Antifibrotic Activity of Diterpenes from *Biota orientalis* Leaves on Hepatic Stellate Cells

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Antifibrotic effect of twelve diterpenes (1-12) from the 90% methanolic fraction of *Biota orientalis* leaves was evaluated employing HSC-T6 cells by assessing cell proliferation and morphological change. Among these diterpenes, totarol (8) and isopimara-8(14),15-dien-19-oic acid (9) dramatically reduced cell proliferation in dose- and time-dependent manner. Furthermore, treatment with these compounds resulted in the different pattern of morphological changes of HSC-T6 cells. Taken together, antiproliferative activity of diterpenes from *B. orientalis* might suggest therapeutic potentials against liver fibrosis.

**Key words:** Diterpene, *Biota orientalis*, HSC-T6, Hepatic stellate cells, Hepatic fibrosis

**INTRODUCTION**

Hepatic fibrosis occurs as the consequences of a sustained wound healing response to chronic liver injury from a variety of causes including viral, autoimmune, drug induced, cholestatic and metabolic diseases (Friedman, 2003). During liver fibrosis, excessive extracellular matrix (ECM) is produced, which leads to liver dysfunction and irreversible cirrhosis. Hepatic stellate cells (HSCs) play important functions in normal liver, such as retinoid storage, remodeling of ECM and production of growth factors and cytokines. However, in response to liver damage, HSCs undergo a process called "activation", which is associated with liver pathogenesis including hepatic fibrosis (Li and Friedman, 1999). Therefore, suppression of HSC activation has been proposed as therapeutic target against hepatic fibrosis (Sakata *et al.*, 2004).

In the course of screening of antifibrotic activity of natural products, the methanolic extract of the leaves of *Biota orientalis* inhibited significantly cell proliferation. The leaves of *Biota orientalis* (L.) Endl. (Cupressaceae) have been used as a hemostatic, expectorant and hypotensor in Korean folk medicine (Chung and Shin, 1990). A number of terpenes and flavonoids were reported as chemical constituents of this tree (Zhu *et al.*, 2004; Koo *et al.*, 2002; Sung *et al.*, 1998; Yang *et al.*, 1995). In addition, several diterpenes have been reported to exert the neuroprotective activity, platelet activating factor receptor binding inhibitory activity and anticancer activity (Kosuge *et al.*, 1985; Han *et al.*, 1998; Koo *et al.*, 2002). In the present study, we further attempted to investigate the antifibrotic constituents of *B. orientalis* using an immortalized rat hepatic stellate HSC-T6 cell line as an *in vitro* assay system.

**MATERIALS AND METHODS**

**Plant material**

The leaves of *B. orientalis* were purchased from Kyungdong Oriental Herbal Market, Seoul, Korea, 2004 and identified by Dr. Jong Hee Park, a professor of the College of Pharmacy, Pusan National University. A voucher specimen (SNU-0008) has been deposited in the Herbarium of the Medicinal Plant Garden, Seoul National University.

**Extraction and isolation**

Dried plant material (24 kg) was extracted six times with 80% MeOH in an ultrasonic apparatus which, upon removal of the solvent *in vacuo*, yielded a methanolic extract (3.2...
kg). This methanolic extract was then suspended in distilled water and partitioned successively with CH$_2$Cl$_2$. The CH$_2$Cl$_2$ layer was suspended in 90% MeOH and then partitioned with n-hexane. Silica gel column chromatography of 90% MeOH fraction (1.03 kg) was carried out using a mixture of n-hexane-EOAc-MeOH with increasing polarity (n-hexane-EOAc, 10:1→100:0 and EOAc-MeOH, 100:0 →0:100) and yielded twelve subfractions (fr.1 – fr.12). Compound 1 was obtained from fr.1 in crystallized form. Following column chromatography of fr.2 on the Sephadex LH-20 (MeOH-H$_2$O, 9:1) and C18 resin (MeOH-H$_2$O, 9:1) yielded compound 11. Column chromatography of fr.4 over C18 resin (MeOH-H$_2$O, 7:3→10:0) resulted in 6 fractions (fr.4-1 → fr.4-6). Among the six subfractions of fr.4, fr.4-2 yielded compound 5 by additional purification step on the Sephadex LH-20 column chromatography (MeOH-H$_2$O, 9:1). Compound 7 was crystallized from fr.4-3 and compound 8 was isolated from the same fraction by column chromatography using C18 resin (MeOH-H$_2$O, 6:4→10:0) and Sephadex LH-20 (MeOH-H$_2$O, 9:1). Compounds 6, 9, 11 and 12 were isolated from fr.5 and 6 on the Sephadex LH-20 column chromatography (MeOH-H$_2$O, 9:1). Column chromatography of fr.7 on silica gel (n-hexane-EOAc, 10:1→0:100) yielded three subfractions Compounds 2, 3 and 4 were obtained from fr.7-2 by column chromatography over silica gel with a solvent gradient of CHCl$_3$-MeOH (5:1→0:100).

**Culture of HSC-T6 hepatic stellate cells**

An immortalized rat hepatocellular cell line, HSC-T6 was kindly provided by Prof. S. L. Friedman (Columbia University, New York). HSC-T6 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/mL penicillin and 100 µg/mL streptomycin at 37°C in a humidified atmosphere of 95% air-5% CO$_2$.

Compounds were dissolved in dimethylsulfoxide (DMSO). Our preliminary study showed that DMSO at a final concentration of 0.1% in media did not affect the cell viability. HSC-T6 cells were treated with vehicle or compounds for 48 h or as indicated.

**Measurement of inhibitory activity of cell proliferation**

Cell proliferation was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). Inhibitory activity of compounds on cell proliferation (% of control) was calculated as 100 × (Absorbance of compound-treated/Absorbance of control).

**Measurement of LDH release**

Lactate dehydrogenase (LDH) release from cells was measured to assess cytotoxicity or necrosis (Anechabala et al., 1999). LDH release (%) was calculated as 100 × (Absorbance in medium/Absorbance in both cell culture supernatant and medium).

**Observation of cellular morphology**

HSC-T6 cells were incubated with vehicle or compounds for 48 h. Cells were observed with phase contrast microscope.

**Statistical analysis**

The evaluation of statistical significance was determined by the Student’s t-test with a value of p<0.05 or less considered to be statistically significant.

**RESULTS**

**Isolation of diterpenes from B. orientalis**

We previously isolated several diterpenes from 90% MeOH fraction of B. orientalis leaves (Sung et al., 1998; Koo et al., 2002). Further investigation of the 90% MeOH fraction of B. orientalis resulted in the isolation of twelve diterpenes by repetitive column chromatography and recrystallization. The structures of these diterpenes were identified by comparing spectroscopic data with previously reported information as pinusolid (1), pinusolidic acid (2), 15-hydroxy-8(17),13-labdadien-19-oic acid (isocupressic acid) (3), trans-communic acid (4), 15-methoxypinusolidic acid (5), 8(17),13,16-labdatriene-19-oic acid (daniellic acid) (6), cedrol (7), totarol (8), isopimara-8(14),15-dien-19-oic acid (9), isopimara-7,15-dien-18-oic acid (isopimaric acid) (10), isopimara-7,15-dien-3β-ol (11) and isopimara-7,15-dien-3β,18-diol (12) (Fig. 1) (Yang et al., 1995; Yang and Han, 1998; Sung et al., 1998; Koo et al., 2002; Fang et al., 1989; Haueuser et al., 1961; Sy and Brown, 1998; Bruno et al., 1986).

**Effects of diterpenes on cell proliferation in HSC-T6 cells**

We have attempted to elucidate the antifibrotic activity of these diterpenes in HSC-T6 cells by assessing the effect on cell proliferation using MTT assay. Among the compounds tested, 8 and 9 dramatically inhibited cell proliferation at a concentration of 100 µM for 48 h incubation (9.6 and 38.4% of control, respectively) (Fig. 2). Other diterpenes, however, showed weak activity. Our present study also showed that 8 and 9 decreased the HSC proliferation in dose- and time-dependent manner (Figs. 3A and 3B). In addition, 8 significantly increased LDH release in the culture medium, whereas 9 did not affect LDH release significantly (Fig. 3C).

**Effects of 8 and 9 on cellular morphology in HSC-T6 cells**

Since 8 and 9 exerted potent inhibitory activity, we further
observed the cell morphology under a phase contrast microscope. As shown in Figs. 4A and 4D, HSC-T6 cells cultured in the absence of compounds exhibited flattened and membranous processes, representing myofibroblastic morphology. However, the morphology of HSC-T6 cells was changed by the treatment with 8 or 9. The morphology

Fig. 1. Diterpenes isolated from the leaves of *B. orientalis*

Fig. 2. Effect of diterpenes isolated from *B. orientalis* on cell proliferation in HSC-T6 cells. HSC-T6 cells were incubated with diterpenes at the concentration of 100 µM for 48 h. Cell proliferation was measured by the MTT assay. Results are expressed as the mean ± S.D. of three independent experiments, each performed using triplicate wells. *p<0.05, **p<0.01, ***p<0.001 compared with control.
Antifibrotic Activity of Diterpenes on Hepatic Stellate Cells

Fig. 3. Concentration- and time-dependent effect of 8 and 9 on cell proliferation and cytotoxicity in HSC-T6 cells. HSC-T6 cells were incubated with 8 or 9 at the concentrations ranging from 1 to 100 µM for 48 h (A), or 100 µM for indicated time (B). Cell proliferation was measured by the MTT assay. (C) Cytotoxicity or necrosis was measured by LDH release. Results are expressed as the mean ± S.D. of three independent experiments, each performed using triplicate wells. *p<0.05, **p<0.01, ***p<0.001 compared with control.

Fig. 4. Effects of 8 and 9 on cellular morphology in HSC-T6 cells. HSC-T6 cells were incubated with vehicle ((A) and (D)), 8 ((B) and (C)); 10 and 100 µM, respectively) and 9 ((E) and (F); 10 and 100 µM, respectively) for 48 h. Cells were observed with phase contrast microscope (original magnification ×100).

of HSC-T6 cells treated with 9 was changed to slender cell shape at the concentrations ranging from 10 to 100 µM (Figs. 4E and 4F). Interestingly, 8 exerted differential effect on cell morphology as the concentration increased. As shown in Fig. 4B, HSC-T6 cells treated with 10 µM of 8 showed slender cell shape, similar to 9. However, HSC-
T6 cells treated with 100 µM of 8 showed the morphological feature of necrosis such as membrane breakdown and lysis (Fig. 4C).

**DISCUSSION**

HSCs are considered to play a key role in the pathogenesis of liver fibrosis. During liver fibrogenesis, HSCs are activated and acquire a myofibroblast-like phenotype which is accompanied by increased proliferation and ECM synthesis (Sario et al., 2002). HSCs activation in vivo can be induced by many factors such as cytokines and soluble factors derived from damaged hepatocytes and Kupffer cells. HSCs activation can be also induced in various conditions in vitro. Culturing HSCs on uncoated plastic plates is known to cause spontaneous activation, leading to myoblastic phenotype, mimicking the process seen in vivo. In addition, HSCs activation can be further promoted by serum and cytokines, such as TGF-β1, PDGF and TNF-α, ROS and other factors (Chen and Zhang, 2003; Wu and Zern, 2000). In the present study, we employed HSC-T6 hepatic stellate cell line for the assessment of antifibrotic activity. HSC-T6 cell line is immortalized rat hepatic stellate cells and has been known to retain all features of activated stellate cells, including expression of desmin, α-smooth muscle actin, and glial fibrillary acidic protein, and it can esterify retinol into retinyl esters (Vogel et al., 2000). Thus, we evaluated antifibrotic activity employing HSC-T6 cells by assessing the inhibitory activity of cell proliferation using MTT assay and observing cell morphology.

In the present study, we isolated twelve diterpenes from *B. orientalis* leaves and demonstrated the antifibrotic effects of these diterpenes using HSC-T6 cells. Our present study showed that two diterpenes, 8 and 9 from *B. orientalis*, exerted significant inhibitory activity of HSC proliferation (Fig. 3A and 3B). Generally, elimination of HSC can be achieved by various pathways, such as inhibition of cell proliferation and/or induction of cell death. As shown in Fig. 4, the morphology observed under microscope suggested that the antifibrotic activities of these two compounds are somewhat different, which is also supported by LDH release (Fig. 3C) and Trypan Blue exclusion test (data not shown). Compound 8 reduced cell proliferation in part by necrosis as demonstrated by cell morphology as well as increase of LDH release in the culture medium. In consistent with our results, 8 has been reported to exert the antibacterial activity via perturbation of membrane structure (Micol et al., 2001; Evan et al., 2000). Interestingly, however, the antifibrotic activity of 9 may not due to direct toxic effect, since the treatment of HSC with compound 9 was associated neither with increased LDH release in the culture medium (Fig. 3C) nor with modification of the Trypan Blue exclusion test with respect to control cells (data not shown). Therefore, we suppose that the antifibrotic activity of 9 might be exerted by interference in cell proliferation, which needs to be clarified by further investigation.

Twelve diterpenes evaluated in the present study can be further divided into three groups according to their structure; 1-6 belong to labdanes-type diterpene, 8 to totarane-type diterpene and 9-12 to isopimarane-type diterpene. As shown in Fig. 2, labdanes-type diterpenes exerted weak inhibitory activity against HSC proliferation, whereas 8, totarane-type diterpene, showed the significant inhibitory effects on HSC proliferation. Meanwhile, isopimarane-type diterpenes exert different effect. Among isopimarane-type diterpenes, 9, containing the olefinic bond at C-8 and C-14, exerted potent inhibitory activity whereas 10-12 with olefinic bond at C-7 and C-8 showed less activity. It has been reported that acanthoic acid, (-)-pimara-9(11),15-dien-19-oic acid, a isopimarane-type diterpene possessing the olefinic bond at C-9 and C-11, showed antifibrotic effect (Cai et al., 2003; Kang et al., 1996). Therefore, although more derivatives should be assessed for relevant relationship between the structure and activity, our study suggested that the position of the double bond in isopimarane-type diterpenes can be a determinant for exerting antifibrotic activity.

In summary, we isolated twelve diterpenes with different skeleton from *B. orientalis* and evaluated the antifibrotic activity of these diterpenes together with their structure activity relationship using HSC-T6 cells. In particular, 8, a totarane-type diterpene, and 9, an isopimarane-type diterpene, exhibited potent inhibitory activities on cell proliferation, probably mediated by different pathway. Thus, it will be of interest to test further whether these diterpenes exert antifibrotic effects in vivo, for example, in animal models of liver fibrosis, to explore their therapeutic potentials. This will provide further insight into the design of new approaches to liver fibrosis.

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