The Anticancer Effect of 2'-3'-dehydrosalannol on Triple-negative Breast Cancer Cells

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Abstract. *Background: Triple-negative breast cancer (TNBC) accounts for 15-20% of all breast tumors and these breast tumors are usually aggressive and highly metastatic. Unfortunately, treatment options for TNBCs are limited; we have identified a novel molecule, 2'-3'-dehydrosalannol (DHS) and in this study we investigated the anticancer effect of DHS against TNBC cells. Materials and Methods: TNBC (MDA-MB 231; MDA-MB 468) cells were treated with DHS and its effect on cell viability, apoptosis and molecular mechanisms were analyzed. Results: DHS inhibited growth and induced apoptosis in TNBC cell lines. Molecular analysis suggested that DHS inhibited cathepsin-mediated pro-survival signaling [pAKT: phosphorylated protein kinase B; BCL-2: B-cell lymphoma 2 and cyclin D1] and induced pro-apoptotic markers such as BAX [BCL-2-associated X protein] and cleaved caspase-3. Conclusion: Our results demonstrate that DHS inhibits cathepsin-mediated pro-survival signaling which resulted in growth arrest of TNBC cells. These findings suggest that DHS may be a promising agent for the prevention and treatment of TNBC.*

Breast cancer is one of the most common types of cancer in women. Each year it affects more than one million women worldwide (208,000 cases in the U.S.) and kills 400,000 patients (40,000 in the U.S.) (1). Although breast cancer mortality has steadily decreased since 1990, it remains the leading cause of cancer death among women aged 20-59 years (1). Women with triple-negative breast cancer (TNBC) have a tendency to be younger than their non-triple-negative counterparts. TNBC is associated with a higher risk of metastasis, a shorter time to recurrence, and a much shorter median time from relapse to death than non-TNBC (2, 3). This is a key challenge for clinicians trying to palliate an incurable disease and extend life. Hence, there is an immediate need to develop effective TNBC treatments.

The development and progression of breast cancer is characterized by aberrant activity in several regulatory pathways in mammary cells and the surrounding stromal tissue. One such pathway is the phosphoinositol-3-kinase (PI3K)/AKT pathway. Expression of phosphorylated AKT (pAKT) is increased in 55-76% of human breast cancer tissue samples (4) and correlates with clinical aggressiveness and progression (4, 5). AKT is a Ser/Thr kinase that functionally modulates numerous substrates that regulate cell proliferation, survival and invasion (6, 7). Down-regulation of constitutively active AKT (by PI3K inhibitor wortmannin and LY294002) prevents cell survival and resistance to chemotherapeutic agents. Thus, specific inhibition of AKT by small molecules may be a valid approach to prevent or treat not only TNBC, but also other human malignancies.

Cathepsin B belongs to the cysteine family of proteins and activation of cathepsin B has been associated with progression and invasion of various types of cancer (8), including breast cancer (9). Increased expression of cathepsin correlates with poor prognosis of breast cancer (9). Recent studies have demonstrated that cathepsin B activated AKT, which resulted in inhibition of the Forkhead box O3a (FOXO3a) function in glioma cells (10). Hence, cathepsin could be an attractive target to inhibit AKT-mediated pro-survival signaling in TNBC.

Nature has been a source of medical treatments for thousands of years, and plant-based systems continue to play an essential role in the primary health care of 80% of the world's population (11, 12). *Azadirachta indica* (neem) is well known in Asian and African countries as a versatile medicinal plant, having a wide spectrum of biological activities (13, 14). Neem leaf extracts, which are non-toxic

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and non-mutagenic, have been shown to possess anti-inflammatory, antioxidant, anticarcinogenic, and potent immunostimulant activities in many types of cancer cells (15-17). It has also been reported that neem extract inhibited PI3K/AKT signaling and induced apoptosis in prostate cancer cells (18). We have identified 2'-3'-dehydrosalannol (DHS), a potent molecule from the ethanolic extract of neem leaves (Figure 1). We, here, investigated the effects of 2'-3'-dehydrosalannol on the growth and induction of apoptosis in TNBC cells.

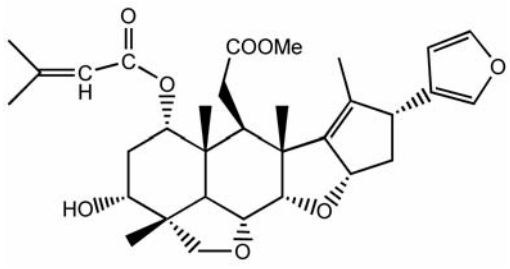

Figure 1. *Structure of 2'-3'-dehydrosalannol.*

Materials and Methods

Reagents. Antibodies against pAKT(Ser 473), AKT, cyclin D1, $p27^{KIP1}$, pFOXO3a (Thr 32), Integrin β3, cathepsin B, cleaved caspase3, Poly (ADP-ribose) Polymerase (PARP), B-cell lymphoma 2 (BCL-2), BCL-2 homologous antagonist/killer (BAK), and BCL-2–associated X protein (BAX) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA) and FOXO3a from Abcam (Cambridge, MA, USA). 2'-3'-Dehydrosalannol was purchased from Asthagiri Herbal Research Foundation (Chennai, India). Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) assay kit was purchased from Promega (Madison, WI, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture. Human TNBC cell lines (MDA-MB 231 and MDA-MB 468) were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in RPMI-1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Mediatech Inc. Manassas, VA, USA). All cells were cultured in a humidified atmosphere of 5% CO_2 at 37˚C.

Cell viability assay. MDA-MB 231 and MDA-MB 468 cells were seeded in 24-well culture plates (6×10^4 cells/ml). After overnight incubation, cells were treated with different concentrations of DHS (0, 20, 40, 60, 80, 100 μM/ml) for 24 h and cell viability was measured using the trypan blue assay. The assay was performed in triplicates.

TUNEL assay. MDA-MB 231 and MDA-MB 468 cells were grown on coverslips and were pretreated with DHS at 75 and 100 μM for 24 h. TUNEL staining was performed according to the manufacturer's recommendations. The coverslips were then mounted and images were acquired using a confocal laser scanning microscope (NIKON C2 Si, Japan). We counted a minimum of 500 cells per slide and the cells that exhibited green fluorescence were considered as cells undergoing apoptosis.

Western blot analysis. MDA-MB 231 cells were treated with 75 and 100 μM of DHS for 24 h; after treatment, the cells were harvested in lysis buffer containing protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). Proteins were separated by gel electrophoresis and transferred onto Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The PVDF membranes were incubated overnight with appropriate primary antibody at 4˚C. Appropriate secondary antibodies were added and the membranes were incubated for 1 h at room temperature and protein expression was visualized with enhanced chemiluminescence kit (Pierce, Rockford, IL, USA) using a Fuji imaging system (Fuji LAS 4000, Fuji Film Life Sciences, Stanford, CT, USA).

Statistical analysis. All experiments were carried out at least thrice to ascertain the reproducibility of the results. The apoptosis and the cell viability data shown, are the mean of four measurements from each of the three experiments (total of 12 readings)±SEM. The student's *t*-test was used to calculate statistical significance between control and treatment groups.

Results

DHS inhibited cell proliferation and induced apoptosis in both MDA-MB 231 and MDA-MB 468 cells. Firstly, we determined the dose-response kinetics of DHS on two TNBC cell lines, MDA-MB 231 and MDA-MB 468. As seen in Figure 2A, DHS inhibited the growth in a dose-dependent manner and even the lowest concentration (20 μM) of DHS treatment significantly suppressed the growth of both TNBC cell lines. Next, we determined whether DHS reduced cell viability by inducing apoptosis, we treated both cell lines with DHS for 24 h and performed the TUNEL assay. Apoptosis was induced in MDA-MB 231 (13% and 30%) and in MDA-MB 468 (18.3% and 45.3%) cells when treated with 75 and 100 μM, respectively, of DHS for 24 h (Figure 2B). These data suggest that DHS inhibits cell proliferation by inducing apoptosis of TNBC cells.

DHS down-regulated the pro-survival mechanism in MDA-MB 231 cells. AKT activation is correlated with disease progression and AKT is indeed highly expressed in TNBC cells. Hence, we determined whether DHS inhibits AKT expression in TNBC cells. As seen in Figure 3A, DHS inhibited the phosphorylation of AKT without altering the total level of AKT in MDA-MB 231 cells. Activation of AKT results in phosphorylation of FOXO proteins leading to inhibition of pro-apoptotic signaling in many cancer models. Inhibition of AKT by DHS resulted in down-regulation of phosphorylation of FOXO3a with a concomitant increase of total FOXO3a, in MDA-MB 231 cells (Figure 3B).

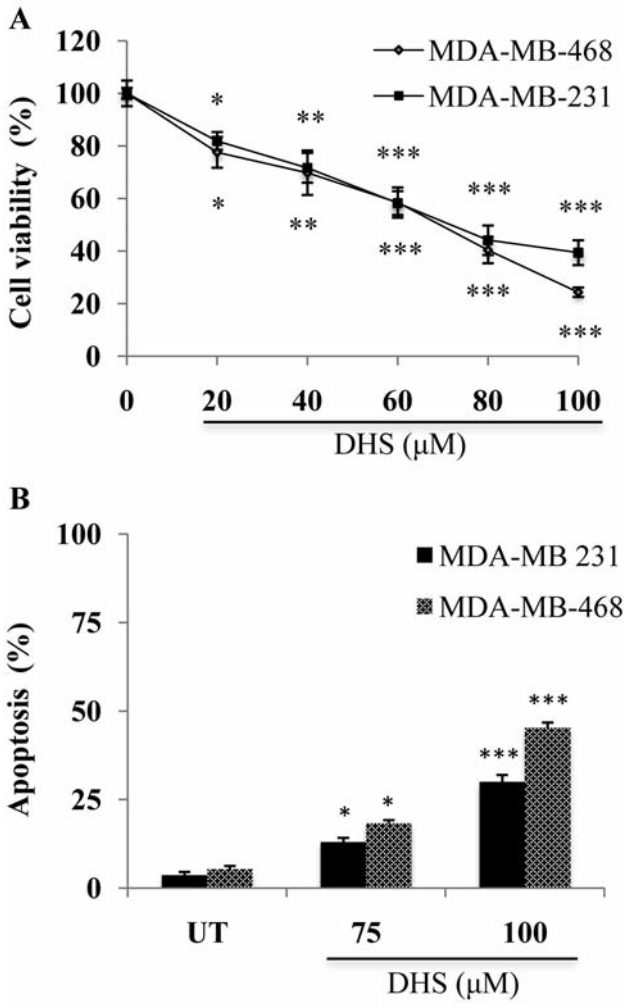

Figure 2. *Effects of DHS on cell viability and apoptosis in triple-negative breast cancer cells. A: MDA-MB 231 and MDA-MB 468 cells were treated with DHS for 24 h and cell viability was assessed by the trypan blue exclusion assay. Data represent the mean±S.E.M (n=6). B: MDA-MB 231 and MDA-MB 468 cells were treated with DHS for 24 h and apoptosis was measured by the TUNEL assay. *p<0.05, **p<0.01 and ***p<0.001 compared to untreated (UT) controls.*

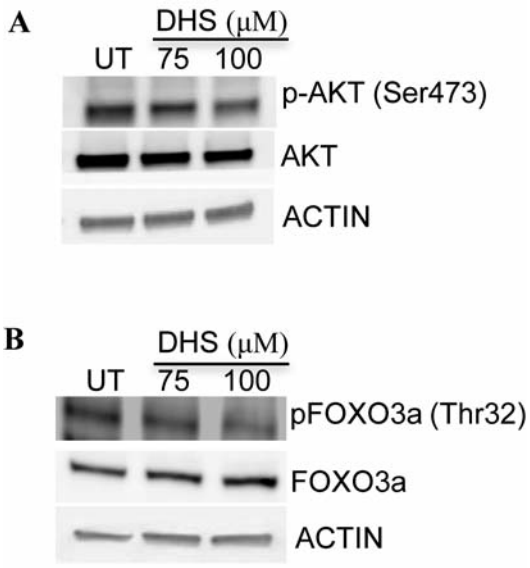

Figure 3. *DHS inhibits phosphorylation of protein kinase B (AKT) and Forkhead box O3-a (FOXO3a). A: MDA-MB 231 cells were treated with DHS (75 and 100 μM) for 24 h, and pAKT and AKT expressions were then measured in cell lysates by western blot analysis. B: Western blot analysis showing the down-regulation of pFOXO3a in MDA-MB 231 cells. UT: Untreated.*

Activated FOXO transcriptionally regulates various cell cycle checkpoint proteins, which in turn induce cell cycle arrest at G_1, by up-regulating cyclin-dependent kinase (CDK) inhibitors $p27^{KIP1}$ and by down-regulating cyclin D1 expression (19, 20). Similarly in our studies, we found that DHS induced $p27^{KIP1}$ expression (Figure 4A) and down-regulated cyclin D1 expression (Figure 4B) in MDA-MB 231 cells. It was reported that cathepsin is upstream of AKT signaling, and that it regulates AKT activation. Increased expression of cathepsin was correlated with poor prognosis of breast cancer (21). In our results, DHS significantly inhibited the expression of both cathepsin B and its downstream target integrin β3, suggesting that DHS may target cathpesin B signaling in TNBC (Figure 4C).

DHS induced expression of pro-apoptotic proteins in TNBC cells. DHS induced apoptosis in TNBC cells; hence we determined apoptotic markers such as BAK, BAX, cleaved caspase 3 and PARP in MDA-MB 231 cells. Our results indicate that DHS treatment up-regulates expression of BAK (Figure 5A), BAX (Figure 5B), cleaved caspase-3 (Figure 5C) and PARP (Figure 5D) in MDA-MB 231 cells in a dose–dependent manner. Next, we observed that DHS down-regulated BCL-2 (Figure 5E) expression in MDA-MB 231 cells, suggesting that DHS may be potent inducer of apoptosis in TNBC cells.

Discussion

In general, TNBC is aggressive in nature, with a specific molecular profile and with limited treatment options, which lead to poor prognosis and high mortality rates. We are the first group to demonstrate the anticancer effect of DHS, an active ingredient of neem leaf extract, against TNBC cells. In our studies DHS effectively inhibited cell growth and induced apoptosis of TNBC cells.

Increased expression of pAKT has been always implicated in the aggressiveness of the disease (22-24) and correlated

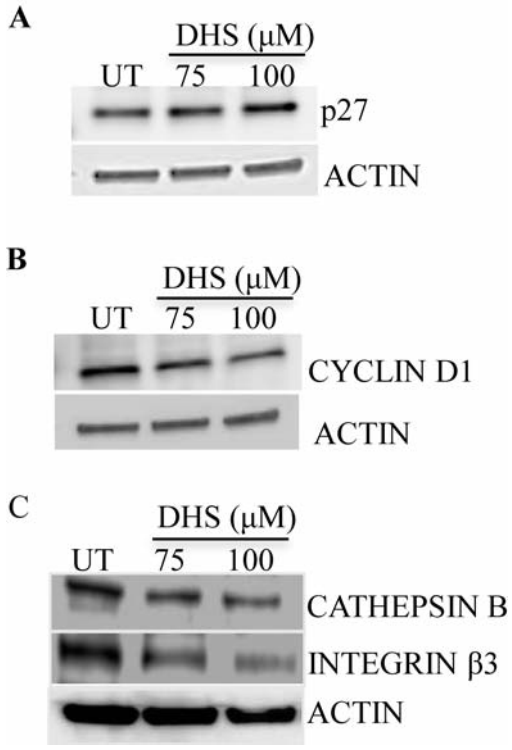

Figure 4. *DHS inhibits pro-survival signaling in MDA-MB 231 cells. A: DHS up-regulated the p27^{KIP1} expression, and inhibited expressions of cyclin D1 (B), cathepsin B and integrin β3 (C) in MDA-MB 231 cells. UT: Untreated.*

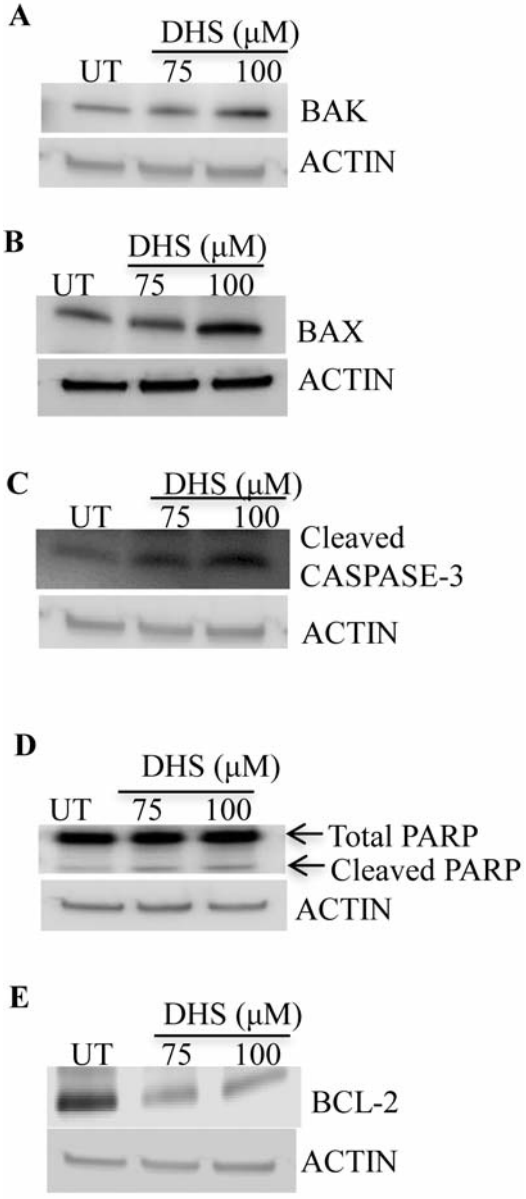

Figure 5. *Effect of DHS on pro-apoptotic signaling in MDA-MB 231 cells. Induction of BCL-2 homologous antagonist/killer (BAK) (A) and BCL-2–associated X protein (BAX) expression (B) cleavage of caspase-3 (C) and activation of Poly (ADP-ribose) polymerase (PARP) (D) by DHS. DHS inhibited the expression of B-cell lymphoma 2 (BCL-2) (E). UT: Untreated.*

with cell proliferation and survival in breast cancer cells (25). Inactivation of FOXO3a by overexpression of AKT leads to loss of its function in cell cycle arrest and apoptosis (26). In our studies, DHS inhibited phosphorylation of AKT which in turn resulted in reduced phosphorylation of FOXO3a, suggesting that DHS restores the FOXO3a function in MDA-MB 231 cells. Earlier studies have also shown that activation of FOXO3a inhibits cell cycle arrest by down-regulating cyclin D1 expression in cancer cells (27). Furthermore, it has been illustrated that ectopic expression of cyclin D1 overcomes FOXO3a-induced cell cycle arrest (27). Hence, FOXO3a regulates CDK family proteins and arrests the cells in the G_1 phase of cell cycle by up-regulating p27^{KIP1} expression and by inhibiting cyclin D1 expression. Our data demonstrate that DHS induced cell cycle arrest in the G_1 phase (data not shown) and increased p27^{KIP1} expression, as well as down-regulated cyclin D1 expression in TNBC cells.

Cathepsin B plays a major role in metastasis by degrading the extracellular matrix; previous work has demonstrated that cysteine cathepsins are effectors of invasive growth and angiogenesis during multistage tumorigenesis. Increased expression of cathepsin B has been implicated in poor prognosis of breast cancer (12) and it has also been demonstrated that ablation of cathepsin B delayed lung metastasis in the PyMT transgenic breast carcinoma model. Inhibition of cathepsin B also caused marked induction of apoptosis by down-regulating the pAKT expression in cancer cells (14). In our studies, inhibition of cathepsin B and pAKT expression correlates with published findings and it

also suggests that cathepsin B may be upstream of AKT signaling; hence, inhibition of cathepsin B may be another strategy to block the function of AKT in cancer cells.

Caspases are a family of cysteine proteases that play a vital role in sequential execution of apoptosis. Caspases usually exist in an inactive form which needs to be cleaved to become active and affect apoptosis. PARP, is a substrate for caspases and is involved in the repair of DNA damage. The capacity of PARP to repair DNA damage is inhibited when it is cleaved by caspase-3 (27). Many cancer drugs cause programmed cell death by activating extrinsic and intrinsic pathways through the caspase family of proteins, which in turn induce cleavage of PARP (28). DHS induced pro-apoptotic machinery by activating caspase-3 and PARP in TNBC cells. Furthermore, DHS also increased the protein expression of pro-apoptotic genes *BAK* and *BAX*, while it simultaneously reduced the expression of antiapoptotic factor BCL-2. These findings clearly demonstrate that DHS induces apoptosis of TNBC cells.

In summary, our results demonstrate that a novel herbal molecule, DHS, significantly inhibited the growth of TNBC cells by inhibiting cathepsin-mediated AKT signaling. Further studies are required to confirm efficacy of the drug *in vivo* as well as the molecular mechanism involved in order to bring this agent to clinical settings.

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