Skin cancer chemopreventive agent, α-santalol, induces apoptotic death of human epidermoid carcinoma A431 cells via caspase activation together with dissipation of mitochondrial membrane potential and cytochrome c release

Manjinder Kaur1, Chapla Agarwal1,2, Rana P. Singh1, Xiangming Guan3, Chandradhar Dwivedi3 and Rajesh Agarwal1,2,*

1Department of Pharmaceutical Sciences, School of Pharmacy, 2University of Colorado Cancer Center, University of Colorado Health Sciences Center, Denver, CO 80262, USA and 3Department of Pharmaceutical Sciences,
College of Pharmacy, South Dakota State University, Brookings, SD 57007, USA

*To whom correspondence should be addressed
Email: rajesh.agarwal@uchsc.edu

α-Santalol, an active component of sandalwood oil, has been studied in detail in recent years for its skin cancer preventive efficacy in murine models of skin carcinogenesis; however, the mechanism of its efficacy is not defined. Two major biological events responsible for the clonal expansion of transformed/initiated cells into tumors are uncontrolled growth and loss of apoptotic death. Accordingly, in the present study, employing human epidermoid carcinoma A431 cells, we assessed whether α-santalol causes cell growth inhibition and/or cell death by apoptosis. Treatment of cells with α-santalol at concentrations of 25–75 μM resulted in a concentration- and a time-dependent decrease in cell number, which was largely due to cell death. Fluorescence-activated cell sorting analysis of Annexin V/propidium iodide (PI) stained cells revealed that α-santalol induces a strong apoptosis as early as 3 h post-treatment, which increases further in a concentration- and a time-dependent manner up to 12 h. Mechanistic studies showed an involvement of caspase-3 activation and poly(ADP-ribose) polymerase cleavage through activation of upstream caspase-8 and -9. Further, the treatment of cells with α-santalol also led to disruption of the mitochondrial membrane potential and cytochrome c release into the cytosol, thereby implicating the involvement of the mitochondrial pathway. Pre-treatment of cells with caspase-8 or -9 inhibitor, pan caspase inhibitor or cycloheximide totally blocked α-santalol-caused caspase-3 activity and cleavage, but only partially reversed apoptotic cell death. This suggests involvement of both caspase-dependent and -independent pathways, at least under caspase inhibiting conditions, in α-santalol-caused apoptosis. Together, this study for the first time identifies the apoptotic effect of α-santalol, and defines the mechanism of apoptotic cascade activated by this agent in A431 cells, which might be contributing to its overall cancer preventive efficacy in mouse skin cancer models.

Introduction

The incidence of non-melanoma skin cancers (NMSCs) has been increasing worldwide at an alarming rate; more than 1 million new cases are reported annually in the USA alone where exposure to solar radiation, both occupational and recreational, is the major etiologic factor for this malignancy (1). This alarming statistical information clearly suggests that the sunscreen usage as a primary preventive measure against NMSCs is not sufficient (2), and additional approaches and strategies are needed, which are far more efficacious and cost effective in preventing and controlling this malignancy. One such approach involves identification and characterization of chemopreventive agents against NMSCs, which could complement available preventive measures against NMSCs.

Cancer chemoprevention by naturally occurring agents, especially phytochemicals, minerals and vitamins, has shown promising results against various malignancies in a number of studies under both in vitro and in vivo conditions (3,4). In terms of mechanism of efficacy, beginning with their effect in blocking the activation of carcinogens and induction of detoxification pathways in 1980s (5,6), studies in more recent years have also shown the modulation of mitogenic and survival signaling cascades leading to cell-cycle arrest and apoptosis induction (7,8). Overall, more than 1000 different phytochemicals from both nutritive and non-nutritive sources have shown cancer chemopreventive efficacy, and about 400 of them are currently under further investigation (9). Many of these phytochemicals have also shown chemopreventive effects against skin carcinogenesis (10–14). One such phytochemical is α-santalol, a major component of sandalwood oil (Santalum album Linn, Indian sandalwood) that is traditionally used in the treatment of various skin ailments, and is shown to induce glutathione-S transferase and acid soluble sulfhydryl levels (15). Topical application of sandalwood oil has been shown to prevent skin tumor development initiated by 7,12-dimethylbenz[a]anthracene and promoted by 12-O-tetradecanoyl phorbol-13-acetate (16,17). A similar effect has also been observed with α-santalol (18); however, the mechanism of its efficacy is not known. Since two major biological events responsible for tumorigenesis include uncontrolled growth and loss of apoptotic death of transformed cells, employing human epidermoid carcinoma A431 cells, here we assessed whether α-santalol causes cell growth inhibition and/or cell death by apoptosis. The results obtained convincingly suggest a decrease in cell number largely due to an apoptotic effect of α-santalol. In the mechanistic studies, we identified the involvement of mitochondrial damage-cytochrome c release-caspase activation in the apoptotic response of α-santalol; however, an inhibition of caspase activation also led to apoptosis by a mechanism yet to be identified.

Abbreviations: CHX, cycloheximide; DEVD, Asp-Glu-Val-Asp; FACS, fluorescence-activated cell sorting; PARP, poly (ADP-ribose) polymerase; PI, propidium iodide; pNA, p-nitroanilide; Z-IETD.fmk, benzoyloxycarbonyl-Ile-Glu-Leu-Glu(Thr)-Asp(Ome)-fluoromethylketone; Z-LEHD.fmk, benzoyloxycarbonyl-Leu-Glu(Thr)-Asp(Ome)-fluoromethylketone; Z.VAD.fmk, benzoyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.
Materials and methods

Reagents

α-Santalol was purified from sandalwood oil and characterized as reported earlier (18). Total and cleaved caspase-3, cleaved poly(ADP-ribose) polymerase (PARP), BID primary antibodies and peroxidase-conjugated secondary antibody were from Cell Signaling Technology (Beverly, MA). Cytochrome c antibody was from BD Pharmingen (San Diego, CA). Different caspase inhibitors, Z-VAD.fmk, benzyloxy-carbonyl-Val-Leu-Lys-Asp(Ome)-fluoromethylketone (Z-DEVD.fmk) and benzyloxy-carbonyl-Leu-Glu-Asp(Ome)-fluoromethylketone (Z-LEHD.fmk) were purchased from Enzyme Systems Products (Livermore, CA). Annexin V-propidium iodide (PI) and JC-1 dye were from Molecular Probes (Eugene, OR). The ECL detection system was from Amersham (Piscataway, NJ). Pefabloc SC (AEROS) was from Roche Diagnostics (Indianapolis, IN). Other reagents were obtained in their highest purity grade available commercially.

Cell culture and growth assay

Human epidermoid carcinoma A431 cells were cultured and maintained in DMEM supplemented with 10% heat inactivated fetal calf serum, 100 U of penicillin G and 100 U of streptomycin sulfamate under 5% CO2 and 95% humidified air atmosphere. Cells were plated overnight at a density of 2 x 10^5 cells/60 mm dish. The following day, the culture medium was replaced with DMEM containing 10% fetal calf serum, 50 µM α-santalol at the final concentration ranging from 0 to 75 µM; all treatments were done employing stock solution in DMSO and the control had an equal volume of DMSO (up to 0.1%, v/v) as in the treatments. Adherent and non-adherent cells were collected at the end of the desired treatments, and cells were counted using a hemocytometer after Trypan blue staining to differentiate live and dead cells.

MTT assay

Cells were plated at a density of 1500 cells/well in 96-well plate. Next day, cells were treated with either DMSO alone or α-santalol (0–75 µM) in DMSO for 12–48 h. At the end of each treatment time, 20 µl of MTT stock solution (5 mg/ml) was added to each well and incubated for another 5 h. Thereafter, media from each well was aspirated out and 200 µl of DMSO was added to each well. Absorbance was measured at 550 nm in the presence of an appropriate blank.

BrdU incorporation assay

Cells were seeded and treated with either DMSO alone or α-santalol (0–75 µM) as described for the MTT assay. At the end of each treatment period, the effect of α-santalol on cell proliferation was assessed in terms of BrdU incorporation using colorimetric ELISA kit (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, cells were labeled with BrdU for another 3 h at 37°C post-treatment time followed by DNA denaturation as per protocol provided by manufacturer. Subsequently, cells were incubated with anti-BrdU antibody, followed by incubation with substrate. BrdU incorporation was measured by taking the absorbance at 450 nm. A blank reading was subtracted from experimental readings before final calculations.

Annexin V/PI staining

Adherent and non-adherent cells following desired treatments were collected after brief trypsinization, and cell pellets were washed twice with ice cold PBS. The cells were then stained with Annexin V/PI as specified in the protocol provided with Vybrant Apoptosis Assay Kit 2 (Molecular Probes, Eugene, OR), followed by fluorescence-activated cell sorting (FACS) analysis as reported earlier (19).

Colorimetric caspase-3, -9 and -8 activity assay

Activity of various caspases was measured using ApoTarget Kit (BioSource International, Camarillo, CA) following Vendor’s protocol, where caspase activation leads to the cleavage of respective colorimetric substrate peptides conjugated to p-nitroaniline (pNA) [caspase-3: DEVD(Asp-Glu-Val-Asp)-pNA, caspase-8: IETD-pNA and caspase-9: LEHD-pNA]. Briefly, A431 cells were plated at a density of 2 x 10^5 cells/60 mm dishes culture dish for overnight. Next day, culture medium was replaced with the one containing α-santalol (0, 25, 50 or 75 µM) with or without 2h or 4h pre-treatment with specific caspase inhibitor. At the end of the desired treatments, the cells were harvested, washed twice with PBS and lysed for 30 min on ice in the lysis buffer provided in the kit. Lysates were cleared by centrifugation at 14,000 r.p.m. at 4°C, and the activity of the specific caspase was measured in the clear lysates essentially as described in the protocol by measuring the OD at 405 nm in a microplate reader.

Immunoblotting

A431 cells were plated and treated as described above, except these treatments also included a 2 h pre-treatment with specific caspase inhibitor, 1 h pre-treatment with serine protease inhibitor, pefabloc and/or 1 h pre-treatment with cycloheximide (CHX). At the end of the desired treatments, cells were harvested, washed twice with PBS, and cell lysates were prepared as reported recently (20). Protein estimation in cell lysates was carried out using a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). An equal amount of protein from cell lysates was subjected to SDS-PAGE followed by western immunoblotting. The membranes were then blocked with 5% skimmed milk for 1 h in TBS (10 mM Tris, 100 mM NaCl, 0.1% Tween 20) at room temperature or overnight at 4°C, and probed for total and/or cleaved caspase-3, cleaved PARP, BID and β-actin, followed by appropriate HRP conjugated secondary antibody and ECL detection.

Analysis of mitochondrial membrane potential

Mitochondrial damage was assessed by JC-1 staining as described earlier (21). Briefly, cells were treated with 75 µM α-santalol for 6 and 12 h, cells were trypsinized and washed with PBS, and then incubated with JC-1 (10 µg/ml in PBS) at 37°C for 10 min. Stained cells were washed twice with PBS, pelleted down in 0.5 ml of PBS followed by FACS analysis, and mitochondrial function was assessed as JC-1 green (uncoupled mitochondria, detector FL-1) or red (intact mitochondria, detector FL-2) fluorescence. Valinomycin (10 µg/ml) treated cells were used as positive control.

Cytochrome c release

Cytochrome c release from mitochondria was measured in A431 cells after treatment of cells with α-santalol. Briefly, subsequent to treatment, cells were harvested with trypsinized, followed by two washings with ice cold PBS, and then re-suspended in permeabilization buffer (20 nM HEPES-KOH, pH 7.4, 50 mM KCl, 210 mM mannitol, 70 mM sucrose, 5 mM MgCl2, 1 mM DTT, 0.1 mM PMSF) containing complete protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN) for 20 min on ice. Cells were then homogenized with a glass Dounce and Pestle, and were kept on ice for an additional 20 min. Homogenates were centrifuged at 14,000 r.p.m. for 15 min at 4°C, and supernatants were collected and further clarified by centrifugation at 14,000 r.p.m. for 30 min at 4°C, resulting in cytosolic extracts. They were then analyzed for cytosolic cytochrome c by SDS-PAGE and immunoblotting as detailed above.

Statistical analysis

Data were analyzed with Jandel Scientific SigmaStat 2.0 software (San Rafael, CA), and the Student’s t-test was employed to assess the statistical significance of the difference between different treatment groups.

Results

α-Santalol treatment strongly decreases A431 cell population

First we assessed the effect of α-santalol on A431 cell growth where cells were treated with varying concentrations of the agent for 24 and 48 h, and then counted using a hemocytometer. As shown in Figure 1, α-santalol treatment on A431

![Graph showing cell number vs. treatment time](image)

**Fig. 1.** α-Santalol induces time- and concentration-dependent growth inhibition in A431 cells. Cells were treated with α-santalol (0–75 µM) for 24–48 h. At the end of the respective treatments, cells were collected after brief trypsinization, and counted using a hemocytometer. Values are mean ± SD of three observations. *P < 0.05; $P < 0.001 indicate statistical significance in α-santalol-treated groups as compared with the control.
cells resulted in a strong decrease in cell number in a concentration- and time-dependent manner. Compared with DMSO-treated controls, α-santalol concentrations in the range of 25-75 μM resulted in 20-82% and 14-96% decrease ($P < 0.05-0.001$) in cell number following 24 and 48 h treatments, respectively (Figure 1). We also observed an increasing number of dead cells with the increase in α-santalol concentrations (data not shown). Therefore, further experiments were performed to investigate whether α-santalol-caused decrease in cell number involves inhibition of cell proliferation and/or induction of apoptotic cell death.

**α-Santalol strongly reduces viability of A431 cells**

In similar treatments as above, cell viability and cell proliferation were assessed by MTT and BrdU incorporation assays. In the MTT assay, a concentration- and time-dependent decrease in OD$_{550}$ nm was observed following α-santalol treatment (Figure 2A). α-Santalol (25–75 μM) treatment for 12, 24 and 48 h resulted in a 7–23, 9–41 and 14–56% ($P < 0.05-0.001$) decrease in cell viability, respectively (Figure 2B). In the BrdU incorporation assay, α-santalol (25–75 μM) treatment for 12, 24 and 48 h resulted in a 6–45, 10–42 and 19–48% ($P < 0.05-0.001$) inhibition in cell proliferation or DNA synthesis, respectively (Figure 2C and D). Further, we calculated the ratio of BrdU incorporation to viable cells for each treatment that indicated the inhibitory effect of α-santalol on cell proliferation only at 12 h of treatment (23–28% at 50–75 μM doses), whereas no inhibition in cell proliferation was observed at 24 and 48 h of treatment (Figure 2E). These results suggest that at an early treatment time (12 h), α-santalol

**Fig. 2.** Effect of α-santalol on cell viability and DNA synthesis in A431 cells. Cells were treated with α-santalol (0–75 μM) for 12–48 h. At the end of respective treatments (A and B) MTT and (C and D) BrdU incorporation assays were performed as detailed in the Materials and methods. Values are mean ± SD of three samples in each treatment. (E) Ratio of proliferating cells to viable cells. *, $P < 0.05$; #, $P < 0.01$; $S$, $P < 0.001$ indicate statistical significance in α-santalol-treated groups as compared with the control.
inhibits cell proliferation; however, at higher treatment times, the decrease in cell number by α-santalol is mainly due to the decrease in cell viability.

α-Santalol induces concentration- and time-dependent apoptotic cell death
As we also observed cell death by α-santalol in trypan blue dye exclusion assay employed during determination of cell number, we next assessed whether the cell death observed is an apoptotic response of the agent. In similar α-santalol treatments, as for determination of cell number assay, Annexin V/PI staining and FACS analysis showed a strong apoptotic activity following longer treatment times (data not shown), which led us to conduct a study employing lower α-santalol concentrations (0–75 μM) and earlier time points of treatments (3–24 h). As shown in Figure 3A, α-santalol caused a strong apoptotic cell death in both concentration- and time-dependent manner. Compared with the DMSO-treated control A431 cells showing 1.4–1.6% apoptotic cell population in 3–12 h, α-santalol treatments for 3–12 h at 25, 50 and 75 μM concentrations resulted in 3–7, 7–23 and 14–31% (P < 0.05–0.001) apoptotic cell death, accounting for 2–5-, 5–15- and 10–21-fold apoptosis induction over controls, respectively (Figure 3A). The treatment of cells at 25–75 μM concentrations of α-santalol for 24 h resulted in a comparable apoptotic response with that observed after 12 h of treatment (Figure 3A), and accordingly, the molecular mechanistic studies were performed using 6 and 12 h treatment times. These results suggest that apoptotic cell death could be a major contributor in overall cell growth inhibition caused by α-santalol.

α-Santalol-induced apoptotic cell death involves caspase-3 and PARP cleavages
As caspase-3 followed by PARP cleavage is the key event in the process of apoptosis, and as these events are also employed as markers of apoptosis induction (22,23), we next examined the effect of α-santalol treatment on the activation of caspase-3 by western immunoblotting. As shown in Figure 3B, α-santalol treatment of the cells led to a time- and concentration-dependent activation of caspase-3, where its cleavage was evident following 6 h post-treatment of the agent at 50 (a very faint band) and 75 (a strong band for cleaved caspase) μM concentrations. Much stronger cleaved caspase-3 bands were observed at these concentrations of α-santalol after 12 h of treatment where 75 μM concentration specifically showed very strong bands at ~19 and 17 kDa (Figure 3B). Based on the observed cleaved caspase-3 levels by α-santalol and the fact that PARP is a major substrate of activated caspase-3 (23), we next examined the levels of cleaved PARP in the total cell lysates used for cleaved caspase-3 blotting. As shown in Figure 3B, consistent with our cleaved caspase-3 results, we also observed modest to very strong levels of cleaved PARP (89 kDa) in α-santalol-treated lysates.

α-Santalol-caused caspase-3 activation associates with upstream caspase-9 and -8
In a classical apoptotic cascade, activation of caspase-3, an executioner caspase, is known to occur via two pathways: an extrinsic pathway with caspase-8 being the initiator or an intrinsic pathway where caspase activation cascade is mediated by caspase-9 (24). Based on our results showing that α-santalol causes strong cleavage of caspase-3, we also assessed whether caspase-9 and -8 are activated as upstream effectors leading to
Materials and methods. Data are shown as fold increase over control, and treatments for 12 h (Figure 4C), respectively. 

1.4 m (Figure 4B), and a 1.3 for 12 h, and then enzymatic activity in cell lysates towards tetrapeptide and -8 activities in A431 cells. Cells were treated with DMSO controls, caspase-3 activation. As shown in Figure 4A, compared with DMSO-treated controls showing 1.9 ± 0.1 and 3.0 ± 0.3% cells with green fluorescence, α-santalol treatment at 75 µM concentration resulted in 3.9 ± 0.6 and 8.4 ± 3.2% (P < 0.01-0.05) cells positive for green fluorescence following 6- and 12-h treatments accounting for ~2-3-fold increase over controls, respectively (Figure 5A). When the cytosolic fractions prepared from DMSO or α-santalol-treated cells were analyzed by immunoblotting, consistent with our other observations, α-santalol caused a concentration- and a time-dependent increase in cytosolic cytochrome c levels without noticeable changes in actin used as a loading control (Figure 5B).

α-Santalol disrupts the mitochondrial membrane potential and induces cytochrome c release into cytosol

Loss of mitochondrial membrane potential and subsequent cytochrome c release into the cytosol from the mitochondria is one of the mechanisms of caspase activation in an overall apoptotic cell death (25). Based on our results showing that α-santalol causes strong apoptotic death together with caspase activation and caspase-3 cleavage in A431 cells, next we assessed its effect on the mitochondrial membrane potential followed by cytochrome c release in the cytosol. To investigate the loss of the mitochondrial membrane potential, cells were stained with a mitochondria-specific dye, JC-1, followed by FACS analysis. This assay is based on the principle that with a decline in the mitochondrial membrane potential, the fluorescence of JC-1 dye increases at 530 nm (FL-1) in its monomeric form with a reduction at 590 nm (FL-2) as aggregates (26). Consistent with our caspase activation and apoptotic death results, α-santalol treatment of cells resulted in a time-dependent increase in the percentage of cells positive for JC-1 monomers (Figure 5A). Compared with DMSO-treated controls showing 1.9 ± 0.1 and 3.0 ± 0.3% cells with green fluorescence, α-santalol treatment at 75 µM concentration resulted in 3.9 ± 0.6 and 8.4 ± 3.2% (P < 0.01-0.05) cells positive for green fluorescence following 6- and 12-h treatments accounting for ~2-3-fold increase over controls, respectively (Figure 5A). When the cytosolic fractions prepared from DMSO or α-santalol-treated cells were analyzed by immunoblotting, consistent with our other observations, α-santalol caused a concentration- and a time-dependent increase in cytosolic cytochrome c levels without noticeable changes in actin used as a loading control (Figure 5B).

α-Santalol-caused caspase-3 activation and apoptosis are mediated via caspase-9 and -8 activation

Taken together the results in Figure 4 showing induction of caspase-9 and -8 activities together with a strong induction in caspase-3 activity by α-santalol in A431 cells, it could be suggested that activation of both caspase-9 and -8 contributes to an overall activation of caspase-3 and associated apoptotic response in A431 cells. To support this suggestion and to further establish the involvement of caspase-9 and -8 pathways in caspase-3 activation and apoptosis, we next conducted several additional studies employing specific inhibitors of caspase-9 and -8, and assessing caspase-3 activity and cleavage, and apoptotic death in α-santalol-treated cells. As the data show in Figure 6A, pre-treatment of cells with caspase-8 inhibitor Z-LEHD.fmk followed by α-santalol resulted in almost comparable caspase-3 activity as in DMSO-treated control, as opposed to a 3-fold induction (P < 0.001) in caspase-3 activity in the α-santalol alone-treated cells, suggesting the involvement of caspase-8 activity by this agent in caspase-3 activation. Interestingly, pre-treatment of cells with caspase-9 inhibitor Z-LEHD.fmk also showed a complete inhibition in α-santalol-caused increase in caspase-3 activity, which convincingly also supports the involvement of this caspase as an upstream effector (Figure 6A). Similarly, pre-treatment of cells with a combination of both caspase-8 and -9 inhibitors also showed complete inhibition in α-santalol-induced caspase-3 activity (Figure 6A). Consistent with caspase-3 activity results, pre-treatment of cells with caspase-8 and/or caspase-9 inhibitors also resulted in a complete inhibition in α-santalol-induced caspase-3 cleavages; however, in all the inhibitor treatments we observed the
formation of some new bands just above the 19 kDa cleaved caspase-3 band, and interestingly these new bands showed reactivity to both total and cleaved caspase-3 antibodies (Figure 6B). The actin immunoblotting showed equal protein loading in each case (Figure 6B). In the studies relating the role of caspase-8 and -9 activation in caspase-3 activation and apoptosis by α-santalol, as shown in Figure 6C, the pre-treatment of cells with either of the two inhibitors resulted in a partial reversal of α-santalol-induced apoptotic death. Interestingly and surprisingly, when cells were treated with both the inhibitors, the observed partial reversal (when they are used alone) in apoptosis by α-santalol was totally lost, and comparable apoptotic cell death was observed as with α-santalol alone (Figure 6C). It is important to emphasize here that since caspase-8 and -9 inhibitors alone and in combination caused complete inhibition in α-santalol-induced caspase-3 activity (Figure 6A) and cleaved caspase-3 bands at 19 and 17 kDa (Figure 6B), the observed partial reversal in apoptosis by these inhibitors alone and a complete lack of apoptosis reversal when they are used in combination, suggest that in the absence of caspase activation (including caspase-8 and -9 upstream and caspase-3 as an effector), α-santalol causes apoptotic death of A431 cells by different mechanism(s).

Further, we observed that caspase-8 and -9 regulate the activation of each other in response to α-santalol treatment of A431 cells (Figure 7A). Only higher dose of α-santalol (75 μM) at 24 h treatment showed a very faint band for cleaved BID (Figure 7B); activated jurkat cell lysate was used as positive control, which showed a strong band for cleaved BID (15 kDa) (Figure 7B). These results suggested a possible role of α-santalol-induced caspase-8 activation in mitochondrial apoptosis pathway. However, in other treatments, death signal for the mitochondrial damage is more likely mediated via independent of caspase-8 pathway. More detailed mechanistic studies are needed in future to further decipher these possibilities and identify additional pathways of apoptosis induction by this agent.

**Role of serine and cysteine proteases in α-santalol-caused apoptotic cell death**

Recent studies have shown that under caspase-inhibiting conditions, serine proteases mediate apoptosis (27). Based on our results showing that inhibiting caspase-9 and -8, employing their specific inhibitors, inhibits α-santalol-induced caspase-3 activity completely, only partially reversed apoptosis and their combinations showed a total lack in apoptosis reversal by α-santalol. We performed several additional studies to assess the role of both serine and cysteine proteases in α-santalol-induced apoptotic death of A431 cells. As shown in Figure 8A, pre-treatment of cells with the pan-caspase inhibitor Z-VAD.fmk resulted in a complete inhibition of α-santalol-caused caspase-3 activity; however, in terms of caspase-3 cleavage, similar band patterns were observed as in the case of caspase-9 and -8 inhibitor studies (Figure 8B). Compared with the controls, α-santalol treatments at 50 and 75 μM concentrations resulted in strong cleaved caspase-3 bands at 17 and 19 kDa, which shifted to a little higher mobility, and a new band, immunoreactive to the cleaved caspase-3-specific antibody, was visualized at ~24 kDa in the Z-VAD.fmk-treated samples in an α-santalol concentration-dependent manner (Figure 8B). Consistent with these observations in Z-VAD.fmk inhibitor studies and caspase-9 and -8 inhibitor studies towards caspase-3 activity and cleavage, as shown by data in Figure 8D, Z-VAD.fmk pre-treatment of cells only partially reversed α-santalol-caused apoptosis, further suggesting and confirming the involvement of other pathways at least when cysteine proteases are in inhibiting conditions.

Based on the above observations, next we focused our attention on the involvement of serine proteases in α-santalol-induced apoptotic cell death, under both caspase activating
and inhibiting conditions, employing a serine protease inhibitor pefabloc (300 µM) either alone or in combination with Z-VAD.fmk. As shown in Figure 8C, compared with α-santalol, pre-treatment of cells with pefabloc resulted in a partial, although strong, reduction in α-santalol-caused cleavage of caspase-3, as evidenced by both total and cleaved caspase-3-specific antibodies, suggesting a partial involvement of serine proteases in α-santalol-caused caspase-3 cleavage. However, caspase-3 cleavage was completely blocked by pan-caspase inhibitor Z-VAD.fmk either alone or in combination with pefabloc (Figure 8C), further suggesting the involvement of both serine and cysteine proteases in α-santalol-caused caspase-3 cleavage. In terms of apoptotic death, pre-treatment of cells with pefabloc increased, although statistically not significant, the apoptotic cell death to 34.9 ± 5.72% as compared with 28.2 ± 5.86 in 75 µM α-santalol-alone treated cells (Figure 8D). In the studies where pefabloc was used in combination with Z-VAD.fmk, no additional reversal in α-santalol-caused apoptotic death was observed, when compared with Z-VAD.fmk alone plus agent-treated cells (Figure 8D), suggesting that pefabloc partially inhibits α-santalol-caused caspase-3 cleavage, however, it does not play any role in the apoptosis induction by this agent. Furthermore, these results also suggest that under caspase-inhibiting conditions, there is an alternative mechanism causing apoptotic death of A431 cells by α-santalol.

Fig. 6. α-Santalol induced caspase-3 activation and apoptotic cell death is mediated via both caspase-9 and -8 pathways in A431 cells. Cells were treated with α-santalol (75 µM) in the presence of caspase-8 and/or -9-specific inhibitors (CI) each at 50 µM concentration for 12 h. (A) Caspase-3 activity was measured towards chromogenic substrate, DEVD-pNA. (B) Cleaved caspase-3 levels were analyzed by western immunoblotting. (C) Apoptotic cell death was measured by FACS analysis of Annexin V/PI stained cells. In each case, bars represent mean ± SD of three samples. *, P < 0.05; #, P < 0.01; $, P < 0.001 indicate statistical significance in α-santalol-treated groups as compared with control or inhibitor treated groups. C, control; α-S, α-santalol; CI8, caspase-8 inhibitor; CI9, caspase-9 inhibitor; N.S., not significant.
cell lysates were prepared. Total and cleaved levels of BID were analyzed by western immunoblotting as detailed in the Materials and methods. In each case, bars represent mean ± SD of three samples.

### Discussion

α-Santalol, a naturally occurring terpenoid, has been shown to be effective against skin carcinogenesis in mouse models (18); however, mechanisms of its efficacy are not yet studied. Inhibition of cell proliferation and induction of apoptosis are two key events, which control the outcome of chemopreventive efficacy of an agent under investigation (6,7). In this regard, it is important to highlight here that in most cancers, the apoptosis pathway in tumor cells is compromised with a distinct survival advantage over the normal cell (7,33). Strategies, therefore, are also needed that lead to induction of apoptotic cell death in both transformed and tumor cells. Consistent with these notions, major findings of the present study are that α-santalol induces both growth inhibition and apoptotic death of human epidermoid carcinoma A431 cells.

A fundamental biochemical event marking the start of apoptosis is activation of caspases, which then dismantle the cell in a systematic fashion in such a manner that neighboring cells and tissues are unaffected (34), and PARP cleavage is considered as one of the markers of caspase activation (23). Accordingly, to elucidate the molecular mechanisms underlying the induction of apoptosis by α-santalol, first we focused our efforts on caspase activation. Employing different approaches and assays, we observed that α-santalol causes caspase-3 activation and cleavage that led to PARP cleavage and apoptotic death of A431 cells. In additional studies, whereas induction of caspase-3 activity and cleavage was completely blocked by a cell permeable pan-caspase inhibitor, Z-VAD.fmkk, α-santalol induced phosphatidylserine externalization, as measured by Annexin V/PI staining, did not. This suggests the involvement of caspase-independent mechanisms in α-santalol-induced apoptotic death.

**CHX inhibits α-santalol-induced caspase-3 cleavage but partially blocks apoptosis**

Several studies in recent years have shown that CHX pre-treatment inhibits apoptosis by various agents (28–30), and that it prevents caspase-3 activation (31,32). Based on our results showing that α-santalol causes caspase-3 cleavage and apoptosis, but under caspase inhibiting conditions apoptosis is not completely reversed, we also conducted studies to assess whether CHX reverses α-santalol-caused caspase-3 activation and apoptosis in A431 cells. Pre-incubation of A431 cells with CHX (0.5 μg/ml) completely blocked α-santalol induced caspase-3 cleavage (Figure 9A); however, apoptotic cell death was inhibited by ~50% (Figure 9B). This further supports the involvement of caspase-independent mechanisms in α-santalol-induced apoptotic death.

**Fig. 7.** Effect of α-santalol and/or caspase inhibitors on caspase activity and BID cleavage in A431 cells. (A) Cells were treated with α-santalol (75 μM) in the presence or absence of caspase-8 and -9-specific inhibitors (50 μM) for 12 h. Caspase-8 and -9 activities were measured using chromogenic substrates as detailed in the Materials and methods. In each case, bars represent mean ± SD of three samples. (B) Cells were treated with α-santalol (0–75 μM) for 12–24 h, and cell lysates were prepared. Total and cleaved levels of BID were analyzed by western immunoblotting as detailed in the Materials and methods. * P < 0.05; #: P < 0.01; $: P < 0.001 indicate statistical significance in CI + α-santalol treated groups as compared with α-santalol-treated groups alone. C, control; α-S, α-santalol; CI8, caspase-8 inhibitor; CI9, caspase-9 inhibitor.
that commits the cell to die by apoptosis (35). Release of cytochrome c is known to cause formation of apoptosome by interacting with adaptor protein Apaf-1, leading to conformational changes favorable for dATP/ATP binding (36) that leads to the formation of cytochrome c and the Apaf-1 heptamer, which then recruits procaspase-9 and activates it to caspase-9 (35). Once activated, caspase-9 can activate downstream effector caspases-3, -6 and -7 (35,37). Consistent with these studies, α-santalol caused disruption in mitochondrial membrane potential as well as cytochrome c release into the cytosol and the activation of caspase-9. This suggests that disruption in mitochondrial membrane potential–cytochrome c release–caspase-9 activation could be one mechanism of α-santalol-caused caspase-3 activation and apoptosis induction in A431 cells. However, there was concomitant and almost similar induction in caspase-8 activity too suggesting an additional
mechanism(s) of caspase-3 activation by α-santalol. To address this issue and study the direct involvement of caspase-9 and/or -8 in caspase-3 activation and apoptosis by α-santalol, the selective inhibitor approach was employed, which showed complete inhibition of α-santalol-caused caspase-3 activity and cleavage in the presence of caspase-8 and/or -9-specific inhibitors Z-IETD.fmk and Z-LEHD.fmk, respectively. However, none of these inhibitors was able to completely reverse α-santalol-induced apoptosis, and interestingly when both inhibitors were used together, there was no effect in reversing apoptosis. These results suggest that α-santalol induced apoptotic cell death may be mediated by both extrinsic and intrinsic pathways of apoptosis, and that at least under caspase inhibiting conditions, an alternative mechanism is involved. Further, in the presence of α-santalol, caspase-8 and -9 regulate the activation of each other, suggesting a positive feedback loop for their activation. α-Santalol at a higher dose and treatment time showed the cleavage of BID, which is known to be cleaved by activated caspase-8 and associated with mitochondrial membrane depolarization, suggesting the role of α-santalol-induced caspase-8 activation in the mitochondrial apoptosis pathway. However, at early treatment time, we did not observe any BID cleavage, suggesting that the death signal for the mitochondrial damage is more likely also mediated via the independent of caspase-8 pathway.

Recent studies have shown the existence of both caspase-dependent and -independent pathways of apoptosis downstream of mitochondrial damage (38). For example, cell death with organelle dysfunction but no measurable caspase activity has been observed upon deletion of downstream effectors of apoptosis, Apaf-1 or caspase-9 (39). Furthermore, several studies have implicated the existence of certain proteins in mitochondria-like apoptosis inducing factor, endoG and Omi/HtrA2, which can mediate caspase-independent apoptosis (40–42). In other mechanisms implicating caspase-independent apoptosis, serine proteases other than Omi/HtrA2, GranzymeA and B are thought to be involved under caspase inhibiting conditions (27). Thus, it is plausible that caspase-independent pathways of apoptosis may override the caspase-dependent pathways under caspase inhibiting conditions. This plausibility is in accord with our results where we observed that in the presence of both the inhibitors (caspase-8 and caspase-9) together, the extent of apoptosis reversed back to that observed in the presence of α-santalol alone.

It is also being increasingly recognized that under caspase inhibiting conditions, or under caspase activation failure, the cell utilizes other default pathways towards self-destruction (43). One such pathway is via serine proteases, which induces apoptosis under caspase inhibiting conditions (27). To delineate whether α-santalol-induced apoptotic death involves serine proteases under caspase-activating or inhibiting conditions, we used the broad-spectrum serine protease inhibitor pefabloc without or with pan-caspase inhibitor Z-VAD.fmk and assessed α-santalol induced apoptotic cell death. We observed that pefabloc was unable to inhibit apoptosis even under caspase inhibiting conditions. Since caspase-3 activation is considered as a point of no return for cells to die, we next examined the effect of these inhibitors on caspase-3 cleavage. Interestingly, we found that although none of these inhibitors was completely able to protect the cell from α-santalol-induced apoptosis either alone or in combination, yet caspase-3 cleavage was completely abolished by pan-caspase inhibitor and to a large extent by serine proteases inhibitor at the concentrations used in this study. These results thus rule out the possibility of serine proteases involvement in α-santalol induced apoptotic cell death. Together, based on our completed studies, other speculation might be the activation of some other proteases like granzymes, lysosomal cathepsins or some matrix metalloproteinases, which are often implicated in caspase-independent cell death (43). More studies would be needed in future to support (or rule out) this speculation.

Suppression of apoptotic cell death induced by various agents has also been shown to occur following pre-treatment with CHX in various cell lines (28–30). In the present study, pre-treatment with CHX led to a significant but not complete reversal of α-santalol induced apoptotic cell death, even though caspase-3 cleavage was completely inhibited. These findings are consistent with previous reports showing that CHX inhibits caspase-3 activation (31,32). The observed incomplete reversal of apoptotic cell death in spite of complete blockage of caspase-3 processing by both benzoyloxy carbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD.fmk) and CHX pre-treatment further implicates and supports the involvement of caspase-independent pathways of apoptosis, and is in concord with recent studies reporting the existence of caspase-independent pathways of apoptosis induced by diverse stimuli (44,45) implicating the involvement of non-caspase proteases (46–49). In conclusion, α-santalol inhibits growth and causes apoptotic death of A431 cells. The mechanism of apoptosis is primarily via the intrinsic pathway involving perturbation of mitochondria and cytochrome c release, although involvement of caspase-independent pathways cannot be ruled out specifically under caspase inhibiting conditions.
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References


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