7,3′,4′-Trihydroxyisoflavone, a Metabolite of the Soy Isoflavone Daidzein, Suppresses Ultraviolet B-induced Skin Cancer by Targeting Cot and MKK4*§

Nonmelanoma skin cancer is one of the most frequently occurring cancers in the United States. Chronic exposure to UVB irradiation is a major cause of this cancer. Daidzein, along with genistein, is a major isoflavone found in soybeans; however, little is known about the chemopreventive effects of daidzein and its metabolites in UVB-induced skin cancer. Here, we found that 7,3′,4′-trihydroxyisoflavone (THIF), a major metabolite of daidzein, effectively inhibits UVB-induced cyclooxygenase 2 (COX-2) expression through the inhibition of NF-κB transcription activity in mouse skin epidermal JB6 P+ cells. In contrast, daidzein had no effect on COX-2 expression levels. Data from Western blot and kinase assays showed that 7,3′,4′-THIF inhibited Cot and MKK4 activity, thereby suppressing UVB-induced phosphorylation of mitogen-activated protein kinases. Pull-down assays indicated that 7,3′,4′-THIF competed with ATP to inhibit Cot or MKK4 activity. Topical application of 7,3′,4′-THIF clearly suppressed the incidence and multiplicity of UVB-induced tumors in hairless mouse skin. Hairless mouse skin results also showed that 7,3′,4′-THIF inhibits Cot or MKK4 kinase activity directly, resulting in suppressed UVB-induced COX-2 expression. A docking study revealed that 7,3′,4′-THIF, but not daidzein, easily docked to the ATP binding site of Cot and MKK4, which is located between the N- and C-lobes of the kinase domain. Collectively, these results provide insight into the biological actions of 7,3′,4′-THIF, a potential skin cancer chemopreventive agent.

Nonmelanoma skin cancer is one of the most frequently occurring cancers in the United States (1). UV irradiation from sunlight is the major etiologic factor in the development of nonmelanoma skin cancers, including squamous cell carcinomas and basal cell carcinomas (2). Among the forms of solar irradiation, UVB (290–320 nm) exhibits highly mutagenic and carcinogenic effects in animal experiments, as compared with UVA (320–400 nm) (3). Prolonged exposure to UVB irradiation causes the development of benign epidermal tumors, most of which become skin carcinomas because UVB functions as a complete carcinogen (4). Therefore, targeting UVB-induced molecular and signaling mechanisms might be effective approaches for the chemoprevention of skin cancer.

Cyclooxygenase 2 (COX-2)*§ is an essential enzyme mediating the conversion of arachidonic acid to prostaglandin, the inducible isof orm of cyclooxygenase (1). The inflammatory process affects numerous human malignancies, including skin cancer, by promoting epidermal hyperproliferation and hyperplasia through the secretion of various inflammatory factors, such as prostaglandin E2 (2). In human skin, increased COX-2 expression is observed in response to acute UVB irradiation (5). Similarly, in the mouse model, COX-2 is overexpressed in hyperplastic skin, benign tumors, and malignant tumors following chronic UVB irradiation (2). Indeed, celecoxib, a COX-2-selective inhibitor, in dietary or topical treatment in mice following UVB exposure, attenuated the number and volume of tumors (2). Therefore, regulating the expression of COX-2 might be a potential protection strategy against skin cancer.

Cot was initially identified in a human thyroid carcinoma cell line and in moloney murine leukemia virus-induced rat T-cell lymphomas (Tpl2) (6). Stimulation of Cot causes the activation of a p38 MAPK, JNKs, and transcription factors NF-κB and

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‡ The abbreviations used are: COX-2, cyclooxygenase 2; THIF, trihydroxyisoflavone; MKK, mitogen-activated protein kinase kinase; MEM, minimum Eagle’s medium; MOPS, 4-morpholinepropanesulfonic acid.
nuclear factor of activated T cells (7). Numerous studies have shown that Cot mediates cytokine production, such as TNF-α, and leads to several inflammatory diseases, such as rheumatoid arthritis. Recently, several studies have revealed that the activation of Cot is observed in tumor cells, such as T cell neoplasia cell lines (7). Cot also positively regulates COX-2 expression by LPS in macrophages (8). However, the involvement of Cot in UVB-induced COX-2 expression in skin has not been reported.

The mitogen-activated protein kinase kinase 4 (MKK4), a dual-specificity kinase, plays a critical role in the SAPK signaling pathway. The SAPK pathways, including p38 MAPK and JNKs, is activated in response to environmental stressors and extracellular stimuli (9). MKK3 and MKK6 are specific upstream kinases of p38, whereas MKK4 activates both p38 and JNKs (10–12). Phosphorylation of p38 and JNKs mediates the inflammatory response, thereby inducing the eukaryotic transcription factor NF-κB activation (13). In mouse skin, UV irradiation activates p38 and JNKs, which contribute to the development of skin cancer (13). Therefore, regulating the upstream molecules of p38 and JNKs is one possible strategy for the chemoprevention of skin carcinogenesis.

Daidzein and genistein are naturally occurring isoflavones in soy foods. In particular, genistein has received attention as a potential anticarcinogenic compound and has been intensely studied, whereas daidzein and its metabolites have been less well studied. Here, we investigated the chemopreventive effects of 7,3′,4′-trihydroxyisoflavone (THIF), a major metabolite of daidzein, against UVB-induced skin cancer. We report that 7,3′,4′-THIF, but not daidzein, is an ATP-competitive inhibitor of Cot and MKK4, and subsequently suppresses UVB-induced COX-2 expression in JB6 P+ mouse epidermal cells. In a mouse skin tumorigenesis model, 7,3′,4′-THIF strongly suppressed the incidence, multiplicity, and volume of UVB-induced mouse skin tumors. Consistent with the tumor data, 7,3′,4′-THIF clearly attenuated UVB-induced COX-2 expression in hairless mouse skin. Furthermore, 7,3′,4′-THIF directly bound with Cot or MKK4, resulting in the suppression of Cot or MKK4 activity in hairless mouse skin.

**MATERIALS AND METHODS**

**Chemicals—**7,3′,4′-THIF was obtained from the Indofine Chemical Co., Inc. (Hillsborough, NJ), and daidzein was purchased from Sigma. The antibodies against phosphorylated ERKs (Thr-202/Tyr-204), total ERKs, total p38, phosphorylated NF-κB, phosphorylated c-Jun NH2-terminal kinases (JNKs; Thr-183/Tyr-185), and total JNKs were purchased from Cell Signaling Technology (Danvers, MA). The antibody against phosphorylated p38 (Tyr-180/Tyr-182) was from BD Biosciences, and Cot (Thr-209) was purchased from Invitrogen. The Cot1 and MKK4 kinase assay kits were obtained from Cell Signaling Technology. CNBr-Sepharose 4B, glutathione-Sepharose 4B, [γ-32P]ATP, and the chemiluminescence detection kit were purchased from Amersham Biosciences. The protein assay kit was obtained from Bio-Rad. G418 and the luciferase assay substrate were purchased from Promega (Madison, WI).

**Cell Culture—**JB6 P+ mouse epidermal (JB6 P+) cell lines were cultured in monolayers at 37 °C in a 5% CO2 incubator in MEM containing 5% FBS, 2 mM l-glutamine, and 25 µg/ml gentamicin. The cells were stably transfected with an NF-κB luciferase reporter plasmid and maintained in MEM supplemented with 5% FBS containing 200 µg/ml G418.

**UVB Irradiation—**UVB irradiation was carried out in a UVB chamber with a transilluminator emitting UVB light protons and fitted with a Kodakel K6808 filter (Eastman Kodak, Rochester, NY) that eliminates all wavelengths below 290 nm. This lamp is one of the most frequently used UVB sources for the study of skin carcinogenesis. Irradiation energy was measured using a UVX radiometer (UVX-31) from UVP (Upland, CA).

**Luciferase Assay for COX-2 Promoter Activity or NF-κB Transcription Activity—**COX-2 (14) or NF-κB (15, 16) luciferase reporter-transfected JB6 P+ cells were constructed as described earlier. The cells (8 × 105), suspended in 100 µl of 5% FBS/MEM, were added to each well of a 96-well plate and incubated at 37 °C in a humidified atmosphere of 5% CO2. When cells reached 80 to 90% confluence, they were starved in 0.1% FBS/MEM for an additional 24 h. The cells were then treated for 1 h with 7,3′,4′-THIF or daidzein and then exposed to UVB for 24 h. After treatment, cells were disrupted with 100 µl of lysis buffer (0.1 M potassium phosphate buffer (pH 7.8), 1% Triton X-100, 1 mM DTT, 2 mM EDTA), and the luciferase activity was measured using a luminometer (Luminoskan Ascent, Thermo Electron).

**Western Blot Analysis—**After the cells (1.5 × 106) were cultured in a 10-cm dish for 48 h, they were starved in 0.1% FBS/MEM for an additional 24 h. They were then treated with 7,3′,4′-THIF for 1 h before exposure to UVB (4 kJ/m2) and harvested 30 min later. The harvested cells were disrupted, and the supernatant fractions were boiled for 5 min. The protein concentration was determined using a dye-binding protein assay kit according to the manufacturer’s protocol. Lysate protein (30 µg) was subjected to 10% SDS-PAGE and transferred to a polyvinylidine difluoride membrane. After blotting, the membrane was incubated with the specific primary antibody at 4 °C overnight. Protein bands were visualized by a chemiluminescence detection kit after hybridization with a horseradish peroxidase-conjugated secondary antibody.

**In Vitro MKK4 and Cot1 Kinase Assays—**The in vitro kinase assays were performed in accordance with the instructions provided by Cell Signaling Technology. Briefly, for the MKK4 assay, 40 ng/µl active MKK4 recombinant murine protein (55.8% purity and 964 units/mg activity) and 7,3′,4′-THIF (20, 40, or 60 µM) were reacted at 30 °C for 10 min. For each reaction, 5 µl of 2 × kinase buffer (10 mM MOPS (pH 7.2), 5 mM β-glycerol phosphate, 2 mM EGTA, 0.8 mM EDTA, 10 mM MgCl2, 0.1 mM DTT), 5 µl of 250 µM ATP, and 0.2 µg/µl of the inactive JNK2 were added. The mixtures were incubated at 30 °C for 15 min. A 5-µl aliquot was removed from the reaction mixture containing 10 µl of 2 mg/ml of ATF-2 substrate peptide, 5 µl of 2 × kinase buffer, and 5 µl of 0.16 µCi/ml [32P]ATP solution, and incubated at 30 °C for 15 min. Then, 20-µl aliquots were transferred onto p81 filter paper and washed three times with 1% phosphoric acid for 5 min per wash and once with acetone for 5 min. Radioactive incorporation was determined using a scintillation counter (LS6500; Beckman Coulter). Each experiment was performed three times. For the Cot1 assay, 150 ng/µl active Cot1 recombinant human protein (62%
purity and 276 units/mg activity) and 7,3',4'-THIF (20, 40, or 60 μM) were reacted at 30 °C for 10 min. For each reaction, 5 μl of 2× kinase buffer, 1 μg/ml of p65 as substrate, and 5 μl of 0.16 μCi/μl [32P]ATP solution were added. The mixture was reacted at 30 °C for 15 min. Then, 20-μl aliquots were transferred onto p81 filter paper and washed three times with 1% phosphoric acid for 5 min per wash and once with acetone for 2 min. Radioactive incorporation was determined using a scintillation counter (LS6500, Beckman Coulter). Each experiment was performed separately three times.

Ex Vivo MKK4 and Cot Immunoprecipitation and Kinase Assays—JB6 P+ cells were cultured to 80% confluence and then serum-starved in 0.1% FBS/MEM for 24 h at 37 °C. Cells were either treated with 7,3',4'-THIF or left untreated for 1 h, exposed to 4 kJ/m² UVB, harvested after 30 min, disrupted with lysis buffer (20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, 1 mM EGTA, 1 mM Triton X-100, 1 mM β-glycerophosphate, 1 mg/ml leupeptin, 1 mM Na3VO4, 1 mM PMSF), and finally centrifuged at 14,000 rpm for 10 min in a microcentrifuge. The lysates, each containing 500 μg of protein, were used for immunoprecipitation with an antibody against MKK4 or Cot and then incubated at 4 °C for 4 h. Protein A/G Plus-agarose beads were then added, and the mixture was continuously rotated overnight at 4 °C. The beads were then washed three times with kinase buffer (20 mM MOPS (pH 7.2), 25 mM β-glycero phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM DTT), and then a kinase reaction was performed in the same manner as for in vitro kinase assays.

In Vitro and ex Vivo Pull-down Assays—Recombinant MKK4 or Cot1 (2 μg) or a JB6 P+ cellular supernatant fraction (500 μg protein) was incubated with 7,3',4'-THIF-Sepharose 4B (or Sepharose 4B as a negative control) beads (100 μl, 50% slurry) in reaction buffer (50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM EGTA, 0.01% Nonidet P-40, 2 μg/ml BSA, 0.02 mM PMSF, 1× protease inhibitor mixture). After incubation with gentle rocking overnight at 4 °C, the beads were washed five times with buffer (50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, 0.02 mM PMSF), and the proteins bound to the beads were analyzyed by immunoblotting.

Mouse Skin Tumorigenesis Analysis—SKH-1 hairless mice (5 weeks of age; mean body weight, 25 g) were purchased from the Institute of Laboratory Animal Resources at Seoul National University. ICR mice were maintained under the same conditions as SKH-1 hairless mice described above. The dorsal areas of ICR mice were shaved before sample treatment. Each group was topically treated with 7,3',4'-THIF (40 or 160 nmol) or daidzein (160 nmol) in 200 μl of acetone 1 h before UVB (0.5 J/cm²) irradiation. The protein extract for Western blot analysis was prepared as described above.

In Vivo Kinase and Pull-down Assays—For the in vivo Cot or MKK4 immunoprecipitation and kinase assay, mice were treated with 7,3',4'-THIF (10 or 40 nmol) in 200 μl of acetone, and dorsal skin was prepared as for in vivo Western blotting. Proteins were extracted as above and centrifuged at 12,000 rpm for 20 min. 700 μg of protein from mouse skin extract was mixed with protein-A/G beads (20 μl) for 1 h at 4 °C. The mixture was processed, and radioactive incorporation was determined as for the ex vivo assay described above. Data are presented as the mean of data points from five mice in each group.

For the in vivo pull-down assay, mice received a topical application of 200 μl of acetone alone or 7,3',4'-THIF (10 or 40 nmol, respectively) in 200 μl of acetone on their backs 1 h before UVB irradiation. Dorsal skin was prepared as described above for the in vivo Western blotting, and proteins were extracted as described above for the Cot or MKK4 immunoprecipitation and kinase assays. Then 500 μg of protein from mouse skin extract were incubated with 7,3',4'-THIF-Sepharose 4B (or Sepharose 4B alone as a control) beads (100 μl, 50% slurry) in reaction buffer as described for the ex vivo pull-down assay. Beads were incubated and washed, and proteins bound to the beads were analyzed by Western blotting as described above.

Molecular Modeling—The homology model structures of Cot and MKK4 were generated by Geno3D using the coordinates of Mst1 and MKK7 (PDB codes 3COM and 2DYL), respectively. Insight II (Accelrys, Inc., San Diego, CA) was used for the docking study and structure analysis.

Statistical Analysis—When necessary, data were expressed as mean ± S.D., and analysis of variance was used for multiple
A probability value of \( p < 0.05 \) was used as the criterion for statistical significance.

**RESULTS**

7,3’,4’-Trihydroxyisoflavone (7,3’,4’-THIF) suppresses skin cancer by inhibiting UVB-induced COX-2 expression by suppressing COX-2 promoter activity and NF-κB transcriptional activity in JB6 P + cells. The cells were treated with 7,3’,4’-THIF or daidzein at the indicated concentrations for 1 h before exposure to UVB (4 kJ/m²) and harvested 4 h later. The levels of COX-2 expression were then determined by Western blot analysis, as described under “Materials and Methods,” using specific antibodies against the corresponding COX-2 and β-actin proteins.

For statistical comparisons, a probability value of \( p < 0.05 \) was used as the criterion for statistical significance.

**RESULTS**

7,3’,4’-THIF Inhibits UVB-induced COX-2 Expression by Suppressing COX-2 Promoter Activity and NF-κB Transcriptional Activity in JB6 P + Cells, Whereas Daidzein Has No Effect—As aberrant COX-2 expression is intimately involved in multiple malignancies, including skin cancer (17), we first examined the possible inhibitory effects of 7,3’,4’-THIF on UVB-induced COX-2 expression in JB6 P + cells. Treatment with 7,3’,4’-THIF dose-dependently inhibited UVB-induced COX-2 expression in these cells (Fig. 1B, left panel), whereas daidzein, even up to 60 μM, had no effect (Fig. 1B, right panel). Consistent with the Western blotting results, 7,3’,4’-THIF strongly suppressed UVB-induced COX-2 promoter activity in JB6 P + cells stably transfected with a COX-2 luciferase reporter plasmid in a dose-dependent manner (Fig. 1C, left panel), but daidzein had no effect (Fig. 1C, right panel).
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and p38 pathways stimulated by UVB in JB6 P

**NF-κB** is an important transcription factor regulating COX-2 expression in the skin (15). Thus, to investigate whether 7,3′,4′-THIF down-regulates UVB-induced COX-2 expression through the inhibition of the NF-κB transcription factor, we measured NF-κB transcription activity by using cells stably transfected with the NF-κB luciferase reporter plasmid. 7,3′,4′-THIF significantly attenuated UVB-induced NF-κB transcription activity (Fig. 1D, left panel). Compared with daidzein, treatment with 7,3′,4′-THIF at low concentrations inhibited NF-κB transcription activity more effectively (Fig. 1D, right panel).

**7,3′,4′-THIF Suppresses UVB-induced Phosphorylation of JNKs and p38 MAPK in JB6 P+ Cells**—JNKs and p38 are generally referred to as stress-activated MAP kinases. We examined whether 7,3′,4′-THIF blocks the activation of the JNKs and p38 pathways stimulated by UVB in JB6 P+ cells. Western blotting data showed that 7,3′,4′-THIF inhibited UVB-induced phosphorylation of JNKs and p38 dose-dependently but had no effect on the phosphorylation of ERKs (Fig. 2A, left panels).

Also, 7,3′,4′-THIF did not affect IκB kinase beta activity (data not shown). Recently, several studies indicated that stimulation of Cot leads to the activation of JNKs, p38, and NF-κB (7). MKK3 and MKK6 activate p38, whereas MKK4 can activate both JNKs and p38 (18). Thus, we further investigated the effects of 7,3′,4′-THIF on the upstream regulatory proteins of the JNKs and p38 pathways. Our results revealed that 7,3′,4′-THIF inhibited both Cot and MKK4 activity in vitro (Fig. 2B, upper panels), but it did not affect the phosphorylation level of MKK4 or Cot in JB6 P+ cells (Fig. 2A, right panels). Consistent with the results from the in vitro kinase assay, an ex vivo kinase assay also revealed that 7,3′,4′-THIF inhibited UVB-induced MKK4 and Cot activity in JB6 P+ cells (Fig. 2C). In contrast, 7,3′,4′-THIF had no effect on either the phosphorylation level of MKK3/6 (Fig. 2A, right panels) or the in vitro kinase activity of MKK3 and MKK6 (Fig. 2B, lower panels). We next examined whether 7,3′,4′-THIF had an effect on the activity of various MAP kinases. The in vitro kinase assay showed that 7,3′,4′-THIF had no effect on ERKs, p38α, or JNK1 kinase activity as

![FIGURE 2. 7,3′,4′-THIF suppresses UVB-induced phosphorylation of JNKs and p38 through the direct inhibition of both Cot and MKK4. A, 7,3′,4′-THIF inhibited UVB-induced phosphorylation of JNKs and p38 in JB6 P+ cells. Cells were treated with 7,3′,4′-THIF (0.20, 40, or 60 μM) for 1 h before being exposed to UVB (4 kJ/m²) and harvested 30 min later. The cells were disrupted, and the levels of phosphorylated and total proteins were determined by Western blot analysis, as described under “Materials and Methods,” using specific antibodies against the respective phosphorylated and total proteins. Data are representative of three independent experiments that gave similar results. B, 7,3′,4′-THIF inhibited both Cot and MKK4 in vitro. In contrast, MKK3 and MKK6 activity was not affected by 7,3′,4′-THIF. The in vitro kinase assay was performed as described under “Materials and Methods,” and kinase activity is expressed as percent inhibition relative to the activity of the untreated kinase control. C, 7,3′,4′-THIF inhibited both Cot and MKK4 activity ex vivo. In the ex vivo Cot or MKK4 kinase assay, cells were pretreated with 7,3′,4′-THIF at the indicated concentrations (0, 20, 40, or 60 μM) for 1 h and then exposed to UVB (4 kJ/m²) and harvested after 30 min. Cells were used for immunoprecipitation, and the kinase assay was performed. Kinase activity is expressed as percent inhibition relative to the activity of UVB-treated cells. The average 32P count was determined from three separate experiments, and the data are presented as mean ± S.D. In the ex vivo kinase assays, the asterisks indicate a significant decrease in kinase activity between the groups treated with 7,3′,4′-THIF and cells treated with UVB only. **, p < 0.01; ***, p < 0.001.

D, 7,3′,4′-THIF did not affect ERKs or MSK1 activity in vitro. The in vitro kinase assay was performed as described under “Materials and Methods,” and kinase activity is expressed as percent inhibition relative to the activity of the untreated kinase control. For the in vitro kinase assays (B and D), the asterisks indicate a significant decrease in kinase activity between the groups treated with active Cot (or MKK4) and 7,3′,4′-THIF and the group treated with active Cot (or MKK4) alone. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

![FIGURE 3. 7,3′,4′-THIF directly binds with Cot and MKK4. A, 7,3′,4′-THIF specifically binds with Cot or MKK4 in vitro. The Cot (or MKK4) 7,3′,4′-THIF binding was confirmed by immunoblotting using an antibody against Cot (left panel) or MKK4 (right panel). 1st lane (input control), Cot, or MKK4 protein standard; 2nd lane (control), Sepharose 4B was used to pull down Cot or MKK4, as described under “Materials and Methods;” 3rd lane, 7,3′,4′-THIF-Sepharose 4B affinity beads were used to pull down Cot or MKK4. B, 7,3′,4′-THIF directly binds with either Cot and MKK4 ex vivo. Binding was confirmed by immunoblotting using an antibody against Cot (left panel) or MKK4 (right panel). 1st lane (input control), whole cell lysates from JB6 P+ cells; 2nd lane (control), a lysate of JB6 P+ cells precipitated with Sepharose 4B beads; 3rd lane, whole cell lysates from JB6 P+ cells precipitated with 7,3′,4′-THIF-Sepharose 4B affinity beads. C, 7,3′,4′-THIF competes with ATP to bind with Cot and MKK4. Active Cot or MKK4 (0.2 μg) was incubated with ATP at different concentrations (0, 10, or 100 μM) and 100 μl of 7,3′,4′-THIF-Sepharose 4B or 100 μl of Sepharose 4B (as a negative control) in reaction buffer to a final volume of 500 μl. The mixtures were incubated at 4 °C overnight with shaking. After washing, the pulldown proteins were detected by Western blotting. 1st lane (input control), Cot or MKK4 protein standard; 2nd lane, (negative control), Cot or MKK4 bound with Sepharose 4B; 3rd lane (positive control), Cot (left panel) or MKK4 (right panel) binding with 7,3′,4′-THIF-Sepharose 4B. Each experiment was performed three times, and representative blots are shown.

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A

Control
UVB (0.18 J/cm²)

7,3′,4′-THIF (10 nmol)
7,3′,4′-THIF (40 nmol)

B

% of tumor bearing mice

15 20 25 27

UVB (0.18 J/cm²)
7,3′,4′-THIF 10 nmol
7,3′,4′-THIF 40 nmol

C

No. of tumors per mouse

15 20 25 30

UVB (0.18 J/cm²)
7,3′,4′-THIF 10 nmol
7,3′,4′-THIF 40 nmol

D

Tumor volume (mm³)

10 40

UVB (0.18 J/cm²)
7,3′,4′-THIF (10 nmol)
7,3′,4′-THIF (40 nmol)

FIGURE 4. 7,3′,4′-THIF Inhibits UVB-induced Skin Carcinogenesis in the SKH-1 Hairless Mouse. Control mice (n = 12) received a topical treatment of 200 µl acetone (no UVB), and experimental mice (n = 12) were treated with 200 µl acetone before UVB (0.18 J/cm²) exposure (3 days/week for 27 weeks). The mice in the third and fourth groups received a topical application of 7,3′,4′-THIF (10 or 40 nmol, respectively, per mouse in 200 µl acetone) on the dorsal surface 1 h before UVB (0.18 J/cm²) irradiation 3 days/week for 27 weeks. The incidence of skin tumors was recorded weekly, and tumors were defined as an outgrowth of >1 mm in diameter persisting for 2 weeks or longer. Tumor incidence and multiplicity were recorded every week until the end of the experiment at 27 weeks. A, appearance of skin tumors. B, 7,3′,4′-THIF retarded the incidence of skin tumors compared with the UVB-only treated group. C, 7,3′,4′-THIF strongly inhibits UVB-induced tumor multiplicity in SKH-1 hairless mice. D, 7,3′,4′-THIF reduces UVB-induced tumor volume in SKH-1 hairless mice. At the end of the study, the dimensions of all tumors on each mouse were recorded. Tumor volumes were calculated using the hemiellipsoid model formula: tumor volume = 1/2(4/3)(π/2)(l/2)(w/2)(h), wherein l is length, w is width, and h is height. The asterisks indicate a significant decrease in tumor incidence or volume between the group treated with UVB alone and the group treated with 7,3′,4′-THIF. **, p < 0.05; ***, p < 0.001.

well as no effect on activity of MSK1, a downstream protein of p38 and ERKs (Fig. 2D). These results indicate that the inhibition of UVB-induced COX-2 expression by 7,3′,4′-THIF was mainly caused by the suppression of both Cot and MKK4 activity.

7,3′,4′-THIF Directly Binds with Either Cot or MKK4 Competitively Binds with ATP—To determine whether the inhibition of Cot1 and MKK4 activities by 7,3′,4′-THIF was caused by direct interaction, we performed an in vitro pull-down assay. We detected Cot1 or MKK4 in 7,3′,4′-THIF-Sepharose 4B beads but not in Sepharose 4B beads alone (Fig. 3A). We also observed ex vivo binding between 7,3′,4′-THIF and Cot or MKK4 in JB6 P+ cell lysates (Fig. 3B). However, 7,3′,4′-THIF did not bind with MKK3 (data not shown). ATP treatment blocked the binding ability of 7,3′,4′-THIF with Cot1 and MKK4 in a dose-dependent manner, suggesting that 7,3′,4′-THIF binds with Cot1 or MKK4 in competition with ATP (Fig. 3C). These results indicate that the inhibition of Cot1 or MKK4 activity by 7,3′,4′-THIF occurs through the direct binding of 7,3′,4′-THIF with both proteins.

7,3′,4′-THIF Represses UVB-induced Skin Tumorigenesis in the SKH-1 Hairless Mouse Model—To evaluate the chemopreventive activity of 7,3′,4′-THIF in vivo, we studied the effects of 7,3′,4′-THIF in the two-stage, UVB-induced skin carcinogenesis model. Data show that 7,3′,4′-THIF treatment suppressed UVB-induced skin cancer development in mice (Fig. 4A). Tumor incidence was also clearly attenuated by topical application of 7,3′,4′-THIF (10 or 40 nmol) prior to UVB irradiation (Fig. 4B). In the UVB-only treated mouse group, the onset of tumors was observed at the 16th week and reached 100% by the 20th week. However, in the 10- or 40-nmol 7,3′,4′-THIF-treated mouse groups, the onset of tumors was delayed until the 17th or 19th week and approached 100% only by the 24th week. Topical application of 7,3′,4′-THIF (10 or 40 nmol) prior to UVB irradiation also clearly suppressed tumor multiplicity throughout the duration of the experiment (Fig. 4C). Furthermore, the volume of tumors was markedly suppressed by 7,3′,4′-THIF treatment (Fig. 4D). Collectively, these results reveal that 7,3′,4′-THIF may serve as an effective chemopreventive agent against UVB-mediated skin tumorigenesis.

7,3′,4′-THIF Inhibits UVB-induced COX-2 Expression and Cot and MKK4 Activity by Directly Binding with Cot or MKK4 in SKH-1 Hairless Mouse Skin—To further confirm the inhibitory effect of 7,3′,4′-THIF on UVB-induced COX-2 expression in an in vivo model, we examined the level of COX-2 expression in SKH-1 hairless mouse skin. Consistent with the results from the JB6 P+ cells, the Western blotting data showed that the levels of COX-2 expression in the 7,3′,4′-THIF-treated groups
**DISCUSSION**

Isoflavones are most abundant in soybeans but also exist in significant amounts in various other beans, legumes, sprouts, and clover. In plants, isoflavones are present as glycoside compounds, and on ingestion, the aglycons are easily detached from glucoside following reductive metabolism by intestinal bacteria (19). Several cellular and mouse studies have reported that soy extract (17) or isoflavones (20) have a photoprotective effect in skin. Recently, numerous studies have shown that isoflavones are subject to oxidative bio-transformation in the rat liver (21) and in humans (22) through hepatic metabolism. One of the major products of the hepatic metabolism of daidzein is 7,3',4'-THIF (19). However, the effects of this hepatic metabolite on UVB-induced skin cancer have not been reported. We found that 7,3',4'-THIF had a stronger inhibitory effect on UVB-induced COX-2 expression in JB6 P+ cells than did 6,7,4'-THIF, another metabolite of daidzein (data not shown). In contrast, daidzein had no effect on UVB-induced COX-2 expression in JB6 P+ cells. In the present study, we demonstrated a strong chemopreventive effect of 7,3',4'-THIF on
UVB-induced skin cancer and suggested a molecular mechanism and targets.

The aberrant expression of COX-2 is a property of epithelial cancers, including skin cancer, in mice and humans (23). Moreover, COX-2-deficient mice have a reduced tumor incidence and multiplicity response to 7,12-dimethylbenz(a)anthracene and 12-O-tetradecanoylphorbol-13-acetate treatment (24, 25).

Therefore, the development of a natural inhibitor suppressing the aberrant expression of COX-2 is a promising strategy in the chemoprevention of skin cancer. Our results showed that 7,3',4'-THIF exerts potent anti-tumor and anti-inflammatory effects. NF-κB is an important transcription factor in tumor-promoting processes such as inflammation and proliferation, and it is an important factor in the development of skin cancer.
Two estimated NF-κB binding sites are present in the promoter region of COX-2, and COX-2 expression is positively regulated by NF-κB. Our results showed that 7,3',4'-THIF inhibits UVB-induced COX-2 expression by attenuating COX-2 promoter activity, which was attributed to the inhibition of NF-κB transcription activation.

Research data indicate that MAP kinase phosphorylation triggers signals from the cell surface, activating various transcription factors, thereby contributing to the regulation of target gene expression. JNKs and p38 are generally recognized as stress-activated MAP kinases. Several kinds of MKKs can phosphorylate JNKs, and p38-MKK3 and MKK6 activate p38, whereas MKK4 can activate both JNKs and p38 MAPK (18). Increased levels of MKK4 are positively related to the increased proliferation and invasion of several cancer cell lines (26). Our results showed that 7,3',4'-THIF suppressed UVB-induced phosphorylation of p38 and JNKs and was also effective at inhibiting MKK4 activity in vitro and ex vivo, resulting in the inhibition of COX-2 expression.

The serine/threonine protein kinase Cot has been studied mainly as a mediator of the inflammation or macrophage signaling pathways (27, 28). Recent studies showed that Cot is up-regulated in a variety of cancers and that Cot positively regulates COX-2 expression and neoplastic cell transformation through transcriptional transactivation. Our previous study showed that Cot represents a novel serine/threonine kinase directly interacting with histone H3, resulting in the increased cell transformation (29). Cot regulates NF-κB transcriptional activity by directly binding to p65 and triggering its phosphorylation (30). Our preliminary data showed that the Cot inhibitor down-regulated UVB-induced COX-2 expression by suppressing COX-2 promoter activity and NF-κB transcription activity (data not shown). We then examined whether 7,3',4'-THIF affected Cot activity, and the results showed that 7,3',4'-THIF clearly suppressed Cot1 activity in vitro and ex vivo by directly binding with Cot1 competitively with ATP. Taken together, these results indicate that the inhibition of COX-2 expression by 7,3',4'-THIF was attributable to the suppression of Cot and MKK4 activity.

The SKH-1 hairless mouse is an excellent model to study the appearance of skin tumors induced by chronic UV radiation (31). The topical application of 7,3',4'-THIF strongly suppressed the incidence, multiplicity, and volume of UVB-induced skin cancer development in the SKH-1 hairless mouse model. Induction of COX-2 expression after skin exposure to UVB was substantial, and 7,3',4'-THIF strongly inhibited the UVB-induced COX-2 expression in hairless mouse skin. Furthermore, 7,3',4'-THIF directly bound with Cot or MKK4, resulting in suppression of Cot or MKK4 activity in both JB6 P+ cells and hairless mouse skin. Collectively, these results suggest that 7,3',4'-THIF suppresses UVB-induced skin cancer by directly targeting Cot and MKK4.

To investigate the molecular basis of Cot and MKK4 inhibition by 7,3',4'-THIF, we carried out a docking study using the homology model structures of the kinase domain of Cot, derived from the crystal structure of Mst1, which has 50% homology in amino acid sequence, and MKK4, derived from the crystal structure of MKK7 that has 66% homology. Homology modeling was used because neither the structure of Cot nor MKK4 is available as yet. The kinase domains of Cot and MKK4 consist of an N-lobe and a C-lobe. The N- and C-lobes are linked through a loop, which is called a "hinge region." The backbone of this loop interacts with the adenine moiety of ATP through hydrogen bonding. Considering the experimental result showing that 7,3',4'-THIF is an ATP-competitive inhibitor of Cot and MKK4, we docked the compound to the ATP binding site of each of the two kinases. 7,3',4'-THIF easily docked to the Cot or MKK4 ATP binding site, which is located between the N- and C-lobes of the kinase domain. In the model structure of Cot, complexed with 7,3',4'-THIF, the hydroxyl group at the 3' and 4' positions of 7,3',4'-THIF can form hydrogen bonds with the backbone of Glu-208 and Gly-210 in the hinge region of Cot (Fig. 6A). The hydroxyl group at the 7 position could form a hydrogen bond with the backbone carbonyl group of Gln-173. In addition, the inhibitor would be sandwiched in the ATP binding site by the side chains of the hydrophobic residues, including Ala-165, Met-207, and Val-152 from the N-lobe and Met-262, Val-260, and Val-269 from the C-lobe. In the model structure of MKK4, complexed with 7,3',4'-THIF, the hydroxyl groups at the 3' and 4' positions on 7,3',4'-THIF could form hydrogen bonds with the backbone of Glu-179 and Met-181 in the hinge region of MKK4 (Fig. 6B). The carbonyl group at the 4 position could make a hydrogen bond with the side chain of Lys-187. 7,3',4'-THIF could form van der Waals interactions at the ATP binding site with hydrophobic residues, including Ala-120, Met-178, Ile-108, Val-116, Cys-156, and Leu-236. Because of the lack of a hydroxyl group at the 3' position of daidzein, its interaction with the hinge regions of Cot and MKK4 would be weaker than that of 7,3',4'-THIF, and thus daidzein could not effectively inhibit either of these kinases. Further studies by x-ray crystallography to determine the inhibitor complex structures could elucidate the exact binding mode of 7,3',4'-THIF to Cot and MKK4.

In summary, 7,3',4'-THIF, but not daidzein, inhibits UVB-induced COX-2 expression in JB6 P+ cells. The inhibition is mediated mainly by the blockage of the JNKs and p38 pathways activation and subsequent suppression of NF-κB activity. 7,3',4'-THIF binds with Cot and MKK4 and strongly inhibits their kinase activity. The chemopreventive effect of 7,3',4'-THIF was confirmed in the hairless mouse model because the in vivo data showed that 7,3',4'-THIF strongly suppressed UVB-induced tumor incidence, multiplicity, and tumor volume. Consistent with the tumor data, 7,3',4'-THIF clearly inhibited UVB-induced COX-2 expression in hairless mouse skin, and 7,3',4'-THIF directly bound with Cot or MKK4, resulting in the suppression of Cot or MKK4 activity in hairless mouse skin. Collectively, these results suggest that Cot and MKK4 are the main molecular targets of 7,3',4'-THIF in the suppression of UVB-mediated skin cancer. These results provide insight into the biological actions of 7,3',4'-THIF and the molecular basis for the development of new chemoprotective agents.

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