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NFAT Transcription Factor Inhibitory Constituents from *Cnidium officinale*

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Abstract – Four hundred varieties of plant extracts were screened for inhibitory activity against the NFAT transcription factor which plays an important role in inducing immune response. Among them, the MeOH extract of *Cnidium officinale* showed potent activity, and the activity-guided separation yielded butylidenephthalide, senkyunolide A and falcarindiol as the active constituents. The IC_{50} value of butylidenephthalide was 1.3×10^{-4} M and was similar to that of senkyunolide A (2.1×10^{-4} M). Interestingly, falcarindiol showed higher activity (IC_{50}, 2.6×10^{-5} M) than the two phthalides.

Keywords – *Cnidium officinale*, inhibitory activity against NFAT transcription factor, butylidenephthalide, senkyunolide A, falcarindiol

Introduction

Nuclear factor of activated T cells (NFAT), a cytoplasmic protein, is dephosphorylated by the Ca^{2+} activated calcineurin and then migrates to the nucleus to induce transcription of genes required for T-cell activation, including the IL-2 gene (Winter and Harris, 1993; Abbas *et al.*, 1997). Activation of NFAT normally plays a significant role in inducing the immune response. However, excessive activation provokes immunopathological reactions including autoimmunity, transplant rejection and hypersensitivity (Abbas *et al.*, 1997). Therefore, modulation of NFAT transcription seems to be useful in the therapy of immune diseases. We screened four hundred varieties of plant extracts to search for NFAT transcription factor regulators, and found potent inhibitory activity in the MeOH extract of *Cnidium officinale* rhizomes. *C. officinale* is well known as a crude drug having haemodynamic and analgesic effects in Oriental medicines. Various phthalide derivatives and acidic polysaccharides have been isolated from this plant (Pushan, *et al.*, 1984; Naito *et al.*, 1992) and they showed centrally acting muscle relaxant effects and anti-complement activities (Ozaki *et al.*, 1989; Tomoda *et al.*, 1994). To identify specific NFAT transcription factor regulators from this extract, we isolated butylidenephthalide, senkyunolide A and falcarindiol by the bioactivity-guided fractionation. Their inhibitory activities against the NFAT transcription factor are reported herein.

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Materials and Methods

General experimental procedures – Melting points were measured using an Electrothermal 9100 and are uncorrected. IR was recorded with Perkin-Elmer 780 Jasco Report-100 IR spectrometer. FAB and EI-MS spectra were recorded using a JEOL JMS-HX 110A tandem and a Hewlett-Packard 5889A mass spectrometer, respectively. The ^{1}H- (300 MHz), ^{13}C-NMR and DEPT (75 MHz) spectra were recorded on a Bruker DRX-300 NMR instrument and the chemical shifts are quoted with TMS as an internal standard. Column chromatography was carried out on Kieselgel 60 (Merck No. 9385).

Plant materials – Rhizomes of *C. officinale* used for this study were purchased from a crude drug store, Daejeon (Korea) in January 2000. A voucher specimen (CNU 20122) was deposited at the herbarium in the College of Pharmacy, Chungnam National University.

Extraction and isolation – The dried rhizomes (2.6 kg) were extracted with MeOH three times to afford a crude extract (270 g). This extract was suspended with water and successively partitioned with CH_2Cl_2 and EtOAc. The CH_2Cl_2 extract (112 g) was chromatographed on a silica gel column and eluted with *n*-hexane-EtOAc (15:1-1:1, step gradient) to yield 11 fractions. Fractions 4 and 6 were further chromatographed on a reverse phase C-18 column

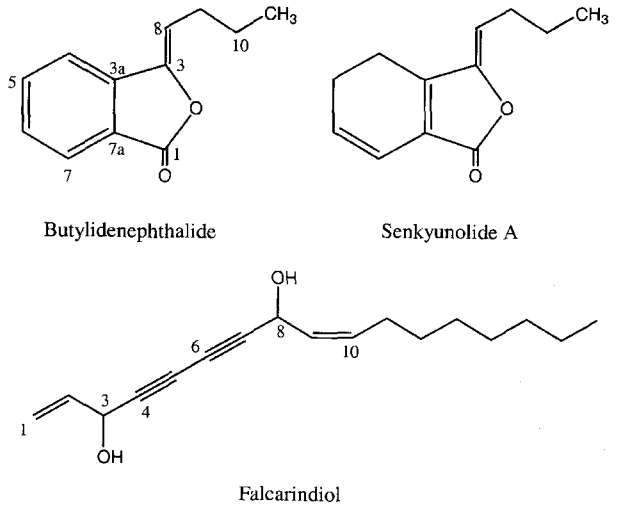

Fig. 1. NFAT transcription factor imhibitory constituents from *C. officinale.*

using MeOH-H_2O (6:4) to yield butylidenephthalide (27 mg) and senkyunolide (1.5 g), respectively. Fraction 8 was subjected to a Silica gel column with CH_2Cl_2-MeOH (35:1-20:1, step gradient) to give 4 fractions. From subfraction 3, falcarindiol (127 mg) was separated using a YMC gel column (MeOH-H_2O, 7:3). The identification of these compounds, as shown in Fig. 1, was confirmed by the reported references for butylidenephthalide, senkyunolide A (Yamagishi and Kaneshima, 1977) and falcarindiol (Kozawa *et al.*, 1983; Baba *et al.*, 1987).

Butylidenephthalide – Pale white crystal, IR (KBr) cm^{-1}: 2950 (C-H), 1765 (C=O), EI-MS *m/z*: 188(M^+, $C_{12}H_{12}O_2$), ^{1}H-NMR (300 MHz, $CDCl_3$) δ : 0.99 (3H, t, *J*=7.36, CH_3), 1.56 (2H, m, H-10), 2.45 (2H, m, H-9), 5.64 (1H, t, *J*=7.83 , H-8), 7.50 (1H, m, H-4), 7.65 (2H, m, H-5 and H-6), 7.88 (1H, m, H-7). ^{13}C-NMR (75 MHz, $CDCl_3$) δ : 13.6 (C-11), 22.5 (C-10), 27.8 (C-9), 109.4 (C-8), 119.6 (C-4), 124.4 (C-7a), 125.2 (C-7), 129.3 (C-6), 134.2 (C-5), 139.6 (C-3), 145.7 (C-3a), 167.2 (C-1).

Senkyunolide A – Yellow oil, IR (KBr) cm^{-1}: 2950 (C-H), 1750 (C=O), EI-MS *m/z*: 192 (M^+, $C_{12}H_{16}O_2$), ^{1}H-NMR (300 MHz, $CDCl_3$) δ : 0.92 (3H, t, *J*=7.1 Hz, CH_3), 1.38 (4H, m, H-9 and H-10), 1.51 (1H, m, H-8a), 1.87 (1H, m, H-8b), 2.46 (4H, m, H-4 and H-5), 4.91 (1H, dd, *J*=7.8, 3.7 Hz, H-3), 5.90 (1H, m, H-6), 6.20 (1H, m, H-7). ^{13}C-NMR (75 MHz, $CDCl_3$) δ : 13.8 (C-11), 20.8 (C-4), 22.3 (C-10), 22.4 (C-5), 26.7 (C-9), 31.9 (C-8), 82.5 (C-3), 116.9 (C-7), 124.5 (C-7a), 128.3 (C-6), 161.4 (C-3a), 171.2 (C-1).

Falcarindiol – Yellow oil, IR (KBr) cm^{-1}: 3400 (OH), $[\alpha]_D^{25}$ + 194.5 (c=0.5, MeOH), EI-MS *m/z*: 260 (M^+, $C_{17}H_{24}O_2$), ^{1}H-NMR (300 MHz, $CDCl_3$) δ : 0.90 (3H, t, *J*=6.7 Hz, H-17), 1.26 (10H, brs, H-12, H-13, H-14, H-15, H-16), 2.10 (2H, m, *J*=6.8 Hz, H-11), 4.94 (1H, d, *J*=4.7 Hz, H-3), 5.22 (1H, d, *J*=8.2 Hz, H-8), 5.26 (1H, d, *J*=9.1 Hz, H-1a), 5.48 (1H, m, H-1b), 5.61 (1H, m, H-10), 5.94 (1H, m). ^{13}C-NMR (75 MHz, $CDCl_3$) δ : 14.1 (C-17), 22.6 (C-16), 27.7 (C-11), 29.1 (C-13, 14, 15) 31.8 (C-12), 58.6 (C-8), 63.4 (C-3), 68.7 (C-6), 70.3 (C-5), 78.2 (C-7), 79.8 (C-4), 117.0 (C-1), 127.6 (C-9), 134.7 (C-10), 135.8 (C-2).

Preparation of buffers and reagents – RPMI 1640 without phenol red (11835-030, Gibco. BRL) was mixed with 0.5% fetal bovine serum and 1% penicillin-streptomycin. Phoboal 12-myristate 13-acetate (25 ηg/ml) and ionomycin (0.5 μM) as a stimulator were dissolved in DMSO. *P*-Nitrophenylphosphate (120 mM) as a substrate was dissolved with SEAP buffer (1 M diethanolamine, 0.5 mM $MgCl_2$, 10 mM homoarginine).

Preparation of cells and samples – The Jurkat T cell line containing an NFAT dependent transcriptional reporter gene, pCMV·SEAP was maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were harvested by centrifugation, washed once in PBS and resuspended in RPMI 1640 without phenol red. Each sample was dissolved in DMSO and diluted in RPMI 1640 without phenol red.

Inhibitory activity against NFAT transcription factor – Inhibitory activity against NFAT transcription factor was determined by the modified alkaline phosphatase (SEAP) assay (Yang *et al.*, 1997). For assay, 100 μl of cells (1×10^4 cells/well) were incubated with 50 μl of sample and 50 μl of stimulator at 37°C for 18 hours. The reaction mixture was centrifuged and 100 μl of supernatant were transferred to an eppendorf tube and heated at 65°C for 1 hour. The heated sample was transferred to a flat-bottomed microplate and incubated with 50 μl of SEAP buffer and 50 μl of substrate at 37°C for 4 hours. After incubation of the reaction mixture, optical density was measured at 405 nm by using a microplate reader. Inhibitory activity against NFAT transcription factor was determined as a mean of triplicate tests per concentration and expressed as percent inhibition of the control. The IC_{50} value was defined as the final concentration of the inhibitor required to block 50% NFAT transcription. Cyclosporin A was used as the positive control (IC_{50}, 1.2×10^{-9} M).

Cell viability – Using a MTT cell proliferation Kit (465007, Roche), cell viability was determined by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan.

Results and Discussion

About four hundred samples of plant extracts were screened for inhibitory activity against the NFAT transcription

Table 1. IC_{50} values on NFAT transcription factor from *C. officinale*

Samples	IC_{50} value
MeOH extract	69.8 μg/ml
CH_2Cl_2 fraction	11.2 μg/ml
butylidenephthalide	1.3×10^{-4} M
senkyunolide A	2.1×10^{-4} M
falcarindiol	2.6×10^{-5} M

factor. As shown in Table 1, the MeOH extract of *C. officinale* showed a significant activity against the NFAT transcription factor without affecting cell viability (IC_{50}, 69.8 μg/ml). The MeOH extract was suspended in water and then consecutively partitioned with CH_2Cl_2 and EtOAc. The highest activity was obtained from the CH_2Cl_2 fraction (IC_{50}, 11.2 μg/ml). The CH_2Cl_2 extract was subjected to a subsequent column chromatographic purification process by using activity-guided separation. Senkyunolide A (which was renamed from senkyunolide (Kobayashi *et al.*, 1984)), butylidenephthalide and falcarindiol were isolated as active compounds. These compounds have been reported to have various pharmacological activities : for example, antifungal activity against *Mycocentrospora acerina* (Garrod and Lewis, 1982); antimutagenic activity on Trp-p-1 (Miyazawa, *et al.*, 1996); and antiproliferative activity on primary cultures of mouse aorta smooth muscle cells (Kobayashi, *et al.*, 1992). In this study, we first determine their inhibitory activities against NFAT transcription factor, which may be related to their pharmacological effects. The IC_{50} values of butylidenephthalide and senkyunolide A are 1.3×10^{-4} M and 2.1×10^{-4} M, respectively, and that of falcarindiol is 2.6×10^{-5} M showing the highest inhibitory activity. Although the structure-activity relationship was not investigated in this study, the polyacetylenic compound may be more potent inhibitor than two isolated phthalides against the NFAT transcription factor. These results suggest that butylidenephthalide, senkyunolide A and falcarindiol may be responsible for the therapeutic efficacy of *C. officinale* in NFAT-related immune diseases.

Acknowledgments

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