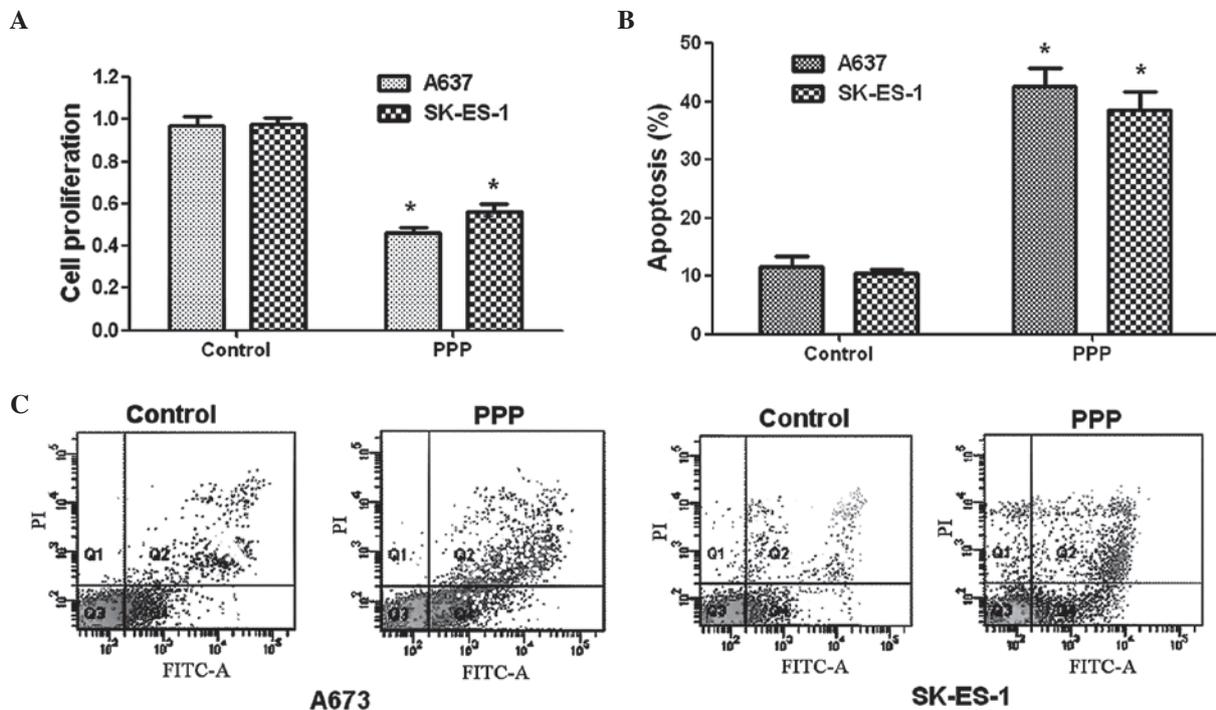


Figure 2. Effects of PPP on cell proliferation and apoptosis. (A) Cell proliferation was determined using a bromodeoxyuridine cell staining kit, and was inhibited by PPP. (B) Cell apoptosis was determined by Annexin V/PI staining, and was induced by PPP. (C) The distribution of apoptotic cells induced by PPP. The data are presented as the mean \pm standard deviation. * $P < 0.05$, vs. the control group. PPP, picropodophyllin; PI, propidium iodide.

the A673 and SK-ES-1 ES cell lines. As shown in Fig. 1A, PPP inhibited A673 and SK-ES-1 cell viability in a dose-dependent manner, and the half maximal inhibitory concentration (IC_{50}) values for the A673 and SK-ES-1 cell lines were 0.42 and 0.48 μM , respectively. Further analysis determined that PPP exhibited time-dependent inhibitory effects on A673 and SK-ES-1 cell viability. As shown in Fig. 1B, the percentage of A673 and SK-ES-1 cell viability declined significantly at 24 h, and the rates of cell viability were 49 and 52%, respectively, as compared with the control ($P < 0.05$). These results suggest that PPP inhibits cell viability in a dose- and time-dependent manner.

Effects of PPP on cell proliferation. To evaluate the effects of PPP on ES cell proliferation, a BrdU cell staining kit was used. As shown in Fig. 2A, PPP was able to inhibit >50% of A673 cells at an value IC_{50} of 0.42 μM . Although the effect of PPP on the SK-ES-1 cells was more marked than that on the A673 cells, cell viability still decreased by 44% in the A673 cells. These results suggest that PPP is able to inhibit ES cell survival.

PPP treatment induces ES cell apoptosis. To further evaluate the effect of PPP on cell apoptosis, Annexin V/PI double staining was performed on the A673 and SK-ES-1 cells. The

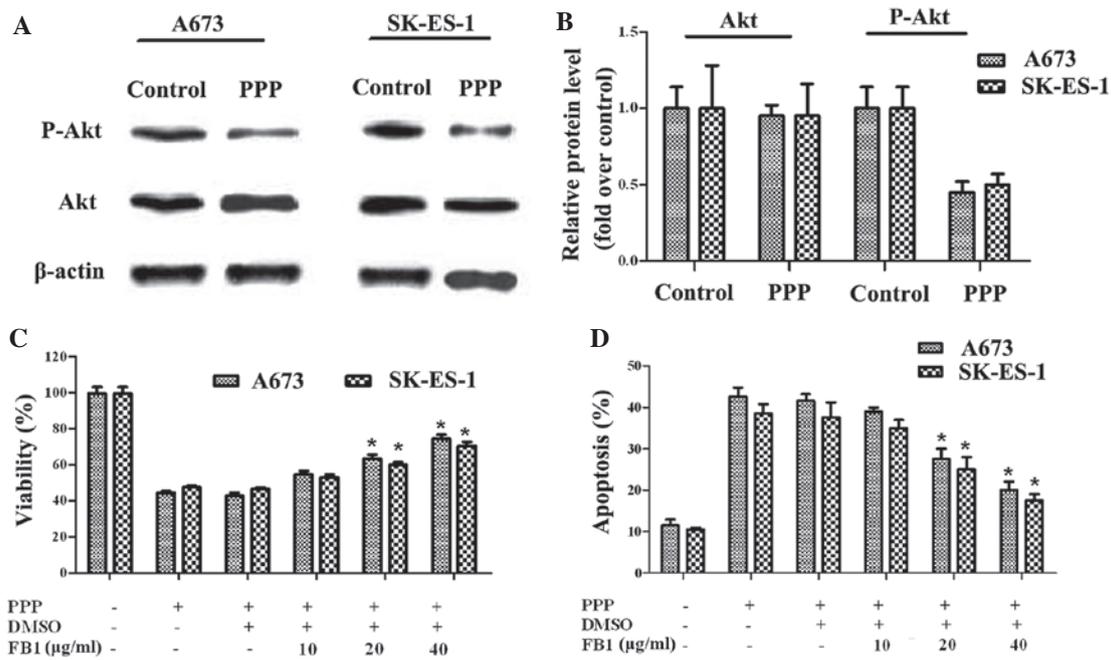


Figure 3. PPP affects Akt phosphorylation in A673 and SK-ES-1 cell lines. (A) The effects of PPP on Akt phosphorylation, as determined by western blotting. (B) The quantification of Akt and p-Akt protein expression levels in A673 and SK-ES-1 cell lines. (C) The effects of FB1 on cell viability, as determined by an MTT assay. (D) The effects of FB1 on PPP-induced apoptosis. * $P < 0.01$, vs. the control and # $P < 0.05$, vs. the PPP-treated group. PPP, picropodophyllin; p, phosphorylated; FB1, fumonisins B1; DMSO, dimethyl sulfoxide.

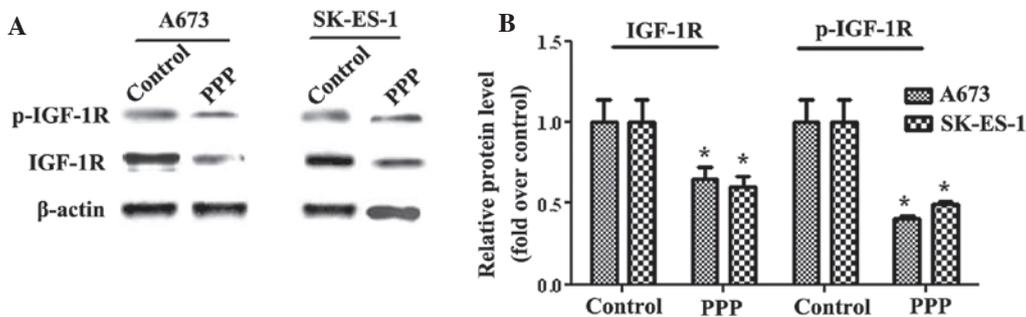


Figure 4. PPP decreased the expression levels of IGF-1R and p-IGF-1R in A673 and SK-ES-1 cell lines. (A) Following treatment with PPP, the expression levels of IGF-1R and p-IGF-1R were measured by western blotting. (B) The relative protein expression levels of IGF-1R and p-IGF-1R in the A673 and SK-ES-1 cell lines. * $P < 0.01$, vs. the control. PPP, picropodophyllin; p, phosphorylated; IGF-1R, insulin-like growth factor 1 receptor.

SK-ES-1 cell apoptotic rates were significantly increased from 10.1 (control) to 38.5% (PPP-treated) (Fig. 2B). The apoptotic rates induced by PPP are shown in Fig. 2C. The apoptotic rates in the A673 cells were significantly increased from 11.2% (control) to 40.8% (PPP-treated) following treatment with 0.42 μ M PPP. These data demonstrated the effect of PPP on cell apoptosis in the A673 and SK-ES-1 cells.

PPP blocks ES cell growth through Akt signaling. The Akt signaling pathway has an important role in cell progression, including proliferation and apoptosis. The activation of Akt improves the survival of ES cell lines (24). To explore the mechanism underlying the effects of PPP on cell growth, the effects of PPP on Akt expression were detected in the A673 and SK-ES-1 cell lines. As shown in Fig. 3A and B, the total Akt levels remained unchanged, whereas the phosphorylation levels of Akt markedly decreased in the two cell lines. Therefore, PPP may inhibit ES cell growth via Akt signaling. To further

verify this hypothesis, FB1, a specific activator of Akt, was used for subsequent study. Following pre-treatment with FB1, the viability of the A673 and SK-ES-1 cells was significantly increased. The viability of the A673 cells increased from 42.8 to 63.5%, following the addition of 20 μ g/ml FB1. In addition, PPP-inhibited cell viability in the SK-ES-1 cells increased from 44 to 53, 60 and 70.5%, respectively, following treatment with various doses of FB1 (Fig. 3C). Conversely, PPP-induced cell apoptosis was decreased from 42.55 to 20% (40 μ g/ml FB1-treated groups) in the A673 cells, following treatment with FB1. Furthermore, apoptotic rates in the SK-ES-1 cells also decreased from 38.55 (PPP-treated) to 17.5% (40 μ g/ml FB1-treated; Fig. 3D). These results suggest that PPP markedly inhibits A673 and SK-ES-1 cell growth by blocking Akt signaling.

Effects of PPP on IGF-1R activation. IGF-1R is over-expressed in various tumors, including breast tumors,

prostate tumors and myeloma (25). In addition, IGF-1R has an important role in the prevention of apoptosis by inducing the Akt signaling transduction cascade (26). To further investigate the PPP-regulated Akt signaling pathway in ES, the expression levels of IGF-1R were analyzed. Total IGF-1R expression levels in the A673 cells decreased by ~35% compared with untreated cells, whereas SK-ES-1 cells exhibited a 40% decrease in IGF-1R expression (Fig. 4). Similarly, the phosphorylation levels of IGF-1R decreased by >50%, as compared with those of the control cells in the two cell lines. The A673 cells exhibited a 59% decrease in the phosphorylation levels of IGF-1R, and the SK-ES-1 cells a 50.5% decrease, as compared with the control group. As an inhibitor of IGF-1R, PPP significantly reduced the expression and phosphorylation levels of IGF-1R. These results suggest that PPP regulates ES cell growth via the IGF-1R/Akt signaling pathway.

Discussion

ES is a relatively rare type of malignancy predominantly occurring between the ages of four and 25 (27,28). The aim of current research is to acquire a greater understanding of the biological pathogenesis of ES, and to identify an effective drug for the treatment of ES (29). Previous studies demonstrated that PPP inhibits numerous types of cancers, including osteosarcoma and human multiple myeloma (30,31); however, no research has been performed to date on ES. To the best of our knowledge, the present study is the first to investigate the function of PPP in ES. The results of the present study demonstrated that PPP induces proliferation inhibition and apoptotic enhancement in human ES cell lines. Therefore, PPP may be effective in the inhibition of ES, and merits further investigation.

Numerous molecular studies have demonstrated that the Akt signal transduction cascade usually participates in ES cell progression, cell apoptosis, cell proliferation and drug susceptibility (32-34). As an IGF-1R inhibitor, PPP was found to have Akt inhibitory effects in neuroblastoma cell lines (35). Furthermore, the efficacy of PPP against multiple myeloma has also been demonstrated (31). To further investigate the mechanism underlying the cell growth inhibitory effects of PPP, the present study investigated the Akt signaling pathway in ES. The results indicated that PPP induced downregulation of p-Akt expression by ~50%. The inhibitory effect of PPP on p-Akt was recovered following treatment with an Akt-specific activator, Fb1. These results demonstrated that PPP was indeed able to inhibit human ES survival by blocking the Akt signaling pathway.

Recently, IGF signaling has become a potential target for novel anticancer agents (36,37). IGF-1R is an activator of the Akt signaling pathway. The IGF-1R-mediated Akt signaling pathway exhibited anticancer effects in various types of cancer (38,39). Baumgarten *et al* (40) suggested that IGF-1R signaling may be required for Akt activation. Previous studies have also demonstrated the association between IGF-1R and Akt (41,42). In the present study, PPP appeared to block IGF-1R phosphorylation. Consequently, it was hypothesized that PPP may inhibit ES growth by inhibiting the IGF-1R-Akt signaling pathway.

In conclusion, the present study demonstrated the effectiveness of PPP in human ES. The inhibition of cell survival, as well as the effective induction of apoptosis, led to further investigation of the underlying mechanism. The IGF-1R/Akt signaling pathway was shown to involve the PPP-induced survival in ES cells. Therefore, the application of PPP may provide a novel therapeutic strategy for the treatment of ES.

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