Effects of α-Santalol on Proapoptotic Caspases and p53 Expression in UVB Irradiated Mouse Skin

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Abstract. *Background: Cancer chemoprevention by naturally occurring agents, especially phytochemicals, minerals and vitamins has shown promising results against various malignancies in a number of studies both under in vitro and in vivo conditions. One such phytochemical, α-santalol, a major component of sandalwood oil, is effective in preventing skin cancer in both chemically and UVB-induced skin cancer development in CD-1, SENCAR and SKH-1 mice; however, the mechanism of its efficacy is not fully understood. The objective of the present investigation was to study the effects of α-santalol on apoptosis proteins and p53 in UVB-induced skin tumor development in SKH-1 mice to elucidate the mechanism of action. Materials and Methods: Female SKH-1 mice were divided into two groups: Group 1, which served as control received topical application of acetone (0.1 ml) one hour before UVB treatment; Group 2 received α-santalol (0.1 ml, 5% w/v in acetone, topical) one hour prior to UVB treatment. UVB-induced promotion was continued for 30 weeks. Results: Pre-treatment with α-santalol one hour prior to UVB exposure significantly ($p<0.05$) reduced tumor incidence and multiplicity, and resulted in a significant ($p<0.05$) increase in apoptosis proteins, caspase-3 and -8 levels and tumor suppressor protein, p53. Conclusion: These results suggest that α-santalol prevents skin cancer development by inducing proapoptotic proteins via an extrinsic pathway and increasing p53.*

Skin cancer is the most common type of cancer in the United States and its incidence is expected to increase substantially because of increased recreational exposure to sunlight and depletion of the ozone layer (1). Most of the more than 1 million cases of non-melanoma skin cancer diagnosed yearly in the United States are considered to be sun-related, accounting for 10,710 estimated deaths in 2007 (2).

The two major biological events that occur during tumor development are uncontrolled growth and loss of apoptotic death (3). Apoptosis is a controlled physiological process of cell elimination of fundamental importance to all multicellular organisms (4-6). The tumor suppressor gene, p53, is a well-studied potent regulator which becomes mutated under various cancer conditions (7). The strategies that prevent tumor progression using naturally occurring agents, especially phytochemicals, minerals and vitamins have substantially increased over the recent years (3, 8). Recent studies (3-6) have emphasized the importance of phytochemicals playing a major role in modulating mitogenic and survival signaling cascades leading to cell cycle arrest and induction of apoptosis. The search for such beneficial natural agents led to the identification of one phytochemical, α-santalol, which is distilled from sandalwood oil. Topical application of sandalwood oil or α-santalol has been shown to prevent skin tumor development in chemically and UVB-induced skin carcinogenesis in experimental animal studies (9-13); however, the exact mechanism of its efficacy has not been completely elucidated. Our *in vitro* studies (4) have indicated that α-santalol induces apoptotic proteins (caspases) in human epidermoid carcinoma A431 cells.

The main objective of the present investigation was to study the effects of α-santalol pretreatment on UVB-induced changes in apoptotic proteins (procaspases and caspases 3 and 8) and the tumor suppressor protein p53, to elucidate the possible mechanism of action.

Materials and Methods

Chemicals. Sandalwood oil (SW oil), distributed by NOW Foods (Glendale Heights, IL, USA), was purchased from a local store. Primary antibodies against procaspase-3 and cleaved caspase-3 were obtained from Cayman Chemical (Ann Arbor, MI, USA), while that for caspase-8 was obtained from BD-Biosciences Pharmigen (San Diego, CA, USA) and for p53 from Abcam Inc.

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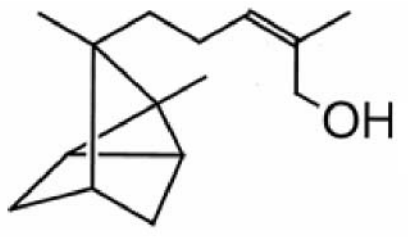

Figure 1. *Structure of α-santalol.*

(Cambridge, MA, USA). Secondary antibodies conjugated to horseradish peroxidase and protein assay kit were bought from Pierce Biotechnology Inc. (Rockford, IL, USA) and the detection kit from Amersham Biosciences (CA, USA). Other reagents and chemicals were obtained in their highest purity grade available commercially.

Isolation and characterization of α-santalol. α-Santalol (Figure 1) was isolated by sandalwood oil distillation and characterized by NMR and GC-MS analysis (11).

Animals. Female SKH-1 mice (5 weeks old) were purchased from Charles River Laboratories (Wilmington, MA, USA). All mice were housed in the College of Pharmacy animal facility under climate-controlled environment with a 12 h light/dark cycle. Mice (10 mice per cage) were allowed free access to food pellets and water placed inside the food chamber on top of the cage cover. The experimental protocol was approved by Institutional Animal Care and Use Committee.

Female SKH-1 mice were divided into two groups; each group consisted of ten mice. The UVB-induced tumor protocol was used as reported elsewhere (13) and is presented in Table I. Mice in both groups were weighed and tumors were counted once a week throughout the experiment.

Determination of caspase-3 and -8, and p53. Mice from the tumorigenesis protocol were sacrificed by cervical dislocation and the epidermis was collected from both groups. The fat and tumors in the skin of these groups were scraped by scalpel, and the epidermis was homogenized by an OMNI GLH-115 homogenizer (Omni International, Inc., Warrenton, VA, USA) in 0.1 mM Tris-HCl (pH 7.4) containing 0.15 M sodium chloride. The epidermal homogenate was filtered through a cheesecloth and the filtrate was centrifuged at 10,000xg for 20 min in a Beckman J2-21 Centrifuge (Fullerton, CA, USA). This pellet was combined with 5% sodium dodecyl sulfate containing 1% 100 mM phenylmethylsulphonylfluoride, 0.5% leupeptin, and 0.5% pepstatin and was allowed to pass through a 25G needle and centrifuged at 13,000xg for 20 min. The supernatant was collected, allowed to pass through a 25G needle again and heated in heating block (100˚C) for 5 min. Finally proteins were collected and used for determination of caspase-3 and -8, and p53 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting analysis, as described by Zhang *et al.* (14). Equal amounts of proteins (60 μg) were loaded onto 12.5% sodium dodecyl sulfate-polyacrylamide gel and electrophoresis was run to separate the proteins. The proteins in gels were transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat milk in Tris-buffered saline (10 mM Tris and 100 mM NaCl) for 1 h, and probed for primary antibodies against caspase-3 and -8, and p53 followed by horseradish peroxidase (HRP)-conjugated secondary antibody and electrochemiluminescence (ECL) detection. The Western blots were quantified using a UVP Biochem Gel Documentation System (UVP, Inc., Upland, CA, USA).

Table I. *UVB-induced skin tumorigenesis protocol.*

Group	Initiation for 14 days	Resting period	Promotion for 30 weeks
Control	Acetone (100 μl) 1 h before UVB*	1 week	Acetone (100 μl) 1 h before UVB* twice a week
α-Santalol	α-Santalol** (100 μl) 1 h prior to UVB*	1 week	α-Santalol** (100 μl) 1 h prior to UVB* twice a week

*UVB radiation (180 mJ/cm^2); **α-Santalol (5% w/v in 100 μl acetone).

Protein assay. Protein was assayed using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA) with albumin as a standard.

Statistical analysis. The software INSTAT (Graph Pad, San Diego, CA, USA) was used to analyze the data. A Chi square test was used to compare the tumor incidence in both groups. ANOVA followed by Student's *t*-test was used to compare the tumor multiplicity, weight gain, caspase-3 and -8, and p53 levels in both groups. Significance in all the cases was considered at $p<0.05$.

Results

Effects of α-santalol on tumor incidence and multiplicity. Effects of α-santalol pretreatment on UVB-induced skin tumor incidence is shown in Figure 2a. Tumors appeared in control and the α-santalol pretreated group by the 10th and 17th week respectively. α-Santalol treatment delayed the appearance of tumors by 7 weeks. However, tumor incidence was 100% in both groups by week 24. Tumor incidence was significantly ($p<0.05$) lower in the α-santalol pretreated group until the 19th week of promotion compared to the control group.

The effect of α-santalol pretreatment on UVB-induced skin tumor multiplicity is given in Figure 2b. α-Santalol pretreatment significantly ($p<0.05$) reduced tumor multiplicity throughout the duration of the experiment. Control mice had 19.6 tumors/mice whereas α-santalol pretreated mice had 11.3 tumors/mice at 30 weeks of promotion. The effect of α-santalol pretreatment on UVB-induced skin tumor development in SKH-1 mice are consistent with our previous report (13). There was no significant difference in weight gain between the control and the α-santalol pretreated group (data not shown).

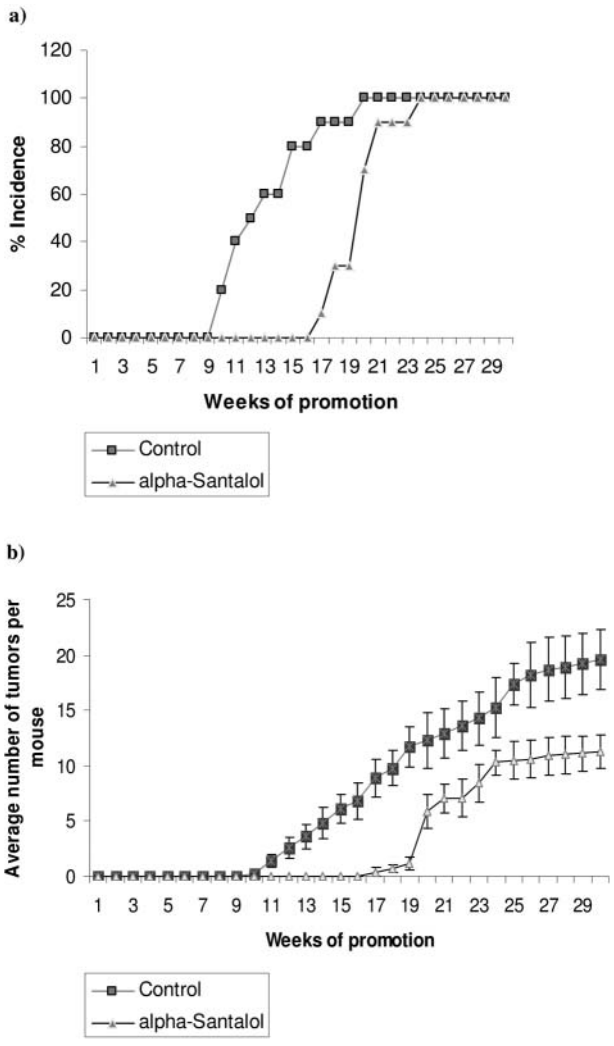

Figure 2. *Effect of α-santalol on (a) tumor incidence and (b) tumor multiplicity in UVB-initiated and promoted skin cancer in SKH-1 mice. Values represent mean±SE from ten mice.*

Effect of α-santalol on procaspases and caspases. The key event involved in apoptosis is the activation of caspase-3. In the present study, Western blot analysis revealed a convincingly significant ($p<0.05$) activation of caspase-3 by α-santalol pretreatment. Application of α-santalol one hour before UVB exposure up-regulated the levels of pro-caspase-3 to 185.78±16.28% and caspase-3 to 472.06±182.08% compared to control mice.

The classical apoptotic process occurs *via* two pathways: the extrinsic and intrinsic pathway. The extrinsic pathway involves caspase-8 as an initiator. Based on the above results, a further study was conducted to understand the pathway behind caspase-3 activation and to assess if upstream effector caspases are being activated. A significant increase ($p<0.05$) in pro-caspase-8 and caspase-8 levels were observed in the α-santalol pretreated group. The values of pro-caspase-8 and caspase-8 were 197.49±50.41% and 235.29±30.10% of the control group respectively in the α-santalol pretreated group.

Effects of α-santalol on p53. There was a significant ($p<0.05$) increase in p53 levels in the α-santalol pretreated group compared to the control group. The levels of p53 were found to be 198.02±17.8% that of the control in the α-santalol pretreated group.

Discussion

Cancer is a major cause of mortality and morbidity in developed as well as developing countries (16). The primary cause of skin cancer is the cumulative lifetime exposure of the skin to UV radiation, especially to that of UVB (17). The credibility of chemoprevention as a serious and practical approach to intervention in order to arrest or reverse the process of carcinogenesis has been enhanced within the past few years (18). To combat a disease as complicated and multifactorial as cancer is, a putative chemopreventive agent must exhibit diverse mechanistic properties to modulate the carcinogenic process leading to cell cycle arrest and apoptosis induction (3, 19).

Sandalwood oil, which has been used since ancient times by Ayurvedic practitioners to treat various skin ailments, was shown to bring about a significant decrease in skin papilloma development (11-13). α-Santalol (Figure 1), a sesquiterpene, is a major constituent of sandalwood oil. Previous studies on α-santalol have shown its chemopreventive effects against skin carcinogenesis in mouse models (9-13). Further *in vitro* studies related to understanding the molecular mechanism behind the chemoprotective activity have shown that α-santalol induces both growth inhibition and apoptotic death of human epidermoid carcinoma A431 cells (4). Studies also showed that α-santalol acts as an antiperoxidant in lowering UVB-induced skin tumor development in SKH-1 mice (20).

Previous studies in our laboratory on the distribution of α-santalol have concluded that the chemopreventive effects of α-santalol are not due to its blocking ability but possibly due to its systemic absorption into the skin (13).

As the fundamental biochemical event which marks the start of apoptosis is the activation of caspases, we therefore investigated the effects of α-santalol towards the activation of caspases (4). The Western blot studies have implied that α-santalol activates the major apoptotic proteins, caspase-3 and -8, suggesting that α-santalol might cause apoptotic death through the extrinsic pathway. This activation of caspases is significant in α-santalol pretreated mice indicating that pretreatment of mice with α-santalol can potentially induce apoptotic cell death. These results are in

agreement with our previous report (4) on the effects of α-santalol on human epidermoid carcinoma A431 cells.

Tumor suppressors are the physiological barriers against clonal expansion and are thus able to arrest uncontrolled cell proliferation by oncogenes. The p53 tumor suppressor gene is involved in the cell cycle arrest and activation of programmed cell death. Mutations in the p53 gene have been detected in 50% of all human cancers and in almost all skin carcinomas (21). Inactivation of the p53 gene plays an important role in the induction of skin cancer by UV radiation. Analysis of mutations in the p53 gene has established an unequivocal connection between UV exposure, DNA damage and skin carcinogenesis. Normally, there is little p53 protein in the cell, but in response to UV damage, high levels of p53 are induced. Therefore, p53 aids in the DNA repair or elimination of cells that have excessive DNA damage through increased apoptosis (21). Western blot studies of p53 protein of skin samples have shown a significant increase in p53 levels in the α-santalol pretreated groups in comparison with the control groups, which alludes that α-santalol enhances the levels of tumor suppressor proteins and thus proves its chemopreventive activity.

In conclusion, α-santalol elicits its chemopreventive effects through multiple mechanisms. The chemopreventive effects of α-santalol could be attributed to the induction of apoptotic and tumor suppressor proteins in addition to antiperoxidant and antiprolifeative effects. The mechanism of apoptosis may be primarily *via* the extrinsic pathway, although involvement of caspase-independent pathways cannot be ruled out. Further studies on various signaling pathways involved in carcinogenesis are needed to explain the mechanism of chemopreventive effects of α-santalol.

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References

1 F'guyer S, Afaq F and Mukhtar H: Photochemoprevention of skin cancer by botanical agents. Photodermatol Photoimmunol Photomed *19(2)*: 56-72, 2003.

2 Cancer Facts and Figures 2007. American Cancer Society Publication http://www.cancer.org.

3 Kaur M, Agarwal C, Singh RP, Guan X, Dwivedi C and Agarwal R: Skin cancer chemopreventive agent, {alpha}-santalol, induces apoptotic death of human epidermoid carcinoma A431 cells *via* caspase activation together with dissipation of mitochondrial membrane potential and cytochrome *c* release. Carcinogenesis *26(2)*: 369-380, 2005.

4 Shi Y: Caspase activation, inhibition, and reactivation: a mechanistic view. Protein Sci *13(8)*: 1979-1987, 2004.

5 Proskuryakov SY, Konoplyannikov AG and Gabai VL: Necrosis: a specific form of programmed cell death? Exp Cell Res *283(1)*: 1-16, 2003.

6 Au JL, Panchal N, Li D and Gan Y: Apoptosis: a new pharmacodynamic endpoint. Pharm Res *14(12)*: 1659-1671, 1997.

7 Sinicrope FA and Gill S: Role of cyclooxygenase-2 in colorectal cancer. Cancer Metastasis Rev *23(1-2)*: 63-75, 2004.

8 Manson MM: Cancer prevention – the potential for diet to modulate molecular signaling. Trends Mol Med *9*: 11-18, 2003.

9 Dwivedi C and Abu-Ghazaleh A: Chemopreventive effects of sandalwood oil on skin papillomas in mice. Eur J Cancer Prev *6(4)*: 399-401, 1997.

10 Dwivedi C and Zhang Y: Sandalwood oil prevent skin tumor development in CD-1 mice. Eur J Cancer Prev *8(5)*: 449-455, 1999.

11 Dwivedi C, Guan X, Harmsen WL, Voss AL, Goetz-Parten DE, Koopman EM, Johnson KM, Valluri HB and Matthees DP: Chemopreventive effects of alpha-santalol on skin tumor development in CD-1 and SENCAR mice. Cancer Epidemiol Biomarkers Prev *12(2)*: 151-156, 2003.

12 Dwivedi C, Maydew ER, Hora JJ, Ramaeker DM and Guan X: Chemopreventive effects of various concentrations of alpha-santalol on skin cancer development in CD-1 mice. Eur J Cancer Prev *14(5)*: 473-476, 2005.

13 Dwivedi C, Valluri HB, Guan X and Agarwal R: Chemopreventive effects of α-santalol on UVB-induced skin cancer development. Carcinogenesis *27(9)*: 1917-1922, 2006.

14 Zhang X, Kundoor K, Khalifa S, Zeman D, Fahmy H and Dwivedi C: Chemopreventive effects of sarcophine-diol on skin tumor development. Cancer Lett *253*: 53-59, 2007.

15 Kopnin BP: Targets of oncogenes and tumor suppressors: key for understanding basic mechanisms of carcinogenesis. Biochemistry (Mosc) *65(1)*: 2-27, 2000.

16 Shukla Y and Pal SK: Complimentary and alternative cancer therapies; past, present and the future scenario. Asian Pac J Cancer Prev *5(1)*: 3-14, 2004.

17 Boukamp P: UV-induced skin cancer: similarities – variations. J Dtsch Dermatol Ges *3(7)*: 493-503, 2005.

18 Sporn MB and Suh N: Chemoprevention of cancer. Carcinogenesis *21(3)*: 525-530, 2000.

19 Gescher A, Pastorino U, Plummer SM and Manson MM: Suppression of tumor development by substances derived from the diet-mechanisms and clinical implications. Br J Clin Pharmacol *45(1)*: 1-12, 1998.

20 Bommareddy A, Hora J, Cornish B and Dwivedi C: Chemoprevention by α-santalol on UVB radiation-induced skin tumor development in mice. Anticancer Res *27(4B)*: 2185-2188, 2007.

21 Benjamin CL and Ananthaswamy HN: p53 and the pathogenesis of skin cancer. Toxicol Appl Pharmacol *224(3)*: 241-248, 2007.

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