Chemopreventive Effects of Honokiol on UVB-induced Skin Cancer Development

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Abstract. *Background: Skin cancer is the most prevalent of all cancer types and its incidence is expected to increase substantially. Chemoprevention involves the administration of chemical agents to prevent initiation, promotion and/or progression that occurs during neoplastic development. Honokiol, a plant lignan isolated from bark and seed cones of Magnolia officinalis, has been shown to have chemopreventive effects on chemically induced skin cancer development. Aim: The objective of this investigation was to study the chemopreventive effects of honokiol on UVB-induced skin tumor development in SKH-1 mice, a model relevant to humans, and to elucidate the possible role of apoptotic proteins involved in the prevention of skin tumor development. Materials and Methods: Female SKH-1 mice were divided into two groups. Group 1 received acetone (0.2 ml, topical) and Group 2 received honokiol (30 μg in 0.2 ml acetone, topical) one hour before UVB treatment. Tumor initiation and promotion were carried out by UVB radiation (30 mJ/cm^2/day), 5 days a week for 30 weeks. Tumor counts and mouse weights were taken weekly. Results: The honokiol-pretreated group exhibited a 45% reduction in tumor multiplicity as compared to the control group. Mechanistic studies showed the possible involvement of caspase-3, caspase-8, caspase-9, poly (ADP-ribose) polymerase (PARP) and p53 activation ($p<0.05$) leading to the induction of DNA fragmentation and apoptosis. Conclusion: Pretreatment with honokiol, at concentrations in micrograms per application compared with milligram applications of other potential chemopreventive agents, prevents UVB-induced skin cancer development, possibly by activating proapoptotic proteins through both intrinsic and extrinsic pathways.*

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Skin cancer is the most common cancer in the United States and is frequently diagnosed in Caucasians (1, 2). One among five Americans is estimated to develop skin cancer (1). Each year more than a million cases of skin cancer are diagnosed, leading to over 10000 deaths annually (2). Non melanoma skin cancer (NMSC), including basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), especially occurring in light skinned people as a result of sun exposure, are not lethal and are easily cured (3). The major causative agent in skin cancer is UV radiation from sunlight (2, 3). Chronic exposure to UV radiations leads to skin cancer (4). Both experimental and epidemiological evidences suggest UVB (320-400 nm of solar radiation) as important component of solar radiation responsible for skin cancer. UVB acts as complete carcinogen by initiating and promoting cancer growth (4, 5). Occurrence of NMSC is directly related to the exposure to UVB radiation and inversely with pigmentation of skin (1).

UVB exposure leads to the mutations in DNA that include pyrimidine-pyrimidone photoproducts and cyclobutane dimers. If these lesions are not repaired, they lead to C to T and CC to TT transition mutations known as UVB signature mutations (6,7). In human SCCs, these UVB signature mutations were seen in tumor suppressor genes, oncogenes and genes involved in regulation of skin cell proliferation. This DNA damage causes a rapid increase in *p53*, a tumor suppressor gene, by phosphorylation and nuclear translocation, and results in the synthesis of Cip1/p21, thereby decreasing cell replication by causing cell cycle arrest, and in extensive cases this repair is by apoptosis. Those cells which escape this defense mechanism are transformed to UVB initiated epidermal keratinocytes (7-9). Mutations in *p53* gene appear in SCC caused by UV radiation (10). *p53* is a tumor suppressor gene which is involved in up regulation of BAX, a proapoptotic protein and down-regulation of BCL-2, an antiapoptotic protein (11). Apoptosis plays an important role in homeostasis. Two major pathways of apoptosis include the death receptor pathway (extrinsic pathway) and the mitochondrial pathway (intrinsic pathway). In the extrinsic pathway, death ligand binds to death receptor on the cell surface and activates caspase-8 which then

activates caspase-3 resulting in apoptosis. In the intrinsic pathway, mitochondria are involved in the activation of caspase-9 by forming apoptosomes and thereby activating caspase-3, an executioner caspase (12-14). Activated caspase-3 can break the poly (ADP-ribose) polymerase (PARP) thereby causing in DNA fragmentation leading to apoptosis.

Chemoprevention of skin cancer involves administration of chemical agents to prevent various events during the multistage process of neoplastic development (7, 12, 15). Chemoprevention by using natural compounds has gained importance in recent years (5, 7, 12). More than 1000 phytochemicals have shown chemopreventive effects (17). A major limitation of phytochemicals is that most of them are still in the preclinical stages and are not effective in human skin cancer. As a result, the search for new agents that could inhibit NMSC (7, 18, 19) continues. In our laboratory, studies have been carried out on two naturally occurring phytochemicals: α-santalol obtained from sandalwood oil and sarcophine-diol obtained from soft coral, *Sarcophyton glaucum*, which showed chemopreventive effects in skin cancer (7, 12, 20, 21). Honokiol is a naturally occurring biphenol isolated from the bark and seed cones of *Magnolia officinalis*. Studies have demonstrated that honokiol inhibits angiogenesis and tumor growth (22). Honokiol down-regulates BCL-x_L, an antiapoptotic protein (23), inhibits tissue necrotic factor expression (24), and potentiates apoptosis and inhibits invasion through modulation of nuclear factor kappa B (NF-κB) pathway (25). In addition, studies also demonstrated honokiol was effective in prostate cancer, colon cancer and multiple myeloma (26-28). Honokiol inhibits skin tumor promotion in 7,12- dimethylbenz(α)anthracene (DMBA)-initiated and 12-*O*-tetradecanoyl phorbol-13-acetate (TPA)-promoted skin carcinogenesis model (29).

The purpose of this investigation was to study the chemopreventive effects of honokiol on UVB-induced skin carcinogenesis in which UV acts as complete carcinogen by initiating and promoting skin cancer in SKH-1 mice, a model relevant to human cancer. In this study, mice were exposed to a UVB dose of 30 mJ/cm^2/day for five days a week which is a physiologically relevant dose that is in close range of human exposure to sunlight that can cause skin cancer (30, 31). Loss of apoptosis is one of the major biological events responsible for tumorigenesis. Caspase-dependent apoptosis and p53, a key regulating protein involved in promoting tumorigenesis, were investigated in the present study to elucidate possible mechanisms of action.

Materials and Methods

Chemicals. Chemicals were purchased from the sources indicated in parentheses. Honokiol (Nacalai Tesque, Kyoto, Japan); primary antibody caspase-3 (Cell Signaling Technology, Inc., Beverly, MA, USA); caspase-8, caspase-9 and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA); horse radish peroxidase (HRP) conjugated goat anti-rabbit and anti-mouse antibodies (BD Biosciences, Rockville, MD, USA); ECL kit (Amersham Biosciences, Piscataway, NJ, USA); sodium chloride, phenylmethylsulphonylfluride (PMSF) (Sigma chemicals, St.Louis, MO, USA); leupeptin and pepstatin (Roche Diagnostics GmbH, Mannheim, Germany); acrylamide (Bio Rad Laboratories, Hercules, CA, USA); nitrocellulose membrane (Bioexpress, Kaysville, UT, USA); In Situ Cell Death Detection kit, fluorescein (Roche Applied Sciences, Mannheim, Germany).

Animals. Female SKH-1 mice were purchased from Charles River Laboratories (Wilmington, MA, USA). Mice were housed in a climate controlled environment at the College of Pharmacy of South Dakota State University animal facility. All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC). Mice were acclimatized for 2 weeks and were provided with free access to food and water during experiment. IACUC guidelines were followed in the handling and care of animals.

UVB exposure source. Four FS-40-T-12-UVB sunlamps were used as the UVB light source. Daavlin Flex Control manufactured by Daavlin Corporation (Bryan, OH, USA) integrating dosimeters were used to control UVB exposure dose.

Experimental design. Female SKH-1 mice of five weeks old were divided into two groups of 20 each. Group 1 was assigned as control and group 2 as honokiol-treated group. Group 1 received 200 μl of acetone and group 2 received 30 μg of honokiol in 200 μl of acetone topically. Carcinogenesis was initiated and promoted by UVB as detailed by Gu *et al*. (31). Topical treatment of honokiol was given 1 h before UVB exposure of 30 mJ/cm^2 for 5 days/week. The experiment was carried out for 30 weeks. Body weights and tumor counts were recorded for 30 weeks on a weekly basis. Mice were euthanized at the end of experiment by cervical dislocation. Results were evaluated for tumor incidence, size and multiplicity. Epidermal and tumor samples were collected and used for histopathological, immunohistological and Western blotting analysis.

Histopathological analysis. Skin with tumor samples from mice were fixed in 10% neutral buffered formalin and processed for paraffin-wax embedded sectioning of 4-6 μm thick, stained with hemotoxylin and eosin dye and observed for the histology of the tissue under light microscope.

Western blotting. Epidermis was collected from mice. Tumors and fat from epidermis were removed by scalpel and homogenized in 0.1 mM Tris-HCl (pH 7.4). The homogenate was filtered and centrifuged at 10000g for 45 min in a Beckman J2-21 centrifuge (Beckman, USA). This cell pellet was combined with 5% SDS, 0.5% leupeptin and pepstatin and 1% PMSF. The supernatant was passed through a 25G needle and centrifuged at 13000 xg for 20 min.

Sixty micrograms of protein were separated on 12.5% SDS-PAGE gel and proteins from the gel were transferred onto nitrocellulose membrane overnight at 4˚C. After transfer, membranes were blocked in 5% non-fat milk in tris-buffer solution (TBS) for 1 h and probed for primary antibody (caspase -3 and -8, PARP, *p53*) in TBS overnight at 4˚C. After washing blot for 3 times with TTBS (0.1 % Tween 20, 10 mM tris) for 10 min each, the membrane was incubated with HRP-conjugated secondary antibody for 1 h. The blot was washed with TTBS again for 3 times and was developed with ECL kit. Proteins were quantified by using a UVP biochem gel documentation system (UVP, Inc., Upland, CA, USA).

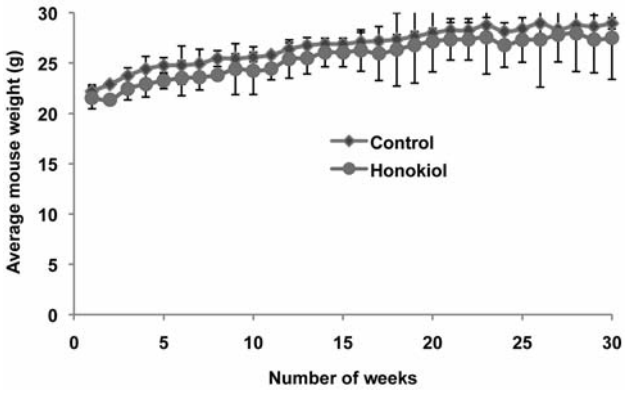

Figure 1. *Effects of honokiol pretreatment on weight gain of SKH-1 mice. Each point indicates mean±SE. No significant difference was seen between control and honokiol-treated groups.*

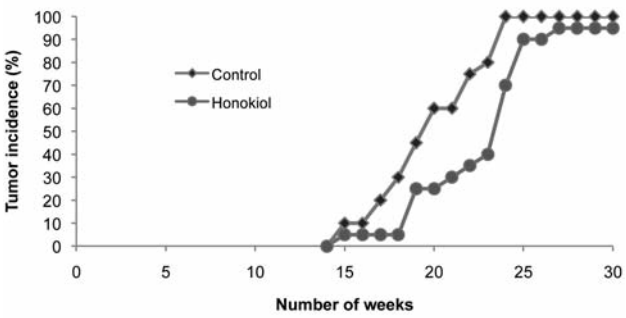

Figure 2. *Effects of honokiol pretreatment on tumor incidence in SKH-1 mice. No significant difference was observed in tumor incidence between control and honokiol-treated groups at the end of the experiment. Significant difference ($p<0.05$) was observed in honokiol-treated mice from weeks 17-26.*

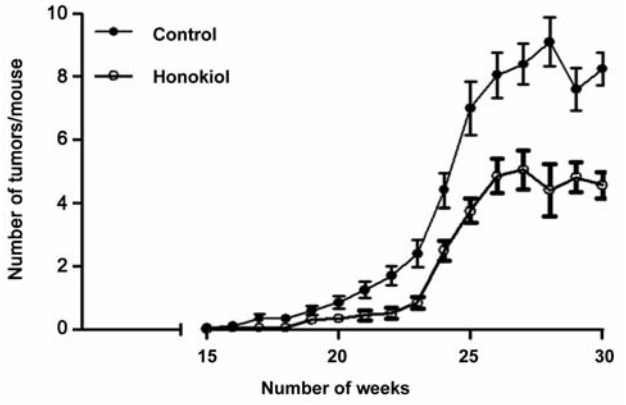

Figure 3. *Effects of honokiol treatment on tumor multiplicity of SKH-1 mice. Tumor multiplicity was significantly lower ($p<0.05$) in the honokiol-pretreated group compared to control from 21-30 weeks. Each point represents the mean tumor number per mouse±SE from 20 mice.*

Protein assay. Protein was assayed using BCA protein assay kit (Pierce, Rockford, IL, USA) with albumin as standard.

Tumor area. Effects of honokiol on tumor area were quantified by using images which were taken at the end of 30 weeks of experiment. Tumor surface area for each mouse was calculated by using digital photographs of mice. By using Photoshop CS3 (Adobe systems, San Jose, CA, USA) tumor boundaries were determined and areas were measured using Image-Pro Plus 5.1 (Media Cybernetics, Inc, Bethesda, MD, USA) with the measure-area feature as described elsewhere (20).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Immunohistological studies for detection of apoptosis in tissue sections were conducted on paraffin embedded sections of skin by using *In Situ* Cell Death Detection kit (Roche Diagnostic GmbH). TUNEL assay was performed using the manufacturer's protocol. Briefly slides were deparaffinized using xylene and rehydrated by washing with different descending grades of alcohols. Then these rehydrated sections were incubated with permeabilization solution containing 0.1% Triton X, 0.1% sodium citrate in a humidified chamber for 8 min followed by incubation with TUNEL reaction mixture containing TdT solution (enzyme solution) and fluorescein isothiocyanate (FITC)-labeled dUTP (Labeled solution) for 60 min in a humidified chamber. After washing with PBS, they were counterstained with propidium iodide (PI). Negative controls were prepared by replacing TUNEL reaction mixture with PBS, and positive control by incubating tissue sections in recombinant DNAse I solution for 10 min to induce DNA strand-breaks before labeling procedures. The slides were mounted and analyzed under confocal microscopy using excitation range of 450-500 nm and detection in the range of 515-565 nm

Statistical analysis. INSTAT software (Graph Pad, San Diego, CA, USA) was used to analyze data. Chi-square analysis was used for determining significance for tumor incidence. Student *t*-test was used to compare tumor multiplicity, apoptosis and caspases. Significance in all experiments was considered at $p<0.05$.

Results

Effects of honokiol treatment on weight gain. The effects of honokiol pretreatment on weight gain are shown in Figure 1. Topical application of honokiol did not have any significant effect on weight gain when compared with control groups throughout the experiment. This indicates that honokiol treatment did not affect normal growth and development of mice.

Effects of honokiol treatment on tumor incidence. The effects of honokiol on tumor incidence are shown in Figure 2. First tumor appeared in the control group at 15 weeks and eventually tumor incidence was 100% in the control and 90% in the honokiol-treated groups at the end of 30 weeks. There was no significant difference in tumor incidence between the control and honokiol-treated groups at the end of 30 weeks. However, the results showed that pretreatment with honokiol significantly ($p<0.05$) reduced tumor incidence from 17 to 26 weeks.

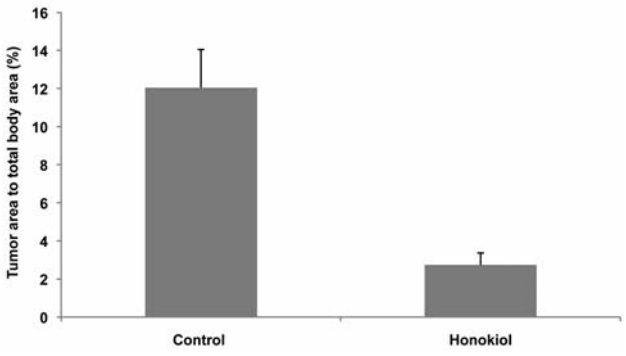

Figure 4. *Effects of honokiol on tumor area in SKH-1 mice. Honokiol the treatment significantly ($p<0.05$) reduced the percentage ratio of tumor area to total back area (mean±SE).*

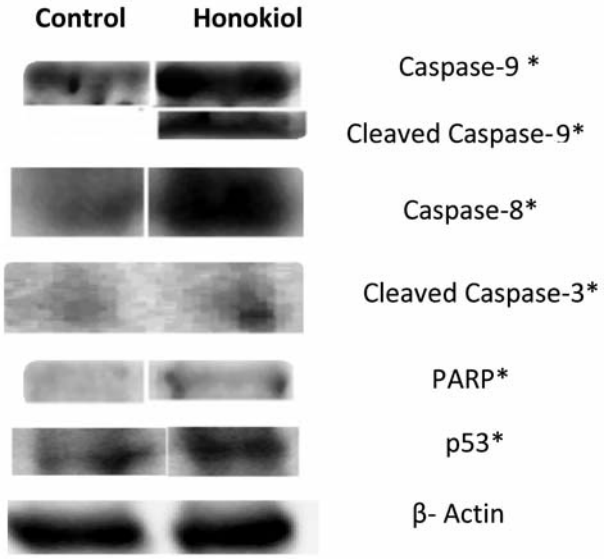

Figure 5. *Western blots showing the effects of honokiol on caspase-8, caspase-9, caspase-3, PARP and p53 in SKH-1 mice. Equal loading is verified by using β-actin for each membrane. *Significant difference ($p<0.05$).*

Effects of honokiol pretreatment on tumor multiplicity. The effects of honokiol on tumor multiplicity are shown in Figure 3. Topical application of honokiol showed protection against UVB-induced tumor development in skin of SKH-1 mice. First tumor appeared in the control group at week 15, and by 22 weeks, all mice in both groups had tumors. The mean number of tumors in the control and honokiol-treated group was 9.2 and 4.5 respectively at the end of study. Tumor multiplicity significantly ($p<0.05$) decreased from 21-30 weeks by 45% with honokiol treatment.

Effects of honokiol pretreatment on the ratio of total tumor area to total back area. The effects of honokiol pretreatment on tumor area to total back area ratio in SKH-1 mice are shown in Figure 4. In control and honokiol-treated groups, the mean ratio of total tumor area to total back area was 12.0% and 2.0% respectively. Honokiol pretreatment resulted in a 78% decrease (significant at $p<0.05$) in the mean ratio of tumor area to total back area at the end of study.

SCC induced by UVB radiation in SKH-1 mice. Histopathological examination of tumor progression indicated that the origin of tumor was proliferative stratified epidermis (Data not shown). Normal skin is 2-3 layers thick compared to neoplastic epidermis, which is 6-8 layers. Mild dyskeratosis was seen in the region of the stratum spinosum. A thick layer of keratin covered the surface of epidermis which was compatible with orthokeratotic hyperkeratosis. All these findings suggest SCC.

Effects of honokiol on apoptosis. Effects of the topical treatment of honokiol on caspases and PARP in UVB irradiated mouse skin are presented in Figure 5. Topical application of honokiol modulated expression of caspase-3, caspase-8 and caspase-9, which was demonstrated by Western blotting analysis. Honokiol pretreatment increased expressions of caspase-3 and caspase-8 compared to control. Honokiol treatment also elevated expression of PARP (85 kDa). Honokiol pretreatment increased the expression of cleaved caspase-3 (17 kDa). It also increased expressions of caspase-8 at 18 kDa and caspase-9 at 10 kDa compared to the control group. Increased expressions of caspase-3, -8, and -9 and PARP were statistically significant ($p<0.05$).

Effects of honokiol on p53 expression. Topical application of honokiol increased ($p<0.05$) expression of p53 by Western blotting and immunohistological analysis as shown in Figure 5. p53 is important for apoptosis and lack of its expression indicates a high risk of tumor development (10, 11). Significant increase in expression of p53 at $p<0.05$ was observed in the honokiol-treated group.

Honokiol induces DNA fragmentation in cells. Honokiol pretreatment caused an increase in DNA fragmentation, which is hallmark of apoptosis in skin. In the control group which was treated with acetone, very few cells showed DNA fragmentation compared to the honokiol-treated group. Green fluorescence is seen in DNA fragmented cells and red PI staining is seen in normal cells. In the control group, as shown in Figure 6, very few cells were stained with green color and in the honokiol-pretreated group, an increase in DNA-fragmented cells (green fluorescence) was observed. These observations suggest that honokiol pretreatment induced DNA fragmentation in cells.

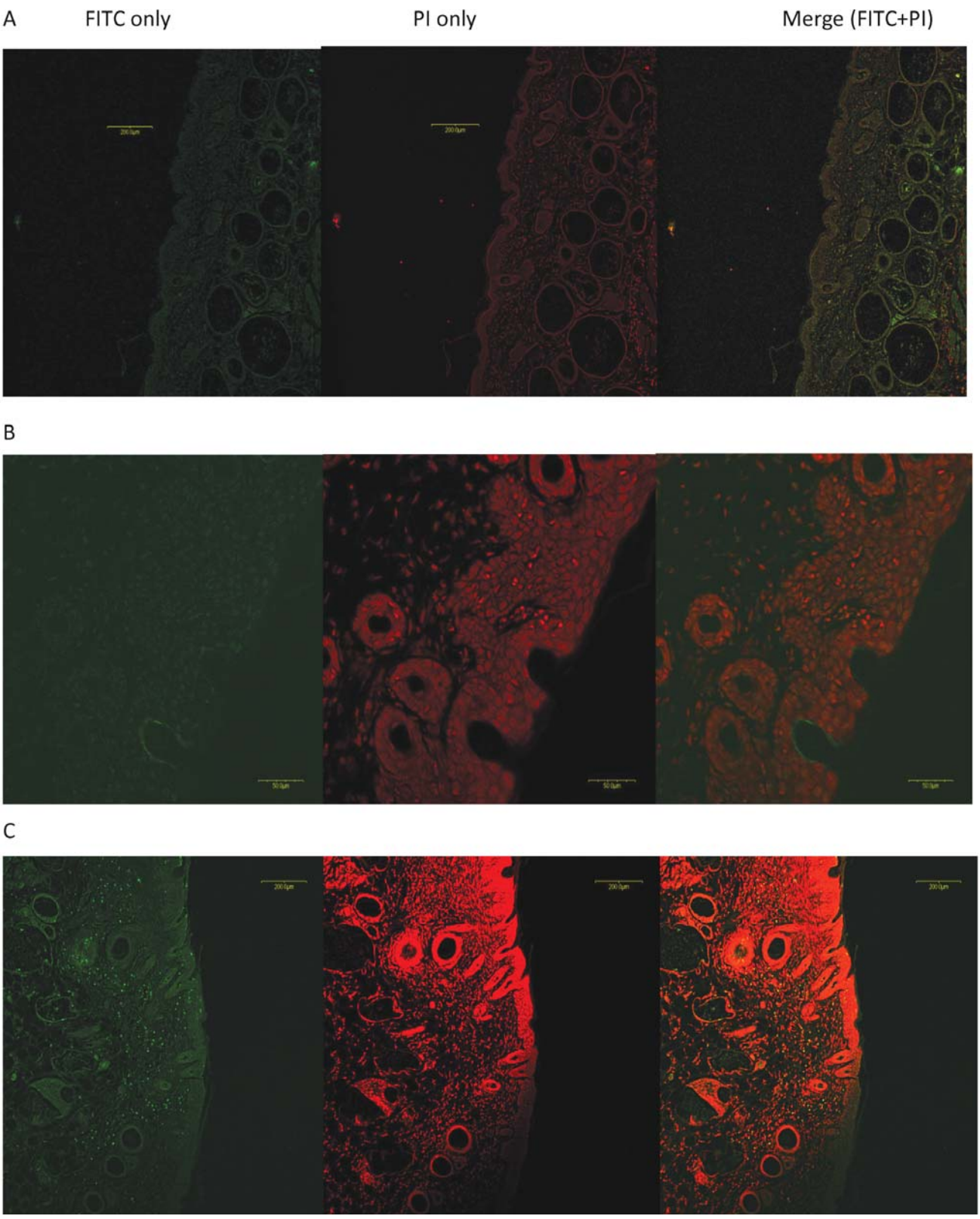

Figure 6. *Effects of honokiol on apoptosis by TUNEL assay. Mechanistic studies were carried out on skin sections to determine DNA fragmentation. Apoptotic cells were stained with green fluorescent dye (FITC) which were then counterstained with red (PI) dye. Image A: Control mouse skin; B: positive control (DNA fragmentation induced); C: honokiol-treated group.*

Discussion

The increased incidence of NMSCs suggests usage of sunscreens alone is not sufficient in preventing skin cancer, additional approaches and strategies are required and one such approach is usage of chemopreventive agents (32). Cancer chemoprevention by using natural compounds such as phytochemicals, vitamins and minerals is a promising strategy for controlling and preventing cancer development (15-17). Molecules that reverse or stop progression of premalignant cells in which damage has already occurred are referred to as chemopreventive agents. Many laboratories have been investigating chemopreventive effects of natural substances against skin cancer development (5, 7, 12, 30, 39). In our laboratory, chemopreventive effects of α-santalol and sarcophine-diol were investigated on skin cancer in both DMBA initiated-TPA promoted mouse models and UVB-induced mouse models (7, 12, 20, 21). Honokiol, a phytochemical obtained from the magnolia plant have been shown to decrease tumor development in the DMBA-TPA treated mouse model (29).

Honokiol is a hydroxylated biphenyl compound from bark and seed cones of magnolia species, most commonly used in traditional Chinese medicine for the treatment of fever, headache, typhoid fever and stroke (35). Studies have shown that honokiol induces apoptosis, thereby producing antiagiogenic and antineoplastic effects (22-25). Neolignans from *Magnolia officinalis* delayed papilloma formation in skin treated with TPA (29). This observation prompted us to investigate the effects of honokiol on UVB-induced skin carcinogenesis model since the major etiological factor for NMSCs is exposure of skin to UVB radiation (2, 3). A UVB dose of 30 mJ/cm^2/day was used in our experiment as it is has more translational significance compared with previous studies that used higher doses (30, 31). The present study demonstrated that topical application of honokiol at a concentration of 30 μg /200 μl of acetone significantly (p<0.05) reduced skin tumor development. It delayed the onset of tumorigenesis and reduced the tumor number compared to that of the control. It did not significantly decrease tumor incidence at the end of experiment but did decrease between 17-26 weeks and the percentage of tumor area was reduced. Pretreatment with honokiol is effective in inhibiting UVB-induced skin carcinogenesis at a very low concentration (30 μg per applications) as compared to other reported chemopreventive agents with comparable effects at milligrams per applications. For example, topical application of α-santalol inhibited UVB-induced skin cancer development in mice at 5 mg per application (8, 21); topical application of epigallocatechin-3-gallate (EGCG) at 1 mg/cm^2 skin area per application prevented photocarcinogenesis in wild-type (C_3H/HeN) mice (38); topical application of silymarin protected from photocarcinogenesis in SKH-1 mice at 9 mg per application (5), and silibinin at 9 mg per application prevented UV-induced skin damage in SKH-1 hairless mice (39).

Chemopreventive effects are modulated by two major events: inhibition of cell proliferation and induction of apoptosis (33, 34). We focused our investigation on apoptosis. Our data indicate that honokiol induced apoptosis by both extrinsic and intrinsic pathways, significantly up regulating caspase-8, caspase-9, caspase-3 and PARP cleavage, which is involved in DNA fragmentation and apoptosis. DNA fragmentation is a hallmark of apoptosis which commits cell to die. Our results suggest that honokiol inhibit UVB-induced skin carcinogenesis by inducing apoptosis and DNA fragmentation leading to apoptosis.

In summary, we found that honokiol treatment increases apoptosis through both extrinsic and intrinsic pathways thereby producing protective effects against photocarcinogenesis. Honokiol has a great potential as an effective and potent chemopreventive agents for skin cancer. Further studies on concentration and time responses, pre and post treatment and various signaling pathways involved are needed to fully evaluate the chemopreventive effects of honokiol on skin cancer development.

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