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Falcarindiol, a Polyacetylenic Compound Isolated from *Peucedanum japonicum*, Inhibits Mammalian DNA Topoisomerase I

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Abstract A methanol extract of the root of *Peucedanum japonicum*, used as a medicinal herb, showed an inhibitory effect on mammalian topoisomerase I activity. The methanol extract was suspended in ethyl acetate, and a topoisomerase I inhibitor in the organic soluble fraction was then isolated by silica gel and thin layer chromatography. The topoisomerase I inhibitory compound was identified as falcarindiol based on the analysis of EI-MS, ^{1}H and ^{13}C NMR spectroscopy. This inhibitor showed cytotoxicity against human leukemia Jurkat T and HL60 cells with an IC_{50} value of 7 μg/ml. These results suggest the possibility of falcarindiol as a new anticancer agent which can be expected to have a synergistic effect on other anticancer drugs. In addition, the present data show that falcarindiol has antifungal, yet not antibacterial, activity.

Key words: Falcarindiol, DNA topoisomerase I inhibitor, *Peucedanum japonicum*, cytotoxicity, antifungal activity

DNA topoisomerases are nuclear enzymes that alter the DNA topology required for replication, transcription, recombination, and segregation of daughter chromosomes [4, 22-24]. Eukaryotic cells have two types of topoisomerases, topoisomerase I and II. Topoisomerase I catalyzes the passage of the DNA strand through a transient single-strand break in the absence of any high energy cofactor, while topoisomerase II catalyzes the passage of DNA double strands through a transient double-strand break in the presence of ATP.

In recent years, topoisomerase I has been considered as an attractive target for antitumor agents [11, 20]. The expression of topoisomerase I is enhanced in several types of leukemia, lymphoma, and colon carcinoma cells [2, 18]. Topoisomerase I-targeted drugs such as plant alkaloid camptothecin (CPT) and its derivatives, including topotecan, 9-amino-CPT, and CPT-11, are used in cancer chemotherapy [5, 21]. CPT inhibits topoisomerase I by stimulating the cleavable complex formation between DNA and the enzyme [6, 17]. Recently, some compounds including β-lapachone, bulgarein, cryptotanshinone, and diospyrin have also been reported to be topoisomerase I inhibitors [3, 9, 10, 19]. These results have prompted us to screen topoisomerase I inhibitors in medicinal herbs. This paper reports on the isolation of falcarindiol as a novel inhibitor of eukaryotic topoisomerase I from the root of *P. japonicum*, which has been used as a medicinal drug for wind phobia, evil wind, gout, and spasms [15].

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Materials and Methods

Plant Materials and Enzymes

Medicinal plants used in this study were obtained from a medicinal market in Taegu City. Calf thymus topoisomerase I and human topoisomerase II were purchased from Takara Shuzo (Japan) and TopoGen (U.S.A.), respectively.

General Procedure

For the analysis of the structure, the electron impact mass spectrum (EI-MS) was obtained using a micromass Quattro II spectrometer (England). The nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DMX600 (600 MHz, Germany). The NMR spectra were measured in $CDCl_3$ using tetramethylsilane (TMS) as an internal reference.

Topoisomerase Assays

The topoisomerase I activity was monitored by DNA relaxation assay. The relaxation assay was carried out in 20 μl of the reaction mixture containing 35 mM Tris-HCl (pH 8.0), 72 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 5 mM spermidine, 0.01% bovine serum albumin, supercoiled

pGBT9 (0.4 μg), and topoisomerase I (1 unit). One unit of topoisomerase I activity was defined as the minimum amount of the enzyme required for complete relaxation of 0.4 μg of supercoiled DNA. In some experiments, the reaction mixtures were supplemented with falcarindiol or CPT [1 μl in dimethysulfoxide (DMSO)]. After incubation at 37°C for 15 min, the reactions were terminated by 5 μl of a stop buffer containing 5% SDS, 50 mM EDTA, 20% Ficoll, 0.1 mg/ml bromophenol blue, and 0.1 mg/ml of xylene cyanol, and then the DNA samples were electrophoresed in 0.7% agarose gel. The gels were stained with ethidium bromide (5 μg/ml) and photographed. The DNA relaxation activity of topoisomerase II was assayed as described above, except that ATP (1 mM) and topoisomerase II were added to the reaction mixture.

Purification of Topoisomerase I Inhibitor

The methanol extracts of 50 medicinal plants were screened for topoisomerase I inhibitor using a DNA relaxation assay, and *P. japonicum* was selected for the purification of the topoisomerase I inhibitor. To isolate the topoisomerase I inhibitor from *P. japonicum*, the powdered dried root (4 kg) of *P. japonicum* was stirred with methanol (20 l) overnight at room temperature and the supernatant was concentrated under reduced pressure to produce a methanol (MeOH) extract. The MeOH extract was suspended in ethyl acetate (EtOAc, 500 ml) and separated into a supernatant and pellet. Subsequently, the soluble fraction with the topoisomerase I inhibitory activity was concentrated under reduced pressure and then applied to a silica gel column (Merck, 5.8 × 67 cm). The materials absorbed on the resin were eluted by 5-stepwise gradient solvent ($CHCl_3$:MeOH= 40:1, 35:1, 30:1, 25:1, 20:1, v/v). The active fractions were collected and re-chromatographed on a silica gel column (Merck, 3×70 cm) using hexane-EtOAc (3:1, v/v) as a mobile phase. The fractions having topoisomerase I inhibitory activity were pooled and further separated on a preparative TLC plate (Merck) using hexane-EtOAc-acetic acid (30:20:1) as the developing solvent. The silica containing the inhibitor was scraped off the plate, and the inhibitor was extracted with EtOAc (Fig. 1). After filtration and concentration, the purified compound was dissolved in DMSO and stored at -20°C.

Peucedanum japonicum (4 kg)
↓
Methanol extraction (20 l)
↓ Discard pellet
↓ Concentrate
EtOAc extraction (500 ml)
↓ Discard pellet
↓ Concentrate
Silica gel column chromatography
↓ Stepwise elution with $CHCl_3$-MeOH (40:1, 35:1, 30:1, 25:1, 20:1, v/v)
Silica gel column chromatography
↓ Elution with hexane-ethyl acetate (3:1, v/v)
Preparative TLC
↓ Develop with hexane-ethyl acetate-acetic acid (30:20:1, v/v/v)
↓ Extract with ethyl acetate
Falcarindiol (200 mg)

Fig. 1. Purification scheme for inhibitor of topoisomerase I from *Peucedanum japonicum*.

Cytotoxicity Assay

A cytotoxicity assay was carried out according to Pauweis *et al.* [16]. Human leukemia Jurkat T cells and HL60 cells were maintained in RPMI 1640 medium (Sigma) with 10% heat-inactivated fetal calf serum, 20 mM HEPES (pH 7.0), 100 μg/ml gentamycin, and 5×10^{-5} M β-mercaptoethanol. The growing cells were plated in a 96-well plate at 5×10^4 cells per well with 100 μl RPMI 1640. Subsequently, a serial dilution of falcarindiol ranging in concentration from 0.7 to 12 μg/ml was added. The cells were incubated for 44 h at 37°C in an atmosphere containing 5% (v/v) CO_2, then 50 μl of an MTT solution (1.1 mg/ml, Sigma) was added to the wells for 4 h. The cells were harvested by centrifugation and dissolved in 150 μl DMSO. The optical density at 540 nm was measured using an ELISA reader (Bio-Rad).

Antimicrobial Activity Assay

E. coli was grown in an LB broth (1% tryptone, 0.5% yeast extract, 1% NaCl) at 37°C and *S. cerevisiae* in a YPAD medium (1% yeast extract, 2% peptone, 0.004% adenine sulfate, 2% glucose) at 30°C. The antibacterial and antifungal activities of falcarindiol were tested using a growth inhibition assay. A diluent of falcarindiol was added to the broth media containing fresh inoculum, and the *E. coli* and *S. cerevisiae* were then cultured while shaking for 24 h. The initial absorbance of *E. coli* and *S. cerevisiae* was 0.04 and 0.05 at 600 nm, respectively. The growth inhibition was determined by measuring the turbidity of the medium at 600 nm.

RESULTS AND DISCUSSION

Purification and Structure Determination of Topoisomerase I Inhibitor

A topoisomerase I inhibitor was purified from the root of *P. japonicum*, as described in Materials and Methods (Fig. 1). The purity of the compound was confirmed by developing on a normal phase TLC plate using two different solvent systems (hexane-EtOAc-acetic acid=30: 20:1, $CHCl_3$-MeOH= 20:1). The active principle was obtained as a colorless oil.

Table 1. NMR data for isolated compound.[a]

No	^{13}C (multiplicity)	^{1}H (multiplicity, *J* in Hz)
1	117.37 (*t*)	5.28 (*ddd*, 1.0, 1.5, 10.5)
		5.50 (*ddd*, 1.0, 1.5, 17.5)
2	135.80 (*d*)	5.94 (*d*, 8.8)
3	63.52 (*d*)	4.95 (*brd*, 5.5)
4	78.24 (*s*)	-
5	70.32 (*s*)	-
6	68.72 (*s*)	-
7	79.87 (*s*)	-
8	58.63 (*d*)	5.21 (*brd*, 8.0)
9	127.66 (*d*)	5.52 (*ddt*, 1.5, 8.0, 10.5)
10	134.73 (*d*)	5.62 (*ddt*, 1.0, 7.5, 10.5)
11	27.71 (*t*)	2.11 (*dt*, 1.5, 7.5)
12	29.22 (*t*)[b]	1.39 (*brt*, 7.5)
13	29.12 (*t*)[b]	1.28 (*m*)
14	29.17 (*t*)[b]	1.28 (*m*)
15	31.80 (*t*)	1.28 (*m*)
16	22.65 (*t*)	1.28 (*m*)
17	14.10 (*q*)	0.88 (*brt*, 7.0)

[a]δ in ppm, $CDCl_3$.
[b]Assignments could be exchanged.

The ultraviolet (UV) spectrum of the purified compound showed absorption maxima at about 232, 245, 257, 266, and 283 nm. The molecular ion peak $[M]^+$ was found at *m/z* 260 in EI-MS. The fragment ion at *m/z* 242 indicated the presence of a hydroxyl group. From a DEPT (distortionless enhancement by polarization transfer) analysis, one methyl, seven methylene, five methine, and four quaternary carbons were detected. Based on a combination of the DEPT with the mass data, the molecular formula was determined to be $C_{17}H_{24}O_2$. The methylene resonances originating from the hydrocarbon moiety appeared at δ 22.65-31.80 ppm in ^{13}C NMR. In addition, four characteristic *sp* carbon signals at δ 68.72-79.87 ppm, four sp^2 carbons at δ 117.37-135.80, and two oxygenated carbons at δ 63.52 and 58.63 ppm were found (Table 1). An HMQC (heteronuclear multiple quantum correlation) analysis revealed the presence of a terminal alkene carbon at δ 117.37 ppm. These data strongly suggested that the compound was a derivative of polyunsaturated fatty alcohol containing double and triple bonds in its structure. Based on the coupling patterns in the ^{1}H NMR and a careful analysis of the ^{1}H -^{1}H COSY spectrum, the connectivity of C1-C2-C3 and C8-C9-C10-C11 were established. Considering these results and the HMBC (heteronuclear multiple bond connectivity), the inhibitor was tentatively identified as falcarindiol, which is one of the representative polyacetylene compounds commonly found in Umbelliferae plants (Fig. 2). The chemical structure of the inhibitor was finally verified by a comparison with the spectral data of previous studies [8, 13].

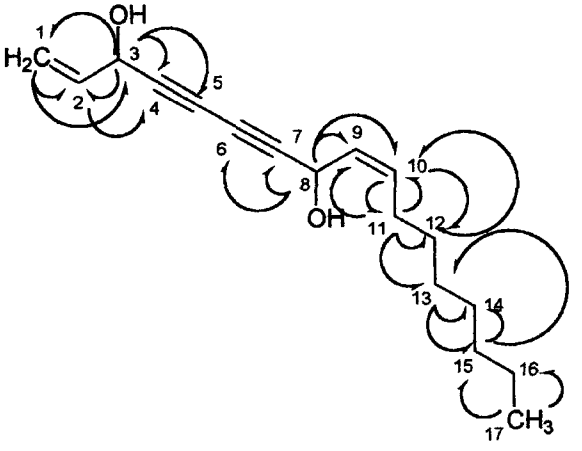

Fig. 2. Structure and summarized HMBC data of falcarindiol isolated from *P. japonicum*. Arrows indicate correlations between ^{1}H and ^{13}C.

Inhibition of Mammalian DNA Topoisomerase I Activity by Falcarindiol

The inhibition of topoisomerase I activity by falcarindiol was studied using the DNA relaxation assay as described in Materials and Methods (Fig. 3). Falcarindiol inhibited the topoisomerase I activity in a dose-dependent fashion. Little topoisomerase I inhibitory activity of falcarindiol was observed at a concentration of 10 μg/ml, however, it was clear that falcarindiol inhibited the topoisomerase I activity at concentrations above 30 μg/ml. To obtain quantitative data, the amount of relaxed DNA was measured by scanning the photograph with an image analyzer (B. I. System). The IC_{50} value of falcarindiol was approximately 150 μg/ml. Under these conditions, the topoisomerase I activity was inhibited by CPT with an IC_{50} of about 1 μg/ml (data not shown), in agreement with previous report [6]. To investigate whether mammalian DNA topoisomerase II was a target of falcarindiol, human topoisomerase IIα was used in an *in vitro*

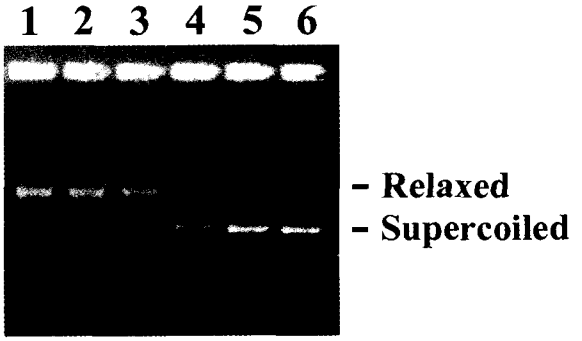

Fig. 3. Effect of falcarindiol on calf thymus topoisomerase I activity.
Supercoiled DNA (0.4 μg) was incubated with 1 unit of topoisomerase I in the presence of falcarindiol (lanes 2-5) and then analyzed by agarose gel electrophoresis. The control reactions contained an equivalent volume of DMSO in the presence (lane 1) or absence (lane 6) of the enzyme. Lane 2, 10 μg/ml; lane 3, 30 μg/ml; lane 4, 90 μg/ml; lane 5, 270 μg/ml.

DNA relaxation assay. Falcarindiol failed to inhibit the DNA relaxation activity of topoisomerase II at a concentration of 270 μg/ml (data not shown). The restriction enzyme *Eco*RI was not inhibited by falcarindiol (data not shown). These results indicate that the inhibition of topoisomerase I activity by falcarindiol is relatively specific.

Cytotoxicity of Falcarindiol Against Human Leukemia Cells

Since a topoisomerase I inhibitor can be used as an anticancer drug [5, 11, 20, 21], the cytotoxic effect of falcarindiol on human cancer cells was investigated. The cytotoxicity of falcarindiol was determined by an MTT assay using human leukemia Jurkat T cells, as described in Materials and Methods. Figure 4 shows the effect of falcarindiol on the growth of Jurkat T cells. The falcarindiol-induced inhibition of cell growth appeared to be concentration-dependent in the cell lines tested. About 40% of the cells were killed in 6 μg/ml after 44 h. The IC_{50} value against Jurkat T cells was estimated at 7 μg/ml. The cytotoxicity of falcarindiol was also tested using HL60 human promyelocytic leukemia cells, and a similar IC_{50} value was obtained (Fig. 4).

Antifungal Activity of Falcarindiol Against *S. cerevisiae*

The antimicrobial activity of falcarindiol against *E. coli* and *S. cerevisiae* was investigated. As shown in Fig. 5, *S. cerevisiae* was sensitive to the cytotoxicity of falcarindiol at a concentration of 6.3 μg/ml, with the IC_{50} value of 9 μg/ml. However, the antibacterial activity of falcarindiol against *E. coli* could not be detected even at a high concentration of 25 μg/ml. Because yeast DNA topoisomerase I is very similar to its mammalian counterparts, these results

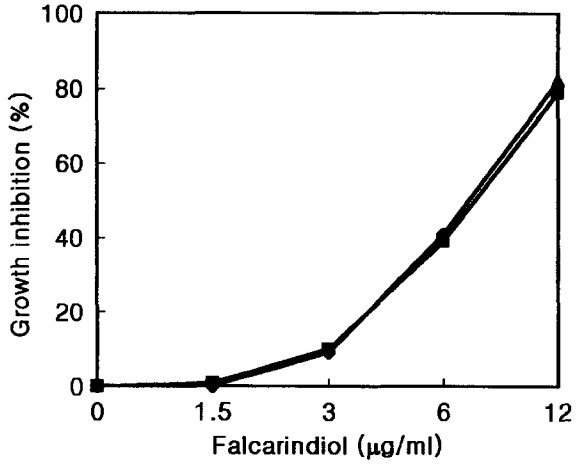

Fig. 4. Cytotoxicity of falcarindiol against human leukemia cells. Human leukemia Jurkat T (◆) and HL60 (■) cells were incubated with various concentrations of falcarindiol for 44 h. The cytotoxicity was measured by a standard MTT assay as described in Materials and Methods. The percentage cell growth inhibition=(1-A/B) × 100 (A: OD_{540} of the tumor cells in a medium with falcarindiol; B: OD_{540} of the tumor cells in a medium without falcarindiol).

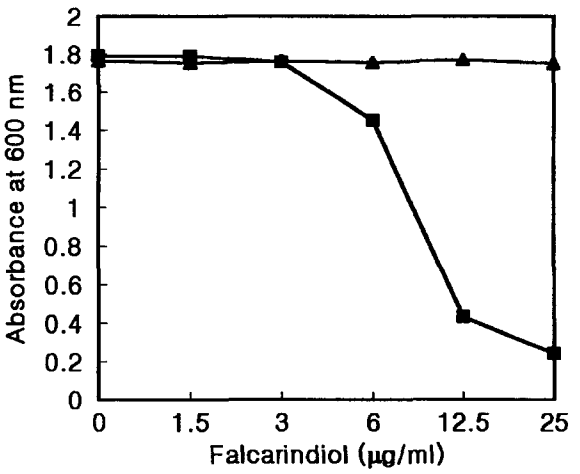

Fig. 5. Antifungal activity of falcarindiol against *S. cerevisiae*. *E. coli* (▲) and *S. cerevisiae* (■) were cultured in the broth media (LB broth for *E. coli*; YPAD medium for *S. cerevisiae*) with a diluent of falcarindiol for 24 h at 37°C and 30°C, respectively. The growth inhibition was determined by measuring the turbidity of the medium at 600 nm.

seem to indicate that the antifungal activity of falcarindiol is due to topoisomerase I inhibition in yeast cells.

This study demonstrated that falcarindiol, a polyacetylene compound isolated from *P. japonicum*, inhibits topoisomerase I activity and tumor cell growth. Falcarindiol has been isolated from *Anthriscus sylvestris* [7], *Heracleum moellendorffii* [14], *Angelica furcijuga* [12], *Dendropanax arboreus* [1], and *P. praeruptorum* [13]. However, no reports on the purification of falcarindiol from *P. japonicum* have previously appeared. Falcarindiol has already been identified as an antiproliferative compound [1, 7, 14], antimutagenic compound [13], or nitric oxide production inhibitory compound [12]. However, this paper reports an another inhibitory effect of falcarindiol on topoisomerase I. In addition, the present results suggest that the cytotoxicity of falcarindiol is due to its inhibitory effect on topoisomerase I in the cells.

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