Arch Pharm Res Vol 25, No 5, 640-642, 2002

Archives of Pharmacal Research
http://apr.psk.or.kr

Antiangiogenic Activity of *Bupleurum longiradiatum* on human umbilical venous endothelial cells

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(Received April 20, 2002)

The ethyl acetate fraction of *Bupleurum longiradiatum* was found to have an inhibitory effect on the tube-like formation of human umbilical venous endothelial (HUVE) cells. The active compounds, isolated from the fraction, were identified as acetylbupleurotoxin (**P1**) and bupleurotoxin (**P2**). The compounds **P1** and **P2** completely inhibited the tube-like formation of HUVE cells at 30 μg/ml, below the cytotoxic concentration. But, they did not exhibit antitumor activity on BDF1 mice bearing Lewis lung carcinoma cells despite their antiangiogenic activity.

Key words: Angiogenesis, *Bupleurum longiradiatum*, Acetylbupleurotoxin, Bupleurotoxin

INTRODUCTION

Recently, we reported that some Korean and Vietnamese plants showed inhibitory effects on tube-like formation of human umbilical venous endothelial (HUVE) cells (Bae *et al.*, 2001: Nam *et. al.*, in press). Among them, it was found that the methanol extracts of *Aristolochia manshuriensis, Ephedra sinica, Adonis amurensis*, and *Bupleurum longiradiatum* showed the potent inhibitory effects. Their active principles were found to be aristolochic acid from *A. manshuriensis* (unpublished data), alkaloid fraction from *E. sinica* (Nam *et al.*, 2002), and cymarilic acid from *A. amurensis* (You *et al.*, in press).

In this paper we report the isolation, identification, and antiangiogenic activity of two polyacetylenic compounds from the root of *Bupleurum longiradiatum*, one of the plants which showed the potent inhibitory effect. In addition, the antitumor activity of the isolates on BDF1 mice bearing Lewis lung carcinoma cells would be evaluated.

MATERIALS AND METHODS

Plant material

The root of *B. longiradiatum* was collected at Mt. Deokyou, Muju, Jeonbuk, Korea on August, 2001. This plant was identified by one of the authors (K.H. Bea). The voucher specimen (CNUP-0049) is deposited in the herbarium of College of Pharmacy, Chungnam National University.

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Extraction and isolation

The air-dried root (250 g) was refluxed with methanol (3 L × 3) for 6 h. The methanol extract was concentrated to yield a light brown residue (25 g), which was suspended in water. The suspension was extracted by hexane, ethyl acetate, successively, to yield 11.5 g of the hexane fraction, 4 g of the ethyl acetate fraction. The ethyl acetate fraction (4 g), which exhibited bioactivity, was chromatographed over silica gel (100 g, 70-230 mesh, Merck) with hexane-ethyl acetate (3:1) to give 5 fractions. The elutes were monitored by TLC (hexane:ethyl acetate, 3:1; silica gel, 0.25 mm, Merck). The chromatogram was sprayed with 10% H_2SO_4, followed by heating. The second (43 mg) and the fourth fraction (55 mg), which also exhibited the activity, were rechromatographed over silica gel (80 g) with hexane-ethyl acetate (cyclohexane:ethyl acetate, 5:1 → 1:1) to give **P1** (18 mg, colorless oil), and **P2** (25 mg, colorless oil). Compounds **P1**, **P2** were purified by semi-preparative HPLC (ODS2 C_{18}, 10 × 250 mm, CH_3OH:H_2O = 65:45, 1.5 mL/min, t_R = 75 min and t_R = 62 min, respectively) with UV detection at 230 nm.

^{1}H-NMR of P1: 6.69 (1H, dd, J = 15.5, 10.8 Hz), 6.23 (1H, dd, J = 10.9, 6.42 Hz), 6.12 (1H, dd, J = 14.9, 11.0 Hz), 5.88-5.83 (1H, m), 5.68 (1H, d, J = 10.9 Hz), 5.58(1H, d, J = 15.4 Hz), 4.92-4.87 (1H, m), 4.43 (2H, d, J = 6.4 Hz), 2.17-2.12 (2H, m), 2.03 (3H, s), 1.69-1.62 (2H, m), 1.57-

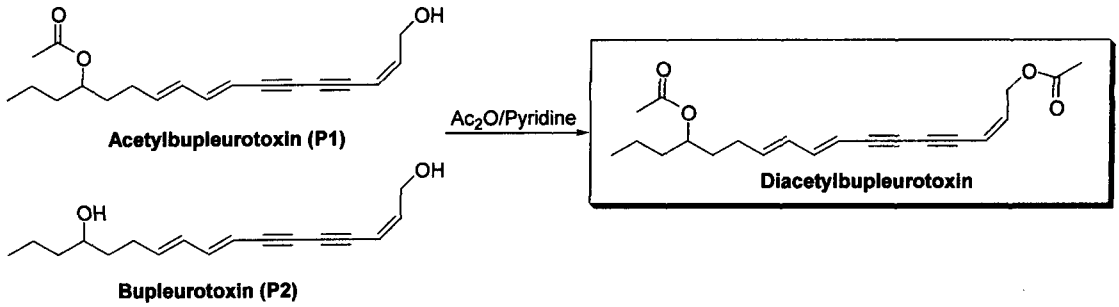

Fig. 1. Acetylation of P1 and P2 gave diacetylbupleurotoxin

1.44 (2H, m), 1.37-1.27 (2H, m), 0.91 (3H, t, J = 7.3 Hz)

Acetylation of P2: To a solution of **P2** (20 mg) in acetic anhydride (1.5 mL) was added pyridine (1.2 mL), and then the solution was stirred for 2 h at ambient temperature. The reaction mixture was poured into ice water (100 mL), and extracted with ethyl acetate (100 mL). The organic layer was washed with water (100 mL × 3), brine (100 mL × 3), dried over anhydrous sodium sulfate, and concentrated *in vacuo* to give crude product. Silica gel column chromatography (hexane:EA = 6:1) of it gave pure compound (15 mg, 80% yield) as a colorless oil.

^{1}H-NMR: 6.70 (1H, dd, J = 15.4, 11.2 Hz), 6.16-6.09 (2H, m), 5.87 (1H, dd, J = 13.8, 6.8 Hz), 5.76 (1H, dd, J = 9.8, 1.60 Hz), 5.58 (1H, d, J = 15.6 Hz), 4.92-4.83 (2H, m), 2.25-2.12 (2H, m), 2.09 (3H, s), 2.03 (3H, s), 1.68-1.62 (2H, m), 1.53-1.46 (2H, m), 1.36-1.28 (2H, m), 0.91 (3H, t, J = 7.2 Hz).

Acetylation of P1: Target compound was synthesized as the same method described above. The acetylation product was purified as above to give 13 mg as a colorless oil (yield: 88%) and showed the same ^{1}H-NMR pattern as that of the acetylation product of **P2**.

Tube-like formation activity

The tube-like formation assay was measured as described previously (Kim *et al.*, 2002). The total length of the tube structure was measured using Adobe Photoshop TM[6] software. The total capability length per well was given as the average value from four fields.

Antitumor activity

The antitumor activity was measured as described previously (Kim *et al.*, 2002). Bupleurotoxin (20, 40, 60, 80, 100 mg/kg/day) was intraperitoneally injected on the 1st and 8th day.

RESULTS AND DISCUSSION

The compounds **P1** and **P2** were identified as acetylbupleurotoxin and bupleurotoxin, respectively, by spectroscopic methods employing ^{1}H, ^{13}C NMR, UV, and IR spectrometry, in comparison with published data (Zhao *et al.*, 1985; 1987). In every step of purification of **P2**, the major peak was accompanied with a small amount of an unknown compound which was eluted earlier than **P2.**

Table 1. Inhibitory effect of subfractions from ethyl acetate fraction of the root of *B. longiradiatum* on tube-like formation of HUVECs.

Sample[a]	Inhibition[b]	Sample	Inhibition
BE1	+	BE4	+++
BE2	+++	BE5	+
BE3	+	EA fr.[c]	+++

[a]Final concentration (50 μg/mL), [b]Inhibitory effect: +++ (> 90%), ++ (70-89%), + (50-69%), - (< 50%). [c]Ethyl acetate fraction of the root of *B. longiradiatum*.

Table 2. Inhibitory effect of **P1** and **P2** on tube-like formation of HUVECs.

Sample	Conc. (μg/mL)	Inhibition[a]	Sample	Conc. (μg/mL)	Inhibition
P1	50	+++	P2	50	+++
	30	+++		30	+++
	10	+		10	+

[a]Inhibitory effect: +++ (> 90%), ++ (70-89%), + (50-69%), - (< 50%)

Table 3. *In vivo* antitumor activity of bupleurotoxin, active component from the root of *B. longiradiatum* on BDF1 mice bearing LLC tumors.

Sample	Schedule[a]	Dose (mg/kg/day)	Inhibition ratio (%)[c]
Bupleurotoxin	q7d × 2[a] (day 1 and 8)	20	N.A.[d]
	q7d × 2	40	N.A.
	q7d × 2	60	25.3
	q7d × 2	80	Toxic[e]
	q7d × 2	100	Toxic
Etoposide	q4d × 3[b] (day 1, 5, and 9)	36	71.2

[a]q7d × 2: Sample was administered on the 1st and 8th day. [b]q7d × 2: Sample was administered on the 1st, 5th, and 9th day. [c]Inhibition ratio was determined as described previously (Kim *et al.*, 2002). [d]N.A.; not active. [e]Toxic: All mice tested died .

After peracetylation of **P1** and **P2**, their NMR-data were compared. Acetylation of **P1** afforded diacetylbupleurotoxin which was identical with peracetylation product of **P2** (Fig. 1). Presense of two hydroxy groups seemed to make **P2** unstable.

Inhibitory effect of *B. longiradiatum* on the tube-like formation of HUVECs

The methanol extract of the aerial part did not show the inhibitory activity, while the methanol extract of the root exhibited a remarkable inhibitory effect on the tube-like formation of HUVECs. The methanol extract of the root was partitioned into hexane, ethyl acetate and water fractions. The ethyl acetate fraction showed the strongest effect was chormatographed over a silica gel column to give five fractions (BE1-BE5). Among them, BE2 and BE4, the most active fractions (Table 1) were purified by HPLC to afford the active compounds **P1** and **P2**. They showed inhibitory effects on tube-like formation of HUVECs at a non cytotoxic concentration (30 μg/mL) as shown in Table 2.

Although, it was reported previously that the methanol extract of the root of *B. longiradiatum* exhibited the inhibitory effect on monoamince oxidase (Kim *et al.*, 1998), and two polyacetylenic compounds from the root showed a strong toxicity in mice (Zhao *et al.* 1985, 1987), this is the first report on polyacetylenes showing an inhibitory effect on angiogenesis.

In vivo antitumor activity

The antitumor assay was performed using BDF1 mice bearing Lewis lung carcinoma cells. Bupuleurotoxin (**P2**) was intraperitoneally injected daily with a dose of 20, 40, 60, 80, or 100 mg/kg on the 1st and 8th day, but **P2** was found to show a negligible antitumor activity (IR, 25.3%) at a dose of 60 mg/kg in spite of its antiangiogenic activity. Moreover, at higher doses (80, or 100 mg/kg), all mice tested died, indicating that bupuleurotoxin was too toxic to be able to be used as an antitumor agent.

For use as anticancer agents, chemical manipulation of their structures would be required.

ACKNOWLEDGEMENTS

This study was supported by a grant of Korea Research Foundation (1999-042-F6104).

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