Survivin Down-regulation by α-Santalol Is Not Mediated Through PI3K–AKT Pathway in Human Breast Cancer Cells

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Abstract. *Background: α-Santalol, a terpenoid found in sandalwood oil, has been shown to inhibit cancer cell growth in vitro by inducing apoptosis. This study was performed to investigate the anticancer properties of α-santalol associated with the induction of apoptosis in cultured MCF-7 [estrogen receptor (ER)-positive, and wild-type p53)] and MDA-MB-231 (ER-negative and mutant p53) breast cancer cells. Materials and Methods: Expression of major proteins examined in the study were determined using a standard western blot protocol and analyzed by LICOR-Odyssey infrared scanner. Total protein levels of survivin were confirmed by survivin enzyme-linked immunosorbent assay (ELISA) kit. Cell viability was assessed by the trypan blue dye exclusion assay, and caspase-3 activity was determined by caspase-3 (active) ELISA kit. Results: Treatment of breast cancer cells for 6 and 9 h with α-santalol (20, and 40 μM) resulted in statistically significant concentration-dependent down-regulation of survivin. Phosphorylated protein kinase B (pAKT) levels were found to be slightly up-regulated despite the down-regulation of survivin. Pharmacological inhibition of the phosphoinositide 3-kinase - protein kinase B (PI3K-AKT) pathway did not result in a synergistic/additive increase in cell death or caspase-3 activity caused by α-santalol. Conclusion: The study reveals that survivin down-regulation by α-santalol in breast cancer cells is not mediated through the PI3K–AKT pathway.*

Breast cancer remains one of the most commonly diagnosed types of cancer and the second leading cause of cancer-related deaths in women worldwide, despite significant advances in therapeutic approaches (1). It is estimated that one in every ten women will develop breast cancer in their lifetime and that there will be 231,840 new cases of breast cancer and 40,290 deaths due to breast cancer this year alone in the United States (1, 2). Chemotherapy and hormonal therapy are largely ineffective in advanced-stage and refractory breast cancer (3, 4). In addition, patients present with severe side-effects of therapy that may negatively affect the person's quality of life. For these reasons, it is vital novel therapeutic or preventive agents that not only combat cancerous growth, but are also safe to normal, healthy cells are discovered and developed (5).

Studies from our laboratory and others have shown that α-santalol, a naturally-occurring terpenoid from the sandalwood tree, possesses anticancer and cancer-preventive properties. For example, α-santalol was shown to have chemopreventive properties against chemically-induced and UVB-induced skin carcinogenesis (6-10), suppress proliferation of non-melanoma and melanoma skin cancer cells (11), and inhibit the growth of human prostate cancer PC-3 and LNCaP cells irrespective of their androgen or p53 status (12,13). Moreover, α-santalol was found to be non-toxic to normal epithelial cells at the concentrations used in the current study (12). Even though the growth-inhibitory effects of α-santalol against breast cancer cells are not fully understood, we previously showed that α-santalol treatment induces apoptosis and causes cell-cycle arrest in both MCF-7 and MDA-MB-231 cells (14). The present study aimed to explore the mechanistic details associated with the induction of apoptosis by α-santalol using cultured breast cancer cells (MDA-MB-231 and MCF-7 cells).

Materials and Methods

Materials. α-Santalol used in the study was isolated as described by us previously (14). Cell-culture reagents, including RPMI-1640 medium, minimal essential medium (MEM), fetal bovine serum

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(FBS), and penicillin/streptomycin antibiotic mixture were purchased from Invitrogen-Life Technologies (Carlsbad, CA, USA). ELISA kit for survivin activity was purchased from Cell Signaling (Danvers, MA, USA) and caspase-3 activity ELISA kit was purchased from Invitrogen (Camarillo, CA, USA). All the antibodies used in the study were purchased from Cell Signaling.

Cell lines. Cell lines MDA-MB-231 and MCF-7, which were previously authenticated by Research Animal Diagnostic Laboratory (University of Missouri, Columbia, MO, USA) to test for interspecies contamination and alleles for short tandem repeats identifiable in the ATCC database, were a generous gift from Dr. Shivendra V. Singh (University of Pittsburgh Cancer Institute, Pittsburgh). MDA-MB-231 cells were cultured in RPMI Nutrient Mixture supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin antibiotic mixture. The MCF-7 cells were maintained in MEM supplemented with sodium pyruvate (1 mM/l), non-essential amino acids (1 mM/l), 10% (v/v) FBS, and 1% penicillin/streptomycin antibiotic mixtures. Each cell line was maintained in an atmosphere of 95% air and 5% CO_2 at 37˚C. A stock solution of α-santalol was prepared in dimethyl sulfoxide (DMSO) and an equal volume of DMSO (final concentration <0.5%) was added to the controls.

Cell viability assay. The effect of α-santalol on cell viability in MDA-MB-231 cells was determined using Trypan blue dye exclusion assay as described by us previously (12). Briefly, cells (20×10^5) were plated in 12 well plates, followed by overnight incubation, cells were treated with DMSO-control or α-santalol (20, 40 μM) for 24 hours. After the treatment time period, cells were collected and analyzed for cell viability using Trypan blue dye exclusion assay.

Survivin assay. Survivin sandwich ELISA kit was used to detect the endogenous levels of total survivin protein. Briefly, cells (5×10^5) were plated in tissue culture dishes, allowed to attach by overnight incubation and exposed to DMSO-control or α-santalol (20, 40 μM) over 6 and 9 hours. Cells were collected and lysed using 1x cell lysis buffer (provided in the kit) supplemented with 1 mM phenylmethylsulfonyl fluoride. Survivin ELISA was carried out according to the manufacturer's recommendations. Briefly, cell lysates (60 μg) from control and treated groups were diluted with appropriate volume of sample diluent to obtain a total volume of 100 μl of the diluted lysates. The diluted lysates were added to microwells, which were then sealed and incubated (overnight, 4˚C). Following incubation, contents of the wells were discarded by gentle tapping and washed (4 times) with 1x wash buffer and incubated with detection antibody (100 μl, 1 h, 37˚C). After removal of the detection antibody solution, the wells were washed again and incubated with horse radish peroxidase (anti-rabbit antibody (100 μl, 30 min, 37˚C). After aspiration of the secondary antibody, substrate solution (100 μl) was added and plates were again incubated (10 min, 37˚C). The reaction was stopped by adding stop solution (100 μl) and the yellow color developed, an indication of survivin present in the samples, was read using a microplate reader (450 nm) within 30 min after adding the stop solution.

Immunoblotting. Cells treated with 20 or 40 μM of α-santalol or DMSO (control) for 6 or 9 h were processed for immunoblotting. Lysate proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membrane and immunoblotting was performed as described by us previously (12). After blocking with blocking buffer (LI-COR, Lincoln, NE, USA), mixed with 0.1% Tween-20, the membrane was incubated with primary antibodies (survivin, pAKT, total AKT and actin) overnight at 4˚C. Following incubation with appropriate secondary antibody, the immunoreactive bands were visualized using LI-COR-Odyssey infra-red scanner (LI-COR). The blots were re-probed with anti-actin to correct for differences in protein loading.

Caspase-3 activity assay. The effect of α-santalol on caspase-3 activity in MDA-MB-231 cells was determined using a commercially available caspase-3 (active) ELISA kit, as described by us previously (12). Active caspase-3 (ng/mg total protein of cell lysate) was determined and results were expressed as the fold of caspase-3 activity in α-santalol-treated cells relative to that of the DMSO-treated control. ELISA assay for caspase-3 activity was carried out according to the manufacturer's instructions.

Pharmacological inhibition of AKT pathway. Inhibition of AKT pathway was carried out by employing LY294002 (commercially available pharmacological inhibitor of AKT pathway). Briefly, MDA-MB-231 cells (5×10^5) were plated in tissue culture dishes, allowed to attach by overnight incubation. Next day cells were pretreated with 20 μM of LY294002 for 2 h and co-treated with DMSO (control) or 40 μM of α-santalol for 24 h. Cells were then collected and processed for survivin assay and caspase-3 activity assay as described earlier.

Statistical analysis. Each experiment was carried out at least twice with triplicate measurements for quantitative comparisons. Statistical significance of difference in measured variables between control and treated groups was determined by one-way ANOVA followed by Bonferroni's multiple comparison test. Differences were considered significant at $p<0.05$.

Results

α-Santalol treatment reduced total survivin level and protein expression in cultured breast cancer cells. Figure 1 shows the total survivin level in MDA-MB-231 and MCF-7 cells. A dose-dependent statistically significant decrease in survivin level was observed in both cell lines treated with α-santalol for 9 h. Figure 1C and D represent expression of survivin protein in MDA-MB-231 cells treated with α-santalol (at 20, or 40 μM) over 6 and 9 h. As shown in the Figure, survivin expression decreased in a concentration-dependent manner, indicating that α-santalol suppresses the total protein levels of survivin. Based on these results, we reasoned that survivin down-regulation may be modulated through the PI3K–AKT pathway, hence we investigated the expression levels of p-AKT (S473) and total AKT levels. As shown in Figure 1D, expression of pAKT (s473) appeared to be slightly up-regulated, albeit total AKT levels being unaltered when compared using the loading control. Taken together, these results show that α-santalol causes down-regulation of survivin levels in both cell lines irrespective of their ER or p53 status.

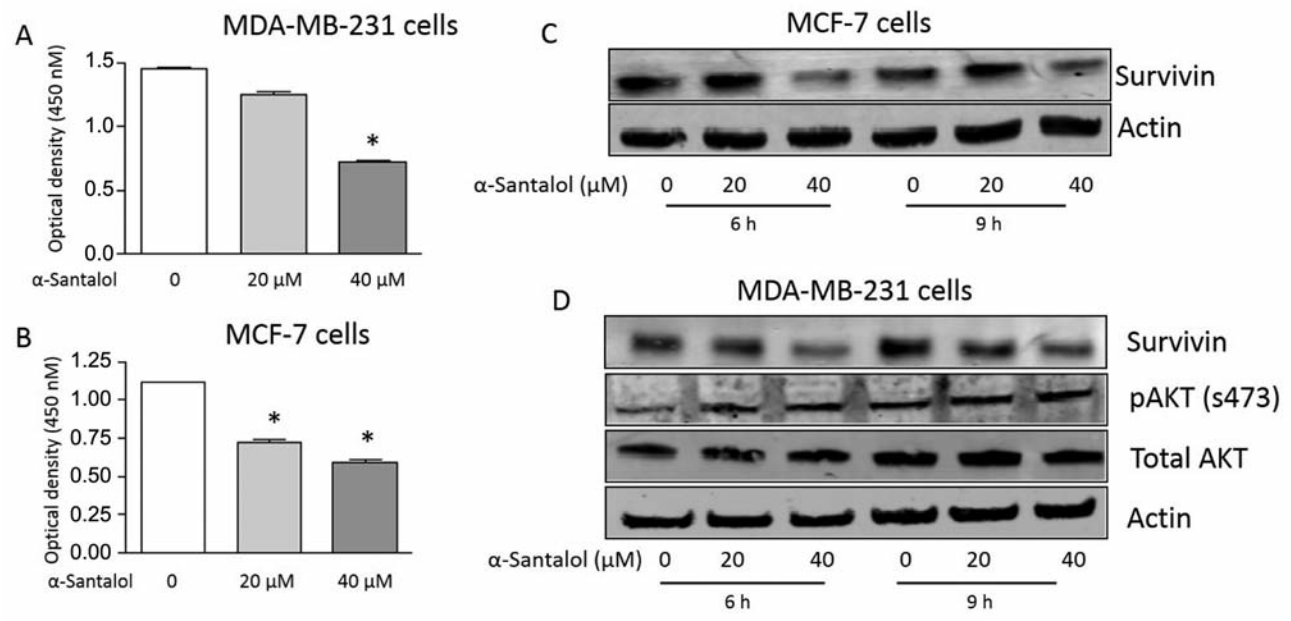

Figure 1. *Effect of α-santalol on total survivin protein levels in MDA-MB-231 cells (A) and MCF-7 cells (B). Immunoblotting for survivin expression in MCF-7 cells (C) and for survivin, phosphorylated protein kinase B (pAKT), and total AKT expression in MDA-MB-231 cells (D). *Significantly different (p<0.05) compared with dimethyl sulfoxide-treated control cells.*

Pharmacological suppression of AKT pathway did not significantly reduce total survivin protein levels. Because studies have shown the involvement of AKT pathway in survivin down-regulation (15, 16), we decided to investigate if there is any association between AKT and survivin. As shown in Figure 2, MDA-MB-231 cells treated with α-santalol exhibited decreased levels of total survivin protein as determined by survivin ELISA assay. However, the total survivin level in cells treated with α-santalol and Ly294002, a pharmacological inhibitor of the AKT pathway, was not significantly different when compared to that observed in cells treated by α-santalol alone. These results indicate that survivin down-regulation caused by α-santalol seems to be independent of the PI3K-AKT pathway.

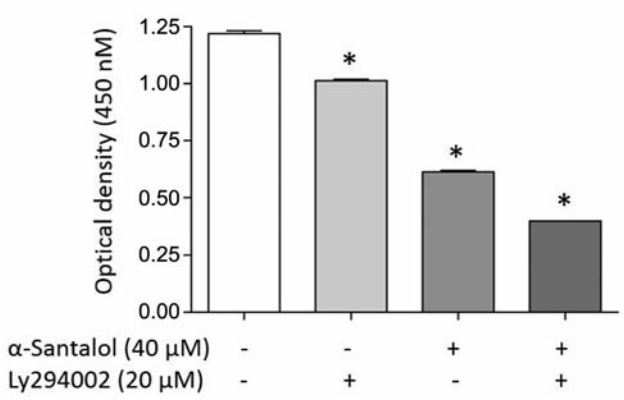

Figure 2. *Effect of α-santalol on survivin protein levels in the presence of pharmacological inhibitor of phoshoinositide-3-kinase-protein kinase B (PI3K–AKT) pathway (Ly294002) in MDA-MB-231 cells. *Significantly different (p<0.05) compared to dimethyl sulfoxide-treated control cells.*

Pharmacological inhibition of the AKT pathway did not offer synergistic/additive effect on α-santalol-induced cell death. Next, we designed experiments to determine whether pharmacological suppression of the AKT pathway has any effect on cell death caused by α-santalol. Figure 3A depicts the morphology of MDA-MB-231 cells treated with DMSO, α-santalol, Ly294002 and a combination of α-santalol and Ly294002. As shown in Figure 3A, cells in the α-santalol-treated groups exhibited characteristics of apoptotic cells compared to those treated with DMSO or Ly294002 alone. Figure 3B represents cell viability of the same cells. As expected, the percentage of cell viability in the α-santalol-treated group was significantly lower compared to that of DMSO- or Ly294002-treated groups. However, there was no statistically significant difference in cell viability between groups treated with α-santalol and the combination of α-santalol and Ly294002, indicating that inhibition of AKT pathway did not offer any additional increase in cell death. Based on these results, it is reasonable to conclude that pharmacological inhibition of the AKT pathway has no synergistic or additive effect on cell death caused by α-santalol.

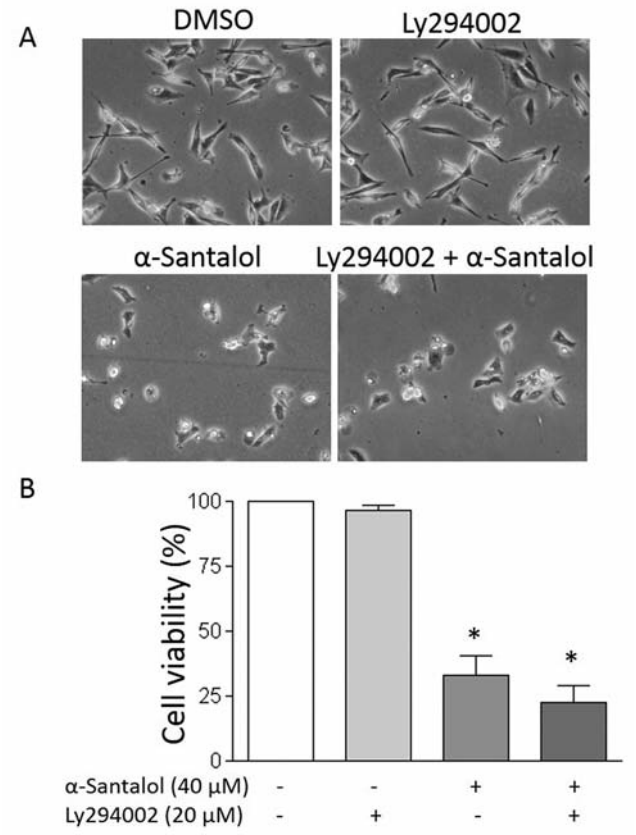

Figure 3. *Effect of α-santalol on MDA-MB-231 cell viability in the presence of Ly294002. A: Microscopic analysis of cell morphology. B: Cell viability of α-santalol-treated MDA-MB-231 cells compared to dimethyl sulfoxide-treated control cells determined by trypan blue dye exclusion assay. *Significantly different (p<0.05) compared to DMSO-treated control cells.*

α-Santalol-mediated caspase-3 activity is independent of the PI3K–AKT pathway. We have shown that α-santalol causes cleavage of caspase-3 in prostate and breast cancer cells (12, 14). We questioned whether α-santalol-induced apoptosis and cleavage of caspase-3 was mediated through the PI3K–AKT pathway, which was speculated based on our unpublished results in a different cancer model (Bommareddy, unpublished observations). As shown in Figure 4, caspase-3 activity was analyzed using ELISA assay in MDA-MB-231 cells treated with DMSO, Ly294002, α-santalol and a combination of α-santalol and Ly294002. The levels of active caspase-3 in α-santalol-treated cells was statistically higher when compared to DMSO- and Ly294002-treated groups. However, the levels of active caspase-3 determined in cells treated with α-santalol alone were not significantly different when compared to those determined in cells treated with the combination of α-santalol and Ly294002 group. These results indicate that α-santalol-mediated caspase-3 activity, and thus apoptotic cell death, may not be regulated through the PI3K–AKT pathway.

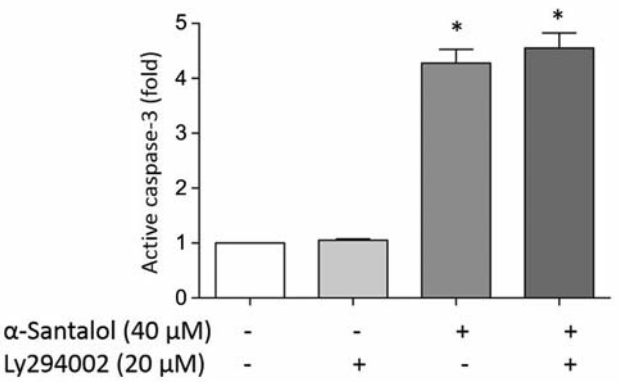

Figure 4. *Caspase-3 activity in MDA-MB-231 cells treated with α-santalol as determined by enzyme-linked immunosorbent assay. *Significantly different (p<0.05) compared to dimethyl sulfoxide-treated control cells.*

Discussion

The present study shows, for the first time, that α-santalol treatment causes down-regulation of total survivin protein irrespective of ER expression in breast cancer cells. Recent studies point-out the role of survivin, a member of the inhibitor of apoptosis family, in a variety of clinical cancer types and its selective expression in tumors compared to normal tissues (15). Modulation of survivin is associated with the activation of various cell survival signaling cascades including PI3K–AKT, mammalian target of rapamycin, mitogen-activated protein kinase, extracellular-signal regulated kinases, vascular endothelial growth factor and several other growth factors and epigenetic pathways (15-17). Overexpression of survivin is correlated with tumor recurrence and therapeutic resistance. For example, it was shown that elevated levels of human epidermal growth factor receptor 3 (ERBB3/HER3) confers paclitaxel resistance in ERBB2-overexpressing breast cancer cells through PI3K–AKT-mediated up-regulation of survivin (18). In addition, studies also showed that inhibition of the PI3K–AKT pathway down-regulates survivin levels and reduces tumor burden. It was shown that inhibition of the PI3K–AKT–survivin pathway synergizes the anti-leukemic properties of nutlin-3 in acute lymphoblastic leukemia cells (19). Similarly, suppression of survivin by vemurafenib and nutlin-3 reduced the cell viability of melanoma cells and tumor growth *in vivo* (20).

In the present study, we explored the association of the PI3K–AKT pathway and survivin down-regulation and identified that the suppression of survivin is not highly dependent on PI3K–AKT pathway in breast cancer cells. We decided to focus on the PI3K–AKT pathway owing to its frequent up-regulation in various types of cancer, including

breast cancer. To our surprise, the cell death caused by α-santalol is not regulated by this pathway as opposed to its direct association in a different cancer model treated with α-santalol (Bommareddy, unpublished observations). We previously found that α-santalol induces apoptotic cell death and causes cell-cycle arrest in breast cancer cells (14). In the present study, we found that α-santalol-mediated caspase-3 activity was not significantly altered upon inhibition of the PI3K–AKT pathway, signifying that α-santalol-mediated growth suppression of breast cancer cells may not be regulated through the PI3K–AKT pathway.

In conclusion, the present study demonstrates that α-santalol-mediated anticancer effects in breast cancer cells may be regulated, in part, through suppression of survivin. It is possible that down-regulation of survivin may occur through a different pathway other than the PI3K–AKT pathway, and it is in our future interest to explore the mechanistic details associated with the down-regulation of survivin in breast cancer cells.

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