Chemopreventive effects of α-santalol on ultraviolet B radiation-induced skin tumor development in SKH-1 hairless mice

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Recent studies from our laboratory have shown the chemopreventive effects of α-santalol against 7,12-dimethylbenzanthracene (DMBA) initiated and 12-O-tetradecanoylphorbol-13-acetate (TPA) promoted skin tumor development in mice. The objective of the present investigation was to study 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 mice. For the tumor studies, 180 mice were divided into three groups of 60 mice each, and each group was divided into two subgroups of 30 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 treated topically on the dorsal skin with α-santalol (5%, w/v in acetone). The tumorigenesis in the first group was initiated with UVB radiation and promoted with TPA; in the second group it was initiated with DMBA and promoted with UVB radiation; and in the third group it was both initiated and promoted with UVB radiation. In each case, the study was terminated at 30 weeks. Topical application of α-santalol significantly (P < 0.05) decreased tumor incidence and multiplicity in all the three protocols, suggesting its chemopreventive efficacy against UVB radiation-caused tumor initiation, tumor promotion and complete carcinogenesis. In a short-term biochemical study, topical application of α-santalol also significantly (P < 0.05) inhibited UVB-induced epidermal ODC activity. Together, for the first time, our findings suggest that α-santalol could be a potential chemopreventive agent against UVB-induced skin tumor development and, therefore, warrants further investigations.

Introduction

Human non-melanoma skin cancers (NMSCs), including both basal and squamous cell carcinomas, are the mostly frequently diagnosed malignancy in the Caucasian, accounting for over 1.3 million new cases each year in the United States alone (1). NMSCs are caused mostly because of repeated sunlight exposure, where ultraviolet B (UVB) radiation is the causal etiologic factor (2). Whereas wavelengths within UVB (290–320 nm of solar radiation) are most carcinogenic, animal studies have clearly demonstrated that UVA (320–400 nm) is also capable of producing skin cancer (3,4). However, compared with UVB, since UVA-induced skin cancers in mice require much greater exposure and longer latency period before tumors are evident (5), UVB is the most frequently used photocarcinogen in animal studies (reviewed in refs. 6 and 7). The initial step in UVB-induced carcinogenesis involves induction of DNA damage [e.g. cyclobutane pyrimidine dimer and pyrimidine (6–4) pyrimidine photoproduction formation], which triggers a rapid increase in p53, enhancing Cip1/p21 synthesis and shutting off cell replication and DNA synthesis, allowing extended time for either DNA repair or apoptosis induction in the cells carrying UVB-damaged DNA (8). Whereas this defense mechanism efficiently removes UVB-damaged cells from the skin epidermis, sometimes damaged cells escape both repair and apoptotic death finally leading to transformed cells known as UVB-initiated epidermal keratinocytes (8). These cells follow multiple additional hits by repeated UVB exposure finally leading to benign followed by malignant skin tumors in mice, where clonal expansion of initiated cells involves both cellular and biochemical alterations including ornithine decarboxylase (ODC) induction, oxidant–antioxidant imbalance, mitogenic and anti-apoptotic signaling cascades, and altered cell cycle and apoptosis regulation (8–10). Taken together, these basic science observations suggest that newer approaches and strategies are needed to inhibit UVB-caused skin alterations that would possibly lead to a reduction in human NMSCs.

Consistent with the above suggestion, several studies have shown that various phytochemicals inhibit UVB-caused alterations in both murine and human skin, and that most of them also prevent UVB-caused skin cancer at least in the mouse model of photocarcinogenesis (9–13). Major limitation, however, is the fact that most of these phytochemicals are either not effective when it comes to human skin cancer or are still at the pre-clinical stage (9–13). This limitation raises the need for the search for newer and additional agents that could inhibit UVB-caused skin damages together with preventing solar radiation-caused NMSC. In this regard, α-santalol has shown promising results in recent studies, where we have reported the chemopreventive effects of both sandalwood oil and α-santalol (an active ingredient, ~61% w/w in sandalwood oil) against 7,12-dimethylbenzanthracene (DMBA) initiated and 12-O-tetradecanoylphorbol-13-acetate (TPA) promoted skin tumor development in mice, together with their inhibitory activity against TPA-caused epidermal ODC induction in mice (14–17). Additional studies by us on the effects of α-santalol in human epidermoid carcinoma A431 cells indicated its apoptotic effects primarily via intrinsic pathway involving perturbation of mitochondria and...
cytochrome c release followed by caspases activation (18). Whereas these studies clearly establish the skin cancer chemopreventive effect of α-santalol together with its apoptotic activity and associated mechanism, the caveat is that the animal models employed in these efficacy and mechanistic studies are not those that mimic human NMSCs. The purpose of the present investigation, therefore, was to study the effects of α-santalol on UVB radiation-induced skin tumor development and epidermal ODC activity in SKH-1 hairless mice, a model that most closely mimics human NMSCs (6,7).

Specifically, we employed UVB irradiation as both tumor initiator and tumor promoter, and a complete carcinogenic as well (reviewed in ref. 10). The rationale for the selection of these three protocols was to dissect out the protective effects of α-santalol at different stages of photocarcinogenesis, for example, (i) UVB-induced tumor initiation stage; (ii) UVB-induced tumor promotion stage; and (iii) UVB-induced tumor initiation and tumor promotion, also known as UVB-induced complete carcinogenesis (6,7).

Materials and methods

Chemicals

DMBA, TPA, pyridoxal phosphate, EDTA disodium salt, dithiothreitol, orinthine, ethanoleamine and methoxyethanol were purchased from Sigma Chemical (St Louis, MO). DL-14C-ornithine was purchased from American Radiolabeled Chemicals (St Louis, MO). Sandalwood oil (NOW Foods, Glendale Heights, IL) was purchased from a local store. All other routine chemicals were obtained from Fisher Scientific (Hanover Park, IL) in the purest form commercially available.

Isolation, characterization and purity of α-santalol

α-Santalol (Figure 1) was isolated from sandalwood oil by distillation under vacuum as described in detail recently (16). On the basis of the NMR spectrum and the boiling point of the distillate, the major component of sandalwood oil is only α-santalol (16). Further, GC–MS analysis, NMR data and mass spectrum of the isolated agent were consistent with the structure of α-santalol (19), as reported recently (16).

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 (20). The UVB exposure dose was controlled using two Daavlin Flex Control Integrating 305 Dosimeters (20). 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.

Long-term α-santalol efficacy experimental design

A single long-term study was designed, and three different UVB-induced tumorigenesis protocols were employed to assess the protective effect of topically applied α-santalol against (i) UVB-induced tumor initiation; (ii) UVB-induced tumor promotion; and (iii) UVB-induced complete carcinogenesis (both tumor initiation and tumor promotion caused by repeated multiple UVB irradiation) as detailed by Wang et al. (21). The selection of this protocol was based on the fact that with limited UVB irradiation the tumor initiation stage of photocarcinogenesis could be achieved as evidenced following multiple TPA applications leading to skin tumors (22). Similarly, following DMBA single-dose tumor initiation, multiple UVB exposures lead to tumor development supporting UVB’s role as tumor promoter, and, finally, combining the two protocols mimics real NMSC induction and development as evidenced in humans (ref. 22 and references therein). Studies by Wang et al. (21) have also shown that once daily exposure of SKH-1 hairless mice with 180 mJ/cm² UVB dose for seven days results in red sunburn lesions of the skin that is also in accord with human skin sunburn. The details of the protocols employed in the present study are summarized schematically in Figure 2.

In the studies assessing the efficacy of α-santalol against UVB-caused tumor initiation, 60 mice were divided into two groups of 30 each, and treated topically with either 100 µl of acetone alone (control) or 100 µl of α-santalol (5%, w/v, in acetone, treated group) per mouse per day. These treatments were continued once daily for 14 days. On the 15th day, mice in both the groups started receiving UVB irradiation at the dose of 180 mJ/cm²/day. The UVB irradiation was continued daily for 10 days, and therefore the total UVB dose employed was 1800 mJ/cm² fractionated in 10 equal doses for 10 days. One week after the last UVB exposure, animals in both the groups were treated topically with TPA (10 nmol in 100 µl acetone/mouse) as tumor promoter. The TPA treatment was continued twice a week up to the end of the experiment at 30 weeks.

In the studies assessing the efficacy of α-santalol against UVB-caused tumor promotion, 60 mice were divided into two groups of 30 each, and treated topically with a single application of DMBA (200 µl of 10%, w/v, in acetone) as tumor initiator. One week following tumor initiation with DMBA, the mice were treated topically with either 100 µl of acetone alone (control group) or 100 µl of α-santalol (5%, w/v, in acetone, treated group) per mouse. One hour after acetone/α-santalol application, the mice in both the groups were irradiated with 180 mJ/cm² dose of UVB. This treatment of acetone/α-santalol followed 1 h after UVB exposure was given twice a week up to the end of the experiment at 30 weeks.

In the studies assessing the efficacy of α-santalol against UVB-caused complete carcinogenesis (both antitumor initiating and antitumor promoting effects), 60 mice were divided into two groups of 30 each, and treated topically with either 100 µl of acetone alone (control group) or 100 µl of α-santalol (5%, w/v, in acetone, treated group) per mouse per day. These treatments were continued once daily for 14 days. On the 15th day, mice in both groups started receiving UVB irradiation at the dose of 180 mJ/cm²/day. UVB irradiation was continued daily for the next 10 days. One week after the last UVB radiation, the mice were treated topically with either 100 µl of acetone alone (control group) or 100 µl of α-santalol (5%, w/v, in acetone, treated group) per mouse. One hour after acetone/α-santalol application, the mice in both the groups were irradiated with 180 mJ/cm² dose of UVB. This treatment of acetone/α-santalol followed 1 h after UVB exposure was given twice a week up to the end of the experiment at 30 weeks. Animals in all three protocols described above 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 in all three protocols.

Short-term α-santalol efficacy experimental design

Four groups of five female SKH-1 hairless mice were used in each group, and a short-term study was designed to assess the efficacy of α-santalol against UVB radiation-induced epidermal ODC activity. The mice in the first group were treated topically with 100 µl of acetone alone (UVB positive control group) and the mice in the second group were treated topically with 100 µl of α-santalol (5%, w/v, in acetone, treated group) per mouse. One hour after acetone/α-santalol application, the mice in both the groups were irradiated with 180 mJ/cm² dose of UVB. In addition to these two groups, the mice in the third and fourth groups were treated with acetone (100 µl) and α-santalol (100 µl, 5%, w/v in acetone) alone, which represented controls for the treatments in first and second group, respectively. Twenty-four hours after UVB radiation, mice in all the groups were killed, and the dorsal epidermis was collected and used for ODC activity assay. Briefly, the epidermis was cleaned and homogenized in an ice-cold phosphate buffer (pH 7.2, 175 mM) containing 0.1 mM pyridoxal phosphate and 0.1 mM EDTA. The homogenates were centrifuged at 105 000× g for 90 min in a ultracentrifuge (Beckman, Optima Ultra LE80K), and the supernatants were used for the ODC activity assay. ODC activity was determined by measuring the formation of 14CO₂.
from $^{14}$C ornithine as described earlier (22), and is reported as nmole $^{14}$CO$_2$ produced/mg protein/h.

**Statistical analysis**

$\chi^2$ and Student’s $t$-test were performed on sample means using INSTAT software (Graph Pad, San Diego, CA). $\chi^2$ was used for analyzing the data on tumor incidence. Student’s $t$-test was used for the data on tumor multiplicity and ODC activity. Significance was considered at $P < 0.05$.

### Results

**Chemopreventive effect of α-santalol against UVB-caused skin tumorigenesis**

In all the experimental protocols, topical application of α-santalol prior to UVB irradiation resulted in a strong protection against UVB-caused tumor initiation, tumor promotion and complete carcinogenesis (Figure 3). In the studies evaluating its effect as antitumor initiating agent against UVB, application of α-santalol for 14 days before the start of UVB irradiation resulted in a strong protection in both tumor incidence and multiplicity throughout the study. Together with a 2 weeks delay in first tumor appearance, tumor incidence was significantly ($P < 0.05$) lower in α-santalol group compared with UVB-alone group (Figure 3D). In terms of tumor multiplicity in this protocol, the α-santalol-treated group started rising after 24 weeks, though these effects were not as strong as observed in anti-initiation protocol. The data on tumor incidence, presented in Figure 3C, show that α-santalol treatment caused a significant ($P < 0.05$) reduction in tumor incidence from 6 to 15 weeks of promotion; however, no difference in tumor incidence was observed thereafter up to the end of the study at 30 weeks. Interestingly, α-santalol treatment caused a significant ($P < 0.05$) decrease in tumor multiplicity starting at 5 weeks up to the end including from Week 17 to 30, where it showed no effect on incidence (Figure 3D). In terms of tumor multiplicity in this protocol, the α-santalol-treated group showed a 37% reduction ($P < 0.05$) as compared with DMBA + UVB alone group of mice (Figure 3D).

A much profound effect of α-santalol was evidenced when it was applied topically before UVB exposures during complete carcinogenesis protocol. Together with a reduction in first tumor appearance by 2 weeks, α-santalol treatment resulted in a significant ($P < 0.05$) decrease in tumor incidence from Week 13 to 30 (Figure 3E). Whereas tumor incidence in the α-santalol-treated group started rising after 24 weeks, it still remained lower than the control group in the UVB-initiated and promoted protocol (Figure 3E). The strongest effect of α-santalol out of all the long-term tumorigenesis studies was observed in terms of its effect on reducing tumor multiplicity in UVB-caused complete carcinogenesis protocol where this agent significantly ($P < 0.05$) decreased the mean number of tumors in UVB-initiated and promoted protocol beginning from 9 weeks of study (Figure 3F). By the end of the protocol at 30 weeks, a 72% decrease ($P < 0.05$) in tumor multiplicity was observed in α-santalol-treated group of mice compared with UVB-alone group (Figure 3F). There was no significant difference in body weight gain profile among all experimental groups without or with α-santalol treatments (data not shown).

**Inhibitory effect of α-santalol on UVB-caused induction of epidermal ODC activity**

We have previously shown that topical application of α-santalol inhibits TPA-caused induction of ODC activity
Fig. 3. Chemopreventive effect of α-santalol against UVB-caused skin tumorigenesis in SKH-1 hairless mice. The details of the protocols are described in Materials and methods. The protective effects of the agent are shown in terms of UVB-caused tumor initiation (A and B), tumor promotion (C and D) and complete carcinogenesis (E and F), in which the left panels represent % tumor incidence and the right panels represent tumor multiplicity as a function of weeks of treatments. In each case, the data shown are from 30 mice per group. For tumor multiplicity, each point represents mean (±SD) of tumors per mouse calculated from 30 mice; standard deviation numbers are smaller than the symbols and cannot be seen in the graph.
in mouse skin that was in accord with its antitumor-promoting efficacy against TPA-caused tumor promotion in DMBA-initiated mouse skin (16,17). Consistent with previous observation and that ODC induction by tumor promoters including UVB irradiation is used as a marker at tumor promotion stage (22), studies were also done here to assess the effect of topical α-santalol application on UVB-caused induction of epidermal ODC activity. As shown in Figure 2, compared with acetone and α-santalol-alone treatment groups that did not have detectable ODC activity, a single UVB exposure caused a strong induction in ODC activity in the exposed mouse epidermis. α-Santalol pretreatment, however, resulted in a 60% reduction (P < 0.05) in UVB-induced epidermal ODC activity.

**Discussion**

With the major aim of the present study being to evaluate α-santalol efficacy against UVB-induced tumorigenesis, our results, for the first time, show a strong chemopreventive potential of this naturally occurring agent against photocarcinogenesis in three different protocols. When the effect of α-santalol was assessed against UVB-caused tumor initiation, this agent showed a strong reduction in both tumor incidence and multiplicity. Similar protective effects, though to a lesser extent, were also observed when α-santalol was applied before each UVB exposure in a tumor promotion protocol. It is interesting that whereas α-santalol did show an inhibitory effect on tumor incidence in the middle of study protocol, the observed inhibition was not sustainable during later treatments in antitumor promotion studies. In contrast to this loss of effect at later time points, the tumor multiplicity results in antitumor promotion protocol clearly show an inhibitory effect of α-santalol during 17–30 weeks of the study, suggesting its inhibitory effect at least on tumor growth. Most effective observation of the present study was the α-santalol’s effect on UVB-caused complete tumorigenesis, specifically a 72% reduction in tumor multiplicity. Since this protocol mimics human NMSC, the results obtained are encouraging, and warrant more studies assessing α-santalol efficacy against NMSC in pre-clinical models and associated mechanisms of action. It is anticipated that successful outcomes from such studies would help conduct a clinical trial with this agent in human population suffering with NMSC or a pre-cancerous stage actinic keratoses for squamous cell carcinoma skin cancer (11).

Overall, the results of the present investigation indicated that α-santalol treatment inhibited skin tumor development, both tumor incidence (to a lesser degree) and multiplicity during UVB-initiated initiation, UVB-promotion and UVB-induced initiation and promotion phases in SKH-1 mice. The effects of α-santalol treatment on tumor multiplicity during both UVB-initiation and promotion phases were higher when compared with α-santalol treatment during either UVB-initiation or UVB-promotion phase only (Figure 3). Induction of epidermal ODC activity was a prominent effect observed after UVB irradiation of skin; however, α-santalol treatment significantly decreased UVB-induced ODC activity in SKH-1 mice epidermis (Figure 4). Preliminary experiments in our laboratory have also indicated that α-santalol inhibits in vitro lipid peroxidation in skin and liver microsomes (data not shown, unpublished observation).

**References**


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