Carcinogenesis vol.31 no.11 pp.2004–2011, 2010
doi:10.1093/carcin/bgq186
Advance Access publication September 7, 2010

Honokiol, a phytochemical from the *Magnolia* plant, inhibits photocarcinogenesis by targeting UVB-induced inflammatory mediators and cell cycle regulators: development of topical formulation

Mudit Vaid[1], Som D.Sharma[1] and Santosh K.Katiyar[1,2,*]

[1]Department of Dermatology, University of Alabama at Birmingham, Birmingham, AL 35294, USA and [2]Birmingham Veteran Affairs Medical Center, Birmingham, AL 35294, USA

*To whom correspondence should be addressed. Department of Dermatology, University of Alabama at Birmingham, 1670 University Boulevard, Volker Hall 557, Birmingham, AL 35294, USA. Tel: +1 205 975 2608; Fax: +1 205 934 5745;
Email: skatiyar@uab.edu

To develop newer and more effective chemopreventive agents for skin cancer, we assessed the effect of honokiol, a phytochemical from the *Magnolia* plant, on ultraviolet (UV) radiation-induced skin tumorigenesis using the SKH-1 hairless mouse model. Topical treatment of mice with honokiol in a hydrophilic cream-based topical formulation before or after UVB (180 mJ/cm^2) irradiation resulted in a significant protection against photocarcinogenesis in terms of tumor multiplicity (28–60%, $P < 0.05$ to <0.001) and tumor volume per tumor-bearing mouse (33–80%, $P < 0.05$ to 0.001, $n = 20$). Honokiol also inhibited and delayed the malignant progression of papillomas to carcinomas. To investigate the *in vivo* molecular targets of honokiol efficacy, tumors and tumor-uninvolved skin samples from the tumor-bearing mice were analyzed for inflammatory mediators, cell cycle regulators and survival signals using immunostaining, western blotting and enzyme-linked immunosorbent assay. Treatment with honokiol significantly inhibited UVB-induced expression of cyclooxygenase-2, prostaglandin E_2 ($P < 0.001$), proliferating cell nuclear antigen and proinflammatory cytokines, such as tumor necrosis factor-α ($P < 0.001$), interleukin (IL)-1β ($P < 0.01$) and IL-6 ($P < 0.001$) in the skin as well as in skin tumors. Western blot analysis revealed that honokiol: (i) inhibited the levels of cyclins D1, D2 and E and associated cyclin-dependent kinases (CDKs)2, CDK4 and CDK6, (ii) upregulated Cip/p21 and Kip/p27 and (iii) inhibited the levels of phosphatidylinositol 3-kinase and the phosphorylation of Akt at Ser^{473} in UVB-induced skin tumors. Together, our results indicate that honokiol holds promise for the prevention of UVB-induced skin cancer by targeting inflammatory mediators, cell cycle regulators and cell survival signals in UVB-exposed skin.

Introduction

The incidence of skin cancer is equivalent to the incidence of malignancies in all other organs combined (1). The continuing increase in life expectancy, the depletion of the ozone layer that allows more solar ultraviolet (UV) radiation to reach the Earth's surface, together with changing dietary habits and lifestyle appear to be contributing factors in the increasing risk of skin cancer. Although many environmental and genetic factors contribute to the development of skin cancer, the most important factor is the chronic exposure of the skin to solar UV radiation (2). More effective chemopreventive strategies that can inhibit or reduce the UV-induced risk of skin cancers in high-risk human population are needed and there is considerable interest in naturally occurring phytochemicals, which can be used as cancer chemopreventive agents and/or as complementary and alternative medicines (2). We therefore examined the effects of honokiol, one such phytochemical, on UV radiation-induced skin tumor development using the SKH-1 hairless mouse model.

Honokiol ($C_{18}H_{18}O_2$), a biphenolic phytochemical (Figure 1A), is one of the bioactive constituents of the bark and leaves of *Magnolia* plant species. It is used in the traditional Japanese medicine 'Saiboku-to' for the treatment of various ailments due to its anxiolytic, antithrombotic, antidepressant, antiemetic and antibacterial properties (3). Honokiol has attracted a great deal of research interest due to these diverse biologic and pharmacological activities that include antibacterial, anti-inflammatory, antifungal, antioxidative and anticarcinogenic effects (3–9). Honokiol is soluble in organic solvents, such as acetone; therefore, to avoid the need for the use of organic solvents in the application of this pharmacological agent, we developed a topical formulation of honokiol by dissolving it in a hydrophilic cream, which was used as a base. We have developed such hydrophilic cream-based topical applications in the past for the application of green tea polyphenols and these are non-toxic in nature (10).

Exposure of the skin to solar UV radiation induces inflammatory responses, oxidative stress, alterations in cell cycle progression and DNA damage, etc., which together have been implicated in various skin diseases, including the development of skin cancers (11–15). UV-induced inflammatory responses are characterized by the development of edema, hyperplastic responses and increases in the levels of cyclooxygenase-2 (COX-2) and prostaglandin (PG) metabolites (11). UV-induced inflammation is considered as an early and important event in tumor promotion and the growth of skin tumors and chronic inflammation plays an important role in all three stages of tumor development, i.e. initiation, promotion and progression (11); thus, the control of UVB-induced inflammatory responses is considered to be an important strategy in reducing the risk of skin cancer. The present study was designed to determine whether topical application of honokiol prevents UVB radiation-induced: (i) photocarcinogenesis in terms of tumor incidence, tumor multiplicity and tumor growth and (ii) malignant progression of papillomas to carcinomas in a mouse model. As UV-induced inflammation and its mediators, such as the induction of COX-2 expression, proinflammatory cytokines and hyperproliferation, along with the alterations in cell cycle progression, have been implicated in skin tumor development, we further sought to determine whether (*iii*) the chemopreventive effects of honokiol on photocarcinogenesis are associated with the inhibition of UVB-induced inflammation and inflammatory mediators and correction of cell cycle progression in mouse skin and skin tumors.

Materials and methods

Animals

The female SKH-1 hairless mice (6–7 weeks old) used in these studies were purchased from Charles River Laboratory (Wilmington, MA). The mice were acclimatized for at least 1 week before experimental use in the animal resource facility and maintained under standard conditions of a 12 h dark/12 h light cycle, a temperature of 24 ± 2°C and relative humidity of 50 ± 10%. The animal protocol used in this study was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Antibodies and chemicals

Antibodies specific for COX-2 and phosphatidylinositol-3 kinase (PI3K) and an immunoassay kit for prostaglandin E_2 (PGE_2) analysis were obtained from Cayman Chemical (Ann Arbor, MI). The primary antibodies specific for proliferating cell nuclear antigen (PCNA), cyclin D1, cyclin D2, cyclin E, CDK2, CDK4, CDK6, Cip1/p21, Kip1/p27 and β-actin and the secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies specific for inducible nitric oxide synthase (iNOS), Akt and p-Akt were purchased from Cell Signaling Technology, Inc. (Beverly, MA).

Abbreviations: CDK, cyclin-dependent kinase; COX-2, cyclooxygenase-2; IL, interleukin; iNOS, inducible nitric oxide synthase; PCNA, proliferating cell nuclear antigen; PG, prostaglandin; PGE_2, prostaglandin E_2; PI3K, phosphatidylinositol-3 kinase; UV, ultraviolet.

© The Author 2010. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oxfordjournals.org

Downloaded from https://academic.oup.com/carcin/article/31/11/2004/2392098 by guest on 05 May 2022

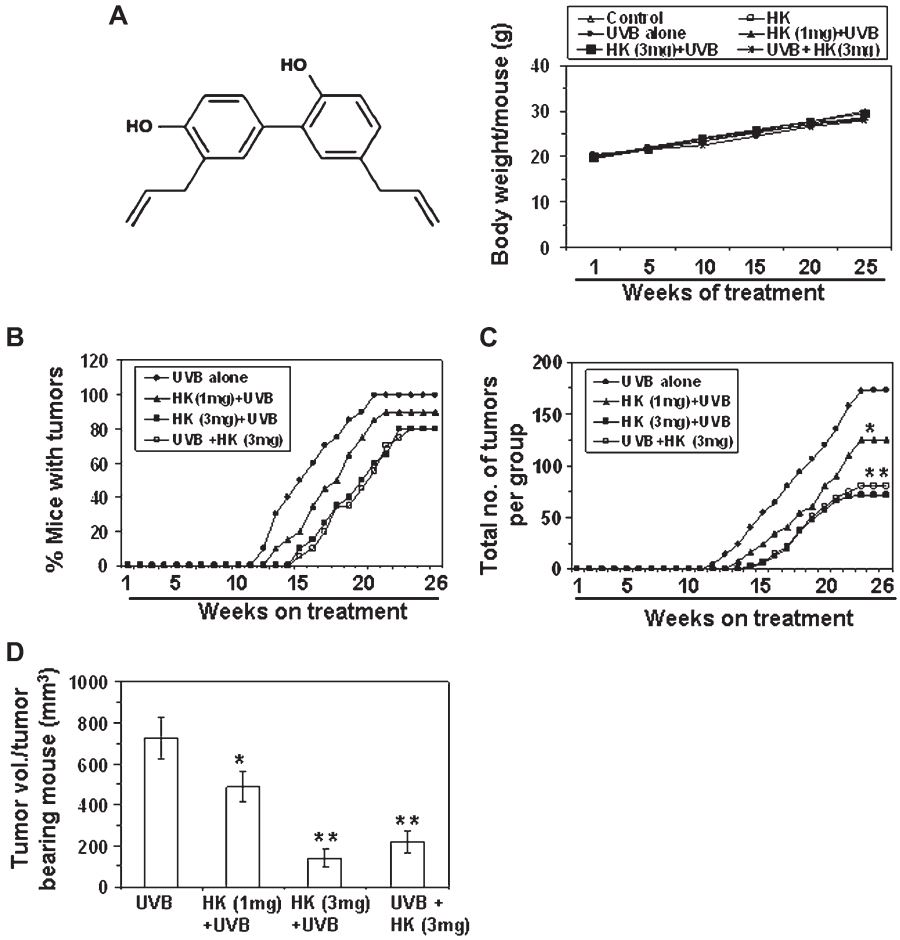

Fig. 1. Honokiol inhibits UVB-induced skin tumor development in SKH-1 hairless mice. Honokiol (HK) was topically applied either before UVB irradiation (HK + UVB) or immediately after UVB irradiation (UVB + HK) of the mouse skin. UVB alone-exposed mice were also treated with the hydrophilic cream before UVB exposure. (**A**) Depicts the molecular structure of honokiol and the mean body weight per mouse in different treatment group was recorded throughout the photocarcinogenesis experiment. The tumor data are presented in terms of the percentage of mice with tumors (**B**) and the total number of tumors per group (**C**) and the tumor volume per tumor-bearing mouse (**D**) are shown as means ± SD. The tumor volume was recorded at the termination of the experiment. The tumor data and body weight per mouse in different treatment groups were plotted as a function of the number of weeks on treatment, $n = 20$. Significant inhibition versus UVB alone at the termination of the photocarcinogenesis experiment, $^{*}P < 0.05$, $^{**}P < 0.001$.

Enzyme-linked immunosorbent assay kits specific for mouse tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 were obtained from BioSource International (Camarillo, CA).

Preparation of topical formulation of honokiol in hydrophilic cream

Purified honokiol was procured from LKT laboratories, Inc. (St Paul, MN). A uniform formulation of honokiol with a hydrophilic cream (Fougera and Co., Melville, NY), which is prescribed for external use as an ointment or cosmetic base, was prepared. The formulation was applied topically (1 or 3 mg honokiol per mouse) onto the dorsal area of the mouse skin either ~30 min before, or immediately after, each exposure to UVB. The formulation was stored at 4°C.

Honokiol treatment, UVB irradiation and photocarcinogenesis protocol

The SKH-1 hairless mice were divided randomly into six separate treatment groups with 20 mice in each group: mice in Group 1 were not UVB-exposed or otherwise treated and served as controls, mice in Group 2 were not UVB-exposed but were treated with honokiol (3 mg per mouse) alone three times per week, mice in Group 3 were exposed to UVB alone with the topical treatment of cream before each exposure of UVB, mice in Group 4 were treated topically with honokiol (1 mg per mouse) at least 30 min before each exposure to UVB, mice in Group 5 were treated topically with honokiol (3 mg per mouse) at least 30 min before each exposure of UVB and mice in Group 6 were treated topically with honokiol (3 mg per mouse) immediately after UVB exposure. The mice that have received honokiol treatment before UVB exposure continued to remain on the skin during the UVB exposure. Topical treatment of honokiol was started at least 14 days before the start of UVB exposure and on alternate days. Mice were UVB irradiated as described earlier (10,14). Briefly, the dorsal skin was exposed to UVB radiation from a band of four FS24T1 UVB lamps (Daavlin; UVA/UVB Research Irradiation Unit, Bryan, OH) equipped with an electronic controller to regulate UV dosage. The UVB lamps primarily emit UVB (290–320 nm, >80% of total energy) and UVA (320–375 nm, <20% of total energy) radiation with peak emission at 314 nm as monitored (14). Short wavelengths of UV (<290 nm) were filtered out using Kodacel cellulose film (Eastman Kodak, Rochester, NY). Under the standard photocarcinogenesis protocol, mice were UVB irradiated (180 mJ/cm^2) three times a week until the end of the protocol.

Evaluation of tumor growth

The UVB-irradiated skin of the mice was examined once a week for tumor appearance. Growths that were >1 mm in diameter and that persisted for at least 2 weeks were defined as tumors and recorded. The dimensions of all the tumors on each mouse were recorded at the termination of the experiment. Tumor volumes were calculated using the hemiellipsoid model formula: tumor volume = 1/2 (4π/3) (l/2) (w/2) h, where l = length, w = width and h = height, as performed earlier (14). At the termination of the experiment, mice were killed and skin and tumor samples were collected for the analysis of various biomarkers of interest. The incidence and multiplicity of carcinoma were also recorded in the same experiment. The diagnosis of carcinoma was confirmed histologically either at the time when carcinoma-bearing mice died or at the termination of the experiment.

Evaluation of non-toxicity of honokiol

Physicochemical observations were recorded to determine potential toxic effects of honokiol in the animals. We determined the weekly consumption of

Downloaded from https://academic.oup.com/carcin/article/31/11/2004/2392098 by guest on 05 May 2022

diet and regular measurement of body weight per mouse and compared these with the control (non-honokiol-treated) group of mice. Any adverse effect, such as inflammation or redness on the skin, etc., was recorded.

Pathological evaluation of skin tumors

At the termination of the experiment, representative biopsies from the skin tumors were collected, fixed in 10% formaldehyde and embedded in paraffin. Sections (5 μm thick) were stained with hematoxylin and eosin for pathological evaluation. Tumors were analyzed in a blinded fashion, and the discrimination between bona fide tumors and non-neoplastic lesions was performed according to the following major criteria: loss of keratinization or keratinized centers, presence of horn pearls and atypical cells.

PGE_2 immunoassay for quantitation of PGE_2

Epidermal skin or tumor samples were homogenized in 100 mM phosphate buffer, pH 7.4, containing 1 mM ethylenediamine tetraacetic acid and 10 μM indomethacin using a polytron homogenizer (Fisher Scientific, Atlanta, GA). The supernatants were collected and the concentration of PGE_2 was determined in supernatants using the Cayman PGE_2 Enzyme Immunoassay Kit (Cayman Chemical) following the manufacturer's instruction.

Assay for proinflammatory cytokines

Epidermal or skin tumor homogenates from each treatment group were used for the analysis of cytokines, such as TNF-α, IL-1β and IL-6, using enzyme-linked immunosorbent assay kits following the manufacturer's instructions and reagents.

Preparation of lysates and western blot analysis

Epidermal or skin tumor lysates for western blot analysis were prepared as described previously (14). Epidermis was separated from the whole skin as described earlier (16). The epidermis or tumor tissue samples were pooled from at least three mice in each group, and three sets of pooled samples from each treatment group were used to prepare lysates, thus $n = 10$. Proteins (25–50 μg) were resolved on 10% Tris–glycine gel and transferred onto nitrocellulose membranes. Membranes were incubated in blocking buffer for 1 h and then incubated with the primary antibodies overnight at 4°C. The membrane was then washed with phosphate-buffered saline and incubated with horseradish peroxidase-conjugated secondary antibody, as detailed (14). Enhanced chemiluminescence reagents were used to visualize the protein bands. To verify equal protein loading on the gel, the blots were stripped and reprobed for β-actin.

Immunohistochemical detection of COX-2- and PCNA-positive cells

Immunohistochemical detection of COX-2 or PCNA in tissue samples was performed following established protocols (17,18). Briefly, 5 μm thick sections were deparaffinized and rehydrated in a graded series of alcohols. Following rehydration, an antigen retrieval process was performed (17,18). The non-specific binding sites were blocked with 1% bovine serum albumin with 2% goat serum in phosphate-buffered saline. The sections were incubated with antibodies specific to COX-2 or PCNA followed by incubation with biotinylated secondary antibody and horseradish peroxidase-conjugated streptavidin. After washing in phosphate-buffered saline, sections were incubated with diaminobenzidine substrate and counterstained with hematoxylin. Representative photomicrographs were taken using an Olympus BX41 microscope fitted with a Qcolor5 digital camera system.

Statistical analysis

Statistical analysis of tumor data was performed at the termination of the experiment. Tumor incidence in the UVB alone and honokiol-treated groups was compared using the χ^2 test. Tumor multiplicity data were analyzed using the Wilcoxon rank-sum test. The data on cytokine levels and PGE_2 are expressed as means ± SD. The statistical significance of difference between the values of control and treatment groups was determined by analysis of variance followed by *post hoc* testing.

Results

Non-toxicity and UV absorption spectrum of honokiol

Our data indicated that there was no significant difference in diet or drinking water consumption on a per day basis between honokiol-treated and untreated animals (data not shown). The treatment of mouse skin with honokiol alone (3 mg per mouse) for 26 weeks did not induce erythema, edema or any other apparent sign of toxicity in the skin. Similarly, there was no significant difference in body weight gain on a weekly basis among the various treatment groups (Figure 1A), further suggesting that honokiol is non-toxic. Molecular structure of honokiol is shown in Figure 1A. UV absorption spectra of honokiol revealed that honokiol does not absorb UVB spectrum (290–320 nm).

Honokiol inhibits UVB-induced skin carcinogenesis

At the termination of the experiment, the tumor incidence was lower by 10 and 20% (not significant), respectively, in the groups of mice treated with 1 or 3 mg per mouse of honokiol before UVB exposure as compared with the group of mice that were not treated with honokiol (Figure 1B). The latency period of the tumors was 3 weeks longer in the honokiol-treated (3 mg per mouse) mice than in the non-honokiol-treated UVB-irradiated mice. As shown in Figure 1C, a total of 173 tumors were recorded in the group of 20 mice that were irradiated with UVB but did not receive honokiol, whereas only 125 tumors were recorded in the group of mice treated with 1 mg honokiol per mouse (28% reduction, $P < 0.05$). The tumor multiplicity (60%, $P < 0.001$) and tumor size were significantly lower (80%, $P < 0.001$) in the group of UVB-irradiated mice that were treated with honokiol (3 mg per mouse) before UVB irradiation than in the control group of mice that were UVB-irradiated but not treated with honokiol. The group of mice that were treated with honokiol immediately after UVB irradiation (3 mg per mouse) resulted in a significant inhibition of tumor multiplicity (54%, $P < 0.001$). The rate of tumor multiplicity in the honokiol (3 mg per mouse)-treated animals was significantly lower ($P < 0.05$, Fisher–Irwin exact test) throughout the experiment than in control animals. Tumor growth was not observed in the skin of mice that were treated with honokiol alone but were not UVB irradiated. When data were compared in terms of average tumor volume/tumor-bearing mouse between honokiol-treated and non-honokiol-treated groups, a significant reduction was observed (1 mg honokiol per mouse = 33%, $P < 0.05$; 3 mg honokiol per mouse = 70–80%, $P < 0.001$) as shown in Figure 1D. Details of tumor data are summarized in Table I.

Inhibition of malignant progression of papillomas to carcinomas

As shown in Figure 2, 70% mice of the non-honokiol-treated UVB-irradiated control mice developed carcinomas as compared with 60% of the mice treated with 1 mg honokiol per mouse and 35% of the mice treated with 3 mg honokiol per mouse (Figure 2A). It was found that a total 24 papillomas had converted into carcinomas in non-honokiol-treated but UVB-irradiated group of mice as compared with 19 (21% reduction, not significant) in the mice treated with 1 mg honokiol per mouse and 9 (62% reduction, $P < 0.001$) in the mice treated with 3 mg honokiol per mouse. When the mice were treated with honokiol (3 mg per mouse) after UVB irradiation, the inhibitory effect of honokiol on conversion of papillomas to carcinomas was very similar to that observed for the group in which mice were treated with honokiol before each exposure to UVB. The inhibitory effect of honokiol, either applied before or after UVB irradiation, in terms of number of carcinoma/carcinoma-bearing mouse also was significant ($P < 0.05$) (Figure 2C). Histologically, these carcinomas were identified as keratoacanthomas and squamous cell carcinomas (data not shown).

As the treatment of mice with 3 mg honokiol per mouse resulted in a significant inhibition of UVB-induced skin tumor development, further studies on molecular targets or chemopreventive mechanism were performed with this dose of honokiol and data were compared with the non-honokiol-treated but UVB-irradiated control group.

Honokiol inhibits UVB-induced COX-2 expression and PGE_2 production in the skin

As UVB-induced skin tumors originate from the epidermis of the skin, we focused on the analysis of the expression of the molecular targets of inflammation in the epidermal layer of the mouse skin. It is worth mentioning that epidermal uptake of honokiol was detected using Liquid Chromatography Tandem Mass Spectrometry (data not shown) and that may target inflammatory biomarkers in UVB-exposed skin. A characteristic response of keratinocytes to UVB radiation is enhanced COX-2 expression and a subsequent increase in the production of PG metabolites in the skin (11,19).

Downloaded from https://academic.oup.com/carcin/article/31/11/2004/2392098 by guest on 05 May 2022

Table I. Protective effect of honokiol on the growth of UVB-induced skin tumors in SKH-1 hairless mice[a]

	UVB alone	HK (1 mg) + UVB	HK (3 mg) + UVB	UVB + HK (3 mg)
Tumor-bearing mice/group, *n*	20	18	16	16
Total tumors/group, *n*	173	125 (28)[b]	72 (58)[c]	80 (54)[c]
Total tumor volume/group (mm^3)	11 618	6017 (48)[c]	1836 (84)[d]	3520 (70)[d]
Total tumor volume/tumor-bearing-mouse (mm^3)	726 ± 101	490 ± 75 (33)[b]	140 ± 45 (80)[d]	220 ± 55 (70)[d]
Mean tumor volme/tumor (mm^3)	62 ± 18	42 ± 13 (32)[b]	23 ± 8 (63)[d]	29 ± 11 (53)[c]

HK + UVB = honokiol was topically applied before UVB irradiation; UVB + HK = honokiol was topically applied after UVB irradiation. UVB alone-exposed mice were also treated with cream before UVB irradiation.

[a]Total number of tumors and tumor volume in different treatment groups were recorded at the end of the experiment at 26th week, $n = 20$. The values in parentheses indicate the percent inhibition.

[b]Significant versus control group, $P < 0.05$.

[c]Significant versus control group, $P < 0.005$.

[d]Significant versus control group, $P < 0.001$.

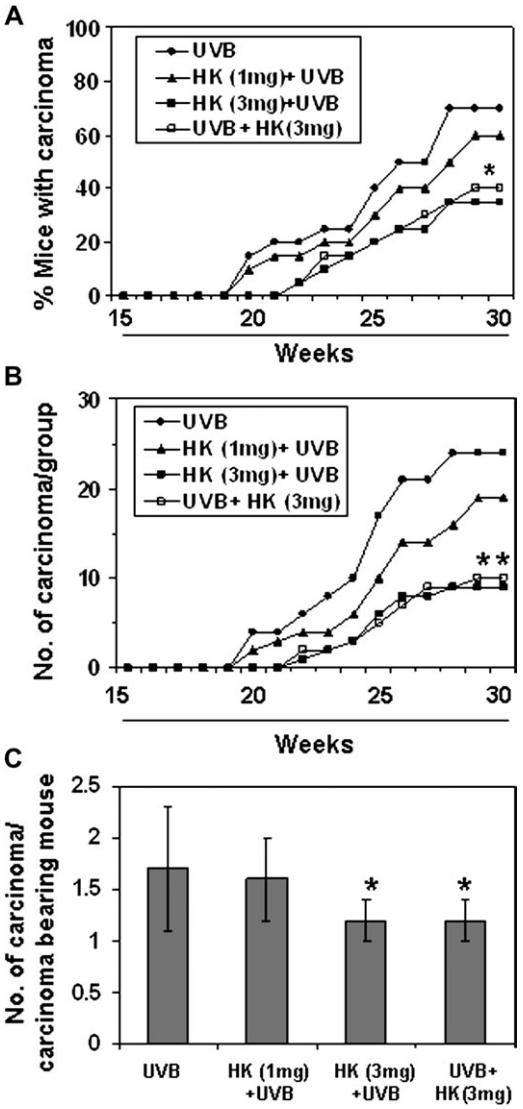

Fig. 2. Honokiol inhibits UVB-induced malignant progression of papillomas to carcinomas in mouse skin. Details of honokiol treatment were similar as described in Figure 1. The carcinoma data are presented in terms of the percent of mice with carcinoma (**A**) and the total number of carcinomas per group (**B**). The carcinoma data in different treatment groups are plotted as a function of the number of weeks on treatment. The number of carcinoma per carcinoma-bearing mouse (**C**) are shown as means ± SD at the termination of the experiment at 30th week of treatment, $n = 20$. Significant inhibition versus UVB alone at the termination of the experiment, $^{*}P < 0.05$, $^{**}P < 0.001$.

Immunohistochemical analysis of COX-2 indicated that chronic exposure of the skin to UVB radiation resulted in greater expression of COX-2 as compared with the skin of the non-UVB-exposed normal skin (Figure 3A). However, treatment of mice with honokiol, whether applied before or after UVB irradiation, resulted in a reduction in the COX-2 expression as compared with the expression in non-honokiol-treated UVB-irradiated mouse skin. The levels of COX-2 expression were verified by western blot analysis, which confirmed that topical treatment of honokiol inhibited chronic UVB irradiation-induced COX-2 expression in the mouse epidermis (Figure 3B). We also determined the levels of PG metabolites in the skin with a particular emphasis on PGE_2 because PGE_2 plays a pivotal role in cutaneous inflammation. The levels of epidermal PGE_2 in UVB-irradiated skin were significantly greater as compared with the skin of the non-UVB-irradiated mouse skin whether or not the skin was treated with honokiol; however, the levels of PGE_2 were significantly lower in the UVB-irradiated mouse skin, which was treated with honokiol whether before (65%, $P < 0.001$) or after UVB irradiation (59%, $P < 0.001$), as shown in Figure 3B.

Honokiol inhibits UVB-induced increases in the levels of PCNA in the skin

Immunohistochemical analyses revealed that exposure of the skin to UVB radiation enhances the proliferation potential of epidermal keratinocytes as indicated by the staining pattern of PCNA in the epidermis as compared with non-UVB-irradiated skin. Treatment of the skin with honokiol inhibited UVB-induced expression of PCNA in skin (Figure 3C). The immunohistochemical data further revealed that the treatment of the mouse skin with honokiol before UVB irradiation is more effective against PCNA than the treatment of honokiol after UVB irradiation of the skin. These data were further confirmed by western blot analysis, as shown in Figure 3B.

Honokiol inhibits UVB-induced increases in the levels of proinflammatory cytokines in the skin

Chronic exposures of the skin to UVB radiation resulted in significantly greater production of proinflammatory cytokines, such as TNF-α ($P < 0.001$), IL-1β ($P < 0.001$) and IL-6 ($P < 0.001$) in the mouse skin (Figure 3D) as compared with the non-UVB-exposed control mice. Topical treatment of mouse skin with honokiol either before or after UVB irradiation resulted in a significant reduction in TNF-α (63 and 55%, $P < 0.001$), IL-1β (55 and 64%, $P < 0.01$) and IL-6 (64 and 57%, $P < 0.001$) as compared with non-honokiol-treated UVB-exposed mouse skin.

Honokiol inhibits UVB-induced biomarkers of inflammation in skin tumors

To further determine whether inhibition of UVB-induced skin tumor development by honokiol is associated with the inhibition of UVB-induced inflammation and its mediators in the skin tumors, various

Downloaded from https://academic.oup.com/carcin/article/31/11/2004/2392098 by guest on 05 May 2022

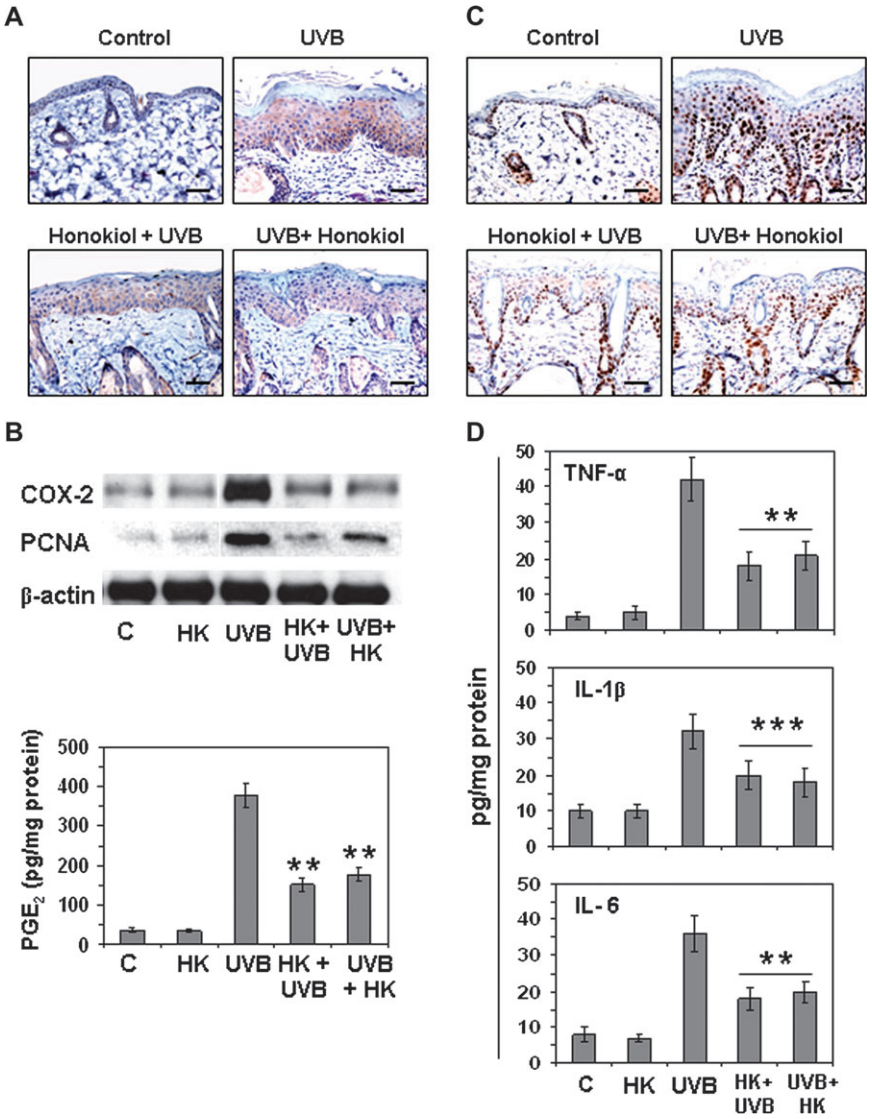

Fig. 3. Honokiol inhibits UVB-induced COX-2 and PCNA expression and PGE_2 production in the mouse skin. Honokiol (HK) was topically applied either before UVB irradiation (HK + UVB) or immediately after UVB irradiation (UVB + HK) of the mouse skin. C, control non-UVB-exposed mouse skin; HK, Honokiol treatment alone. UVB alone-exposed mice were also treated with cream before UVB exposure. (**A**) Immunohistochemical detection of COX-2 expression in chronically UVB-exposed mouse skin. (**B**) The analysis of epidermal COX-2 and PCNA expressions was performed using western blotting, as described in Materials and Methods. A representative blot is shown from three independent experiments with identical observations. The concentration of PGE_2 is expressed in terms of picograms per miligram protein as a mean ± SD, $n = 10$. (**C**) Immunohistochemical detection of PCNA expression in chronically UVB-exposed mouse skin. The COX-2 and PCNA staining appears dark brown, $n = 6$. (**D**) The levels of proinflammatory cytokines, TNF-α, IL-1β and IL-6 were determined in skin homogenate samples using enzyme-linked immunosorbent assay kits and data are presented as the mean ± SD in terms of picograms per miligram protein, $n = 10$. Significant difference versus non-honokiol-treated UVB-exposed controls, $^{***}P < 0.01$, $^{**}P < 0.001$. Scale bar = 50 μm.

biomarkers were analyzed in skin tumors from different treatment groups. Immunohistochemical data revealed that honokiol inhibited UVB-induced expression levels of PCNA in skin tumors in terms of percentage of PCNA-positive cells ($P < 0.001$) (Figure 4A). As shown in Figure 4B, western blot analysis revealed that treatment with honokiol resulted in reduction in the expression levels of PCNA, COX-2 and iNOS. The inhibitory effect of honokiol in tumors was observed whether it was applied before or after UVB irradiation of the skin. The levels of PGE_2 in tumors also were significantly reduced when treated with honokiol either before UVB irradiation (63%, $P < 0.001$) or after UVB irradiation (57%, $P < 0.001$). Treatment of the skin with honokiol either before or after UVB irradiation resulted in a significant inhibition of the expression levels of TNF-α (46 and 35%, $P < 0.001$), IL-1β (28%, $P < 0.01$) and IL-6 (48 and 56%, $P < 0.001$) compared with skin tumors obtained from non-honokiol-treated mice (Figure 4D).

Honokiol modulates cell cycle regulators in UVB-induced skin tumors

As the chemopreventive effect of honokiol was more or less similar whether skin was treated with honokiol before or after UVB irradiation, in further studies, we examined the skin tumors obtained from the mice, which were treated with honokiol before UVB irradiation. Enhanced expression of cell cycle regulatory proteins such as cyclin-dependent kinases (CDKs) and cyclins or decreased expression of CDK inhibitors have been implicated in photocarcinogenesis (20). Based on our results showing an inhibition of tumor growth and cellular proliferation by honokiol in UVB-induced tumorigenesis, we hypothesized that honokiol might have modulatory effects on cell cycle regulatory proteins. As shown in Figure 5, the expression levels of cyclins (cyclin D1, D2 and E) and CDK2, CDK4 and CDK6 were considerably higher in UVB-induced skin tumors compared with non-UVB-irradiated normal skin from age-matched control mice. However, treatment of skin with honokiol before UVB irradiation resulted

Downloaded from https://academic.oup.com/carcin/article/31/11/2004/2392098 by guest on 05 May 2022

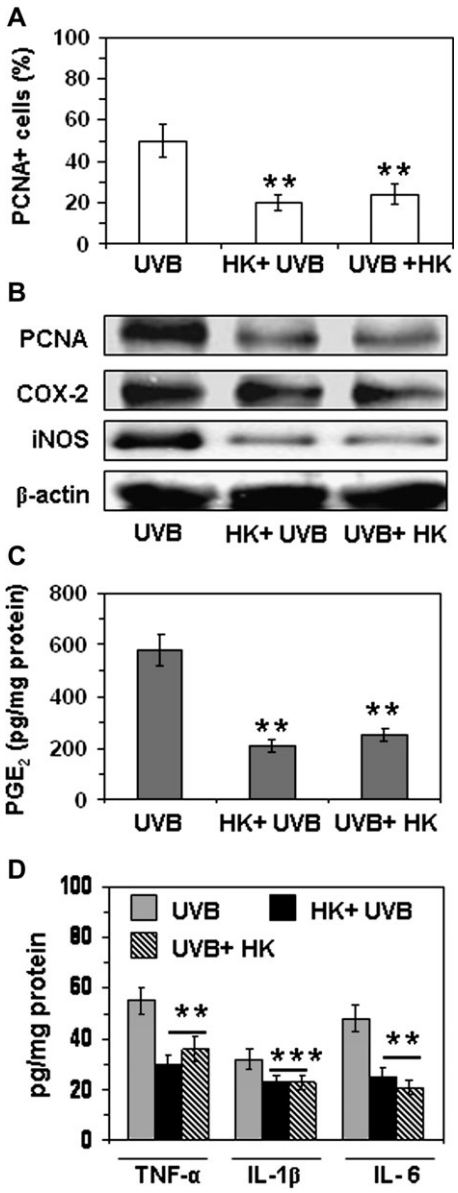

Fig. 4. Honokiol inhibits UVB-induced increases in inflammatory responses and their mediators and PCNA in UVB-induced skin tumors. Honokiol treatments are detailed under Figure 1. (**A**) The levels of PCNA in skin tumors were estimated by immunostaining for PCNA. The PCNA-positive cells in tumors of different treatment groups were counted and summarized in percentage of total cells. Data represent the mean ± SD ($n = 6$). (**B**) The levels of PCNA, COX-2 and iNOS were determined employing western blot analysis. Representative blots are shown from three independent experiments with identical observations. (**C**) The levels of PGE_2 were determined in the tumor homogenate samples using a PGE_2 immunoassay kit. The concentration of PGE_2 is expressed in terms of picograms per miligram protein as a mean ± SD, $n = 10$. (**D**) At the termination of the photocarcinogenesis experiment, tumor homogenates were prepared for the analysis of TNF-α, IL-1β and IL-6 using enzyme-linked immunosorbent assay. The concentration of each cytokine is reported in terms of picograms per miligram protein as a mean ± SD, $n = 10$. Significant inhibition versus UVB alone, $^{***}P < 0.01$, $^{**}P < 0.001$.

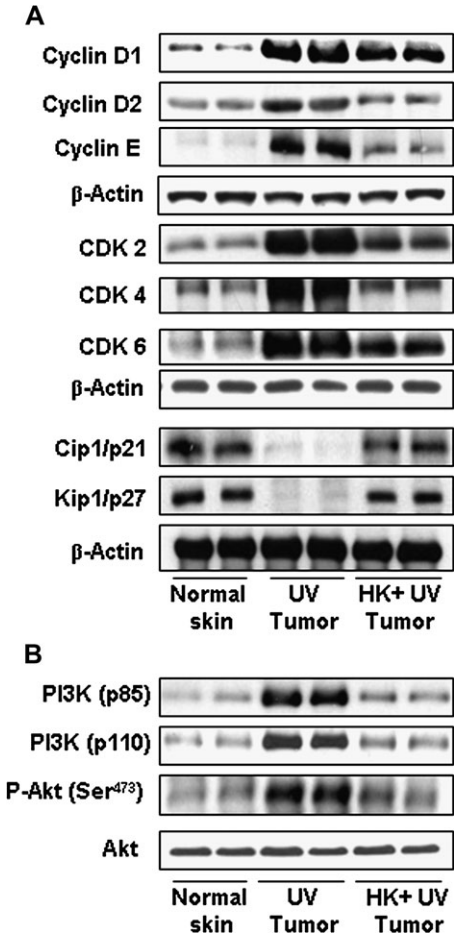

Fig. 5. Effect of honokiol on G_1 phase cell cycle regulatory proteins and cell survival signals in UV-induced skin tumors. (**A**) Lysates from the normal skin and tumors were prepared and then subjected to western blot analysis. Effect of honokiol was determined on the following: the expression of cyclin D1, D2 and E; the expression of CDK 2, CDK 4 and CDK6 and the expression of Kip1/p27 and Cip1/p21. (**B**) Treatment of honokiol inhibits UVB-induced increases of PI3Kp85, PI3Kp110 and p-Akt-Ser^{473} in skin tumors compared with non-honokiol-treated tumors. Representative blots are shown from three independent experiments with almost identical results. Equivalent protein loading on gel was checked by probing stripped blots for β-actin as shown.

in inhibition of UVB-induced expression levels of cyclins (cyclins D1, D2 and E) and CDK2, CDK4 and CDK6 compared with skin tumors obtained from non-honokiol-treated mice. Next, we examined the levels of Cip1/p21, which is a universal inhibitor of cell cycle progression and is transcriptionally activated by p53 after UVB irradiation (21,22). Treatment of honokiol resulted in the enhancement of the levels of Cip1/p21 in skin tumors compared with non-honokiol-treated skin tumors. Kip1/p27 is another important CDK inhibitor that regulates CDK-cyclin activity at G_1–S transition (22). Western blot data revealed that the protein expression of Kip1/p27 was also strongly upregulated in the skin tumors treated with the honokiol compared with those skin tumors that were obtained from non-honokiol-treated mouse skin.

Honokiol inhibits cell survival signals in UVB-induced skin tumors

The risk of UVB radiation-induced skin tumor development can be increased through various signaling pathways including the activation of the cell survival kinases, such as PI3K/Akt (23–25). As expected, the levels of both the catalytic (p110) and the regulatory (p85) subunits of PI3K were enhanced in the UVB-induced skin tumors as compared with the non-UVB-exposed normal mouse skin; however, the levels of the p85 and p110 subunits were greatly reduced in the UV-induced skin tumors from honokiol-treated mice compared with the skin tumors of non-honokiol-treated mice (Figure 5B). Most of the biological effects of PI3K are mediated through the activation of the downstream target Akt, a prosurvival factor. The results of western blot analysis revealed that the treatment of mice with honokiol resulted in reduction of UVB-induced phosphorylation of Akt (Ser^{473})

Downloaded from https://academic.oup.com/carcin/article/31/11/2004/2392098 by guest on 05 May 2022

as compared with the skin tumors of non-honokiol-treated group, whereas the levels of total Akt remained unchanged in all the treatment groups, as indicated by the relative density of the protein bands (Figure 5B).

Discussion

The central finding of this study is that topical treatment of mouse skin with honokiol in a hydrophilic cream-based topical formulation before or after UVB exposure significantly prevents UVB-induced skin tumor development in terms of tumor multiplicity and the growth of the tumors. Similar studies were also conducted by Chilampalli *et al.* (26) wherein chemopreventive effect of honokiol was determined on UVB-induced skin carcinogenesis. The investigators have determined the effect of honokiol (30 μg/200 ml acetone) on 30 mJ/cm^2 UVB-induced skin tumor development in mice while we exposed mice to 180 mJ/cm^2. It is a major difference in selection of UVB dose or exposure of mice. If, in our study, we used 30 mJ/cm^2 UVB dose, then protection must be very high. Second point is that Chilampalli *et al.* have shown the protection in terms of tumor multiplicity by 45%, whereas we have found the protection in terms of tumor multiplicity by 60% if honokiol is topically applied before UVB exposure. Conclusively, both studies have significant differences in the selection of UVB doses. Furthermore, the use of honokiol in hydrophilic cream-based topical formulation may be a better choice for human use and may have better protection from UVB-induced skin tumor development. UV spectra indicate that honokiol does not act as a sunscreen. Furthermore, an excellent work from the research laboratory of Dr Alan Conney has revealed that topical application of moisturizing creams, such as Dermabase, Dermovan, Eucerin, etc. once a day, 5 days a week for 17 weeks to high-risk mice increased the rate of tumor formation and tumor size per mouse (27). In this study, we used a hydrophilic cream as a vehicle for topical application of honokiol, and this cream was obtained from Fougera and Co. We have also used this hydrophilic cream as a vehicle in our prior studies (10). We did not find any effect of this cream in conjunction with UVB exposures of mice when data were compared with UVB carcinogenesis versus cream + UVB carcinogenesis. For this purpose, we had included two groups: (i) UVB alone and (ii) cream + UVB. This particular hydrophilic cream has no effect on UVB-induced skin tumor development compared with non-cream + UVB-induced carcinogenesis. The data which we have included in our figures and manuscript are on cream + UVB-induced carcinogenesis. It was also done because each treatment group of mice received hydrophilic cream as a vehicle and that maintain uniform treatment regimens. Honokiol also inhibits malignant progression of papillomas to carcinomas in mice. Studies have suggested that the transformation of papillomas to carcinomas requires further genetic and epigenetic changes in the papillomas and can be achieved by multiple UVB exposures (11); therefore, it is suggested that honokiol affords protection from further genetic and epigenetic alterations caused by excessive UVB irradiation.

In efforts to determine the molecular targets or chemopreventive mechanism of honokiol in prevention of photocarcinogenesis, we determined the effect of honokiol on multiple targets, which included UVB radiation-induced inflammatory mediators, cell cycle regulators and cell survival signals in UVB-exposed skin and/or skin tumors. UVB-induced chronic inflammation has been implicated in tumor initiation, promotion and progression. One of the most important enzymes in the process of inflammation and tumor development in UV carcinogenesis is inducible COX-2. COX-2 is a rate-limiting enzyme for the generation of PG metabolites from arachidonic acid (28). COX-2 overexpression has been linked to the pathophysiology of inflammation and cancer (29) due to enhanced synthesis of PG metabolites, which have been shown to be a potential contributing factor in the development of non-melanoma skin cancers (30,31). A number of studies have demonstrated overexpression of COX-2 in chronically UVB-irradiated skin, as well as in UVB-induced premalignant lesions and squamous cell carcinomas (14,32,33). A role for COX-2 in photocarcinogenesis is also supported by several studies that demonstrate that inhibition of COX-2 activity by specific inhibitors can partially block carcinogenesis induced by long-term UVB exposures (34,35). In this study, we found that topical application of honokiol before or after UVB exposure significantly inhibited UVB-induced inflammatory responses in terms of inhibition of COX-2 expression, PGE_2 production, iNOS expression and reduction in the levels of epidermal cellular proliferation. The inhibition of proliferating potential of epidermal keratinocytes by honokiol may also be a contributing factor for the inhibitory effects on the growth of UVB-induced skin tumors. Ahn *et al.* have shown that topical treatment of honokiol on the mouse skin suppressed chemical skin tumor promoter-induced inflammatory activity in terms of nuclear factor-kappaB DNA-binding activity compared with untreated group (36).

The chronic and sustained elevated levels of proinflammatory cytokines have been implicated in skin cancer risk (11,37,38); therefore, the higher levels of proinflammatory cytokines would be expected to contribute to the tumor promotion process and thus, the development of tumors would be expected to occur earlier and progress more rapidly. Our data indicated that treatment of the mouse skin with honokiol significantly inhibited UVB-induced expression of proinflammatory cytokines, such as TNF-α, IL-1β and IL-6, in the mouse skin as well as in skin tumors and that may have contributed in inhibition of tumor development.

Skin exposure to UVB also affects cell cycle regulators in the skin. Regulation of cyclin-CDK complexes plays a key role in cell cycle progression at different phases in which CDKs are negatively regulated by a group of functionally related proteins known as CDK inhibitors, such as Kip/Cip family members (39,40). Cip1/p21 is a universal CDK inhibitor and Cip1/p21 binds to PCNA and inhibits PCNA function in DNA replication process (41), whereas Kip1/p27 is upregulated in response to antiproliferative signals. Consistent with these reports, honokiol treatment resulted in an upregulation of Kip1/p27 and Cip1/p21 and reduced the protein levels of CDK2, CDK4 and CDK6, cyclin D1, D2 and E in skin tumors, which may have caused cell cycle arrest in tumor cells. Tumor cell cycle arrest could lead to the reduction in proliferation potential of cells as observed by a decrease in the levels of PCNA in UVB-exposed skin and skin tumors. Thus, it can be suggested that modulation in cell cycle progression and inhibition of cell proliferation could be one of the possible mechanisms through which honokiol inhibits UVB-induced skin tumor development.

Cell survival signals, including PI3K/Akt pathway, have been associated with cellular proliferation and carcinogenesis (42–44). The biological effects of PI3K are mediated through the activation of the downstream target, Akt. Akt is a serine/threonine kinase, which has been identified as an important component of prosurvival signaling pathways (45). The skin tumors augment UVB radiation-induced activation of PI3K and phospho-Akt as compared with the non-UVB-exposed mouse skin. The PI3K/Akt signaling pathway regulates the activity of the transcriptional factor, nuclear factor-kappaB, which in turn is known to regulate several well-known markers of tumor promotion and tumor cell proliferation, e.g. COX-2, iNOS and PCNA (46,47). The data obtained from this study clearly demonstrate that treatment of honokiol inhibits UVB-induced expression of both the regulatory (p85) and the catalytic (p110) subunits of PI3K and inhibits phosphorylation of Akt at Ser^{473} in the skin tumors as compared with the non-honokiol-treated tumors. Thus, the inhibition of PI3K/p-Akt pathway in skin tumors by honokiol may have a role in inhibition of UVB-induced skin tumor growth.

Collectively, the data from this study suggest that honokiol inhibits UV-induced skin tumor development through the inhibition of UVB-induced: (i) inflammation and inflammatory mediators, (ii) cell cycle progression and (iii) cell survival signals in the mouse skin and/or skin tumors. Honokiol can be considered for the development of new strategies for the prevention of UV radiation-induced skin cancers in humans.

Downloaded from https://academic.oup.com/carcin/article/31/11/2004/2392098 by guest on 05 May 2022

Funding

Veterans Administration Merit Review Award (S.K.K.).

Conflict of Interest Statement: None declared.

References

1. Housman,T.S. *et al*. (2003) Skin cancer is among the most costly of all cancers to treat for the Medicare population. *J. Am. Acad. Dermatol.*, **48**, 425–429.
2. Baliga,M.S. *et al*. (2006) Chemoprevention of photocarcinogenesis by selected dietary botanicals. *Photochem. Photobiol. Sci.*, **5**, 243–253.
3. Li,T.S.C. (2002) *Chinese and Related North American Herbs: Phytopharmacology and Therapeutic Values*. CRC Press, Boca Raton, FL.
4. Hahm,E.R. *et al*. (2008) Honokiol, a constituent of oriental medicinal herb magnolia officinalis, inhibits growth of PC-3 xenografts *in vivo* in association with apoptosis induction. *Clin. Cancer Res.*, **14**, 1248–1257.
5. Bai,X. *et al*. (2003) Honokiol, a small molecular weight natural product, inhibits angiogenesis *in vitro* and tumor growth *in vivo*. *J. Biol. Chem.*, **278**, 35501–35507.
6. Munroe,M.E. *et al*. (2007) Honokiol, a natural plant product, inhibits inflammatory signals and alleviates inflammatory arthritis. *J. Immunol.*, **179**, 753–763.
7. Pyo,M.K. *et al*. (2002) Anti-platelet effect of the constituents isolated from the barks and fruits of *Magnolia obovata*. *Arch. Pharm. Res.*, **25**, 325–328.
8. Clark,A.M. *et al*. (1981) Antimicrobial activity of phenolic constituents of *Magnolia grandiflora L. J. Pharm. Sci.*, **70**, 951–952.
9. Park,J. *et al*. (2004) *In vitro* antibacterial and anti-inflammatory effects of honokiol and magnolol against *Propionibacterium sp*. *Eur. J. Pharmacol.*, **496**, 189–195.
10. Mittal,A. *et al*. (2003) Exceptionally high protection of photocarcinogenesis by topical application of (-)-epigallocatechin-3-gallate in hydrophilic cream in SKH-1 hairless mouse model: relationship to inhibition of UVB-induced global DNA hypomethylation. *Neoplasia*, **5**, 555–565.
11. Mukhtar,H. *et al*. (1996) Photocarcinogenesis: mechanisms, models and human health implications. *Photochem. Photobiol.*, **63**, 355–447.
12. Katiyar,S.K. (2006) Oxidative stress and photocarcinogenesis: strategies for prevention. In Singh,K.K. (ed.) *Oxidative Stress, Disease and Cancer*. Imperial College Press, London, UK, pp. 933–964.
13. Katiyar,S.K. *et al*. (2000) Kinetics of UV light-induced cyclobutane pyrimidine dimers in human skin *in vivo*: an immunohistochemical analysis of both epidermis and dermis. *Photochem. Photobiol.*, **72**, 788–793.
14. Meeran,S.M. *et al*. (2009) Inhibition of UVB-induced skin tumor development by drinking green tea polyphenols is mediated through DNA repair and subsequent inhibition of inflammation. *J. Invest. Dermatol.*, **129**, 1258–1270.
15. Rivas,J.M. *et al*. (1994) The role of IL-4, IL-10, and TNF-alpha in the immune suppression induced by ultraviolet radiation. *J. Leukoc. Biol.*, **56**, 769–775.
16. Katiyar,S.K. *et al*. (1999) Prevention of UVB-induced immunosuppression in mice by green tea polyphenol (-)-epigallocatechin-3-gallate may be associated with alterations in IL-10 and IL-12 production. *Carcinogenesis*, **20**, 2117–2124.
17. Meeran,S.M. *et al*. (2008) Interleukin-12-deficiency exacerbates inflammatory responses in UV-irradiated skin and skin tumors. *J. Invest. Dermatol.*, **128**, 2716–2727.
18. Meeran,S.M. *et al*. (2009) Dietary grape seed proanthocyanidins inhibit 12-O-tetradecanoyl phorbol-13-acetate-caused skin tumor promotion in 7, 12-dimethylbenz(a)anthracene-initiated mouse skin, which is associated with the inhibition of inflammatory responses. *Carcinogenesis*, **30**, 520–528.
19. DiGiovanni,J. (1992) Multistage carcinogenesis in mouse skin. *Pharmacol. Ther.*, **54**, 63–128.
20. Hong,J.T. *et al*. (2001) Inhibitory effect of glycolic acid on ultraviolet-induced skin tumorigenesis in SKH-1 hairless mice and its mechanism of action. *Mol. Carcinog.*, **31**, 152–160.
21. Meeran,S.M. *et al*. (2008) Cell cycle control as a basis for cancer chemoprevention through dietary agents. *Front. Biosci.*, **13**, 2191–2202.
22. Graña,X. *et al*. (1995) Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). *Oncogene*, **11**, 211–219.
23. Chun,K.S. *et al*. (2007) Cyclooxygenase-2 inhibits UVB-induced apoptosis in mouse skin by activating the prostaglandin E2 receptors, EP2 and EP4. *Cancer Res.*, **67**, 2015–2021.
24. Luo,J. *et al*. (2003) Targeting the PI3K-Akt pathway in human cancer: rationale and promise. *Cancer Cell*, **4**, 257–262.
25. Nomura,M. *et al*. (2001) Mitogen-and stress-activated protein kinase 1 mediates activation of Akt by ultraviolet B irradiation. *J. Biol. Chem.*, **276**, 25558–25567.
26. Chilampalli,S. *et al*. (2010) Chemopreventive effects of honokiol on UVB-induced skin cancer development. *Anticancer Res.*, **30**, 777–783.
27. Lu,Y.P. *et al*. (2009) Tumorigenic effect of some commonly used moisturizing creams when applied topically to UVB-pretreated high-risk mice. *J. Invest. Dermatol.*, **129**, 468–475.
28. Langenbach,R. *et al*. (1999) Cyclooxygenase-deficient mice. A summary of their characteristics and susceptibilities to inflammation and carcinogenesis. *Ann. N. Y. Acad. Sci.*, **889**, 52–61.
29. Chapple,K.S. *et al*. (2000) Localization of cyclooxygenase-2 in human sporadic colorectal adenomas. *Am. J. Pathol.*, **156**, 545–553.
30. Marks,F. *et al*. (1999) Metabolic targets of cancer chemoprevention: interruption of tumor development by inhibitors of arachidonic acid metabolism. *Recent Results Cancer Res.*, **151**, 45–67.
31. Williams,C.S. *et al*. (1999) The role of cyclooxygenases in inflammation, cancer, and development. *Oncogene*, **18**, 7908–7916.
32. Buckman,S.Y. *et al*. (1998) COX-2 expression is induced by UVB exposure in human skin: implications for the development of skin cancer. *Carcinogenesis*, **19**, 723–729.
33. Athar,M. *et al*. (2001) Ultraviolet B (UVB)-induced COX-2 expression in murine skin: an immunohistochemical study. *Biochem. Biophys. Res. Commun.*, **280**, 1042–1047.
34. Wilgus,T.A. *et al*. (2003) Inhibition of cutaneous ultraviolet light B-mediated inflammation and tumor formation with topical celecoxib treatment. *Mol. Carcinog.*, **38**, 49–58.
35. Pentland,A.P. *et al*. (1999) Reduction of UV-induced skin tumors in hairless mice by selective COX-2 inhibition. *Carcinogenesis*, **20**, 1939–1944.
36. Ahn,K.S. *et al*. (2006) Honokiol potentiates apoptosis, suppresses osteoclastogenesis, and inhibits invasion through modulation of nuclear factor-kappaB activation pathway. *Mol. Cancer Res.*, **4**, 621–633.
37. Scott,K.A. *et al*. (2003) An anti-tumor necrosis factor-alpha antibody inhibits the development of experimental skin tumors. *Mol. Cancer Ther.*, **2**, 445–451.
38. Tron,V.A. *et al*. (1988) Epidermal interleukin-1 is increased in cutaneous T-cell lymphoma. *J. Invest. Dermatol.*, **90**, 378–381.
39. Sherr,C.J. *et al*. (1999) CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.*, **13**, 1501–1512.
40. Morgan,D.O. (1995) Principles of CDK regulation. *Nature*, **374**, 131–134.
41. Fotedar,R. *et al*. (2004) Role of p21WAF1 in the cellular response to UV. *Cell Cycle*, **3**, 134–137.
42. Tyrrell,R.M. (1996) Activation of mammalian gene expression by the UV component of sunlight-from models to reality. *Bioessays*, **18**, 139–148.
43. Saleem,M. *et al*. (2004) Lupeol modulates NF-kappaB and PI3K/Akt pathways and inhibits skin cancer in CD-1 mice. *Oncogene*, **23**, 5203–5214.
44. Osaki,M. *et al*. (2004) Inhibition of the PI3K-Akt signaling pathway enhances the sensitivity of Fas-mediated apoptosis in human gastric carcinoma cell line, MKN-45. *J. Cancer Res. Clin. Oncol.*, **130**, 8–14.
45. Downward,J. (1998) Mechanisms and consequences of activation of protein kinase B/Akt. *Curr. Opin. Cell Biol.*, **10**, 262–267.
46. Callejas,N.A. *et al*. (1999) Requirement of nuclear factor kappaB for the constitutive expression of nitric oxide synthase-2 and cyclooxygenase-2 in rat trophoblasts. *J. Cell Sci.*, **18**, 3147–3155.
47. Carpenter,C.L. *et al*. (1996) Phosphoinositide kinases. *Curr. Opin. Cell Biol.*, **8**, 153–158.

Received June 13, 2010; revised August 19, 2010; accepted August 27, 2010

Downloaded from https://academic.oup.com/carcin/article/31/11/2004/2392098 by guest on 05 May 2022