

Short Communication

Manassantin A and B From *Saururus chinensis* Inhibit Interleukin-6–Induced Signal Transducer and Activator of Transcription 3 Activation in Hep3B CellsJong Sun Chang^{1,†}, Seung Woong Lee^{1,†}, Myo Sun Kim¹, Bo Ra Yun¹, Mi Hye Park¹, Seok-Geun Lee², Su-Jin Park¹, Woo Song Lee¹, and Mun-Chual Rho^{1,*}¹Eco-friendly Biomaterial Research Center, Korea Research Institute of Bioscience and Biotechnology, 1404 Sinjeong-dong, Jeongeup-si, Jeonbuk 580-185, Republic of Korea²Cancer Preventive Material Development Research Center, College of Oriental Medicine, Kyung Hee University, 1 Hoegi-dong, Seoul 130-701, Republic of Korea

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Abstract. Inhibition of interleukin-6 (IL-6) has been postulated to be an effective therapy in the pathogenesis of several inflammatory diseases. The current study was performed to examine potential effects of manassantin A and B isolated from *Saururus chinensis* on the IL-6–induced response to human hepatoma cells. We found that manassantin A and B inhibit signal transducer and activator of transcription 3 (Stat3) activity stimulated by IL-6. We also found that both compounds decreased IL-6–induced Stat3 phosphorylation and nuclear translocation. Both compounds blocked suppressor of cytokine signaling 3 (SOCS-3)-mRNA expression induced by IL-6. In addition, we found that Stat3 inhibitory effects of these compounds could be related to protein tyrosine phosphatase. These findings suggest that manassantin A and B could be useful remedies for treatment of inflammatory diseases by inhibiting IL-6 action.

Keywords: manassantin A and B, interleukin-6, signal transducer and activator of transcription 3 (Stat3)

Interleukin-6 (IL-6) is a key molecule involved in the pathogenesis of several inflammatory diseases and malignancies. IL-6–mediated excessive signal transducer and activator of transcription 3 (Stat3) activation simultaneously promotes the growth of neoplastic epithelium, fuels inflammation, and suppresses the anti-tumor immune response (1). Therefore, use of an IL-6–receptor antagonist or interference with Stat3 signaling is one of the therapeutic strategies to mitigate the pathogenesis of diseases related to IL-6. Indeed, many researchers have been trying to find IL-6 inhibitors and a number of natural compounds and their derivatives including curcumin, curcubitacins, resveratrol as well as magnolol, have been shown to interfere with Stat3 activity (2 – 5).

Saururus chinensis is known as an oriental medicinal

herb and has been used for treatment of various maladies such as edema, gonorrhea, and jaundice. Actually, various compounds isolated from this herb have been reported to have a wide range of biological activities (6 – 8). Especially, many studies noticed biological activities of manassantin A and B isolated from *Saururus chinensis* (9, 10). Based on these reports, we suspected that active compounds of *Saururus chinensis* may be biological response modifiers associated with various human diseases.

In previous reports, we isolated and characterized manassantin A and B from *Saururus chinensis* and showed their biological activity by demonstrating their ability to block tumor necrosis factor α (TNF α)-induced cell adhesion molecules expression in endothelial cells (11). We also reported that these compounds inhibit the effect of phorbol 12-myristate 13-acetate (PMA) on intercellular cell adhesion molecule-1 (ICAM-1) expression, which is very important in inflammatory responses (12). Based on these results, we have suggested that these

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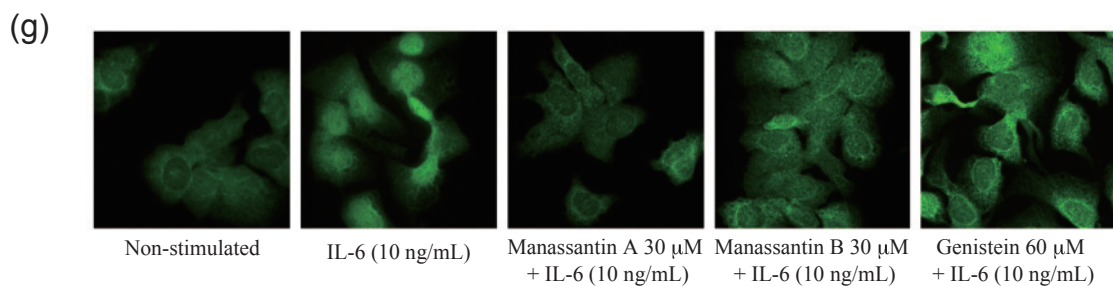
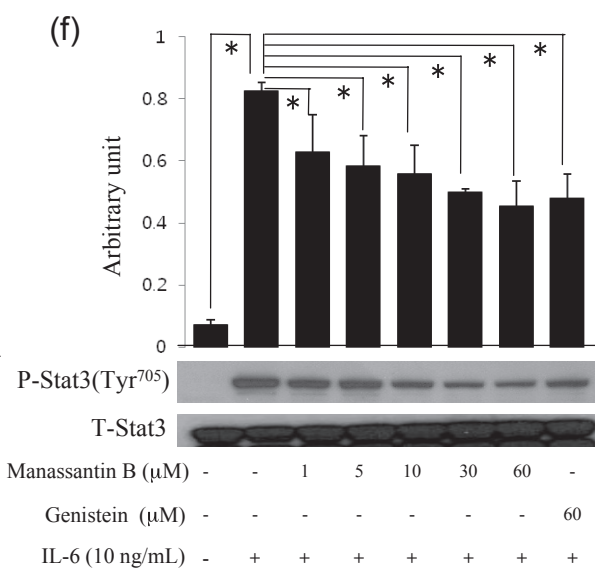
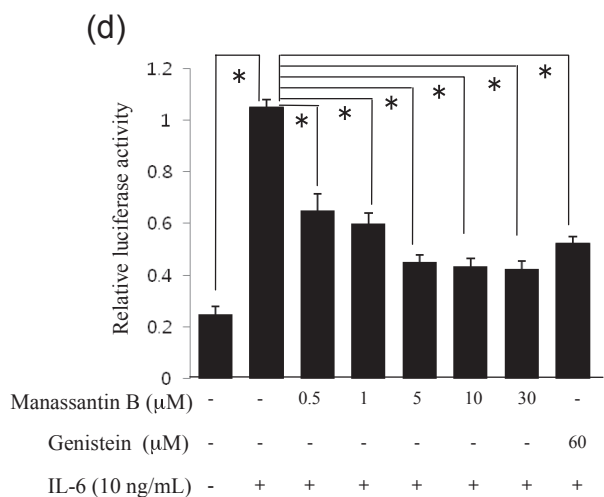
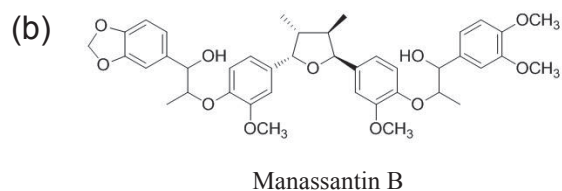
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compounds may have anti-inflammatory effects in some ways. Thus in the present study, we examined potential effects of manassantin A and B on the IL-6 response to human hepatoma cells. Manassantin A and B were isolated from the dried roots of *Saururus chinensis* by repeated column chromatography and semi-preparative HPLC and the purity analyzed by the high performance liquid chromatography-photodiode array detector was 99% as previously reported (11, 12) (Fig. 1: a and b). Human hepatoma Hep3B cells were obtained from American Type Culture Collection (ATCC No. HB-8064; Rockville, MD, USA) and were maintained in a DMEM medium, supplemented with 10% fetal bovine serum, 50 U/mL penicillin, and 50 mg/mL streptomycin, at 37°C in a 5% CO₂ incubator. All cell culture reagents were obtained from GibcoBRL (Life Technologies, Cergy-Pontoise, France). To establish the stable cell line expressing pStat3-Luc, the pStat3-Luc plasmid containing Stat3 binding sites to measure Stat3 activity was purchased from Clontech (Palo Alto, CA, USA). Hep3B cell line transformed with pStat3-Luc was generated by cotransfecting pStat3-Luc with pcDNA3.1/Hygro using lipofectamin plus (Invitrogen, Carlsbad, CA, USA) and selection with hygromycin (100 µg/mL). Expression of luciferase in the clones was confirmed by luciferase assays according to the manufacturer's protocol (Promega Corp., Madison, WI, USA). To examine the effects of manassantin A and B on the IL-6-induced suppressor of cytokine signaling 3 (SOCS-3)-mRNA expression, real-time PCR was performed on a sequence-detection system (StepOne Plus; Applied Biosystems, Foster City, CA, USA). Human SOCS-3 (Hs01000485_g1) primer was obtained by Assay-on-demand from Applied Biosystems. We used 18S rRNA as an endogenous reference to correct for differences in the amount of RNA. For the flow cytometry analysis, recombinant human IL-6 purchased from R&D systems (Minneapolis, MN, USA) was labeled with FITC according to the manufacturer's protocol (Thermo Scientific, Rockford, USA). All experiments in the present study were performed at least three times. Data were expressed as the mean ± S.E.M. Statistical analysis was performed using SigmaPlot Statistical Analysis software (SPCC Inc., Chicago, IL, USA). Differences between group mean values were determined by one-way analysis of variance followed by a two-tailed Student's *t*-test for unpaired samples, assuming equal variances.

Initially, to examine the effect of manassantin A and B on the Stat3 activity, Hep3B cells stably transformed with pStat3-Luc plasmid were stimulated with IL-6 (10 ng/mL) for 12 h in the presence or absence of manassantin A or B and measured Stat3-dependent promoter activity. Their activities were verified with genistein, a

tyrosine kinase inhibitor, as a positive control, which is known to inhibit Stat3 (13). Treatment with IL-6 alone for 12 h increased Stat3-dependent promoter activity (approximately 4.5-fold), and this induction was inhibited by manassantin A and B for 1 h in a dose-dependent manner (Fig. 1: c and d). Both compounds had no cytotoxicity at the highest dose with MTT assays in Hep3B cells (data not shown). Tyrosine phosphorylation and nuclear translocation of activated Stat3 are crucial events for its transcriptional activation by IL-6. We also investigated the inhibitory activities of these compounds on IL-6-induced Stat3 tyrosine phosphorylation and nuclear translocation. As expected, pretreatment with manassantin A and B for 1 h dramatically decreased tyrosine phosphorylation of Stat3 induced by IL-6 in a dose-dependent manner (Fig. 1: e and f). In addition, manassantin A and B blocked nuclear translocation of Stat3 induced by IL-6 as shown in Fig. 1g. These results suggest that both manassantin A and B are antagonists of IL-6 to inhibit Stat3. SOCS can be positively induced through cytokine-elicited Stat signaling. Especially, SOCS-3 is strongly induced by IL-6 stimulation. SOCS-3 is not only an endogenous inhibitor of Stat3 but also a Stat3 transcriptional target (14). Therefore, we investigated whether these compounds affect the SOCS-3-mRNA expression induced by IL-6 with real-time PCR. Consistent with results to block Stat3 activation, both compounds inhibited the IL-6-induced SOCS-3-mRNA expression (Fig. 1h). These results are very similar to those of curcumin that inhibits Stat3 phosphorylation and decreases SOCS-3 expression in several cancer cell lines (2).

Since IL-6-induced Stat3 activation is triggered by IL-6 binding to its receptors, we also investigated whether inhibition of Stat3 activity by these compounds resulted from the blockage of IL-6 binding to IL-6 receptors using flow cytometry. As shown in Fig. 2, a and b, cells treated with FITC-IL-6 for 20 min exhibited significantly higher fluorescence intensity ($74.83 \pm 4.16\%$) than non-stimulated ones ($0.25 \pm 0.05\%$). However, the intensity was not reduced by treatment of manassantin A and B ($69.16 \pm 2.72\%$ and $73.93 \pm 7.09\%$, respectively). These results suggest that manassantin A and B are potential inhibitors of the IL-6-mediated intracellular Stat3 signaling pathway rather being inhibitors of extracellular events like the action of an IL-6-receptor antagonist. Therefore, we determined whether inhibitory effects of manassantin A and B on IL-6-induced Stat3 tyrosine phosphorylation could be due to activation of a protein tyrosine phosphatase, which has been implicated in Stat3 activation (15). Treatment of Hep3B cells with the protein tyrosine phosphatase inhibitor, sodium orthovanadate, partially reversed inhibitory effects of both com-



(h)

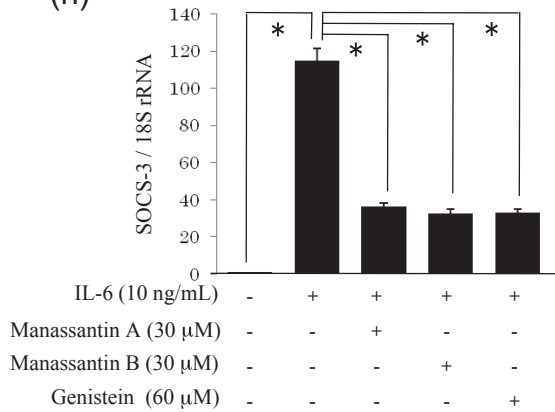
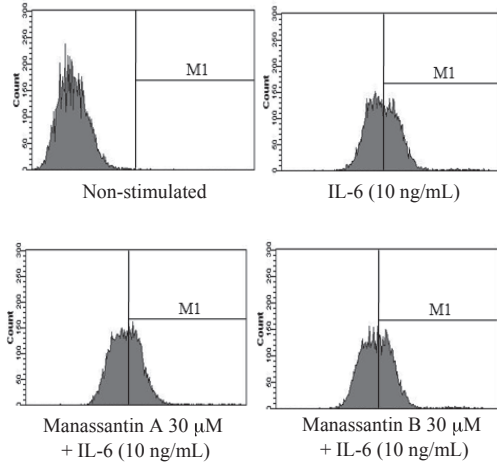


Fig. 1. Manassantin A and B inhibit the IL-6-induced Stat3 activation and SOCS-3-mRNA expression. The chemical structures of manassantin A (a) and manassantin B (b). Hep3B cells stably transformed with pStat3-Luc were treated with IL-6 for 12 h in the presence or absence of manassantin A (c) and manassantin B (d). Results are shown as a relative luciferase activity $\pm$ S.E.M. Hep3B cells were incubated with IL-6 for 20 min in the presence or absence of manassantin A (e) and manassantin B (f). Total cell lysates were isolated and immunoblotted with anti-phospho Stat3 (Tyr⁷⁰⁵) and anti-Stat3 IgG (g). Cells grown on 8-well Nunc LabTek slides were incubated with IL-6 for 4 h in the presence or absence of each compound. Cells were fixed, permeabilized, and reacted with anti-Stat3 IgG and goat polyclonal rabbit-FITC IgG antibody. Subcellular localization of Stat3 was assessed using a Leica DM5000B fluorescence microscope (Leica, Wetzlar, Germany). h) Hep3B cells were treated with IL-6 for 6 h in the presence or absence of manassantin A and B as indicated. Total RNA was isolated and real-time PCR analysis of SOCS-3-mRNA expression was performed. Data are represented as the relative mRNA expression of SOCS-3 normalized to 18S rRNA. All experiments were performed three times. * $P < 0.05$, NS = Not significant.

(a)



(b)

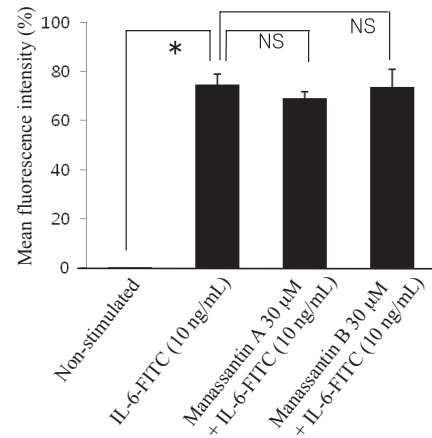


Fig. 2. Effect of manassantin A and B on the IL-6-FITC binding to the cell surface. Hep3B cells were incubated with IL-6-FITC for 20 min in the presence or absence of manassantin A and B and then washed with PBS. Subsequently, the cells were harvested and fixed. a) Then FITC-labeled IL-6 bound cell density (M1) was determined with FACS Aria flow cytometer (BD Bioscience, San Jose, CA, USA). b) Summary of FACSscan data (M1) from three independent experiments. * $P < 0.05$, NS = Not significant.

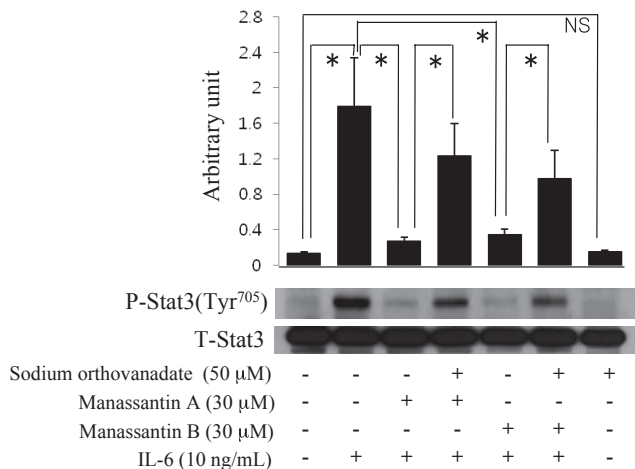


Fig. 3. Effect of manassantin A- and manassantin B-induced protein tyrosine phosphatase activity on IL-6-induced Stat3 phosphorylation. Hep3B cells pretreated with sodium orthovanadate for 1 h were incubated with IL-6 for 20 min in the presence or absence of manassantin A or manassantin B. Total cell lysates were isolated and immunoblotted with anti-phospho Stat3 (Tyr⁷⁰⁵) and anti-Stat3 IgG. All experiments were performed three times. * $P < 0.05$, NS = Not significant.

pounds on IL-6-induced Stat3 tyrosine phosphorylation (Fig. 3). This result suggests that tyrosine phosphatase could be involved in the inhibitory effects of both compounds on the IL-6-induced Stat3 activation.

In summary, we found that manassantin A and B suppresses the IL-6-induced Stat3 activation by blocking phosphorylation and nuclear translocation of STAT3. We also found that inhibitory effects of manassantin A and B on the IL-6-induced Stat3 activation is related to tyrosine phosphatase. Taken together, these results suggest that manassantin A and B may be potentially useful IL-6 antagonists with some benefit to patients who are suffering from IL-6-related inflammatory diseases.

Acknowledgments

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