Chemopreventive effects of combination of honokiol and magnolol with α-santalol on skin cancer developments

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ABSTRACT: α-Santalol is active component of sandalwood oil and has been shown to have chemopreventive effects against chemically and UVB-induced skin cancer development in mice. α-Santalol is also shown to have skin permeation enhancing effects. Honokiol and magnolol isolated from Magnolia officinalis bark extract have also been shown to have chemopreventive effects against chemically and UVB-induced skin cancer in mice. This study was conducted to investigate the combination effects of α-santalol, honokiol and magnolol to study any additive/synergistic effects to lower the doses required for chemoprevention. Pretreatment of combinations of α-santalol with honokiol and magnolol significantly decreased tumor multiplicity up to 75% than control, α-santalol, honokiol and magnolol alone in SKH-1 mice. Combination of α-santalol with honokiol and magnolol also decreased cell viability, proliferation, and enhanced apoptosis in comparison to α-santalol, honokiol and magnolol alone in Human epidermoid carcinoma A431 cells. Overall, the results of present study indicated combinations of α-santalol with honokiol and magnolol could provide chemoprevention of skin cancer at lower doses than given alone.

Keywords: Honokiol, magnolol, chemoprevention, UVB photocarcinogenesis, apoptosis

1. Introduction

Human non melanoma skin cancer including basal cell carcinoma and squamous cell carcinoma is most common type of cancer in the United States for over 1.3 million new cases each year (1). Both experimental and epidemiological evidences suggest UVB acts as complete carcinogen in skin cancer (2,3). Carcinogenesis induced by UV is a multistep process which involves initiation, promotion and progression by which genetic events accumulated with in a cell leading to dysplastic cellular appearance, deregulated cell growth and finally to carcinoma development (4). UVB in mouse models can act as complete carcinogen by initiating and promoting skin cancer.

Chemoprevention involves the administration of natural or synthetic compounds to prevent or reverse the process of carcinogenesis. Cancer chemoprevention by naturally occurring agents, especially the phytochemicals, minerals and vitamins, has shown promising results against various malignancies (5,6). It is promising strategy for carcinoma inhibition before development of invasive tumor (7). In recent years to inhibit or reverse the multistage process, considerable efforts are focused towards natural occurring compounds (5,6). Over 1,000 phytochemicals have shown chemopreventive effects against cancer (6-9). Among such phytochemicals, chemopreventive effects of α-santalol, honokiol and magnolol have been investigated against skin cancer and have shown excellent chemopreventive effects.

α-Santalol is active component of sandalwood oil which constitutes about 61% (w/w) of oil. Studies conducted in our laboratory have shown the topical application of 5% (w/v) of α-santalol inhibited skin tumorigenesis against both chemical (10) and UVB (11) induced animal cancer models. In addition to this, mechanistic studies conducted in human epidermoid carcinoma A431 cells have shown that possible mechanisms involved in chemopreventive effects of α-santalol is by induction of apoptosis through extrinsic and intrinsic pathway and inhibition of cell proliferation through cell cycle arrest at G2/M phase (7,12,13). Honokiol and magnolol are isomers isolated from bark and seed cones of Magnolia officinalis which
are used in traditional Chinese medicine (14). Studies conducted recently in our laboratory have shown that honokiol and magnolol have shown chemopreventive effects against skin cancer development (15,16). Furthermore, the study conducted in our laboratory have shown that α-santalol acts as a permeation enhancer for certain drug such as 5-flurouracil. This study was conducted to investigate if reduced doses of α-santalol produce similar effects in combination of honokiol and magnolol, may enhance the chemopreventive effects of honokiol and magnolol, and may provide additive/synergistic effects against UVB-radiation induced skin cancer development in SKH-1 mice and in vitro in A431 cells.

2. Materials and Methods

2.1. Reagents

α-Santalol was isolated from sandalwood oil and characterized as reported (18). Thiazolyl blue tetrazolium bromide (MTT) and other chemicals of analytical grade were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cell proliferation ELISA kit and In Situ Cell Death Detection Kit were purchased from Roche Diagnostics GmbH (Mannheim, Germany). Vibrant Apoptosis Kit 2 and APO-BrdU TUNEL assay kit were purchased from Molecular Probes (Eugene, OR, USA).

2.2. Isolation characterization and purity of α-santalol

α-Santalol (Figure 1a) was isolated from sandalwood oil by distillation under vacuum as described in detail (18). On the basis of the NMR spectrum and the boiling point of the distillate, the major component of sandalwood oil is α-santalol. Further, GC-MS analysis, NMR data and mass spectrum of the isolated agent were consistent with the structure of α-santalol. Honokiol (Figure 1b) and magnolol (Figure 1c) were purchased from Nacalai tesque (Kyoto, Japan).

2.3. UVB exposure source and animals

UVB light source was four FS-40-T-12-UVB sunlamps (Daavlin, Bryan, OH) emitting 80% radiation within 280-340 nm with a peak at 314 nm. The UVB exposure dose was controlled using two Daavlin Flex Control Integrating 305 Dosimeters. Female SKH-1 hairless mice (five-weeks-old) were purchased from Charles River Laboratories (Wilmington, MA). All animal protocols were approved by the Institutional Animal Care and Use Committee. Mice were housed in the College of Pharmacy, South Dakota State University, animal room facilities (temp. 22 ± 1°C, humidity, 40-60%, light 6:00-18:00 h), and given food and water ad libitum. Mice were acclimatized for 2 weeks before starting the experiment.

2.4. Effects of α-santalol and honokiol on UVB-induced skin cancer development

Female SKH-1 mice were divided in four groups having 20 mice in each group. Group assignment was as follows: group 1, control (200 μL acetone); group 2, α-santalol (5 mg in 200 μL acetone); group 3, honokiol (30 μg in 200 μL acetone); group 4, α-santalol (5 mg in 100 μL acetone) and honokiol (30 μg in 100 μL acetone). The mice in all groups were irradiated with 30 mJ/cm² dose of UVB one hour after the topical application as outlined above. UVB-irradiation was given 5 days a week and the experiment was continued for 30 weeks. Animals were monitored for food and water consumption, and any apparent signs of toxicity such as weight loss or mortality during the entire study period. Skin tumor formation, as evident by an outgrowth > 1 mm in diameter and persisting for ≥2 weeks was recorded. Tumor incidence and multiplicity were recorded weekly until the end of the experiment at 30 weeks.

2.5. Effects of α-santalol and magnolol on UVB-induced skin cancer development

Female SKH-1 mice were divided in four groups having 20 mice in each group. Group assignment was as follows: group 1, control (200 μL acetone); group 2,
α-santalol (5 mg in 200 μL acetone); group 3, magnolol (30 μg in 200 μL acetone); group 4, α-santalol (5 mg in 100 μL acetone) and magnolol (30 μg in 100 μL acetone). Experimental protocol similar to honokiol was carried out for 30 weeks.

2.6. Cell culture

Human epidermoid carcinoma A431 cells were purchased from American Type Culture Collection (Manassas, VA). A431 cells were cultured in DMEM supplemented with 10% FBS, 100 μg/mL penicillin-streptomycin (Invitrogen, Carlsbad, CA) in a humidified atmosphere containing 95% air and 5% CO₂.

2.7. MTT assay for cell viability

Cells (9,000 cells/well) were plated in 96 well plates. After 24 h, cells were treated with different concentrations of control, α-santalol, honokiol, magnolol for 48 h using control as cells treated with growth medium alone. At the end of each treatment, cells were incubated with 10% of MTT stock solution (5 mg/mL) for 4 h. Thereafter medium was aspirated and 150 μL of DMSO (dimethyl sulfoxide) was added to dissolve crystal dye formazan for 1 h. Absorbance was measured at 570 nm with absorbance at 650 nm to correct background for blank (media without cells) using Spectra Max M2 microplate reader.

2.8. BrdU assay for cell proliferation

Bromodeoxyuridine incorporation assay is carried out using ELISA kit using manufactures protocol. Briefly 9,000 cells/well were plated in 96 well plate and treated with different concentrations of control, α-santalol, honokiol, magnolol for 48 h. At the end of each treatment period, cells were labeled with BrdU by incubating for 3 h at 37°C. Then cells were fixed by using FixDenat solution for 30 min followed by incubating fixed cells with anti-BrdU peroxide solution for 90 min. Then cells were rinsed with washing solution and incubated with substrate solution for 20 min. Reaction was stopped using 1M H₂SO₄ and absorbance of samples were measured using microplate reader using 450 nm with absorbance at 690 nm as reference.

2.9. Apoptosis assay

Apoptosis was quantified by using Vibrant Apoptosis Kit 2 as per manufacturer's protocol. Briefly A431 cells (2 × 10⁵) were treated with control, α-santalol, honokiol, magnolol for 48 h. At the end of each treatment cells were washed with ice cold PBS and incubated with annexin V labeled with Alexa 488 and PI for 15 min at room temperature. The stained cells were analyzed by FACS using Cell Quest 3.3 software. The early apoptotic cells were stained with Alexa 488 represented in lower right (LR) quadrant that gave green fluorescence, and late apoptotic cells were stained with both Alexa 488 and PI that gave green and red fluorescence represented in upper right (UR) quadrant.

2.10. Statistical analysis

ANOVA and Tukey test were performed on sample means using INSTAT software (Graph Pad, Sand Diego, CA). Significance was considered at p < 0.05.

3. Results

3.1. α-Santalol and honokiol combination decreased tumor multiplicity

The effects of α-santalol, honokiol and combination of α-santalol and honokiol on UVB-induced tumor multiplicity is shown in Figure 2. In the present study we found that topical application of α-santalol and honokiol combination significantly decreased tumor multiplicity compared to control and mice treated with α-santalol and honokiol alone. We found topical application of α-santalol and honokiol combination resulted in strong protection throughout our study where UVB act as complete carcinogen. At the end of 30 weeks experiment, average number of tumors in various groups was found to be 8.3, 5.6, 4.6, and 3. Furthermore, the combination of α-santalol and honokiol not only provided about 75% decrease in number of tumors as compared to control mice but significantly higher decrease than α-santalol and honokiol alone. These results clearly provide the

Figure 2. Effects of α-santalol, honokiol and combination of α-santalol and honokiol on tumor multiplicity in SKH-1 mice. Mice were irradiated chronically with UVB, 1 h after drug treatment. Group 1 served as control received acetone and group 2, 3, 4 are α-santalol, honokiol, and combination of α-santalol and honokiol. Combination significantly decreased tumor numbers compared to control and mice treated with individual drugs alone. Each data point represents mean tumor number ± SE.
Evidence that \( \alpha \)-santalol significantly enhances the chemopreventive effects of honokiol on UVB-induced cancer development in SKH-1 mice.

### 3.2. \( \alpha \)-Santalol and magnolol combination decreased tumor multiplicity

The effects of \( \alpha \)-santalol, magnolol, and combinations of \( \alpha \)-santalol and magnolol on UVB-induced tumor multiplicity are shown in Figure 3. \( \alpha \)-Santalol and magnolol significantly decreased the average number of tumors when compared with control. A combination of \( \alpha \)-santalol and magnolol topical treatment again provided about 75% decrease in the number of tumors per mice. Furthermore, the combination of \( \alpha \)-santalol and magnolol provided significantly higher decrease in average number of tumors than both mice treated with \( \alpha \)-santalol and magnolol alone. The data again clearly demonstrates the enhancing effects of \( \alpha \)-santalol on the chemopreventive effects of magnolol.

### 3.3. Effects of \( \alpha \)-santalol combination on the effects of honokiol and magnolol on cell viability of A431 cells

The effects of \( \alpha \)-santalol combination on the effects of honokiol and magnolol on cell viability of A431 cells is shown in Figure 4 and 5. \( \alpha \)-Santalol and honokiol treatment (50 \( \mu \)m) alone resulted about 30% decrease in the cell viability of A431 cells (Figure 4). However combination of \( \alpha \)-santalol (50 \( \mu \)m) with honokiol (50 \( \mu \)m) caused a 90% decrease in cell viability of A431 cells. This combination of \( \alpha \)-santalol with honokiol significantly enhances the cytotoxic effects in A431 cells. Similarly, combination of \( \alpha \)-santalol (50 \( \mu \)m) with magnolol (100 \( \mu \)m) significantly enhances the cytotoxic effects in A431 cells (Figure 5).

### 3.4. Effects of \( \alpha \)-santalol combination on the effects of honokiol and magnolol on cell proliferation of A431 cells

Effects of \( \alpha \)-santalol combination on the effects of honokiol on cell proliferation of A431 cell is presented in Figure 6. \( \alpha \)-Santalol (50 \( \mu \)m) treatment caused a reduction of 20 and 40% respecting cell proliferation of A431 cells. However, a combination of \( \alpha \)-santalol (50 \( \mu \)m) with honokiol (50 \( \mu \)m) almost completely stopped the cell proliferation of A431 cells. Although, combination of \( \alpha \)-santalol with magnolol significantly enhances the effects on cell proliferation but not completely as with honokiol (Figure 7).

### 3.5. Effects of \( \alpha \)-santalol combination on the effects of honokiol and magnolol on apoptosis of A431 cells

The effects of \( \alpha \)-santalol combination on the effects...
of honokiol and magnolol on apoptosis in A431 cells is shown in Figures 8 and 9. To investigate whether cell death resulted in response to apoptosis, we determined it by annexin V/PI staining which detects early stages of apoptosis and results were analyzed through FACS. The cells were treated with α-santalol, honokiol, combination of α-santalol and honokiol (50, 50, and 50 + 50 μM) concentrations respectively for 48 h. The results showed that compared to DMSO treated control, apoptotic cells in α-santalol, honokiol, and combination of α-santalol and honokiol were increased. These results suggested that combination of α-santalol and honokiol significantly increased apoptosis compared to cells treated with α-santalol and honokiol alone (Figure 8).

In case of α-santalol and magnolol combination cells were treated with varying concentrations of α-santalol, magnolol, and combination of α-santalol and magnolol (50, 100, and 50 + 100 μM) respectively for 48 h. Similar to α-santalol and honokiol combination, α-santalol and magnolol combination significantly (p < 0.05) increased apoptosis (Figure 9).

4. Discussion

Emulsion, paste, or essential oil of sandalwood (SW) has been used for centuries in India for treatment of inflammatory and eruptive skin diseases (18). Ayurvedic physicians (traditional medical practitioners in India) treat numerous skin lesions in patients with...
and promoted with UVB radiation; and in the third
TPA; in the second group it was initiated with DMBA
was initiated with UVB radiation and promoted with
w/v in acetone). The tumorigenesis in the first group
treated topically on the dorsal skin with
acetone. The
mice. The first subgroup served as control and was
treated topically on the dorsal skin with acetone. The
second subgroup served as experimental and was
induced and 12.0-tetradecahoye phorbol-13-acetate (TPA)-promoted CD-1 mice. SW oil treatment
also decreased TPA-induced epidermal ODC activity in
CD-1 mice (20). SW oil treatment decreased papilloma
incidence and multiplicity in a time- and concentration-dependent manner in CD-1 mice (20). The pretreatment
with 5% SW oil 1 h before DMBA and TPA treatment
provided a maximum reduction in papilloma incidence
and multiplicity (21). Our laboratory isolated α-santalol
from SW oil by distillation and characterized it by
NMR and GC-MS. NMR and GC-MS indicated that
α-santalol is a major component (~61%) of SW oil.

α-Santalol (5% topical) inhibited skin papilloma
development during the promotion phase of DMBA
and TPA protocol in CD-1 and SENCAR strains of
mice. Induction of epidermal ODC activity and DNA
synthesis are some of the prominent effects of TPA
treatment on skin. As expected, α-santalol treatment (5% topical) significantly decreased (p < 0.05) TPA-induced
ODC activity and incorporation of 3H-thymidine in
dNA in the skin of CD-1 and SENCAR strains of mice.
The effects of α-santalol on skin papilloma incidence
and multiplicity are very similar to the effects of SW
oil as reported earlier from our laboratory (20,21).
α-Santalol has a pleasant fragrance, does not produce
any stain, and appears to be nontoxic at concentrations
used in our study. The effectiveness of α-santalol as
chemopreventive agents appears to be very promising
in skin cancer control.

Since UV radiations are the major cause of skin
cancer in humans the effects of α-santalol on ultraviolet
B (UVB) radiation-induced skin tumor development
and UVB-caused increase in epidermal ornithine
decarboxylase (ODC) activity in female hairless SKH-1
cancer in humans the effects of
chemopreventive agents appears to be very promising
in skin cancer control. These findings suggest that α-santalol could be a potential chemopreventive agent against
UVB-induced skin tumor development.

Honokiol and magnolol isolated from the bark of
Magnolia officinalis have been shown to decrease
chemically-induced skin cancer development in mice
(22). We investigated the chemopreventive effects of
honokiol and magnolol on UVB-induced skin
cancer development in mice, a model more relevant
to human. Both honokiol and magnolol provided a
significant protection against UVB-induced skin cancer
development in mice (15,16). Furthermore, mechanistic
studies conducted in vivo in SKH-1 mice and in A431
cells showed that honokiol and magnolol inhibited skin
carcinogenesis by inducing apoptosis and inhibiting cell
proliferation by causing cell cycle arrest (16,17).

Since α-santalol, honokiol and magnolol are isolated
from plants and are relatively expensive, α-santalol,
honokiol and magnolol have a great potential as
chemopreventive agent for the skin cancer development.
We investigated the effects of α-santalol pretreatment
on the effects of honokiol and magnolol on UVB-
induced skin cancer development in mice to evaluate
if pretreatment with α-santalol enhances the effects of
honokiol and magnolol.

The present study, for the first time demonstrated
chemopreventive effects of topical applications of
α-santalol and honokiol and α-santalol and magnolol
combinations on skin tumor development in SKH-1
mice and in A431 cells. Pretreatment of combinations
of α-santalol with honokiol and magnolol significantly
decreased tumor multiplicity up to 75% than control,
α-santalol, honokiol and magnolol alone. Overall,
the results of present study indicated combinations of
α-santalol with honokiol and magnolol at lower doses
than alone provides protection against UVB-induced
skin tumorigenesis in SKH-1 mice possibly by inducing
apoptosis.

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References


