Deoxypodophyllotoxin; The Cytotoxic and Antiangiogenic Component from *Pulsatilla koreana*

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Abstract

The petroleum ether fraction of *Pulsatilla koreana* (Ranunculaceae) was found to have an inhibitory effect on the tube-like formation of human umbilical venous endothelial (HUVE) cells and strong cytotoxic activity against five tumor cell lines. The active component isolated from the fraction was deoxypodophyllotoxin (DPT). The cytotoxic activity against the tumor cells comprising the A549, SK-OV-3, SK-MEL-2, HCT15, and B16F10 cell lines, expressed as ED_{50}, ranged from 6 to 18 ng/ml. 3 ng/ml of DPT, a concentration considerably below the cytotoxic concentration, completely inhibited the tube-like formation of HUVE cells. Furthermore, DPT exhibited an inhibition ratio of 60% on BDF1 mice bearing Lewis lung carcinoma cells. The inhibitory effect on the tube-like formation was suggested to play an important role in antitumor activity.

Pulsatilla koreana Nakai. (Ranuculaceae) is one of the most important herbs in traditional medicine in Korea and has been used for the treatment of amoebic dysentery and malaria [1]. It had been reported that plants from the *Pulsatilla* genus contain ranunculin, anemonin, protoanemonin, triterpenes, and saponins [2], [3], [4]. Among them, protoanemonine and anemonine exhibited mitotoxic activity [5]; ranunculin showed cytotoxic activity against KB cells by inhibiting DNA polymerase [6].

In the course of screening for products showing an inhibitory effect on tube-like formation of human umbilical venous endothelial (HUVE) cells, it was found that the petroleum ether extract of *P. koreana* was reasonably effective. In this study, the active component was isolated from *P. koreana* root and we evaluated its inhibitory effect on tube-like formation of HUVE cells and its cytotoxic activity against various cancer cell lines. Furthermore, the antitumor effect on mice bearing Lewis lung carcinoma cells (LLC) was also evaluated. The relationship between the antitumor activity and the inhibitory effect on tube-like formation of HUVE cells is discussed.

Repeated column chromatography of the petroleum ether extract of *P. koreana* on silica gel followed by preparative HPLC, combined with the bioassay, resulted in the isolation of two sub-

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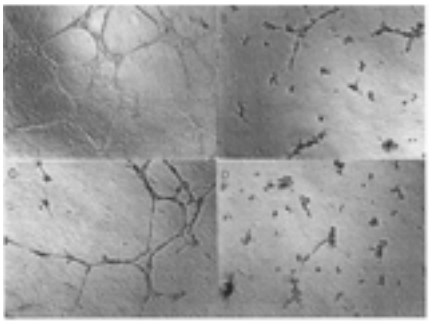
stances. Compounds **1** and **2** were identified as deoxypodophyllotoxin (DPT) and pulsatillic acid, respectively by comparing their spectral data (HR-mass, UV, ^{1}H-, and ^{13}C-NMR) with the reported data [7], [8]. DPT has already been isolated from *Bursera microphylla* [12], *Anthriscus sylvestris* [13], *Juniperus silicicola* [14], *Hernandia ovigera* [15]. It is chemotaxonomically interesting that DPT was first isolated from a Ranuculaceen plant (*Pulsatilla koreana*). Recently, 5-hydroxydeoxypodophyllotoxin (β-peltatin) was isolated from the root of *P. chinensis* [16].

Cytotoxic testing of DPT toward 5 kinds of tumor cell lines including A549, SK-OV-3, SK-MEL-2, HCT15, and B16F10 was carried out with the results summarized in Table **1**. DPT showed potent cytotoxic activity against all of the cell lines (ED_{50}, 6 – 18 ng/ml). Among the human cancer cell lines, A549, a human lung cancer cell line, and HCT15, a human colon cancer cell line, were found to be more sensitive to DPT. Pulsatillic acid exhibited moderate activity (3.2 – 4.8 μg/ml) and was not studied further. The inhibitory effect of DPT on the tube-like formation of HUVE cells was measured (Fig. **1**). DPT inhibited the tube-like formation of HUVE cells at non-cytotoxic concentrations (1 – 3 ng/ml). In comparing the cytotoxic activity (the average ED_{50} values, 12 ng/ml) with the inhibitory activity on the HUVE cells (ED_{100}, 3.0 ng/ml), DPT might be 8 times more potent against to HUVE cells than to the cancer cell lines.

The antitumor assay was performed using BDF1 mice bearing Lewis lung carcinoma (LLC). As shown in Table **2**, DPT was intraperitoneally injected once a day with a daily dose of 20 mg/kg for 14 consecutive days. The tumor volume inhibition ratio was 60%, 58% and 59%, measured on 17th, 19th, and 21st day, respectively. Fig. **2** shows that the antitumor activity of DPT remained unchanged during the period of observation. This effect is remarkable considering that the potency of other anticancer agents generally decrease with time like etoposide does here. Contrary to our results on the solid tumor, Terada and coworkers [17] reported that DPT showed no significant antitumor activity on the life span of CDF1 mice bearing L1210 cells, an ascitic murine leukemia cell line. This means that DPT is more active on a solid tumor than an ascitic one.

Considering the dominance of the antiangiogenic activity over the cytotoxicity and the preferred antitumor activity on solid tumors, it was suggested that inhibition of angiogenesis might be one important antitumor mechanism of DPT against the LLC model. The consistency of the inhibition ratio mentioned above might be also due to the antiangiogenic effect on the antitumor behavior.



A; (-) Control
B; (+) Control (Suramin, 100 μM)
C; DPT (1 ng/ml), 50 % inhibited
D; DPT (3 ng/ml), 100 % inhibited

Fig. **1** Inhibitory effect of deoxypodophyllotoxin on tube-like formation of human umberical venous endotherial (HUVE) cells.

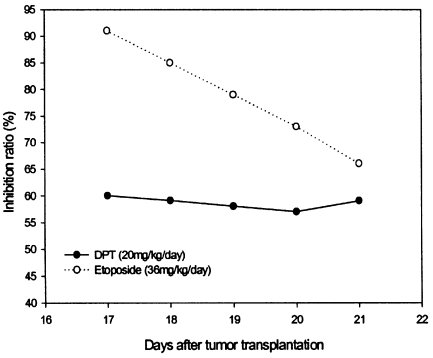


Fig. **2** Time course of antitumor activity of deoxypodophyllotoxin and etoposide.

Materials and Methods

The root of *P. koreana* Nakai was collected in April, 2000 at Keryong mountain near Taejon city, Korea. This plant was identified by Professor Ki-Hwan Bae in the College of Pharmacy, Chungnam National University, where the voucher specimen (CNUP-0349) of the plant was deposited.

The powdered root (1 kg) was refluxed with methanol (3 L × 3) for 24 h. The methanol extract was evaporated to yield a light brown residue (98 g), which was suspended in water. The suspension was partitioned into petroleum ether, ether, butanol successively to yield 10.3 g in the petroleum ether fraction, 21.9 g in the ether fraction, and 35 g in the butanol fraction. The petroleum ether fraction (10.3 g), which exhibited bioactivity was chromatographed over silica gel (400 g, 230 – 400 mesh, Merck) with cyclohexane-EtOAc step gradient (cyclohexane/EtOAc, 50 : 1 ; 40 : 1 ; 30 : 1 ; 20 : 1 ; 10 : 1 ; 8 : 1; 5 : 1 ; 4 : 1 ; 3 : 1 ;

Table **1** Cytotoxicity of active compounds (DPT, pulsatillic acid) from *Pulsatilla koreana*

Compounds	*ED_{50} (ng/ml)*[a]				
	A549	*SK-MEL-2*	*SK-OV-3*	*HCT15*	*B16F10*
DPT	6	13.6	18	8.9	6.8
PA[b]	3200	4800	N.T.[c]	N.T.	N.T.
Etoposide	480	760	N.T.	N.T.	N.T.

[a] ED_{50} value was defined as the concentration of compounds needed to reduce a 50% of absorbance relative to the vehicle-treated controls.
[b] Pulsatillic acid.
[c] Not tested.

Table **2** *In vitro* antitumor activity of deoxypodophyllotoxin (DPT) on BDF1 mice bearing LLC tumor

Treatments	*Tumor Volume (Inhibition ratio, %)*[c]			
	17 day[a]	*18 day*	*19 day*	*21 day*
Vehicle	2050 ± 580[b] (0)	2445 ± 655 (0)	2895 ± 700 (0)	4195 ± 1005 (0)
DPT	820 ± 370 (60)	1005 ± 125 (59)	1205 ± 455 (58)	1720 ± 510 (59)
Etoposide	185 ± 55 (91)	380 ± 135 (84)	595 ± 160 (79)	1380 ± 445 (67)

[a] Days after tumor transplantation.

[b] Mean (mm^3) ± S.D. (mm^3).

[c] The antitumor activity was determined as inhibition ratio (%) in comparison with untreated control group as described in materials and method. DPT (20 mg/kg/day) and etoposide (36 mg/kg/day) as a positive control were administrated *i.p.*

2 : 1 ; 1 L of each solvent) to give 6 fractions. The eluents were detected by TLC (cyclohexane/EtOAc, 5 : 1; silica gel, 0.25 mm, Merck). The chromatogram was sprayed with 10% H_2SO_4, followed by heating. The fifth fraction (1.5 g), which exhibited the activity, was then rechromatographed over silica gel (500 g) with cyclohexane-EtOAc (cyclohexane/EtOAc, 5 : 1 ; 4 : 1 ; 3 : 1 ; 2 : 1 ; 1 : 1 ; 100 mL of each solvent) to yield four subfractions. Finally, compound **1** (18 mg); colorless amorphous crystal, $[\alpha]_D^{25}$ -113.4° (*c* 0.05, $CHCl_3$), m. p. 166 – 167.8 °C, and compound **2** (25 mg); white powder, m. p. 214.7 – 217.1 °C, were isolated from the third subfraction by semi-preparative HPLC (ODS2 C18, 10 × 250 mm, CH_3CN : H_2O = 55 : 45, 1.5 mL/min, t_R = 18 min and t_R = 25 min, respectively) with UV detection at 230 nm. Compounds **1** and **2** were identified as deoxypodophyllotoxin (DPT) and pulsatillic acid, respectively, and their chemical structure was established by physical and spectroscopic methods (m. p., IR, mass, NMR spectra) comparing with the data reported previously [7], [8].

The cell lines were maintained as a mono-layer in RPMI1640 media supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY), sodium bicarbonate, penicillin G, and streptomycin. The HUVE cells were maintained as a mono-layer in M199 media supplemented with glutamine, sodium bicarbonate, penicillin G, streptomycin, 3 ng/mL basic fibroblast growth factor (bFGF) (GIBCO, Grand Island, NY), 17.5 unit/mL heparin, and 20% fetal bovine serum (GIBCO, Grand Island, NY) at 37 °C under a humidified atmosphere of 5% CO_2.

Cytotoxicity was measured by the sulforhodamine B (SRB) method [9]. Viable cells were seeded in the growth medium (180 μL) into 96 well microtiter plates (3 – 4 × 10^4 cells per each well) and allowed to attach overnight. The test sample (DPT or pulsatillic acid) was dissolved in DMSO and adjusted for the final sample concentrations ranging from 0.3 ng/ml to 10 μg/ml by diluting with the growth medium. Each sample was prepared in triplicate. The final DMSO concentration was adjusted to < 0.1%. After 72 h incubation, the medium was removed and the remaining cells were fixed using 10% trichloroacetic acid (TCA) for 1 h at 4 °C. The TCA-treated cells were washed extensively with water and dried in air. Subsequently, 50 μL of SRB solution (0.4% in acetic acid) were added to each well at room temperature. After standing for 1 h, the wells were washed 3 – 4 times with 1% acetic acid and dried in air. The bound dye was dissolved in Tris base (100 μL of 10 mM). The absorbance of the Tris solution was measured using a micro-plate reader at 520 nm. The ED_{50} value was defined as the concentration of DPT needed to reduce a 50% of absorbance relative to the vehicle-treated controls.

The tube-like formation assay was carried out according to the method reported by Yasumasa et al. [10]. Matrigel (Biomedical Research Products, Bedford, MA, USA) was thawed on ice to prevent premature polymerization. 70 μL aliquots were then placed into individual wells of the 96 well plates and allowed to polymerize at 37 °C for at least 30 min. The cells were removed from the confluent cultures by treatment with trypsin-EDTA (GIBCO, Grand Island, NY). They were then washed with serum-containing medium, and resuspended at 1.1 × 10^5 cells/ml in the HUVEC culture medium. Into each culture well, 20 μL of medium 199 with or without DPT was added. Finally, 180 μL of the cell suspension was added. After incubation for 16 h at 37 °C, in a 5% CO_2 atmosphere, four different phase-contrast microscopic fields (× 100) per well were photographed. The total length of the tube structure was measured using Adobe Photoshop TM® software. The total capability length per well was given as the average value from four fields.

The antitumor activity was measured according to Teruhiro's method (11). 2 × 2 × 2 mm^3 tumor fragments of Lewis lung carcinoma (LLC) were transplanted *s. c.* into the auxillary region of the BDF1 mice. 24 h after tumor transplantation, DPT (20 mg/kg/day) was intraperitoneally injected once a day for 14 consecutive days. The tumor volume (TV) was measured on 17, 18, 19 and 21st day, and calculated according to the following formula:

$$TV = \frac{L\,(mm) \times W^2\,(mm^2)}{2}$$

where L and W represents the length and the width of the tumor mass, respectively.

The inhibition ratio of tumor volume (IRTV) was calculated according to the following formula:

$$IRTV\,(\%) = \frac{\text{Mean TV of control group} - \text{Mean TV of treated group}}{\text{Mean TV of control group}} \times 100.$$

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