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Polyphyllin D, a steroidal saponin from *Paris polyphylla*, inhibits endothelial cell functions *in vitro* and angiogenesis in zebrafish embryos *in vivo*

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ABSTRACT

Ethnopharmacological relevance: Angiogenesis, the process of blood vessel formation, is critical to tumour growth. The importance of angiogenesis in tumour development has lead to the development of anti-angiogenic strategies to inhibit tumour growth. In this study, polyphyllin D (PD), an active component in Chinese herb, *Paris polyphylla*, was evaluated for its potential anti-angiogenic effects.
Materials and methods: The inhibitory effects of PD on three important processes involved in angiogenesis, i.e. proliferation, migration and differentiation were examined using human microvascular endothelial cell line HMEC-1 by MTT assay, scratch assay and tube formation assay, respectively. Using zebrafish embryos as an animal model of angiogenesis, the anti-angiogenic effect of PD was further verified *in vivo*.
Results: PD suppressed the growth of HMEC-1 cells at 0.1–0.4 μM without toxic effects. At 0.3 μM and 0.4 μM, PD significantly inhibited endothelial cell migration and capillary tube formation. About 70% of the zebrafish embryos showed defects in intersegmental vessel formation upon treatment with PD at concentrations of 0.156 μM and 0.313 μM.
Conclusion: The anti-angiogenic effects of PD have been explored in the study which implied a potential therapeutic development of PD in cancer treatment.

1. Introduction

Polyphyllin D (PD) is an active component in the *Liliaceae* family plant *Paris polyphylla* which was used as prescribed medicine in China. The rhizome of the plant is known as Chong-lou and was used to treat tumours of liver, digestive tract, leukemia and symptoms such as sore throat and convulsion [Guo et al., 2008; Yan et al., 2009]. One of its major components, PD, is classified as a steroidal saponin. The systemic name of PD is diosgenyl α-L-rhamnopyranosyl-(1 → 2)-[(α-L-arabinofuranosyl-(1 → 4)-β-D-glucopyranoside)] [Deng et al., 1999]. Our previous findings showed that PD exerted anti-proliferative effects on human breast tumour cells (MCF-7 and MDA-MB-231), human hepatocellular carcinoma cells (HepG2) as well as its multi-drug resistant counterpart R-HepG2 through the induction of apoptosis [Cheung et al., 2005; Lee et al., 2005; Ong et al., 2008]. *In vivo* studies also indicated that PD is effective in reducing the tumour size of human breast tumour xenografts in nude mice with no apparent toxicity to the host [Lee et al., 2005].

Anti-apoptotic effects of PD have been demonstrated previously. In this study, the potential anti-angiogenic effect of PD will be examined. Angiogenesis, the process of new blood vessel formation, is important in the normal development of the embryo and fetus as well as in normal physiological processes including wound healing and reproductive functions [Bussolino et al., 1997; Dvorak, 2005]. Angiogenesis also plays a crucial role in the development of cancer. Small solid tumours at the initial stage are not vascularized. New blood vessel formation might favor the transition from hyperplasia to neoplasia which marks the onset of uncontrolled tumour growth [Gordon et al., 2010]. Vascular endothelial growth factor (VEGF), one of the most specific and potent mitogens for endothelial cells, is known to induce neovascularization of tumours. VEGF is over-expressed in tumour cells and it can activate endothelial and, possibly, also tumour cells to migrate and invade adjacent

Abbreviations: PD, polyphyllin D; VEGF, vascular endothelial growth factor; HMEC, human microvascular endothelial cell; ISV, intersegmental vessel.

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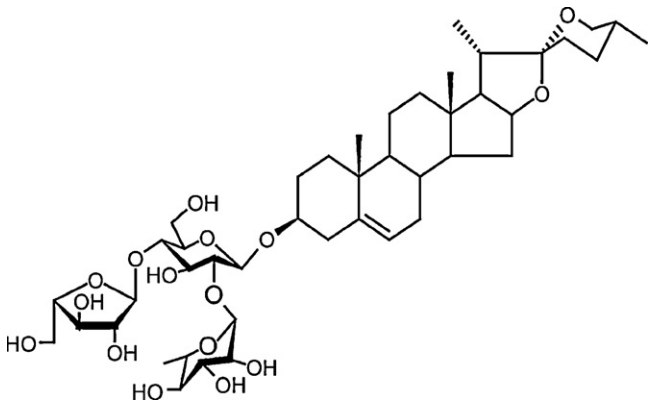

Fig. 1. The chemical structure of PD.

tissue [Murukesh et al., 2010]. Various inhibitors of angiogenesis have been developed to inhibit or slow down the growth of tumours by blocking blood vessel formation, mostly through suppressing VEGF signaling pathways. Bevacizumab (Avastin®) is the first clinically used angiogenesis inhibitor approved by the U.S. Food and Drug Administration (FDA) in 2004 [Shih and Lindley, 2006; Lien and Lowman, 2008]. It is a humanized monoclonal antibody against VEGF which neutralizes and inhibits all active isoforms of VEGF from binding to the VEGF receptors VEGFR-1 (flt-1) and VEGFR-2 (KDR/flk-1). Both receptors are membrane-associated tyrosine kinase receptors responsible for downstream survival and proliferation pathways. Neutralization of VEGF leads to prevention of neovasculature formation and hence limitation of blood supply which might allow a greater capacity for oxygen and chemotherapeutic drugs to reach specific target cells.

In this study, the anti-angiogenic potential of PD was explored, using the human microvascular endothelial cell line HMEC-1 for *in vitro* studies of cell proliferation, migration and tube formation. Moreover, the potential inhibition of angiogenesis by PD was also studied *in vivo* using a zebrafish model.

2. Materials and methods

2.1. Materials

Diosgenyl α-L-rhamnopyranosyl-(1 → 2)-[(α-L-arabinofuranosyl-(1 → 4)-β-D-glucopyranoside)] (PD) was synthesized from diosgenyl-β-D-glucopyranoside as described previously [Li et al., 2001]. The chemical structure of PD is shown in Fig. 1. The method for synthesizing PD was illustrated in our previous publication [Li et al., 2001] in which Nuclear Magnetic Resonance (NMR) was applied to confirm the structure of the final product. PD was dissolved in DMSO at 50 mM as stock solution. Solvent control with 0.0008% DMSO was used in the assays of this study as 0.4 μM of PD was the highest concentration applied. Other chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise specified. Human microvascular endothelial cell line HMEC-1 was purchased from the American Type Culture Collection (Manassas, USA). The cells were maintained in MCDB131 medium supplemented with 10% fetal bovine serum (Invitrogen, CA, USA), 10 ng/mL epidermal growth factor, 1 μg/mL hydrocortisone, penicillin (100 IU/mL) and streptomycin (100 μg/mL) in a humidified incubator with 5% CO_2 at 37 °C.

2.2. Measurement of cell viability by MTT assay

Cell viability was determined by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [Chan et al., 2006]. HMEC-1 cells (1.5×10^4 cells/well) were seeded in 96-well culture plates. After treatment with various concentrations of PD (0.1–0.9 μM) or vehicle (0.0008% DMSO) for 24 h, the medium was replaced by 40 μL of MTT solution (5 mg/mL in PBS). The cells were then incubated for 2 h at 37 °C. Subsequently, MTT solution was removed and 100 μL of DMSO was added. Absorbance was measured at 540 nm using a microplate reader. The percentage of cell viability was calculated against vehicle control.

2.3. Measurement of cell proliferation by direct cell counting

HMEC-1 cells (1.5×10^4 cells/well) were seeded into 24-well plates. After overnight incubation, various concentrations of PD or vehicle were added to the wells. Cells were incubated for 24 h, 48 h or 72 h before harvest by trypsinization. The number of viable cells in each well was determined by trypan blue dye exclusion method.

2.4. Measurement of DNA synthesis

HMEC-1 cells (5×10^3 cells/well) were seeded into 96-well culture plates and synchronized with 1% FBS for 24 h [Noguer et al., 2009]. After treatment with various concentrations of PD or vehicle for 24 h, 0.5 μCi of ^{3}H-thymidine in PBS was added per well and the cells were incubated at 37 °C for 6 h. Then, DNA was harvested on microfilters with a cell harvester (Beckman Coulter, Brea, USA). The amount of DNA synthesized was determined by measuring the radioactivity of the filter using a microplate scintillation counter (Beckman Coulter, Brea, USA).

2.5. Cell cycle analysis

HMEC-1 cells (3×10^5 cells/well) were seeded into 6-well culture dishes. After synchronization, the cells were treated with various concentrations of PD or vehicle for 24 h. Then, the cells were harvested and fixed in 70% ethanol. Before performing flow cytometry, ethanol was removed and the cells were incubated with RNase (8 μg/mL) and PI (10 μg/mL) for 30 min. Cell cycle distribution was then detected using a flow cytometer (BD FACSCanto, BD BioSciences, CA, USA), and the results were analyzed using ModfitLT version 3.0 software.

2.6. Wound healing assay

Wound healing assay, also known as scratch assay, was carried out as described previously [Gebäck et al., 2009]. HMEC-1 cells were grown to full confluence in the wells of 24-well plates. Then, two crosses on cell cultures were scratched with a pipette tip and the wells were washed with PBS to remove detached cells. Cells were then incubated with various concentrations of PD or vehicle for 15 h. Photographs were taken before and after PD or vehicle treatment at 40× magnification by an inverted microscope (Nikon Eclipse TS100). Data set was analyzed with the TScratch software using the default parameter settings [Gebäck et al., 2009]. Four replicates were done in each individual experiment and the presented images are representatives of triplicate experiments with similar outcome.

2.7. Tube formation assay

Each well of 48-well culture plates was coated with 150 μL of Matrigel and allowed to solidify. HMEC-1 cells (5×10^4 cells/well) were then seeded into the wells with the addition of various concentrations of PD or vehicle, and incubated for 24 h. The network of