Src kinase is a direct target of apigenin against UVB-induced skin inflammation

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Apigenin, a flavonoid abundant in various vegetables and fruits, including parsley and onions, has been reported to possess anti-carcinogenic effects. However, the direct molecular target of apigenin and its chemopreventive effect on ultraviolet (UV)-induced skin inflammation are not understood fully. Herein, we examined the anti-inflammatory effect of apigenin and its associated mechanisms in JB6 P+ cell line and SKH-1 hairless mouse model. Apigenin inhibited UVB-induced cyclooxygenase-2 (COX-2) expression, which is a well-known key mediator of inflammation and cancer, and restored the upstream stimulatory factor level in JB6 P+ cells. Immunoblot and kinase assay data demonstrate that Src activity was attenuated by apigenin, and this led to subsequent inhibition of UVB-induced phosphorylation of epidermal growth factor receptor, mitogen-activated protein kinases and Akt signaling. Inhibitory effects of apigenin on UVB-induced signaling were also confirmed in HaCaT human keratinocytes. In addition, in vitro pull-down assays revealed that apigenin binds Src in an adenosine triphosphate-competitive manner. Results using in vivo skin model indicate apigenin significantly inhibits UVB-induced ear edema development, COX-2 expression and Src kinase activity in SKH-1 hairless mice. Collectively, these findings suggest that apigenin exerts potent chemopreventive activity against UVB-induced skin inflammation primarily by targeting Src.

Introduction

Ultraviolet (UV) rays from the sun are well-characterized environmental human carcinogens. Chronic exposure to UV irradiation causes photoaging, inflammation, and ultimately, photocarcinogenesis in human and murine skin (1). Among the UV components, UVB irradiation (290–320 nm) functions as the strongest carcinogen and causes inflammation, DNA damage and cancer in skin (2–4). Several groups have reported that UVB irradiation affects various cellular signaling pathways that are responsible for skin inflammation, including mitogen-activated protein kinase (MAPK) signaling, PI3K/Akt signaling, activator protein-1, nuclear factor-kappa B, upstream stimulatory factor (USF) and cyclic adenosine monophosphate response element binding protein (CREB) (5–9).

Abbreviations: ATP, adenosine triphosphate; COX-2, cyclooxygenase-2; CREB, cyclic adenosine monophosphate response element binding protein; EGFR, epidermal growth factor receptor; FAK, focal adhesion kinase; FBS, fetal bovine serum; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEM, modified Eagle’s medium; USF, upstream stimulatory factor; UV, ultraviolet.

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Cyclooxygenase-2 (COX-2) is a key enzyme in the synthesis of prostaglandins from arachidonic acid (10). Increased production of COX-2 induced by UVB irradiation causes inflammation, cell proliferation, tumor promotion and angiogenesis (11,12). COX-2 overexpression has been observed in several cancers, including skin, colon, stomach, pancreas, lung and breast (13). Hence, UVB-mediated signaling pathways that induce the COX-2 expression could be targets for preventing skin inflammation (14).

Src, a non-receptor tyrosine kinase, has been implicated in regulation of inflammation, differentiation, invasion and angiogenesis (15). Elevated Src kinase activity is responsible for skin tumor promotion, malignancy progression and metastasis (16). Previous studies have demonstrated Src and epidermal growth factor receptor (EGFR) overexpression in many of the same types of tumors, indicating these kinases interact and eventually promote tumourigenesis (17,18). Activated Src kinase and EGFR induce intracellular signaling cascades, predominantly Ras/Raf/MEK/ERK/extracellular signal–regulated kinase (ERK) and PI3K/Akt, and subsequently cause inflammation, proliferation, migration and apoptosis (19). Thus, a strategy that targets elevated Src kinase activity may be an effective approach for preventing skin inflammation and cancer.

Apigenin (4’,5,7-trihydroxyflavone) is a naturally occurring plant flavone that is abundant in various fruits and vegetables, including guava, orange, parsley, onion, chamomile and chili peppers (20,21). Previous studies have shown that apigenin possesses anti-inflammatory, antioxidant and anticarcinogenic properties (20,22–24). In particular, apigenin suppresses UVB-induced COX-2 in skin by regulating USF transcription factors and messenger RNA translation (9,24). However, the upstream direct molecular target of apigenin in regulating skin inflammation and cancer is not fully identified. Herein, we demonstrate that apigenin exerts chemopreventive effects on UVB-induced COX-2 and skin inflammation in JB6 P+ mouse epidermal cells and SKH-1 hairless mice by directly suppressing Src kinase activity.

Materials and methods

Materials

Apigenin (≥95%), fetal bovine serum (FBS) and the antibody against β-actin were purchased from Sigma–Aldrich (St Louis, MO). Modified Eagle’s medium (MEM), gentamicin and t-glutamine were obtained from Life Technologies (Carlsbad, CA). The antibody against COX-2 was from Cayman (Ann Arbor, MI). Antibodies to detect the phosphorylated forms of c-Raf, Akt, EGFR, p90RSK, p70S6K, MSK, MEK, MKK3/6, MKK4, Src, and total Akt, p38, c-Jun-N terminal kinase 1/2 (JNK1/2), MKK3, MEK, p90RSK and p70S6K were purchased from Cell Signaling Biotechnology (Danvers, MA). Antibodies against phosphorylated ERK1/2 and total ERK1/2, USF-2 phosphorylated JNK1/2, c-Raf, EGFR, MSK and MKK4 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The active Src, the Ras assay reagent and antibodies against phosphorylated CREB, phosphorylated focal adhesion kinase (FAK), Src, CREB and FAK were purchased from Upstate Biotechnology (Billerica, MA). Antibody against USF-1 was purchased from Epitomics (Burlingame, CA). Antibody against total Ras and the inhibitor AG1478 were purchased from Calbiochem (Darmstadt, Germany). The inhibitor PP2 was purchased from Tocris Bioscience (Ellisville, MO). C yanogen bromide (CNBr)–sepharose 4B, glutathione–sepharose 4B, [γ-32P] adenosine triphosphate (ATP) and the chemiluminescence detection kit were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Tissue protein extraction reagent was purchased from Thermo Scientific (Lafayette, CO). The protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA), and G418 and the luciferase assay substrate were purchased from Promega (Madison, WI).

Cell culture

The JB6 P+ mouse epidermal cell line and HaCaT human keratinocyte cell line were maintained at 37°C in a humidified atmosphere of 5% CO2 in 5% FBS–MEM and 10% FBS–Dulbecco’s modified Eagle’s medium, respectively.
Luciferase assay for cox-2 activation

COX-2 luciferase reporter-transfected JB6 P+ cells were constructed as described previously (25). The cells were treated for 1 h with apigenin (10, 20 or 40 µM) before exposure to UVB (0.05 J/cm²) and then were incubated for 6 h. Cells were disrupted with lysis buffer, and luciferase activity was measured using a luminometer (Luminoskan Ascent; Thermo Electron, Helsinki, Finland).

Immunoblot assays

Total cell lysates and mouse skin lysates were prepared and subjected to western blot as described previously (25). After cell lysis, the protein concentration was determined using an active Src and EGFR kinase assay (Bio-Rad) as described in the manufacturer’s manual.

Ras activation assay

The Ras activation assay was carried out in accordance with the instructions provided by Upstate Biotechnology. In brief, 1 mg of cell lysates was incubated for 1 h at 4°C with beads coated with a fusion protein consisting of glutathione-S-transferase fused to the Ras-binding domain of Raf-1. After removing the supernatant, beads were washed five times with Mg-containing lysis buffer. The bound proteins were eluted by 2× Laemmli sample buffer, and all samples were subjected to immunoblotting with anti-pan-Ras antibody.

Kinase assays

The in vitro kinase assay was carried out in accordance with the instructions provided by Upstate Biotechnology. In brief, the Src and EGFR kinase assay was performed using an active Src and EGFR protein, Src and EGFR substrate peptide contained in the assay buffer and [γ-32P] ATP solution diluted with magnesium–ATP cocktail buffer. The Src and EGFR kinase assay mixtures were each incubated at 30°C and then aliquots were transferred onto p81 paper and washed with 0.75% phosphoric acid. The radioactive incorporation was determined using a scintillation counter. The effect of apigenin (10, 20 or 40 µM) was evaluated by incubating apigenin with the Src or EGFR kinase reaction mixtures at 30°C for 10 min. Each experiment was performed three times.

For the in vivo Src immunoprecipitation and kinase assay, skin proteins were extracted with tissue protein extraction reagent by Thermo Scientific (Rockford, IL). Mouse skin extracts were mixed with a Src antibody and then sepharose 4B beads were added and gently rocked overnight at 4°C. These tubes were centrifuged and the pellets were washed. Radioactive incorporation was determined as for the kinase assay described above.

Preparation of apigenin–sepharose 4B beads

Sepharose 4B powder was suspended in 1 mM HCl and apigenin was added to the coupling solution (0.1 M NaHCO₃ and 0.5 M NaCl) and mixed on a rotary shaker at 4°C overnight. The procedure was performed as reported earlier (26).

Pull-down assays

For the in vitro pull-down assay, Src was incubated with apigenin–sepharose 4B (or sepharose 4B alone as a control) beads in reaction buffer. After incubation, the beads were washed five times with washing buffer. Proteins bound to the beads were analyzed by immunoblotting.

ATP and apigenin competition assay

Active Src was incubated with 100 µl of apigenin–sepharose 4B or sepharose 4B beads in reaction buffer (see pull-down assay) for 12 h at 4°C, and ATP was added at different concentrations (10 or 100 µM) to a final volume of 500 µl. The samples were washed, and proteins were detected by immunoblotting.

RNA interference

Cells were grown in 60 or 100 mm dishes and transfected with either a Src-specific small interfering RNA oligonucleotide (si-Src; Cat no: MSS238007, MSS238008 and MSS238009; Invitrogen, Grand Island, NY) or scrambled oligonucleotides (si-scrambled; Cat no: RSN-1001 Bioneer, Daejeon, South Korea) using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instructions.

Animals

All animal procedures were conducted in accordance with guidelines provided by Seoul National University. Female SKH-1 hairless mice (5 week old; mean body weight, 25 g) were purchased from the Institute of Laboratory Animal Resources at Seoul National University (Seoul, Korea). Animals were acclimated for 1 week prior to the study and had free access to food and water. The animals were housed in climate-controlled quarters (24°C at 50% humidity) with a 12 h light/dark cycle.

Short-term in vivo model

Skin inflammation was induced in mice utilizing a UVB irradiation system. The UVB light source (Bio-Link crosslinker; Vilber Lourmat, Torcy, France) emitted wavelengths with peak emission at 312 nm. Treatment groups were divided into five groups of five animals each. In the control group, ear and dorsal skin of mice were topically treated with 50 and 200 µl of acetone, respectively. In the UVB group, ear and dorsal skin of mice were topically treated with 50 and 200 µl of acetone, respectively, after UVB (0.18 J/cm²) exposure once every 2 days for total of four times. In the third group, ear and dorsal skin of mice were topically treated with 7.5 nmol of apigenin in 50 µl and 30 nmol of apigenin in 200 µl of acetone, respectively, after UVB (0.18 J/cm²) exposure once every 2 days for total of four times. In the fourth group, ear and dorsal skin of mice were topically treated with 30 nmol of apigenin in 50 µl and 120 nmol of apigenin in 200 µl of acetone, respectively, after UVB (0.18 J/cm²) exposure once every 2 days for total of four times. In the fifth group, ear and dorsal skin of mice were topically treated with 30 nmol of PP2 in 50 µl and 120 nmol of PP2 in 200 µl of acetone, respectively, after UVB (0.18 J/cm²) exposure once every 2 days for total of four times. Both ears of mice were punched at a diameter of 4.2 mm for measurement of ear edema.

Statistical analysis

When necessary, data are expressed as means ± SD, and analysis of variance was used for multiple statistical comparisons. A probability value of P < 0.05 was used as the criterion for statistical significance. All analyses were performed using statistical analysis software.

Results

Apigenin inhibits UVB-induced COX-2 expression and cox-2 promoter activity and modulates transcription factors in JB6 P+ cells

We first examined the possible inhibitory effects of apigenin on UVB-induced COX-2 expression in JB6 P+ cells. Apigenin dose-dependently inhibited UVB-induced COX-2 protein expression (Figure 1B). COX-2 luciferase promoter activity induced by UVB irradiation was also attenuated by apigenin (Figure 1C). As reported previously (9), apigenin restored USF-1, USF-2 and phosphorylated CREB to control levels (Figure 1D). Effect of apigenin on UVB-induced COX-2 and USF-1/2 were similarly observed in HaCaT human keratinocytes (Supplementary Figure 1A, available at Carcinogenesis Online).

Apigenin suppresses UVB-induced phosphorylation of MAPKs and Akt in JB6 P+ cells

COX-2 is upregulated by MAPKs and Akt in UVB-induced signaling (8,27). UVB irradiation (0.05 J/cm²) induced phosphorylation of ERKs, p38, JNKs and Akt was inhibited by apigenin in a dose-dependent manner (Figure 2A). Furthermore, apigenin significantly inhibited UVB-induced phosphorylation of kinases downstream of MAPKs and Akt (Figure 2B).

Apigenin inhibits UVB-induced EGRF signaling, but not Src phosphorylation, in JB6 P+ cells

To identify its upstream molecular targets in UVB-induced signaling, we examined the effect of apigenin on upstream kinases of MAPKs. MEK, MKK4 and MKK3/6 are well-known upstream regulators of MAPKs in UVB signaling (28). Apigenin strongly attenuated UVB-induced phosphorylation of MAPKs including MEK, MKK4 and MKK3/6 (Figure 2C). A previous study demonstrated EGRF activation induced by UVB irradiation upregulated COX-2 protein expression via activation of the Ras/Raf/ERK pathway (29). We next examined whether apigenin affected UVB-induced EGRF signaling. The results revealed that apigenin inhibited UVB-induced activation of c-Raf, Ras and EGRF, but not Src, phosphorylation (Figure 2D). Interestingly, phosphorylation of Tyr845 of EGRF, which is phosphorylated by Src, was dramatically reduced by apigenin treatment. In addition, apigenin showed similar results on UVB-induced MAPKs. Akt, EGRF and Src in HaCaT human keratinocytes (Supplementary Figure 1B, available at Carcinogenesis Online).
Apigenin inhibits skin inflammation by targeting Src

EGFR kinase assay to confirm the effects of apigenin on their activity. Our results revealed that apigenin significantly inhibited Src kinase activity (Figure 3A). However, apigenin had no effect on EGFR kinase activity (Figure 3B). Because apigenin inhibited Src kinase activity, we conducted pull-down assays to determine whether apigenin interacts directly with Src kinase. Src was pulled down by apigenin–sepharose 4B but not by sepharose 4B alone (Figure 3C). Furthermore, the binding of apigenin to Src was decreased in the presence of ATP (Figure 3D), suggesting that apigenin competes with ATP in binding to Src kinase. Taken together, our data indicate that apigenin interacts with Src in an ATP-competitive manner.

**Apigenin suppresses UVB-induced skin inflammation and Src kinase activity in the SKH-1 hairless mouse model.**

To further investigate anti-inflammatory effects of apigenin in vivo, we evaluated its role in SKH-1 hairless mice. Our data show that apigenin treatment suppressed UVB-induced ear edema, a well-known inflammation marker, as represented by the weight and thickness of the mice ears (Figure 4A and B). The immunoblot data determined that COX-2 expression in the apigenin groups was significantly reduced compared with the group treated with UVB irradiation alone (Figure 4C). These data are consistent with the results obtained in JB6 P+ cells (Figure 1A). PP2, a well-known Src inhibitor, significantly reduced UVB-induced ear edema and COX-2 expression in mouse skin (Figure 4A–C). Furthermore, in vivo kinase assays determined that apigenin suppressed Src kinase activity induced by UVB irradiation (Figure 4D), which is again consistent with the results from the JB6 P+ cells (Figure 3A).

**Src inhibitor PP2 suppresses UVB-induced EGFR, Raf and Akt pathway**

To confirm whether Src inhibition suppresses UVB-induced EGFR, Raf and Akt signaling pathways or Akt phosphorylation, we again used the pharmacological inhibitor of Src kinase, PP2. The immunoblot data showed that PP2 treatment suppressed UVB-induced
phosphorylation of EGFR, c-Raf, MEK and Akt but did not affect Src phosphorylation (Figure 5A). Additionally, a previous study demonstrated that the inhibition of Src kinase activity suppressed UVB-induced phosphorylation of MAPKs in the same cell line (30). To further demonstrate the effect of Src suppression in UVB-induced signaling, we analyzed COX-2 and phosphorylation of Src and its downstream signaling after Src knock down. Three sequences of Src small interfering RNA reduced Src expression (Supplementary Figure 2A, available at Carcinogenesis Online). Sequence #1 among Src small interfering RNAs was chosen for further experiments. Src knock down decreased UVB-induced COX-2 expression (Supplementary Figure 2B, available at Carcinogenesis Online). Src knock down attenuated UVB-induced EGFR, MAPKs and Akt phosphorylation (Supplementary Figure 2C, available at Carcinogenesis Online).

**Fig. 2.** Effect of apigenin on UVB-induced signaling in JB6 P+ cells. (A) Apigenin inhibits UVB-induced phosphorylation of ERK, p38, JNK and Akt. (B) Apigenin inhibits UVB-induced phosphorylation of p90RSK, MSK, mammalian target of rapamycin and p70S6K. (C) Apigenin inhibits UVB-induced phosphorylation of MEK, M KK4 and M KK3/6. (D) Apigenin inhibits UVB-induced phosphorylation of EGFR, c-Raf and Ras activation but not phosphorylation of Src. JB6 P+ cells were starved in 0.1% FBS–MEM and then treated or not treated with apigenin at the indicated concentration for 1 h before they were stimulated with UVB (0.05 J/cm²) for 5–30 min depending on the protein. Data are representative of three independent experiments that gave similar results.
Apigenin inhibits skin inflammation by targeting Src

FAK inhibitor PF228 does not affect UVB-induced MAPKs and Akt signaling in JB6P+ cells

Because in a previous study apigenin was reported to modulate Src and FAK (31), we tested the effect of FAK inhibitor PF228 in UVB-induced signaling. Treatment of PF228 did not affect phosphorylation levels of ERK, p38, JNK and Akt in JB6P+ cell (Figure 5B). In addition, UVB irradiation did not induce FAK activation (Figure 5C).

Discussion

Some phytochemicals, which are plant compounds, have been reported to possess substantial anti-inflammatory, antioxidant and anticarcinogenic properties. They are currently hypothesized to act as small molecular inhibitors in cells (32). Identifying the molecular mechanisms and target proteins of phytochemicals is necessary for understanding their chemopreventive effects and ultimately will aid development of chemopreventive agents. Determination of the direct targets of natural phytochemicals will increase confidence in their safety and efficacy and enhance the possibility of their use as a chemopreventive agent for human applications (33).

In this study, we analyzed the inhibitory effect of apigenin on COX-2 and skin inflammation and determined the molecular target. We report that apigenin directly suppressed Src activity, subsequently attenuating downstream signaling pathways that are critical for skin inflammation and carcinogenesis (6). A simplified depiction of the mechanism of apigenin in UVB-induced signaling is proposed in Figure 6B. Our data reveal a novel direct target of apigenin in skin inflammation.

Src, a non-receptor tyrosine kinase, is an oncogenic kinase, and its activity is significantly associated with inflammatory responses (34). In addition, Src is involved in COX-2 expression in various cell types (25,35,36). Multiple lines of evidence suggest inflammation and...
COX-2 promote the development of cancer (37,38). Tyrosine kinases such as Src are considered significant targets for preventing inflammation and cancer (39–41). Our data suggest that apigenin could be a small molecular inhibitor of Src useful for prevention of skin inflammation. In order to further examine how apigenin interacts with c-Src, we docked apigenin in silico to the ATP-binding pocket of c-Src (42–44). Apigenin showed the capability of forming numerous favorable connections and docked within the ATP-binding pocket. Several important hydrogen bonds were formed between the apigenin and backbone the hinge region of c-Src (Figure 6A). The images were generated with the UCSF Chimera program (45).

We assessed COX-2 regulation and the anti-inflammatory effects of apigenin and propose a novel mechanism of action. Our results suggest that Src is a direct target of apigenin and suppresses downstream signaling pathways to decrease COX-2 expression. A previous study reported that a pharmacological Src inhibitor reduced UVB-induced phosphorylation of MAPKs and COX-2 expression (30). In this study, we also demonstrated that a pharmacological Src inhibitor can also

Fig. 4. Effect of apigenin treatment on UVB-induced skin inflammation in SKH-1 hairless mice. (A and B) Apigenin inhibits UVB-induced ear edema development. Female SKH-1 hairless mice were treated on both ear and dorsal skin with apigenin and PP2 at the indicated concentration or acetone alone (as a vehicle) after UVB (0.18 J/cm²) as described in the Materials and methods. (C) Apigenin inhibits UVB-induced COX-2 expression in mouse skin. Three mouse dorsal skin samples were randomly selected from five mouse dorsal skin sample of each group and analyzed for COX-2 expression by immunoblotting. (D) Apigenin inhibits Src kinase activity in SKH-1 hairless mouse dorsal skin extracts. In the Src kinase assay, the dorsal skin protein lysates were prepared from the epidermis, and immunoprecipitation and kinase assays were performed as described in the Materials and methods. PP2 compound was used as a positive control. (***P < 0.001; **P < 0.01, significant differences between groups treated with both apigenin and UVB and the group treated with UVB alone).
Apigenin inhibits skin inflammation by targeting Src suppress phosphorylation of MEK, Raf, EGFR and Akt. The changes in signaling in response to PP2 treatment were identical to those resulting from apigenin treatment in JB6 P+ cells (Figure 2A and D). Some studies have reported that apigenin prevents UVB-induced COX-2 expression through both transcriptional and translational regulatory mechanisms (9,24). Consistent with a previous report (9), we also found UVB-induced upregulation of USF-2 was decreased and UVB-induced downregulation of USF-1 recovered with apigenin treatment. Treatment of apigenin attenuated CREB, which was reported to regulate cox-2 promoter activity (8). Collectively, the marked suppression of COX-2 protein expression can be attributed to direct inhibition of Src, which suppresses the downstream signaling pathway.

Fig. 5. Involvement of Src as an upstream kinase of EGFR and Akt signaling in JB6 P+ cells. (A) PP2 compound suppresses UVB-induced phosphorylation of EGFR, c-Raf, MEK and Akt but not phosphorylation of Src. JB6 P+ cells were treated with PP2 compound at the indicated concentration for 1 h before they were stimulated with UVB (0.05 J/cm²). Data are representative of three independent experiments that gave similar results. (B) PF 228 compound has no effect on phosphorylation of ERK, p38, JNK and Akt. JB6 P+ cells were starved in 0.1% FBS–MEM and then treated or not treated with PF 228 compound at the indicated concentration for 1 h before they were stimulated with UVB (0.05 J/cm²) for 15 min. (C) UVB irradiation has no effect on phosphorylation of FAK. JB6 P+ cells were starved in 0.1% FBS–MEM and then stimulated or not stimulated UVB (0.05 J/cm²) for the indicated time.
that regulates COX-2 expression. Further studies of the preventative effects of apigenin using two-stage tumourigenesis experiments may further elucidate its chemopreventive role in skin cancer.

It has been reported that the activities of Src and FAK are related in some models. In a previous study using prostate cancer cells, apigenin was reported to inhibit FAK/Src activation (31,46,47). Therefore, we evaluated whether FAK plays a role in our UVB-induced skin signaling model. The results showed that FAK inhibitor, PF228 had no effect on UVB-induced MAPKs and Akt. Furthermore, FAK phosphorylation was unaffected by UVB irradiation. These results indicate that FAK probably does not play a major role in the UVB-induced signaling model. Moreover, these data indicate that FAK is not required for the MAPK and Akt signaling pathway-mediated modulation of COX-2 expression induced by UVB irradiation in JB6 P+ cells. Hence, the apigenin inhibition of UVB-induced EGFR, MAPKs and Akt signaling is not due to regulation of FAK activity.

Overall, our study reveals a novel molecular mechanism in which the natural flavone apigenin inhibits UVB-induced COX-2 expression by binding directly to Src. According to our findings, apigenin exerts a significant chemopreventive effect on UVB-induced skin inflammation. In conclusion, apigenin might be useful for treatment of UVB-associated skin inflammation.

Supplementary material

Supplementary Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/.

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