Chemopreventive Effects of α-Santalol on Skin Tumor Development in CD-1 and SENCAR Mice[1]

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Abstract

Studies from our laboratory have indicated skin cancer chemopreventive effects of sandalwood oil in CD-1 mice. The purpose of this investigation was to study the skin cancer chemopreventive effects of α-santalol, a principal component of sandalwood oil in CD-1 and SENCAR mice. α-Santalol was isolated from sandalwood oil by distillation under vacuum and characterized by nuclear magnetic resonance and gas chromatography-mass spectrometry. Chemopreventive effects of α-santalol were determined during initiation and promotion phase in female CD-1 and SENCAR mice. Carcinogenesis was initiated with 7,12-dimethylbenz(*a*)anthracene and promoted with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). The effects of α-santalol treatment on TPA-induced epidermal ornithine decarboxylase (ODC) activity and ^{3}H-thymidine incorporation in epidermal DNA of CD-1 and SENCAR mice were also investigated. α-Santalol treatment during promotion phase delayed the papilloma development by 2 weeks in both CD-1 and SENCAR strains of mice. α-Santalol treatment during promotion phase significantly ($P < 0.05$) decreased the papilloma incidence and multiplicity when compared with control and treatment during initiation phase during 20 weeks of promotion in both CD-1 and SENCAR strains of mice. α-Santalol treatment resulted in a significant ($P < 0.05$) inhibition in TPA-induced ODC activity and incorporation of ^{3}H-thymidine in DNA in the epidermis of both strains of mice. α-Santalol significantly prevents papilloma development during promotion phase of 7,12-dimethylbenz(*a*)anthracene-TPA carcinogenesis protocol in both CD-1 and SENCAR mice, possibly by inhibiting TPA-induced ODC activity and DNA synthesis. α-Santalol could be an effective chemopreventive agent for skin cancer. Additional experimental and clinical studies are needed to investigate the chemopreventive effect of α-santalol in skin cancer.

Introduction

Skin cancer is the most common type of cancer in the United States (1). More than 1 million cases of skin cancer occur in this country each year (2), leading to an estimated 9600 deaths this year.[3] Chemical and UVB radiation-induced carcinogenesis in murine skin and possibly in human skin is a stepwise process of at least three distinct stages: (*a*) initiation; (*b*) promotion; and (*c*) progression (3). Chemoprevention of skin cancer involves the administration of chemical agents to prevent the initiation, and/or promotion, and/or progression events that occur during the multistage process of neoplastic development. Chemopreventive agents are classified into two categories: (*a*) blocking; and (*b*) suppressing agents. The blocking agents prevent carcinogenic agents from reaching or reacting with critical target sites. The suppressing agents prevent the evolution of neoplastic process in cells (4). Classically, the mouse has been considered the laboratory animal most sensitive to skin carcinogenesis by either the complete carcinogenesis protocol or the initiation-promotion protocol (5, 6). Mouse skin tumors can be induced using a multistage model that involves the process defined as initiation and promotion (5, 6). Initiation is accomplished by topical application of a single dose of a skin carcinogen, such as DMBA,[4] and is essentially irreversible. An initiation dose of carcinogen may not produce visible tumors. Visible tumors result only after prolonged and repeated application of a tumor promoter, such as TPA, to initiated skin (7). A wide range of sensitivity to either complete or two stage carcinogenesis has been described in various mouse strains, including several strains essentially resistant to epidermal carcinogenesis (8, 9). The SENCAR mouse is an outbred stock selectively bred for sensitivity to the DMBA-initiation-TPA-promotion protocol and has been reported to be more sensitive to TPA promotion than CD-1 or BALB/c or C57BL/6 mice (9, 10).

A prominent feature among the various biochemical changes observed after topical application of the tumor promoter TPA to mouse skin is the induction of epidermal ODC activity (11, 12). Application of TPA to mouse skin leads to an enhanced accumulation of prostaglandins, which may play a role in ODC induction (13). Binding of phorbol esters to specific receptors is an essential event during tumor promotion and

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[4] The abbreviations used are: DMBA, 7,12-dimethylbenz(*a*)anthracene; ODC, ornithine decarboxylase; GC-MS, gas chromatography-mass spectrometry; NMR, nuclear magnetic resonance; SW, sandalwood; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

cellular differentiation. The specific phorbol ester receptor has been identified as calcium-activated, phospholipid-dependent protein kinase (protein kinase C; Refs. 14–16). The tumor-promoting ability of phorbol esters correlates with their ability to induce RNA, protein, and DNA synthesis in mouse epidermis (17, 18).

A large variety of chemopreventive agents, including non-nutrient dietary constituent blocking and suppressing agents, has been investigated (4). Studies from this laboratory have indicated that the topical application of SW oil for 20 weeks decreased the incidence and multiplicity of skin papillomas in DMBA-initiated and TPA-promoted female CD-1 mice in a concentration- and time-dependent manner. The topical application of SW oil also inhibited TPA-induced ODC activity (19, 20), one of the prominent events during skin cancer promotion. This laboratory has identified α-santalol as a major component (~61%) of SW oil and characterized it by NMR and GC-MS. The purpose of this investigation is to study the effects of α-santalol on DMBA-initiated and TPA-promoted skin tumor development and TPA-induced epidermal ODC activity and DNA synthesis in CD-1 and SENCAR (more skin cancer sensitive) mice.

Materials and Methods

Chemicals. DMBA, TPA, pyridoxal phosphate, calf thymus DNA, EDTA disodium salt, DTT, ornithine, ethanolamine, and methoxyethanol were purchased from Sigma Chemical Co. (St. Louis, MO). DL-^{14}C-ornithine and ^{3}H-thymidine were purchased from American Radiolabeled Chemicals (St. Louis, MO.). SW oil was purchased from a local store and distributed by NOW Foods (Glendale Heights, IL). All routine chemicals were obtained from Fisher Scientific (Hanover Park, IL).

Animals. Female CD-1 mice (5 weeks old) were purchased from Charles River Laboratories (Wilmington, MA), and SENCAR mice were purchased from Frederick Cancer Research and Development Center (Frederick, MD). Institutional guidelines were followed in handling the animals.

Isolation and Characterization of α-Santalol

α-Santalol was isolated from SW oil by distillation under vacuum, BP 95°C/0.5 mm Hg. On the basis of the NMR spectra and the boiling points, the major component of SW oil is α-santalol. GC-MS analysis revealed that two major components are present in SW oil, one accounts for ~61% and the other for ~28%. The NMR data and mass spectrum of the 61% component are consistent with the structure of α-santalol (21). The other major component appears to be one of the isomers of α-santalol. Structure of α-santalol is shown in Fig. 1.

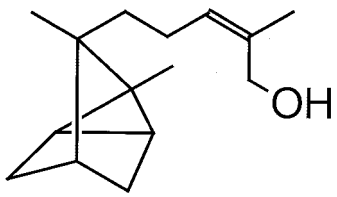

Fig. 1. Structure of α-santalol.

Skin Tumor Protocol

Tumorigenesis protocol as described by Dwivedi and Zhang (20) was used.

Four- to five-week-old female mice (CD-1 and SENCAR) were used. Mice were divided into three groups having 30 mice in each group. Group assignments were as follows:

Group 1, Control; 100 μl of acetone before DMBA and each TPA application.

Group 2, Initiation; 100 μl of α-santalol (5% in acetone, w/v) 1 h before DMBA and 100 μl of acetone 1 h before each TPA application (topical).

Group 3, Promotion; 100 μl of acetone before DMBA and 100 μl of α-santalol (5% in acetone, w/v) topical application 1 h before each TPA treatment throughout the duration of the experiment.

The backs of the mice were carefully shaved with an electric clipper to avoid cuts and bruises. The mice were allowed to rest for 2 days, and only those in the resting phase of the hair growth cycle were used. Tumorigenesis in mice was initiated with topical application of DMBA (200 nmol/100 μl acetone for CD-1 and 10 nmol/100 μl acetone for SENCAR).

Beginning 1 week after initiation, mice in all groups were treated topically with TPA (5 nmol/100 μl acetone for CD-1 and 2 nmol/100 μl acetone for SENCAR) twice a week (Tuesday and Friday) throughout the duration of the experiment (20 weeks). Papilloma counts and group weights were taken on a weekly basis.

Determination of ODC Activity

Three groups of female CD-1 or SENCAR mice having 5 mice in each group were used. The backs of mice were shaved carefully to avoid cuts and bruises. The mice from group 1 were treated with topical application of 100 μl of α-santalol (5% in acetone); groups 2 and 3 were treated with TPA (5 nmol/100 μl acetone for CD-1 and 2 nmol/100 μl acetone for SENCAR, topically). Mice in groups 1 and 2 received 100 μl of acetone 1 h before TPA treatment. Mice in group 3 received topical application of 100 μl of α-santalol (5% in acetone, w/v) 1 h before TPA treatment. Mice were sacrificed 5 h after the topical applications of TPA by guillotine decapitation. The epidermis from the shaved area was used for ODC assay.

The epidermis was cleaned and homogenized in 50 mM phosphate buffer (pH 7.2) containing 0.1 mM pyridoxal phosphate, 0.1 mM pyridoxal phosphate, and 0.1 mM EDTA. The homogenate was centrifuged at 105,000 × *g* for 60 min in Beckman Refrigerated Ultracentrifuge, and the supernatant obtained was used for ODC assay. ODC activity was determined by measuring the production of $^{14}CO_2$ from DL-[1-^{14}C] ornithine as described by Verma and Boutwell (11) and modified by Dwivedi *et al.* (22).

ODC activity was expressed as nanomoles $^{14}CO_2$ produced/μg of protein/h.

Protein Assay

Protein was assayed using the Bio-Rad (Richmond, CA) protein assay kit with albumin as a standard.

Determination of DNA Synthesis

Female CD-1 or SENCAR mice were divided into two groups having 5 mice in each group. The backs of mice were shaved as in the ODC experiment. Mice in both groups were treated with TPA (5 nmol/100 μl acetone for CD-1 and 2 nmol/100 μl acetone for SENCAR), topically. Mice in one group received 100 μl of acetone and in the other group received 100 μl of

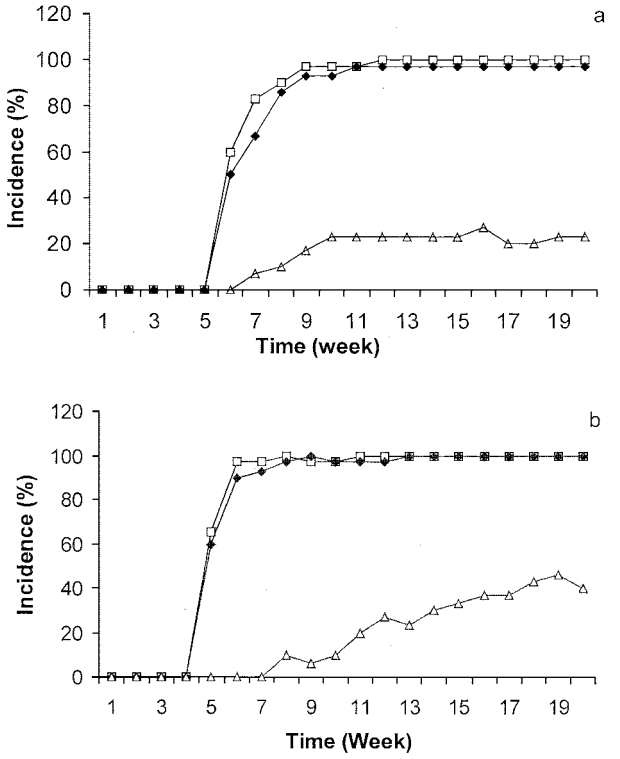

Fig. 2. The effects of α-santalol treatment on tumor incidence in CD-1 mice (*a*) and SENCAR mice (*b*). Control, —□—; initiation, —◆—; promotion, —△—.

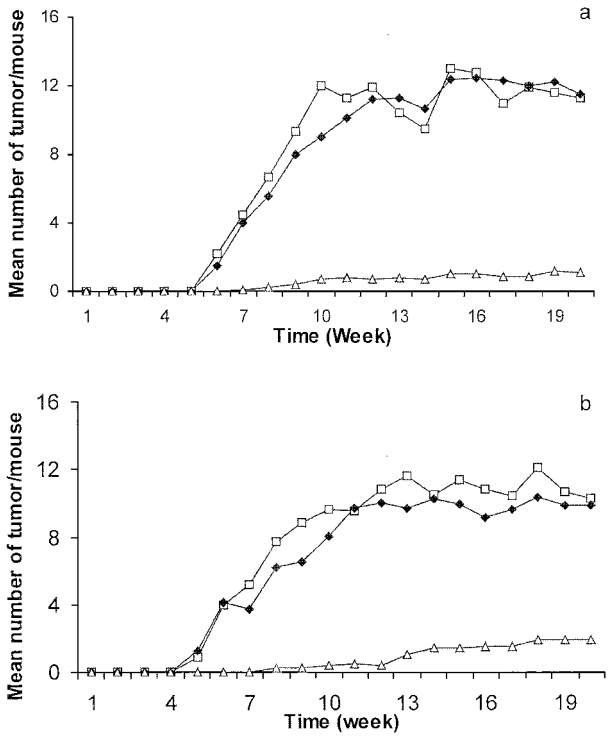

Fig. 3. The effects of α-santalol treatment on tumor multiplicity in CD-1 mice (*a*) and SENCAR mice (*b*). Control, —□—; initiation, —◆—; promotion, —△—.

α-santalol (5% in acetone) 1 h before TPA treatment. Mice in both groups were injected with 10 μl of ^{3}H-thymidine (1 mCi/l ml) i.p. 18 h after the application of TPA. Mice were sacrificed 40 min after the ^{3}H-thymidine injection by cervical dislocation. The isolation of DNA and determination of the incorporation of ^{3}H-thymidine were carried out as described by Huang *et al.* (18) and Smart *et al.* (23). Epidermis was homogenized in 1 mM EDTA. Epidermal homogenate was filtered through cheesecloth. The filtrate was combined with 70% perchloric acid and centrifuged. The resulting pellet was washed with ice-cold 2N perchloric acid and centrifuged; it was washed twice with ice-cold ethanol and centrifuged again, and it was combined with 0.5% perchloric acid and heated at 98°C for 10 min. The samples were placed in ice for 5 min and centrifuged. An aliquot (0.5 ml) from the supernatant was combined with 10 ml of Ecolite (ICN, Costa Mesa, CA) and counted in a Beckman LS6000SE liquid scintillation counter. The data were expressed as dpm/μg epidermal DNA.

DNA determination was made using the method described by Burton (24) using calf thymus DNA as standard.

Statistical Analysis

ANOVA, χ^2, and Student's *t* test were performed on sample means using INSTAT software (Graph Pad, Sand Diego, CA). χ^2 was used for analyzing the data on tumor incidence. ANOVA and Student's *t* test were used for the data on tumor multiplicity, ODC activity, and DNA syntheses. Significance was considered at $P < 0.05$.

Results

The effect of α-santalol treatment on the incidence of development of skin pamillomas in CD-1 mice is shown in Fig. 2*a*. Skin papillomas started appearing in the 6th week after DMBA treatment in the control and initiation groups. α-Santalol treatment during promotion phase delayed the appearance of papillomas by 2 weeks. Skin papilloma incidence was 100, 97, and 23% in control, initiation, and promotion groups, respectively, after 20 weeks of promotion. There was no significant difference in the incidence of papillomas between control and initiation group throughout the duration of the experiment. α-Santalol treatment during promotion phase significantly decreased ($P < 0.05$) the incidence of papillomas from weeks 8 to 20 when compared with the control and initiation groups. The incidence of skin papillomas in various groups of treatment in SENCAR mice is presented in Fig. 2*b*. Skin papillomas appeared during the 5th week in the control and initiation groups, whereas they appeared during the 7th week in the promotion group. Papilloma incidence was 100, 100, and 40% in control, initiation, and promotion groups, respectively. α-Santalol treatment during promotion phase delayed the appearance of papillomas by 2 weeks and significantly decreased ($P < 0.05$) the papilloma incidence when compared with control and initiation groups throughout the duration of the experiment.

The effects of α-santalol treatment on papilloma multiplicity in CD-1 and SENCAR mice are shown in Fig. 3, *a* and *b*, respectively. There was no significant difference in papilloma multiplicity between control and initiation groups throughout 20 weeks of promotion in both strains of mice. α-Santalol treatment during promotion phase resulted in a sig-

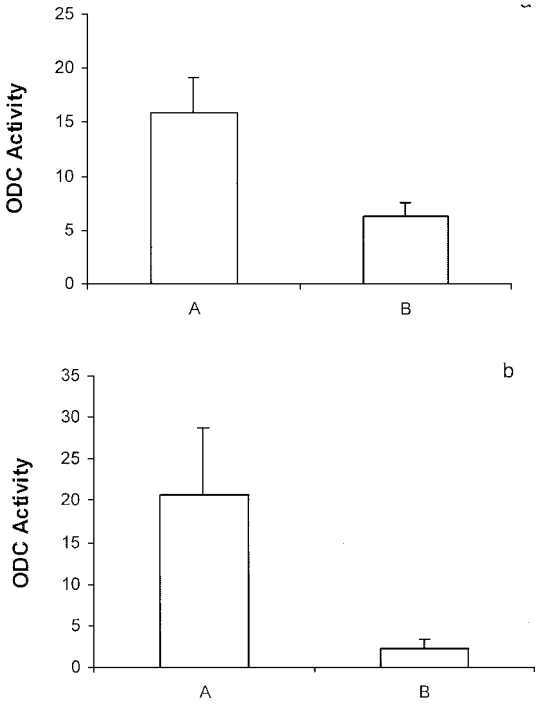

Fig. 4. The effects of α-santalol treatment on TPA-induced epidermal ODC activity in CD-1 mice (*a*) and SENCAR mice (*b*). ODC activity is expressed as nanomoles $^{14}CO_2$ produced/μg of protein/h. A-TPA, B-TPA, and α-santalol. The α-Santalol (only) group did not have detectable ODC activity.

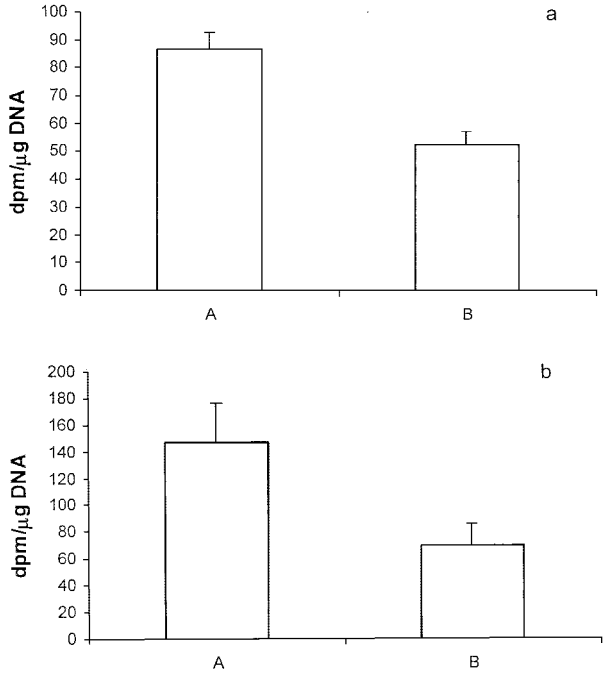

Fig. 5. The effects of α-santalol treatment on the incorporation of ^{3}H-thymidine in epidermal DNA in CD-1 mice (*a*) and SENCAR mice (*b*). A-TPA, B-TPA, and α-santalol.

nificant reduction ($P < 0.05$) in papilloma multiplicity when compared with the control and initiation groups from the 8–20 week period of promotion in both CD-1 and SENCAR mice. The mean numbers of tumors were 11.3, 11.5, and 1.1 and 10.3, 9.9, and 1.9 in control, initiation, and promotion groups of CD-1 and SENCAR mice, respectively.

There was no significant difference in weight gain among all experimental groups throughout the duration of the experiment (data not shown) in both strains of mice. Thus, α-santalol treatment did not influence the normal growth and development of the animals in both strains of mice during the experimental period.

Effects of α-santalol treatment on TPA-induced epidermal ODC activity in CD-1 and SENCAR mice are presented in Fig. 4, *a* and *b*, respectively. α-Santalol treatment resulted in a significant ($P < 0.05$) decrease in TPA-induced epidermal ODC activity in both CD-1 (60% inhibition) and SENCAR (89% inhibition) mice. However, the α-santalol (only) treatment group did not have detectable ODC activity in either strain of mice.

α-Santalol treatment also resulted in a significant ($P < 0.05$) reduction in the TPA-induced incorporation of ^{3}H-thymidine in epidermal DNA in both CD-1 (39% inhibition) and SENCAR (53% inhibition) mice. Data are shown in Fig. 5, *a* and *b*.

Discussion

Chemoprevention of common forms of epithelial cancer during the period of preneoplasia was introduced by Sporn *et al.* (25). Naturally occurring or any other molecule that blocks the DNA damage-initiating carcinogenesis or reversing or stopping the progression of premalignant cells in which such damage had already occurred were referred to as chemopreventive agents. Chemoprevention includes prevention of initiation, promotion, and progression of carcinogenesis to cancer (26). Wattenberg *et al.* (27) has compiled a book from the "Workshop on Cancer Chemoprevention" held in La Jolla, California. A large variety of chemopreventive agents, including nonnutrient dietary agents, has been investigated. The National Cancer Institute has 25 chemopreventive agents in clinical trial and has a need of continuous research on chemoprevention to reduce the incidence of cancer in people who are at high risk and also in general population (26). Chemopreventive effects of many naturally occurring substances against skin cancer development have been investigated in various laboratories (28–38).

SW (Santalum Album Linn, Indian SW), found in southern India and Indonesia is called the "Royal Tree" in India (39). Emulsion, paste, or essential oil of SW has been used for centuries in India for treatment of inflammatory and eruptive skin diseases (40, 41). Ayurvedic physicians (traditional medical practitioners in India) treat numerous skin lesions in patients with SW oil.[5] The essential oil of SW is distilled from the small chips and billets cut out of the heartwood of SW. The oil is extremely viscid, of a light yellow color, and possesses a characteristic pleasant odor. The major constituent of oil is santalol, a mixture of two isomers, α- and β-santalol. Other constitutes of oil are aldehydes, ketones, isovaleric aldehyde, santanone, esters, and free acids (40, 41). Banerjee *et al.* (42) reported that p.o. feeding of SW oil caused an increase in glutathione *S*-transferase activity and acid soluble sulfhydryl levels and suggested possible chemopreventive effects. SW oil inhibits the replication of Herpes simplex viruses-1 and 2 *in*

[5] C. Dwivedi, unpublished observation.

vitro (43). SW oil treatment (100 μl, 5% in acetone, twice a week, topically) significantly decreased papilloma incidence by 67% and multiplicity by 96% in DMBA-initiated and TPA-promoted CD-1 mice. SW oil treatment also decreased TPA-induced epidermal ODC activity in CD-1 mice (19). SW oil treatment decreased papilloma incidence and multiplicity in a time- and concentration-dependent manner in CD-1 mice. The pretreatment with 5% SW oil 1 h before DMBA and TPA treatment provided a maximum reduction in papilloma incidence and multiplicity (20). Our laboratory isolated α-santalol from SW oil by distillation and characterized it by NMR and GC-MS (details in "Materials and Methods"). NMR and GC-MS indicated that α-santalol is a major component (~61%) of SW oil.

The results from this investigation indicated that α-santalol (a major component of SW oil) inhibited skin papilloma development in both tumor incidence and multiplicity only during the promotion phase of DMBA and TPA protocol in both CD-1 and SENCAR strains of mice. α-Santalol treatment did not have any significant effects during initiation phase. Thus, α-santalol may not affect the binding of DMBA to DNA during initiation phase. Induction of epidermal ODC activity and DNA synthesis are some of the prominent effects of TPA treatment on skin. As expected, α-santalol treatment significantly decreased ($P < 0.05$) TPA-induced ODC activity and incorporation of ^{3}H-thymidine in DNA in the skin of both strains of mice. Because α-santalol treatment did not influence DMBA-induced initiation, most likely the effects of α-santalol on TPA-induced promotion are not because of the blocking of absorption of TPA. Preliminary experiments[6] in our laboratory using GC-MS have detected α-santalol within 5 min in serum, skin, and liver of animals receiving topical application of α-santalol. These preliminary observations suggest that α-santalol gets absorbed, and the chemopreventive effects on TPA-induced promotion are likely caused by systematic absorption rather than simply blocking the penetration of TPA. 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 (19, 20). α-Santalol may be the most important constituent in SW oil for these chemopreventive effects.

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.

Additional studies on the chemopreventive effects of α-santalol on UVB-induced initiation, promotion, or initiation-promotion protocol are in progress. Future mechanistic studies are needed to understand the mechanism of action of α-santalol. Although α-santalol is a major (~61%) component of SW oil and may be a major contributor to the chemopreventive effects against TPA-induced promotion, the contributions from other components of SW oil for chemopreventive effects could not be ruled out.

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