

## Antifibrotic Activity of Manassantin B from *Saururus chinensis* in HSC-T6 Hepatic Stellate Cells

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**Abstract** – Manassantin B, a dilignan isolated from *Saururus chinensis*, significantly inhibited proliferation in HSC-T6 cells in concentration- and time-dependent manners. In addition, treatment of HSC-T6 cells with manassantin B changed cell morphology from flattened myofibroblastic membranous morphology, representing activation state, to slender shape, representing quiescent state. Furthermore, manassantin B effectively reduced collagen content in HSC-T6 cells. These results suggested that manassantin B exerted antifibrotic activity in HSC-T6 cells, in part, via inhibition of cell proliferation and decrease of collagen production.

**Key words** – manassantin B, antifibrotic, *Saururus chinensis*, HSC-T6 hepatic stellate cells, collagen

### Introduction

Hepatic fibrosis represents a wound-healing process of the liver in response to acute and chronic insults such as ethanol, viral infection, cholestasis and metabolic diseases (Friedman, 2003). Hepatic stellate cells (HSCs) have been well characterized in liver fibrosis. After a fibrogenic stimulus, HSCs transform from quiescent vitamin A storing cells to activated myofibroblast-like cells (Friedman, 2000). In addition, two major events occur after HSC activation, which substantially contribute to their active role in liver fibrosis. First, activated HSCs are the primary cell type responsible for increased synthesis and deposition of extracellular matrix (ECM) proteins in the liver. Second, they show high proliferative property thereby effectively increasing the population of fibrogenic cells and amplifying the fibrotic response (Tsukada *et al.*, 2006). Therefore, HSCs are considered to play a key role in the pathogenesis of liver fibrosis, which has directed the approach of developing antifibrotic drugs towards targeting HSCs (Wu and Zern, 2000).

*Saururus chinensis* Baill. (Saururaceae) is a perennial herb that has been used for many years to treat edema, jaundice and gonorrhoea in folk medicine (Chung and Shin, 1996). We have been interested in diverse therapeutic potentials of *S. chinensis*. In our previous work on *S. chinensis*, we isolated lignans, sesquilignans, dilignans,

flavonoids and alkaloid, including five new compounds, from the aerial and/or underground parts of *S. chinensis* (Sung *et al.*, 1997; Sung and Kim, 2000; Sung *et al.*, 2001). In addition, we reported the neuroprotective, hepatoprotective and anti-inflammatory activities of the constituents of *S. chinensis* (Sung *et al.*, 2000; Lee *et al.*, 2003a; Kim *et al.*, 2004). Recently, our further study on *S. chinensis* afforded manassantin derivatives from the underground parts of *S. chinensis* (Sung, 2006). Manassantins are dilignans that have been reported to exert diverse biological activities, including antitumor and anti-inflammatory effects (Hodges *et al.*, 2004; Lee *et al.*, 2003b). To date, however, there were no previous studies on antifibrotic activity of manassantins. Thus, in the present study, we attempted to elucidate antifibrotic activity of manassantin B from *S. chinensis* by employing HSC-T6 immortalized rat hepatic stellate cell lines as an *in vitro* system (Fig. 1).

### Experimental

**Chemical** – Supplement for cell culture and other reagents used in the study were obtained from Sigma-Aldrich Co. and 5-bromo-2'-deoxyuridine (BrdU) assay kit was purchased from Roche Diagnostics Co., Manassantin B was isolated from the underground parts of *S. chinensis* and its purity was higher than 95.0% (Sung, 2006).

**Culture of HSC-T6 hepatic stellate cells** – An immortalized rat hepatic stellate cell line, HSC-T6 (Vogel

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*et al.*, 2000), 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<sub>2</sub>. HSC-T6 cells were treated with vehicle or manassantin B for 48 hr or as indicated.

**Assessment of cell proliferation** – Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The effect on cell proliferation was measured by BrdU incorporation assay according to manufacturer's protocol.

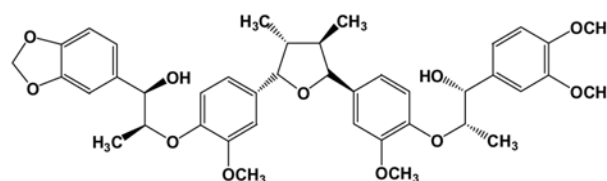
**Measurement of collagen content** – Collagen content was quantified by Sirius Red-based colorimetric assay (Tullberg-Reinert and Jundt, 1999). Cultured HSC-T6 cells were washed with PBS, followed by fixation with Bouin's fluid for 1 hr. After fixation, the fixation fluid was removed and the culture dishes were washed by immersion in running tap water for 15 min. The culture dishes were air dried and stained by Sirius Red dye reagent for 1 hr with mild shaking on a shaker. Thereafter, the solution was removed and the cultures were washed with 0.01 N HCl to remove non-bound dye. The stained material was dissolved in 0.1 N NaOH and absorbance was measured at 550 nm against 0.1 N NaOH as a blank.

**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.

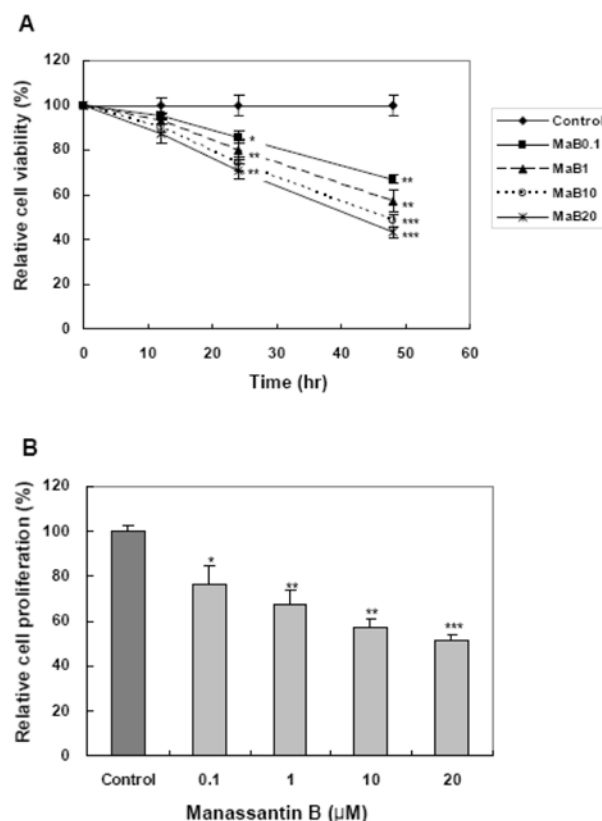
## Results and discussion

High proliferative activity is one of the major characteristics of HSC activation (Friedman, 2000). Therefore, we first investigated the effect of manassantin B on cell proliferation in HSC-T6 cells to assess antifibrotic activity of manassantin B. In our culture system, activated HSCs in serum supplemented DMEM showed high proliferative activity. However, as shown in Fig. 2A, treatment of HSC-T6 cells with manassantin B decreased the cell viability in concentration- and time-dependent manners. When HSC-T6 cells were treated with 20 µM manassantin B for 48 hr, the cell viability was decreased up to 40% compared to that of control. Manassantin B also significantly decreased cell proliferation in HSC-T6 cells as measured by BrdU incorporation (Fig. 2B).

Next we evaluated the morphology of HSC-T6 cells cultured in the presence or absence of manassantin B.



**Fig. 1.** The structure of manassantin B.



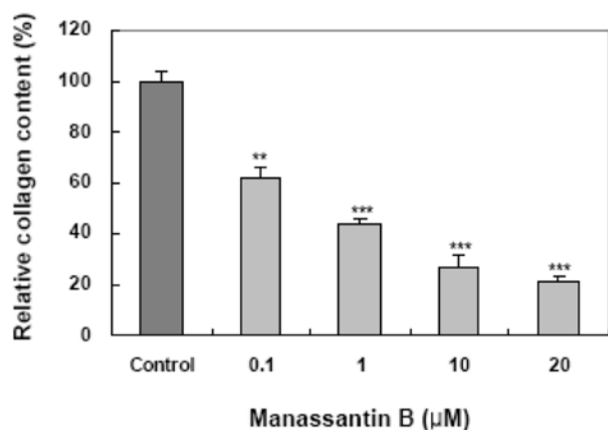
**Fig. 2.** Effect of manassantin B on cell viability (A) and proliferation (B) in HSC-T6 cells.

HSC-T6 cells were treated with manassantin B at concentrations ranging from 0.1 to 20 µM for 48h or as indicated. Cell viability and proliferation were measured by the MTT assay and BrdU assay, respectively. The percent of cell viability and proliferation (%) were calculated as  $100 \times (\text{Absorbance of manassantin B-treated} / \text{Absorbance of control})$ , respectively. Results are expressed as the mean  $\pm$  S.D. ( $n = 3$ ). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared with control.

Culturing HSCs on plastic plates is known to cause spontaneous activation, leading to myofibroblastic phenotype, mimicking the process seen *in vivo* (Chen and Zhang, 2003). Consistent with previous studies, HSC-T6 cells cultured without manassantin B exhibited flattened and membranous processes, representing myofibroblastic morphology (Fig. 4A). However, treatment of HSC-T6 cells with manassantin B resulted in change of cell morphology to slender shape, representing quiescent state

(Fig. 4).

Generally, suppression of HSC population can be achieved by various pathways, such as inhibition of cell proliferation and/or induction of cell death. Our present study clearly showed that the treatment of HSC-T6 cells with manassantin B changed the cell morphology from activation state, representing high proliferative activity, to quiescent state, representing low proliferative activity (Figs. 4E1 and 4E2). In addition, treatment of HSC-T6



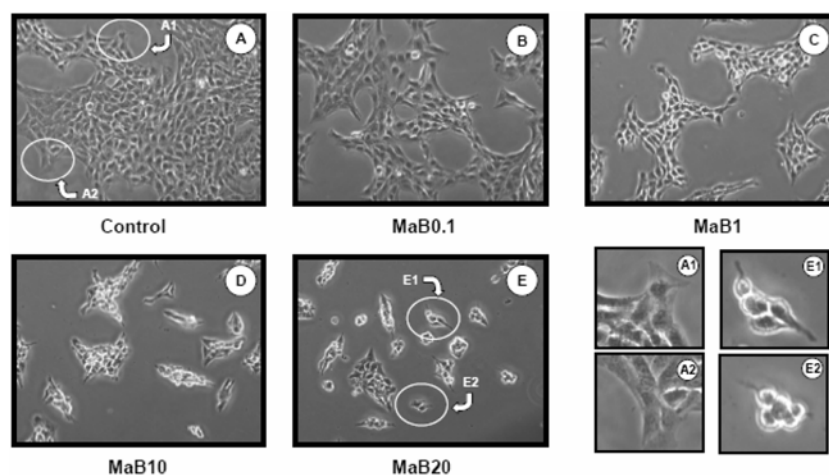
**Fig. 3.** Effect of manassantin B on collagen content of HSC-T6 cells.

HSC-T6 cells were treated with manassantin B at concentrations ranging from 0.1 to 20 µM for 48h. Collagen content was measured by Sirius Red-based colorimetric assay. The percent of collagen content (%) was calculated as  $100 \times (\text{Absorbance of manassantin B-treated} / \text{Absorbance of control})$ . Results are expressed as the mean  $\pm$  S.D. (n = 3). \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 compared with control.

cells with manassantin B was associated neither with increased LDH release in the culture medium nor with modification of the Trypan Blue exclusion test with respect to control cells (data not shown), suggesting that the effect of manassantin B was not caused by direct cytotoxicity. Thus, we carefully suppose that the antifibrotic activity of manassantin B may be achieved by the interference in cell proliferation not by cytotoxicity.

Excessive production and deposition of ECM such as collagen are other important characteristic of HSC activation, and is responsible for liver dysfunction (Friedman, 2000). Therefore, we investigated the effect of manassantin B on collagen production in activated HSC-T6 cells. In our culture system, activated HSC-T6 cells produced high amount of collagen. However, manassantin B dramatically decreased the collagen amount in a concentration-dependent manner (Fig. 3).

Two major events promote the fibrogenic response of HSCs. First, HSCs become directly fibrogenic by changing their pattern of gene expression, to increase synthesis and deposition of ECM. Second, the increased proliferation rate of HSCs effectively amplifies the number of fibrogenic cells, which accelerate the deposition of ECM. In our present study, manassantin B was more effective in the reduction of collagen content than in the inhibition of proliferation of HSC-T6 cells. When HSC-T6 cells were treated with 20 µM manassantin B for 48 hr, the collagen content was decreased up to 20% of control level (Fig. 3), whereas proliferation of HSC-T6 cells was decreased up to about 50% of control levels (Fig. 2B). These results suggest that reduction of collagen content by manassantin



**Fig. 4.** Effect of manassantin B on cell morphology of HSC-T6 cells.

HSC-T6 cells were incubated with vehicle (A) or manassantin B ranging from 0.1 to 20 µM (B-E) for 48 h. A1-A2 and E1-E2 are enlarged pictures of control and 20 µM manassantin B-treated cells, respectively. Cells were observed with phase contrast microscope (original magnification  $\times$  100).

B was achieved by direct inhibition of synthesis and/or deposition of ECM as well as by the decreased cell proliferation in HSC-T6 cells, which needs to be clarified by further investigation.

In conclusion, our present study showed that manassantin B exerted antifibrotic activity by inhibition of cell proliferation and by decrease of collagen content in HSC-T6 cells. Antifibrotic activity of manassantin B was also supported by the morphological changes induced by manassantin B. Thus, it will be of interest to test further whether manassantin B exert antifibrotic effects *in vivo*, for example, in animal models of liver fibrosis, to explore its therapeutic potentials.

### Acknowledgement

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