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INHIBITION OF NF-κB AND CELLULAR INVASION BY NOVEL FLAVONOID DESMAL IN OVARIAN CARCINOMA CELLS

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Abstract – In the course of our screening of NF-κB inhibitors, we isolated known flavonoids that inhibit LPS-induced NO production in mouse monocytic leukemia RAW264.7 cells. Since those flavonoids inhibited the NF-κB activity, we have evaluated the inhibitory activity of plant-derived novel flavonoid, desmal, on NF-κB, and found that it inhibits LPS-induced NO production and NF-κB. It also inhibited cancer cell invasion possibly by the decrease of NF-κB-dependent urokinase-type plasminogen activator. Thus, desmal was found to be a new NF-κB inhibitor having cellular anti-inflammatory and anti-metastatic activities.

Microorganisms and plants are useful sources of antibiotics, anticancer agents, and disease-related enzyme inhibitors. We are screening bioactive metabolites from microorganisms that may suppress inflammation and cancer. We have previously isolated protein-tyrosine kinase inhibitors, protein-tyrosine phosphatase inhibitors, and lipopolysaccharide function inhibitors.

Previously, we designed and synthesized dehydroxymethylepoxyquinomicin (DHMEQ) as a specific inhibitor of NF-κB.[1, 2] DHMEQ showed various anti-inflammatory and anticancer activities in animal disease models.[3] More recently, we have isolated 9-methylstreptimidone from a microorganism as a compound that inhibits LPS-induced NO production.[4] Since the yield of this reported compounds by the producing organism is poor, we designed a simplified structure DTCM-glutarimide.[5] This compound showed anti-inflammatory activity in animal heart transplantation model[6] and ameliorated the animal model of inflammatory bowel disease.[7] Therefore, we are continuing to screen new NF-κB inhibitors from microorganisms and plants. In the course of screening, we have isolated several flavonoids that inhibit LPS-induced NO production. In one hand, we have previously isolated novel flavonoid, desmal, as an inhibitor of protein-tyrosine kinase from a plant of Thailand.[8] In the present research we found that novel flavonoid, desmal, inhibited NF-κB, and that it inhibited NO production in a macrophage-like cell line and cancer cell migration and invasion.

We screened *Streptomyces* culture filtrates for the inhibitors of LPS-induced NO production in mouse macrophage-like cell line RAW264.7 cells. The effect of the culture filtrates on cell death in each cell line was measured using the MTT. Effective culture filtrates without toxicity were selected. Microbial culture filtrates were kindly supplied from Meiji Seika Pharma Co., Ltd., Tokyo, Japan. Fermentation medium was extracted with aqueous acetone. The extract was filtered and evaporated in vacuo to remove the acetone, and the aqueous residue was extracted with ethyl acetate (pH 7.0). Among about 2000 ethyl acetate extract samples AFE72940 inhibited LPS-induced NO production without toxicity. The active substance was then purified and isolated from the obtained microbial culture solution as follows. The extract AFE72940 (2.96 g) was applied to a Sephadex LH-20 column chromatography (CC) eluted with $CHCl_3$-MeOH (1 : 1) to give 8 fractions (Fr.1 – Fr.8), Fr.7 was then purified by HPLC with an ODS column eluted with MeOH : H_2O (50 : 50 – 100 : 0, 0-20 min, 2.5 mL/min) to yield genistein (**1**) (2.5 mg, t_R 16.3 min). Genistein 7-*O*-α-L-rhamnoside (**2**) (26.7 mg, t_R 10.1 min) was obtained from Fr.6, daidzein 6-*O*-methyl-7-*O*-α-L-rhamnoside (**3**) (2.7 mg, t_R 7.2 min) and daidzein 7-*O*-α-L-rhamnoside (**4**) (9.5 mg, t_R 10.6 min) was obtained from Fr.4, by using the same HPLC method.

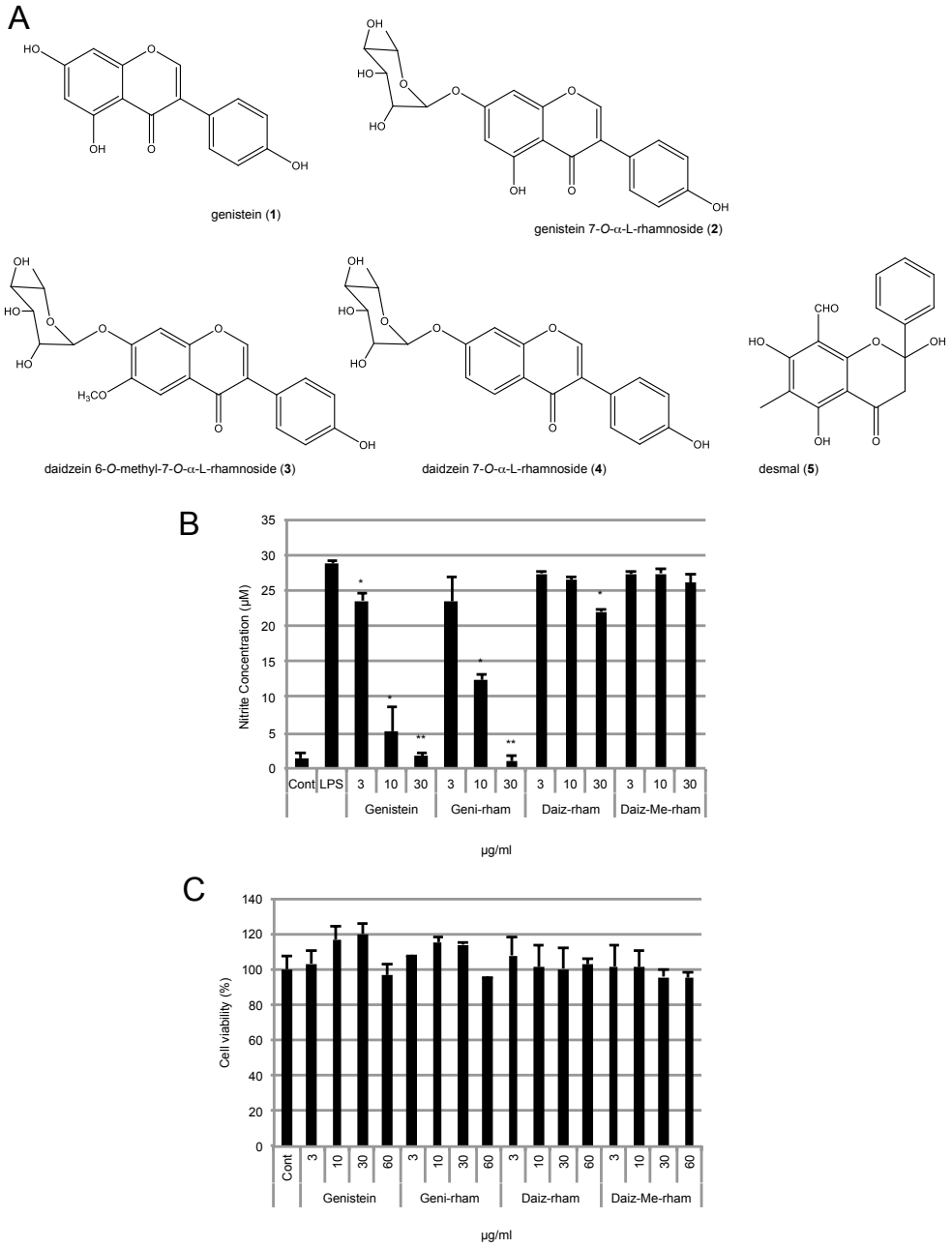

Figure 1. Isolation of known flavonoids from *Streptomyces* culture filtrates. A. Structures of *Streptomyces*-derived flavones and plant-derived desmal. B. Inhibition of LPS-induced NO production by 4 flavonoids isolated from *Streptomyces* in mouse macrophage-like cell line RAW264.7. *, P<0.01; **, P<0.001. C. Effect of *Streptomyces*-derived flavonoids on cell viability in RAW264.7 cells

Thus we obtained 4 reported genistein (**1**), genistein 7-*O*-α-L-rhamnoside (**2**), daidzein 6-*O*-methyl-7-*O*-α-L-rhamnoside (**3**), and daidzein 7-*O*-α-L-rhamnoside (**4**) (Figure 1A). The inhibitory activity and toxicity of these isoflavones are shown in Figures 1B and 1C, respectively. Genistein (**1**) and its glycoside (**2**) showed the potent inhibitory activity at the nontoxic concentrations. Daidzein

7-*O*-α-L-rhamnoside (**4**) showed weaker inhibitory activity, and daidzein 6-*O*-methyl-7-*O*-α-L-rhamnoside (**3**) did not show prominent activity. Anti-inflammatory activities of genistein[9] and daidzein[10] were already reported. We previously isolated a novel flavone desmal (Figure 1A) from a Thai plant *Desmos chinensis* as an inhibitor of protein-tyrosine kinase.[8] Its other biological activity has not been reported. Since genistein and its glycosides showed the inhibitory activity, we tested the inhibitory activity of desmal. The cytotoxicity is shown in Figure 2A, in which desmal of 10 μg/mL is not toxic at all, and that of 30 μg/mL slightly decreases the viability. As shown in Figure 2B, desmal inhibited the NO production at 10-30 μg/mL. NO is produced by inducible NO synthase (iNOS) which is dependent on NF-κB. Then, we studied the effect on LPS-induced NF-κB activation. As a result, it inhibited the activation at 10-30 μg/mL (Figure 2C).

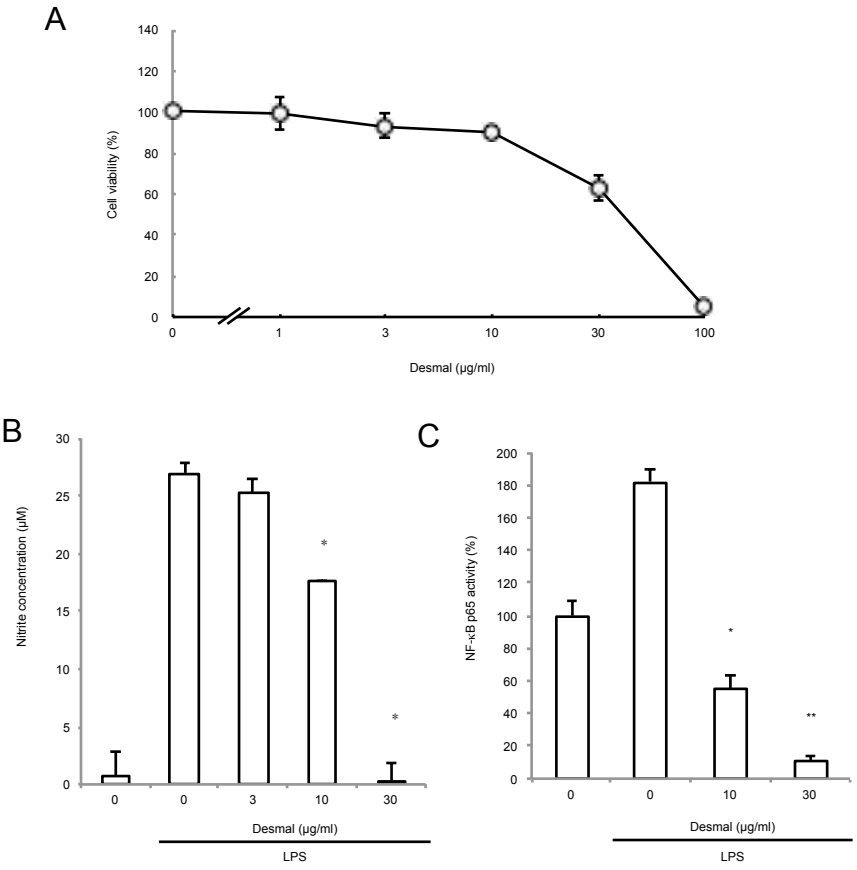

Figure 2. Inhibition of NO production and NF-κB by desmal in RAW264.7 cells. A. Effect of desmal on cell viability. The cell viability was assessed by MTT after 24 h incubation with desmal. B. Inhibition of LPS-induced NO production by desmal. Desmal was added for 0.5 h, then 10 ng/mL LPS was added for 24 h. *, P<0.01. C. Inhibition of LPS-induced NF-κB activation by desmal. Desmal was added for 0.5 h, and then 10 ng/mL LPS was added for 2 h. NF-κB activity was assessed by κB DNA binding of each nuclear extract using the NF-κB p65 Transcription Factor Assay Kit. *, P<0.05; **, P<0.01.

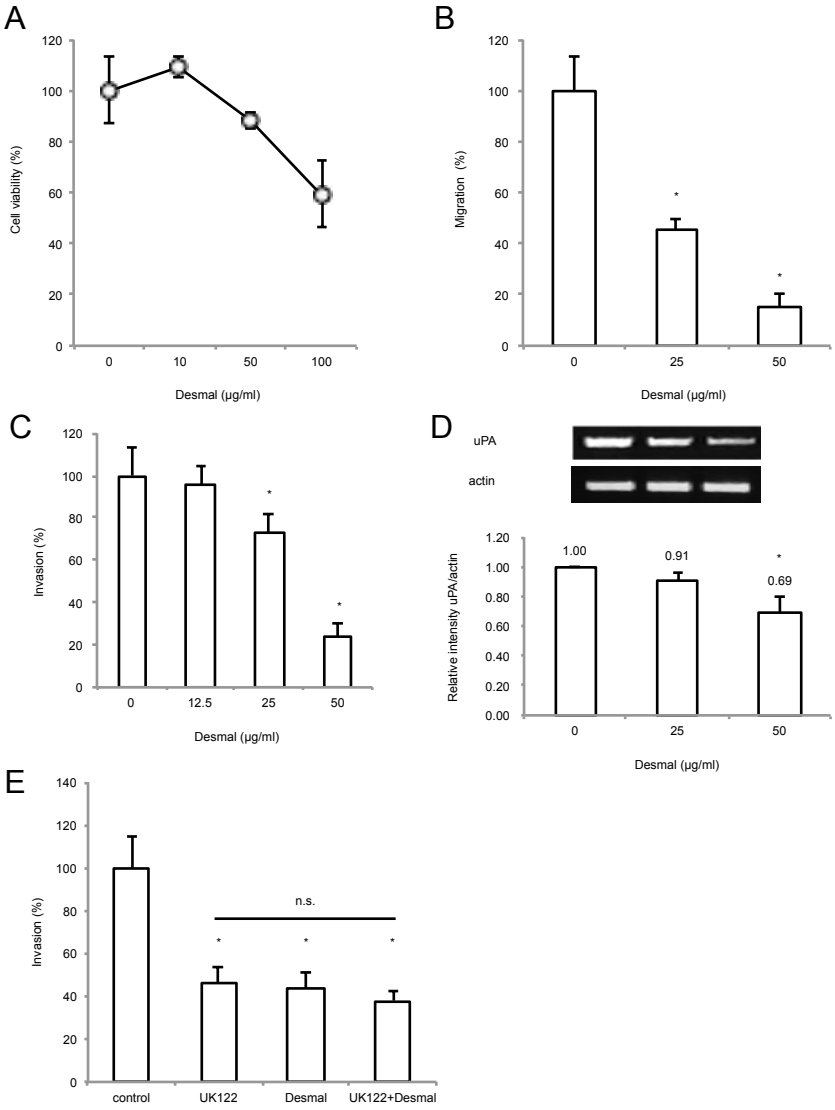

Figure 3. Inhibition of cellular migration and invasion by desmal in ovarian clear cell carcinoma ES-2 cells. A. Effect on cell viability. The cells were incubated with desmal for 24 h, and the viability was assessed by MTT. B. Inhibition of cellular migration. Cellular migration was assessed by the wound-healing assay. Desmal was added for 24 h. *, $P<0.001$. C. Inhibition of cellular invasion. Desmal was added for 24 h, and the invasion was assessed by Matrigel invasion assay. *, $P<0.001$. D. Inhibition of uPA expression by desmal. The cells were incubated with desmal for 24 h, then, uPA expression was measured by PCR. *, $P<0.01$. E. Inhibition of cellular invasion by uPA inhibitor, UK122. The cells were incubated with 30 μM UK122 for 24 h. *, $P<0.001$ against control.

We previously reported that NF-κB inhibitor DHMEQ inhibited cellular invasion in ovarian clear cell carcinoma ES-2 cells.[11] Then, we studied the effect of desmal on cellular migration and invasion in ES-2 cells. ES-2 cells were shown to be more resistant than RAW264.7 cells to desmal, and desmal below 50 μg/mL was not toxic (Figure 3A). Desmal inhibited the migration (Figure 3B) and invasion (Figure 3C) at

the nontoxic concentrations. Urokinase-type plasminogen activator (uPA) is one of the enzymes that accelerate cellular invasion. Desmal was found to lower the uPA mRNA expression (Figure 3D). The uPA expression was measured by PCR as described before.[11] UK122 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), a uPA inhibitor, did not decrease the viability of cells at 30 μM in 24 h (data not shown). It inhibited the invasion at 30 μM, as shown in Figure 3E. UK122 and desmal did not show the additive effect, indicating that desmal is likely to inhibit the invasion via suppression of uPA (Figure 3E).

In conclusion, we obtained 4 reported isoflavones, genistein (**1**), genistein 7-*O*-α-L-rhamnoside (**2**), daidzein 6-*O*-methyl-7-*O*-α-L-rhamnoside (**3**), and daidzein 7-*O*-α-L-rhamnoside (**4**) from *Streptomyces*. The inhibitory activity against NO production and toxicity in mouse monocytic leukemia RAW264.7 cells are evaluated. Compounds (**1**) and (**2**) showed potent inhibitory activity at the nontoxic concentrations. Desmal, which is a new flavonoid isolated from the leaves of Thai plant *Desmos chinensis*, is thus evaluated for NO production and NF-κB inhibitory activities. As the result, desmal inhibits NF-κB with similar activity with genistein. It may be useful as a chemical ligand for the mechanistic study of inflammation and cancer progression. Desmal is known to inhibit protein-tyrosine kinase, and this is the first report on NF-κB inhibitory activity.

EXPERIMENTAL

Materials. Desmal was isolated from the leaves of *Desmos chinensis* collected in Khon Kaen, Thailand, as described before.[8]

Cell culture. Murine macrophage-like cell line RAW264.7 was purchased from Riken Cell Bank, Tsukuba, Japan. The cells were cultured in RPMI-1640 medium (Sigma-Aldrich Co., St Louis, MO) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, and 5% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), and maintained at 37 °C in humidified 95% air, 5% CO_2 atmosphere. Human ovarian clear cell carcinoma ES-2 cells (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and penicillin/streptomycin at 37 °C in a humidified incubator with 5% CO_2.

Cell viability. Cell viability was evaluated by the MTT assay. Cell suspensions at a density of 3 x 10^5 cells per mL were seeded in a 96-well plate and incubated for 1 h. Then each flavonoid at different concentrations were added and further incubated for 24 h. MTT solution was added to each well and incubated for 1 h. Then, the culture medium was replaced by DMSO to dissolve formazan crystals formed. Absorbance was measured at 570 nm with a microplate reader.

Measurement of NO production. RAW264.7 cells were cultured in a 96-well plate at a density of 3 x 10^5 cells per mL. The cells were treated with *Streptomyces* culture filtrate or the indicated flavonoid for 30 min. LPS 10 ng/mL was added and incubation was continued for 24 h. Then, the supernatants were collected, and nitrite was measured colorimetrically after adding 50 μL of Griess reagent consisting of 0.1%, N-naphthyl-ethylenediamine 2HCl, l% sulfanilamide, and 2.5% H_3PO_4 to a 50 μL sample of supernatants. Sodium nitrite was used as the standard. The absorbance of the mixture was measured at 570 nm with a microplate reader.

Measurement of NF-κB activity in cultured RAW264.7 cells. RAW264.7 cells in complete medium (2.5 x 10^6 cells) were grown in 60-mm dishes. The next day, the cells were treated with the desired concentrations of desmal for 30 min and stimulated with 10 ng/mL LPS for 2 h. The nuclear extracts were prepared with a Nuclear Extract Kit (Active Motif Japan, Tokyo, Japan). Then, the DNA binding activity of NF-κB in nuclear extracts was measured with the TransAM NF-κB p65 Transcription Factor Assay Kit (Active Motif, Japan).

Wound healing assay. Cells in 24-well plates were allowed to reach confluence before the surface was uniformly scratched across the center of each well with a pipette tip. The wells were then rinsed twice with serum-free media to remove floating cells and growth media, after which the cells were cultured in serum-free media for 24 h. The initial wounded area and movement of the cells into the scratched area were recorded. Experiments were performed in triplicate in three independent experiments.

Cell invasion assay. ES-2 cells were suspended in 500 μL of serum-free medium containing desmal or the DMSO and seeded into the upper chambers coated with BD Matrigel Basement Membrane Matrix (Corning Inc., Corning, NY). The lower chambers were filled with 750 μL of medium containing 10% FBS and incubated for 24 h at 37 °C in a humidified CO_2 incubator. Then, after fixation of the invading cells, non-invading cells remaining on the upper surface were removed by wiping with a cotton swab. Invading cells attached to the underside were stained with Diff-Quick solution (Sysmex, Kobe, Japan), and counted.

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