The anticancer potential of steroidal saponin, dioscin, isolated from wild yam (*Dioscorea villosa*) root extract in invasive human breast cancer cell line MDA-MB-231 in vitro.

Pranapda Aumsuwan^{1, 2}, Shabana I. Khan^{1,3}, Ikhlas A. Khan^{1,3}, Zulfiqar Ali¹, Bharathi Avula¹, Larry A. Walker^{1,2,4}, Zia Shariat-Madar², William G. Helferich⁵, Benita S. Katzenellenbogen⁶, Asok K. Dasmahapatra^{1,2,4,*}

¹National Center for Natural Product Research, ²Division of Pharmacology, Department of BioMolecular Sciences, ³Division of Pharmacognosy, Department of BioMolecular Sciences, School of Pharmacy, University of Mississippi, University, MS 38677, USA.

⁴University of Mississippi Cancer Institute (Oxford Campus), School of Pharmacy, University of Mississippi, University, MS 38677, USA.

⁵Department of Food Science and Human Nutrition, ⁶Department of Molecular and Integrative Physiology, University of Illinois, Urbana, IL 61801, USA.

* Corresponding Author

Asok K Dasmahapatra

National Center for Natural Product Research

University of Mississippi

University

MS 38677

Voice: 662-915-7077

Fax: 662-915-5148

E-mail: asok@olemiss.edu

Abstract

Previously, we observed that wild yam (*Dioscorea villosa*) root extract (WYRE) was able to activate *GATA3* in human breast cancer cells targeting epigenome. This study aimed to find out if dioscin (DS), a bioactive compound of WYRE, can modulate *GATA3* functions and cellular invasion in human breast cancer cells. MCF-7 and MDA-MB-231 cells were treated in the absence/presence of various concentrations of DS and subjected to gene analysis by RT-qPCR, immunoblotting, and immunocytochemistry. We determined the ability of MDA-MB-231 cells to migrate into wound area and examined the effects of DS on cellular invasion using invasion assay. DS reduced cell viability of both cell lines in a concentration and time-dependent manner. *GATA3* expression was enhanced by DS (5.76 μ M) in MDA-MB-231 cells. DS (5.76 μ M)-treated MDA-MB-231 cells exhibited the morphological characteristic of epithelial-like cells; mRNA expression of *DNMT3A*, *TET2*, *TET3*, *ZFPM2* and *E-cad* were increased while *TET1*, *VIM* and *MMP9* were decreased. Cellular invasion of MDA-MB-231 was reduced by $65 \pm 5\%$ in the presence of 5.76 μ M DS. Our data suggested that DS-mediated pathway could promote *GATA3* expression at transcription and translation levels. We propose that DS has potential to be used as an anti-invasive agent in breast cancer.

Key words: dioscin, *GATA3*, *ZFPM2*, invasion/migration, gene expression

Introduction:

Breast cancer is the most common cancer affecting women worldwide. In the United States an estimated 232,670 female and 2,360 males are diagnosed with breast cancer in 2014 (<http://www.cancer.gov/cancertopics/types/breast>). Due to earlier detection and therapies, the mortality rates in breast cancer patients are decreasing; however, mortality due to recurrence of cancer which develop drug resistance and metastases is very common (Lu et al., 2009; Scully et al., 2012). The mechanisms associated with breast cancer are genetic alterations, including specific gene amplifications, deletions, point mutations, chromosome rearrangements, and aneuploidy; however, like other types of cancer, breast cancer is also driven by epigenetic mechanisms (Atalay, 2013; Byler et al., 2014). Epigenetic alterations do not affect the primary DNA sequence and occur through three mutually interacting events, namely DNA methylation, histone modifications and noncoding RNA interference (Byler et al., 2014). These events modulate chromatin structure, and in turn activate or silence gene expression. Due to reversibility, epigenetic modifications are considered potentially useful in drug development approaches (epi-drugs). The currently available synthetic epi-drugs such as 5-azacytidine (vidaza) and 5'aza-2 cytidine (decitabine) approved for the treatment of myelodysplastic syndrome (Marks 2012; Di Costanzo et al., 2014) are non-specific and have many adverse effects. As an alternative, phytochemicals from natural products are a promising source for the development of novel cancer therapeutics. Because of their potential effectiveness and low toxicity profiles (Crowell, 2005; Khan et al., 2012), several phytochemicals are effective in clinical use for many diseases including cancer (Harvey, 2008; Khan et al, 2012; Bilal et al., 2014).

GATA-binding protein 3 (GATA3) is a member of the GATA family of zinc finger transcription factors (GATA1–GATA6) that binds with high affinity to the consensus DNA site (T/A-GATA-A/G) (Yan et al., 2010). GATA3 is essential in breast glandular cell development. In particular, GATA3 is necessary for embryonic mammary development and is actively involved in maintaining the differentiated state of luminal epithelial cells of the mammary gland in adults (Kouros-Mehr et al. 2006, 2008; Yan et al. 2010). In breast cancer, GATA3 has emerged as a strong and independent predictor of tumor differentiation and clinical outcome (Kouros-Mehr et al. 2006, 2008; Yan et al. 2010, Yoon et al. 2010). The low expression of GATA3 in breast cancer correlated with a poorer outcome related to high tumor grade (poorly differentiated or undifferentiated), larger tumor size, and short disease-free survival (Kouros-Mehr et al. 2006, 2008, Yan et al. 2010, Yoon et al. 2010). The expression of GATA3 is also involved with elevated E-cadherin (E-cad), but decreased vimentin (VIM), N-cadherin (N-cad) and Matrix metalloproteinase 9 (MMP9). This suggests that GATA3 may drive the invasive breast cancer cells to undergo the reversal of epithelial-mesenchymal transition (EMT) leading to the inhibition of metastasis. Importantly, increasing GATA3 expression in the low expressing MDA-MB-231 breast cancer cells led to cancer cell differentiation, decreased their invasive potential in vitro (Yan et al. 2010; Chou et al. 2013, Chu et al. 2012, 2013), and decreased tumor formation in vivo (Yan et al. 2010). Therefore, GATA3 could be highly relevant entity as a tumor suppressor.

Previously, we have observed that wild yam (*Dioscoria villosa*) root extract (WYRE) has the potential to modulate epigenetic events in human breast cancer cell lines (Aumsuwan et al.,

2015). Moreover, WYRE has the ability to activate *GATA3* in both estrogen receptor positive (ER⁺) MCF-7 cells and estrogen receptor negative (ER⁻) MDA-MB-231 cells. We revealed that WYRE induces TET-dependent DNA demethylation leads to alter *GATA3* gene. The goal of this study is to determine whether the component of WYRE would directly activate *GATA3*-dependent pathway. We have isolated several triterpene saponins and one sapogenin from WYRE (Avula et al., 2014) and verified their potential in vitro as anti-cancer agents. Our preliminary data indicate that among these compounds, only four compounds including DS have positive anticancer effects. Because of its significant anticancer effects, we have further focused on anti-invasive activities of DS modulating through *GATA3*.

DS, a plant steroidal glucoside saponin possesses anti-proliferating effects against a number of human cancer cells (Cai et al., 2002; Li et al., 2003). DS is also used as traditional medicine in the treatment of hyper-cholesterolemia, hyperglycemia, hypertriacylglycerolemia and diabetes (McAnuff et al., 2005). Moreover, DS was found to have no subchronic toxicity in Sprague Dawley female rats with an oral administration of 300 mg/kg for three months (Xu et al., 2012). Over the past decade, a series of preclinical and mechanistic studies have shown that DS could inhibit proliferation and induce apoptosis in a variety of tumor cells (Tong et al., 2014; Wang et al., 2014). The anti-tumor activities of DS have been demonstrated via modulation of multiple cell signaling events involving critical molecular functions including growth, differentiation, apoptosis and oncogenesis (Chen et al., 2014). Recently, DS has been shown to promote osteoblastic proliferation and differentiation via *Lrp5* and ER pathway in mouse and human osteoblast-like cells (Zhang et al., 2014). Therefore, these preclinical and mechanistic findings strongly suggest that DS may possess cancer chemotherapeutic potential.

The purpose of this study was to evaluate the anti-cancer activities of DS in two types of human breast cancer cell lines (MCF-7, estrogen receptor positive luminal, and MDA-MB-231, triple negative breast cancer cells) and understand the underlying mechanisms played by DS in breast cancer chemoprevention and suppression of cellular invasion.

Materials and methods:

Plant material.

Dioscin (3 β ,25*R*)-spirost-5-en-3-yl *O*- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 4)]- β -D-glucopyranoside (Figure 1) was isolated at the National Center for Natural Products Research (NCNPR), University of Mississippi, Mississippi, USA; the identity and purity (91.2%) were confirmed by chromatographic (TLC, LC-ELSD) and spectral (IR, 1D- and 2D-NMR, ESI-HRMS) analysis (Avula et al., 2014).

Cell culture and extraction of nucleic acid.

Cell culture conditions (under specific hormone-free experimental conditions) and the cellular isolation of RNA was described previously (Aumsuwan et al., 2015). In brief, human breast adenocarcinoma, MCF-7 (ER⁺) and MDA-MB-231 (ER⁻) cells were purchased from American type culture collection (ATCC). Both cell lines were maintained in phenol red free DMEM-F12 (1:1) medium supplemented with 10% dextran charcoal treated fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 50 U/mL penicillin and 50 μ g/mL streptomycin as Pen-Strep (Life

Technology, Grand Island, NY) and 2 mM of L-glutamine (Life Technology) at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. To maintain both cell lines in identical culture conditions, human recombinant insulin (0.01 mg/ml) was omitted for MCF-7 cells. The cells (~500 x 10³ cells) were allowed to attach in the 25 cm³ culture flasks in 6 mL volume and after 24h treated with various concentrations of DS (1.15-5.76 µM) or 5-azacytidine (5-aza) (250-1000 nM) (Sigma-Aldrich, St. Louis, MO) as a known inhibitor of DNMT enzyme activities (Di Costanzo et al., 2014) for three days. The media of the cells treated with 5-aza were replaced every 24h. After complete removal of the media at 72h of treatment, the cells were trypsinized, resuspended in the medium, washed twice with PBS, and were used for biochemical analysis. RNA was extracted by Trizol reagent (Invitrogen, Carlsberg, CA) as described previously (Aumsuwan et al., 2015). The extracted RNA was dissolved in nuclease-free water. Before cDNA synthesis, RNA samples were treated with RQ1 DNase (Promega, Madison, WI) to ensure removal of contaminating genomic DNA. The RNA concentration was determined by NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA) and the samples were stored at -80 °C until analysis.

Cell viability assay.

MCF-7 and MDA-MB-231 cells in logarithmic growth were seeded in 96 well plates at a density of 2x10⁴ cells/ well and 7.5x10³ cells/ well (for 24 h and 72 h treatments, respectively) and grown at 37 °C in a humidified atmosphere of 95% air and 5% CO₂ for 24 h. Stock DS dissolved in DMSO at concentration of 2 mM and 5-aza at concentration of 1 mM were diluted in serum-free medium supplemented with antibiotics and added to the wells to achieve the desired concentrations in a 250 µL final volume and incubated for 72 h. Incubation with DS was continued either for 24 h (0.72- 23.03 µM) or 72 h (0.04-11.51 µM). DMSO (0.01%) was used as vehicle control. Cell viability was determined by XTT method (Scudiero et al. 1988). Percent cell viability was calculated compared to vehicle control.

Analysis of DS stability in cultured cells and media.

The stability of DS at the end of incubation (72 h) was determined by UPLC analysis of the cell culture media and the cell lysates (Avula et al., 2014). This was needed to confirm that DS is not converted to its aglycone (diosgenin) under experimental conditions. All analyses were performed on a Waters Acquity UPLCTM system (Waters Corp., Milford, MA, USA). The presence of DS and its aglycone (diosgenin) was confirmed in selected ion recording (SIR) mode. This method involved the use of [M+H]⁺ ions of compounds which were observed for standard compounds in the positive ion mode *m/z* at 869 (DS) and 415 (diosgenin).

Quantitative real-time PCR (RT-qPCR).

Gene-specific mRNA analysis was done by RT-qPCR methods based on PCR array (SA BioScience, Valencia, CA) (Aumsuwan et al., 2015). Briefly, the extracted RNA was reverse transcribed to cDNA by iScript Reverse transcriptase following the manufacturer's protocol (Bio-Rad, Hercules, CA). Two µL of cDNA in a final reaction volume of 20 µL was amplified with gene-specific primers or internal controls (ACTB or RPL19) either in PCR array plates (DNMT1, DNMT3A, DNMT3B, GATA3, ZFPM2; E-cad , VIM, and MMP9 or in PCR tubes (TET1, TET2, and TET3). The primers (DNMT1, DNMT3A, DNMT3B, GATA3, ZFPM2; E-

cad, VIM, MMP9, RPL19, and ACTB) used in the PCR array were designed and added to the plates by the manufacturer (SA BioScience), however, for the primers used for ten-eleven-translocation (TET) enzymes (TET1: GenBank locus: NM_030625, sense: 5'-CCATATTATACACA CCTTGGG-3', anti-sense: 5'-CTCCATGAACAGCCAAAAGAG-3'; TET2A: GenBank locus: NM_001127208, sense:5'-GGTGGCATCTCTTACAGAAGC-3', anti-sense:5'-TGTGATTTGAGAGTAAGAGC-3'; TET2B: GenBank locus: NM_017628, Sense:5'-GGGCAGCCTTGTGGATGGCCC-3',Antisense:5'-GGAGCCCAGAGAGAGAAGGTT-3', TET3:GenBank locus:NM_144993, sense5'-ATGGACTCAGGGCCAGTGTAC-3',antisense:5'-GTTCCCAGCCTCACGACTCAT C-3') were purchased from Integrated DNA Technologies (IDT, Corralville, IA) and the nucleotide sequences of the amplified PCR products were analyzed by a commercial source (Retrogen, San Diego, CA) and verified with GenBank data base. The reaction conditions included an initial denaturation at 95 °C for 3 min, one cycle, followed by 40 cycles of denaturation at 94 °C for 30 sec, annealing 60 sec at 60 °C, and extension 120 sec at 72 °C, fluorescence data collection for 1 sec. After the end of each cycle the samples were incubated again for 1 sec at 78 °C and a second set of fluorescence data collection was made to prevent error due to formation of primer dimers, if any. A final extension of one cycle at 72 °C for 10 min was made. The melting curve was constructed by plotting fluorescence data (1 sec) against temperature (65 °C to 95 °C with an interval of 0.2 °C and a holding time of 0.5 sec). Each reaction was performed in duplicate. The cycle threshold or C(t) line was set at the point where the signals surpass background noise and began to increase. This threshold was applied to all wells for consistent analysis. For each sample, the threshold cycle for internal standard (ACTB) amplification (Ct, ACTB) was subtracted from the threshold cycle of the corresponding enzyme, GATA3, ZFPM2, E-cad, VIM, or MMP9 mRNA amplification (Ct, enzyme, GATA3, ZFPM2, E-cad, VIM, or MMP9) to yield ΔCt . For each treatment group, the mean ΔCt of control samples was subtracted from individual samples to yield individual $\Delta\Delta Ct$. Fold induction relative to control samples was calculated with $2^{-\Delta\Delta Ct}$.

Protein extraction and Immunoblotting.

MDA-MB-231 cells were plated in 6-well plates (3×10^5 cells in 4 mL media). After 24 h of plating, cells were treated with DS (1.15- 5.76 μ M) or DMSO (0.01%) for 72 h. After washing twice with ice-cold PBS, cells were treated with RIPA lysis buffer (Santa Cruz Biotech Dallas, TX) for 1h at 4 °C with occasional rocking. The lysate were passed through a 21-gauge needle to shear the DNA and centrifuged at 10,000Xg for 20 min at 4 °C. Supernatants were mixed with equal volume of 2X SDS sample buffer, boiled for 5 min and then separated through 10% SDS-PAGE (Bio-Rad, Hercules, CA). After electrophoresis, proteins were transferred to polyvinylidene fluoride membrane (Bio-Rad) and blocked with 5% nonfat dry milk (Santa Cruz Biotech) for 1 h at room temperature. The membrane was immunoblotted with goat anti-GATA3 polyclonal antibody (1:100) (Santa Cruz Biotechnology, Dallas, TX) overnight at 4 °C. The membrane was washed three times with washing buffer (Life Technology, Grand Island, NY) and incubated with horseradish peroxidase-labeled donkey anti-goat antisera (1:2000) (Santa Cruz Biotech) for 1 h at room temperature. After washing in washing buffer (Life Technology, Grand Island, NY), the membrane was incubated with Western blotting luminal reagent (Santa Cruz Biotech) for detection of GATA3 protein. Also the membrane was incubated with rabbit anti-TET2 (1:100) (Santa Cruz Biotech) and β -actin (1:2400) overnight at 4 °C followed by three times washing in washing buffer and was incubated with biotin-conjugated goat anti-rabbit IgG

for 1 h at room temperature. TET2 and β -actin proteins were visualized with streptavidin-conjugated HRP.

Immunocytochemistry and confocal microscopy.

MCF-7 and MDA-MB-231 cells (40,000 cells/ 500 μ L) were grown on chambered glass slides (MERK Millipore, Billerica, MA). After 24h of incubation, cells were treated with DS (5.76 μ M) or DMSO (0.01%) for 72 h. After incubation, cells were fixed in 4% formaldehyde and blocked for 1 h with blocking buffer (1XPBS, 5% normal goat serum, 0.3% tritonX-100) followed by overnight incubation with rabbit anti-GATA3 polyclonal antibody (1:100) (Santa Cruz Biotech) and antitubulin monoclonal antibody (1:100) (Santa Cruz Biotech), at 4 $^{\circ}$ C. Slides were washed 3 times with PBS for 5 min each and incubated for 1 h with 1:250 diluted goat anti-rabbit IgG fluorescein and goat antitubulin conjugated secondary antibody (Thermoscientific, Pittsburgh, PA). Slides were washed again 3 times for 5 min each with PBS and counterstained with DAPI (Life Technology). Fluorescence was examined by a Zeiss LSM 510 META confocal microscope in the University of Mississippi Light Microscopy Core.

Wound healing assay.

MDA-MB-231 cells were seeded into 24-well tissue culture plate at a density of 3×10^5 cells/well. After 24 h of growth, when the cells reach ~70-80% confluency as a monolayer, a gentle scratch was made with a micropipette tip across the center of the well and washed twice gently with the medium. The cells were treated with various concentrations of DS (1.15-5.76 μ M) or DMSO (0.01%). The extent of wound healing was observed microscopically and photographs of each well were taken under a phase contrast microscope with 4X magnification at 0, 12, 18 and 24 h of treatment. Data were analyzed by using Wimasis Image Analysis software (IBIDI, Verona, WI).

Cell migration assay.

Cell migration was determined by using Boyden chambers with an 8 μ polyethylene terephthalate (PET) membrane and the quantity of cell migration was detected by following the instruction provided by the manufacturer (Trevigen, Gaithersburg, MD). In brief, the experimental cells were allowed to express free receptors twenty four hours in serum-free medium prior to the beginning of the assay. MCF-7 and MDA-MB-231 cells were incubated in serum-free media with various concentrations of DS (1.15-5.76 μ M) or 0.01% DMSO (control) and 5×10^4 cells/ well were transferred onto the top of the invasion chamber. Using access port, 150 μ L of media were transferred to the bottom chamber of each well. The chambers were incubated at 37 $^{\circ}$ C in a humidified environment of 95% air and 5% CO₂ for 24 h. After incubation, the top chamber was transferred to assay chamber plate and added 100 μ L of cell dissociation solution/Calcein-AM to bottom chamber and incubated at 37 $^{\circ}$ C in a humidified atmosphere of 95% air and 5% CO₂ for 1 h. Then, the top chamber was removed and the plates were read by a spectrophotometer at 485 nm excitation and 520 nm emission wave length. Using a standard curve Relative Fluorescence Units (RFU) data were converted to cell numbers and cell migrations was determined as percent of controls.

Cell invasion assay.

Cell invasion was determined by Boyden Chambers with an 8 μ polyethylene terephthalate (PET) membrane that was coated with 0.5X basement membrane extract (BME). MCF-7 and MDA-MB-231 cells preincubated with serum-free medium with DMSO as control and various concentrations of DS at 1.15, 2.30, and 5.76 μ M were transferred onto top invasion chamber with a cell density of 50,000 cells /well. Quantitative determination of cell invasion was detected by fluorescein technique following manufacturer's instruction (Trevigen, Gaithersburg, MD).

Statistics.

The data were presented as mean $\pm$ SEM and $p < 0.05$ was considered as significant. Statistical significance was assessed by unpaired t-test and one-way ANOVA to evaluate differences between control and various treatment groups using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA).

Results:

DS is very stable in culture conditions or in cellular environment.

Biotransformation of DS can result in the production of diosgenin or other saponins either in the culture media or in the cellular environment. We therefore evaluated the presence of DS both in the media as well as in the cells after 72 h of exposure. At the highest concentration of DS (5.76 μ M) diosgenin (biotransformation product of DS) was not detected in the culture media or in the cell lysate. Our findings would appear to support the conclusion that DS is stable under standard tissue culture conditions. Therefore, the effects of DS on invasion, migration, and proliferation of MCF-7 or MDA-MB-231 cell lines were pursued.

DS reduced MCF-7 and MDA-MB-231 cell proliferation.

The anti-cell proliferative activity of DS was determined by treating MCF-7 and MDA-MB-231 cells by DS for 24 (0.72- 23.03 μ M) and 72h (0.04-11.51 μ M) (Figure 2.1 and 2.2). The calculated IC_{50} of DS is 13.70 μ M after 24 h exposure and 3.85 μ M after 72 h exposure for MCF-7 and 6.77 μ M after 24 h and 2.07 μ M after 72 h for MDA-MB-231. The IC_{50} of the control drug 5-aza is 2.25 μ M in MCF-7 cells and 0.75 μ M in MDA-MB-231 cells after 72 h of treatment in identical conditions (Figure 2.3). Our findings indicate that both MCF-7 and MDA-MB-231 cell lines showed a significant sensitivity to DS which was time dependent (Fig 2.1 and 2.2).

Effect of DS and 5-aza on *GATA3* mRNA expression in MCF-7 and MDA-MB-231 cells.

Since *GATA3* has a critical role in regulation and cell proliferation, further investigations were performed to determine the effect of increasing concentration of DS (1.15-5.76 μ M), and 5-aza (0.25-1.00 μ M) on *GATA3* transcript in MCF-7 and MDA-MB-231 cells. It was observed that both DS and 5-aza (the known DNMT synthesis inhibitor) were unable to alter *GATA3* mRNA expression in MCF-7 cells. However, in MDA-MB-231 cells, in comparison to the corresponding controls, DS and 5-aza enhanced *GATA3* mRNA content significantly (Figures 3.1 and 3.2). The increase in *GATA3* mRNA was noticed only with 5.76 μ M DS while 5-aza at

both 0.5 and 1.00 μM was found to enhance *GATA3* mRNA expression in MDA-MB-231 cells. Thus the effect of DS and 5-aza on *GATA3* transcript appear to be cell-line-specific.

Effect of DS on *GATA3* protein expression in MDA-MB-231 cells.

Since higher concentration of DS altered *GATA3* transcription, we tested induction of *GATA3* protein by immunoblotting and immunocytochemical techniques. *GATA3* protein expression was found to be comparatively higher in MCF-7 cells than MDA-MB-231 cells (it was almost undetectable in MDA-MB-231 cells) (Figure 4.1 and 4.3). However, when MDA-MB-231 cells were exposed to DS or 5-aza (1 μM) expression of *GATA3* protein was increased in comparison with the corresponding controls (Figure 4.2). For validation of expression of *GATA3* protein in these cells and to determine the effects of DS, we have used an immunocytochemical technique which is found to be more sensitive than immunoblotting. Our data indicate that immunocytochemical expression of *GATA3* protein as determined by immunoblotting appeared to be lower in MDA-MB-231 cells compared to MCF-7 cells (Figure 4.3); however, when MDA-MB-231 cells were exposed to DS (5.76 μM) for 72 h, immunoreactivity in *GATA3* protein was found to be increased in cell nuclei (Figure 4.3). The analysis of protein expression pattern of *GATA3* followed the same trend as the mRNA expression in MDA-MB-231 cells treated with DS. These data confirmed that the accumulation of *GATA3* protein depends on induction of *GATA3* mRNA in MDA-MB-231 cells.

Effect of DS on *DNMT* and *TET* mRNA expression in MCF-7 and MDA-MB-231 cells and *TET2* protein expression in MDA-MB-231 cells.

While DNA methylation can negatively influence transcription, TET proteins may regulate DNA demethylation. The relationship of DNA methylation/demethylation and *GATA3* expression in MCF-7 and MDA-MB-231 cells by DS was investigated. RT-qPCR analysis of *DNMT* mRNAs indicate that DS (1.15-5.76 μM) was unable to alter *DNMT1*, *DNMT3A* and *DNMT3B* mRNAs in MCF-7 cells while in MDA-MB-231 cells, DS was able to enhance only *DNMT3A* mRNA at 5.76 μM concentration; other *DNMT* mRNAs (*DNMT1* and *DNMT3B*) remained unaltered by DS in this cell line (MDA-MB-231) (Figure 5.1, 5.2 and 5.3). TET analysis by RT- qPCR indicated that *TET1* mRNA was significantly decreased by DS (2.30-5.76 μM) in both MCF-7 and MDA-MB-231 cells when compared with the corresponding controls (Fig.6.1). However, *TET2* and *TET3* mRNAs remained unaltered in MCF-7 cells by DS but increased in MDA-MB-231 cells at highest DS concentration (5.76 μM) used in this study (Figures 6.2 and 6.3). Further analysis of TET2 protein by immunoblotting did not show any significant enhancement by DS (Figure 6.4). These data showed differential induction of *DNMT* and *TET* mRNAs in MDA-MB-231 cells treated with DS.

Effect of DS on cellular transition.

As the cellular transition of breast cancer cells are important steps of metastasis, we examined the cell morphology by immunofluorescence staining technique. As observed under confocal microscope, the shape of MCF-7 cells are cuboidal and MDA-MB-231 cells are spindle-like (Figure 4.3). After DS treatment, highly invasive MDA-MB-231 cells partially respond to DS and both epithelial and mesenchymal cells were coexisting. Some of them are still spindle-like; some of them changed to epithelial-like as in MCF-7 cells (Figure 4.3).

Effect of DS on cellular migration, and invasion.

For cell migration analysis, we used wound healing assay in which a gap is generated on confluent MDA-MB-231 cells monolayer. The length of time required to fill the gap by cells is monitored in the presence of varying concentrations of DS. We found that the gap was completely filled at 18-24 h in control (cells are not treated with DS). When MDA-MB-231 cells were treated with DS, a significant reduction in cell migration was observed within this period (18-24h). This effect was not concentration dependent with a maximal inhibition at in the presence of 1.15-5.76 μM at 24 h (Figure 7.1). The mean cell migration was also calculated in the absence or presence of DS (Figure 7.2). To confirm DS's inhibitory effect on cell migration, we performed cell migration and invasion assay using Boyden's chambers with both MCF-7 and MDA-MB-231 cells (Figure 8). We have observed that like wound healing assay, DS effectively inhibited cell migration (Figure 8.1 and 8.2) and invasion (Figure 8.3) in the MDA-MB-231 cell line. MCF-7 cell migration was significantly lower than MDA-MB-231 cells (Figure 8.1). The random migration (Figure 8.2) and invasion (Figure 8.3) of MCF-7 and MDA-MB-231 cells were also significantly inhibited by DS (5.76 μM). Therefore, DS interferes with basal migration and invasion of MCF-7 and MDA-MB-231 cells.

Effect of DS on *ZFPM2*, *E-cad* (*CDH1*), *VIM*, and *MMP9* mRNA expression in MCF-7 and MDA-MB-231 cells.

A RT-qPCR analyses of *ZFPM2* (a modulator of activity of GATA family proteins) and *E-cad* (a tumor suppressor gene) mRNAs indicate that DS (1.15-5.76 μM) was unable to alter both *ZFPM2* and *E-cad* mRNAs in MCF-7 cells. However, in MDA-MB-231 cells, both *ZFPM2* and *E-cad* mRNAs were found to be increased significantly with 5.76 μM DS after 72 h of treatment (Figures 9.1 and 9.2) suggesting that DS causes an increase in cellular adhesion and modulation of GATA3 activities. *VIM* (a marker of mesenchymal derived cells) and *MMP9* mRNA analyses by RT-qPCR indicated that DS at 5.76 μM concentration was able to reduce *VIM* and *MMP9* mRNAs in both MCF-7 and MDA-MB-231 cells when compared with the corresponding controls (Figures 9.3 and 9.4). DS was also found to be effective in reducing *MMP9* mRNA expression in both MCF-7 and MDA-MB-231 cells even at lower concentration (2.3 μM). Since *VIM* over expression results in an increase motility and invasiveness in both MCF-7 and MDA-MB-231 cells, DS could be considered as a novel antiinvasive agent with therapeutic potential on clinical use.

Discussion:

Previously, we have observed that wild yam root extract (WYRE) is capable of modulating DNA methylation in human breast cancer cell lines (Aumsuwan et al. 2015). However, the exact mechanisms by which WYRE reduced DNA methylation are unknown. Recent evidence has shown that DNA methylation plays a role in gene regulation. TET2 protein plays a role in the DNA demethylation process. Down-regulation or mutations in TET2 enzyme was associated with increased cell proliferation and abnormal differentiation in some cancers. In this study, we provided evidence that DS, a bioactive steroidal saponin compound isolated from wild yam (*Dioscoria villosa*) root, is an antiproliferative, antiinvasive and antimigrative agent (Figure 10). Using the human breast cancer cell lines MCF-7 (Estrogen receptor positive, ER⁺) and MDA-MB-231 (ER negative, ER⁻) under hormone-free environment, we demonstrated that DS altered

the expression of several genes, especially in MDA-MB-231 cells, all of which encode proteins involved in regulation of essential cellular functions, such as cell growth, cell proliferation and cell migration.

During mammary gland development, GATA3 is critical for luminal differentiation and expressed only in the ducts of terminal end buds (TEB) of luminal cells (Kouros-Mehr et al., 2006; 2008). Loss of GATA3 expression has been associated with a worse prognosis in breast cancer patients (Yoon et al., 2010). Moreover, expression of GATA3 is methylation-specific, comparatively higher in ER⁺ non-invasive MCF-7 cells with hypomethylated *GATA3* promoter and insignificant in ER⁻ invasive MDA-MB-231 cells with hypermethylated *GATA3* promoter (Sun et al, 2011). Investigations were performed to determine whether WYRE (Aumsuwan et al., 2015), or its components affect the DNA methylation/demethylation processes.

DS is the precursor of many steroidal saponins and sapogenins (such as diosgenin). Therefore, we initially assessed the biotransformation of DS by measuring levels of its major metabolites in the cell conditioned media and cell lysates. Although DS was present, its metabolites were not detected in the cell conditioned media or in cell lysates under our experimental condition, indicating that DS is a stable molecule in the conditioned culture medium. Based on this finding, we explored the potential inhibitory effect of DS on both MCF-7 and MDA-MB-231 cell lines, and characterized the possible mechanisms of its anticancer effect, especially in MDA-MB-231 cells.

Next, investigations were carried out to determine the effect of DS on cell viability, invasion, migration, and wound healing. Several available reports have documented the cytotoxic potential of DS in a variety of human malignant cell lines (Cai et al., 2002, Gao et al., 2011, Hsieh et al., 2013, Liu et al., 2004, Wang et al., 2001; 2014, Zhao et al., 2014, Chen et al., 2014). Our data indicate that DS has cytotoxic activity and is able to target both MCF-7 (ER⁺) and MDA-MB-231 (ER⁻) cells in a time and concentration-dependent manner. Moreover, the cytotoxic effects of DS appeared to be cell line specific, approximately two times more effective in invasive MDA-MB-231 cells than in non-invasive MCF-7 cells as observed both at 24h (IC₅₀ is 13.70 μ M in MCF-7 and 6.77 in MDA-MB-231) and 72h (IC₅₀ is 3.85 in MCF-7 and 2.05 in MDA-MB-231). Our data further indicate that 5-aza, a known DNMT inhibitor, is also able to show cytotoxicity in these cell lines and the effect is approximately 3-times more potent in MDA-MB-231 cells (IC₅₀=0.75 μ M) than MCF-7 cells (IC₅₀=2.25 μ M) after 72 h exposure. The calculated IC₅₀ for DS in other studies mostly depend on two factors; the cell type used and the duration of DS exposure (Table 1). For example, in HeLa cells after 48 h DS exposure the calculated IC₅₀ is approximately 2-times higher than human prostate cancer cell line LNCaP for an identical period of time (Table 1). In another study with human gastric cancer cells (SGC-7901), the IC₅₀ after 24 h exposure is approximately 3-times higher than the IC₅₀ after 72h exposure (Gao et al., 2011). However, in prostate cancer cells (LNCaP) the calculated IC₅₀ between 12 and 48 h exposure have shown only marginal difference (Chen et al., 2014). Although the proposed mechanisms of cytotoxicity by DS in cancer cells are involved in the induction of apoptosis (Wang et al., 2014) or cell cycle arrest (Man et al., 2010), our previous studies on cytotoxic effects of WYRE using MCF-7 and MDA-MB-231 cells showed specific modification in the epigenome that affects gene expression and functions (Aumsuwan et al., 2015). Our present data on the cytotoxic response of these cell lines to DS and 5-aza are found to be time- and concentration-dependent.

GATA3 plays an important role in altering the tumor microenvironment in breast cancer to promote differentiation, inhibit proliferation, suppress metastasis and deregulate physiologic protein turnover. Studies of human breast cancer demonstrated that GATA3 is predominantly expressed in early stage of well differentiated breast cancers but not in that of advanced invasive cancers. The co-expression of estrogen receptor alpha (ER- α) and GATA3 is shown in breast cancer cells. Demethylation of GATA3 promoter in MDA-MB-231 and other ER⁻ breast cancer cells could enhance GATA3 gene activation and function (Sun et al., 2011). To gain insights into the potential modulatory role of DS in breast cancer cells, we characterized expression pattern of GATA3 in the presence of DS. Treatment of MCF-7 and MDA-MB-231 cells with DS triggered a change in mRNA and protein levels of GATA3. While it remained unaltered in MCF-7 cells, *GATA3* mRNA expression was significantly increased in MDA-MB-231 cells by both DS and 5-aza. Using immunocytochemistry technique, we observed that DS enhanced GATA3 protein expression and its nuclear distribution in highly invasive MDA-MB-231 cells. However, induction of the expression pattern of GATA3 mRNA and protein levels were not concentration-dependent, suggesting that DS might be intracellularly compartmentalized. Further investigations will be needed to confirm this suggestion using the radiolabeled DS.

To improve our understanding of the mechanism of action of DS on GATA3 (a tumor suppressor) expression, we performed RT-qPCR and western blot experiments to explore DS regulation of DNMT and TET expression. DS treatment promoted a significant increase in TET2 and TET3 transcripts but not TET2 protein in MDA-MB-231 cells. The apparent low TET2 protein did not result in a direct decrease in GATA3 transcript, which suggests the involvement of additional mechanism in the process of DS-induced GATA3 stimulation, such as DNMT-mediated increase in global DNA methylation. DS treatment promoted an increase in DNMT3A transcripts in MDA-MB-231 cells. Since DS influenced TET2 and DNMT3A transcripts and on the basis of our findings, we predict that DS modulates cytosine's methylation or demethylation process that may affect GATA3 expression.

Several reports indicate that overexpression of GATA3 is positively associated with a reduction in tumorigenesis and metastasis (Dydenborg et al., 2009; Yan et al., 2010; Chu et al., 2012) and triggers mesenchymal-to-epithelial transition (MET) (Yan et al. 2010; Chu et al., 2013). After excluding cytotoxicity, the effect of DS on the migration and invasiveness of MDA-MB-231 cells was assessed under normal and serum-free culture conditions. Since cancer invasion recruit certain molecules such as adhesion molecules, extracellular matrix protein and proteases to remodel extracellular matrix, we selectively measured the effect of DS on zinc finger protein2 (ZFPM2), E-cad, VIM, and MMP9 transcript expression in MCF-7 and MDA-MB-231 cells. Although its mechanism of action remains controversial (Holmes et al. 1999, Svensson et al. 1999), *ZFPM2* is a member of friend of GATA (FOG) family and a cofactor for GATA family of transcription factors (Tevosian et al., 2000) that modulates the activity of GATA proteins. In this study, we found that DS caused an increase in ZFPM2 transcript, indicating that the DS treatment could influence both GATA and its cofactor expression. Therefore DS can modulate GATA3-dependent pathway.

Down regulation of *E-cad* promotes epithelial-to-mesenchymal transition (EMT) (Yang and Weinberg, 2008). Loss of *E-cad* promotes metastasis through induction of EMT (Onder et al., 2008). Evidence indicates that enhanced *GATA3* could influence E-cad expression after binding GATA-like motif of *E-cad* promoter (Yan et al., 2010). In this study, *E-cad* transcript levels

were consistent with the expression pattern of *GATA3*, *TET* and *ZFPM2* mRNA levels in DS-treated MDA-MB-231 cells, suggesting that DS imposes a dominant regulatory effect on these genes. To study the effect of DS on cell invasion, we assessed *MMP9* transcription pattern in DS-treated MCF-7 and MDA-MB-231 cells. Overexpression of *MMP9* is associated with higher incidence of metastasis (Yousef et al., 2014). Based on the study by Yan et al (2010) which showed that ectopic expression of *GATA3* in MDA-MB-231 cells decreased *MMP9* level, we also observed that DS could reduce *MMP9* in a concentration-dependent manner in these cells, which is known to contribute to angiogenesis and the degradation of basement membrane collagen (Satelli and Li, 2011; Grant and Kyprianou, 2013; Mallini et al., 2014; Yousef et al., 2014). The DS-dependent decrease in *MMP9* resulted in reduced invasiveness and migration of MDA-MB-231 cells. *MMP9* is a zinc-dependent protease. Therefore, the effect of DS on the concentration of zinc need to be explored.

Because *GATA3* over expression influenced MDA-MB-231 cell morphology and ectopic expression of *GATA3* in MDA-MB-231 cells prevents metastasis (Yan et al., 2010), we explored the effect of DS on MDA-MB-231 morphology. Comparison of the morphology of MDA-MB-231 with MCF-7 cells in the presence of DS demonstrated an increased transition of MDA-MB-231 cells from spindle shape (mesenchymal) to cuboidal-like epithelial phenotype. We next analyzed the inhibitory relevance of DS on *VIM*, a potential marker of EMT (Thiery, 2002; Korsching et al., 2005; Chaffer et al., 2006). *VIM* expression was elevated in breast cancer (Satelli and Li, 2011). Mice with the *VIM* deficiency exhibit impaired wound healing (Eckes et al., 2000). In contrast, *VIM* mRNA levels were decreased in DS-treated MCF-7 and MDA-MB-231 cells. Evidence indicates that ectopic expression of *GATA3* in MDA-MB-231 cells decreases *VIM* (Yan et al., 2010). It would be tempting to speculate that decreased *VIM* mRNA is due to overexpression of *GATA3* by DS in these cancer cell lines. NF- κ B is capable of binding to *VIM* promoter (Satelli and Li, 2011). Therefore, the effect of DS on NF- κ B-mediated *VIM* over-expression cannot be ignored. Moreover, the tumor suppressor activity of E-cad which is increased by DS in our study, at least in part, is mediated by binding with its cytoplasmic domain to β -catenin, and preventing the translocation of β -catenin to the nucleus (Yilmaz and Christofori, 2009), where it stimulates transcription of various other genes involved in EMT. For example, β -catenin induces expression of the intermediate filament vimentin in breast cancer cells, leading to the acquisition of a mesenchymal phenotype (Gilles et al., 2003). DS is also able to upregulate β -catenin expression in mouse pre-osteoblast like MC3T3-E1 cells (Zhang et al., 2014). Therefore, interactions of these genes with regard to metastasis is also influenced by DS. Although we are unable to establish the correlation, our data indicate that DS plays an important role in modulating expression of several genes including E-cad, *VIM*, and *MMP9*, at the transcription level that are directly or indirectly involved in invasion, migration, matrix remodeling, and cell morphology in vitro. Further studies are needed to determine which DS-dependent factor (protein or kinase) are involved in this process.

In summary, our data indicate that changes in *TET* and *GATA3* by DS could alter MDA-MB-231 cell invasion, migration, and wound healing (Figure 10). Moreover, it appears that both *TET* and *GATA3* are influenced by DS in parallel, as there is evidence that DS-induced elevation of *TET* reduces proliferation and DS-mediated elevation of *GATA3* suppresses cell invasion, migration, and wound healing, without evidence that one pathway precedes or causes the other in MDA-MB-231. The findings herein support the potential therapeutic utility of DS in invasive breast cancer.

Acknowledgements:

Our sincere thanks go to Dr. Tracy A Brooks, Assistant Professor, Department of BioMolecular Sciences, Pharmacology Division, for her interest and helpful suggestions during this work. This work was supported in part by the United States Department of Agriculture (USDA), Agricultural Research Service Specific Cooperative Agreement no 58-6408-2009 and the National Center for Complementary and Alternative Medicines (NCCAM), the Office of Dietary Supplements (ODS), and the National Cancer Institute (NCI), Grant Number P50AT006268 (IAK, WGH, and BSK). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NCCAM, ODS, NCI or the National Institutes of Health.

References:

- Atalay C. Epigenetics in breast cancer. *Exp Oncol.* 35:246-249(2013).
- Aumsuwan P, Khan SI, Khan IA, Avula B, Walker LA, Helferich WG, Katzenellenbogen BS, Dasmahapatra AK. Evaluation of wild yam (*Dioscorea villosa*) root extract as a potential epigenetic agent in breast cancer cells. *In Vitro Cell Dev Biol Anim.* 51:59-71(2015).
- Avula B., Wang Y-H., Wang M, Ali Z, Smillie TJ, Zweigenbaum, Khan IA. Characterization of steroidal saponin from *Dioscorea villosa* and *D. cayenensis* using ultrahigh performance liquid chromatography/electrospray ionization quadrupole time-of-flight mass spectrometry. *Planta Med.* 80:321-329(2014).
- Bilal I, Chowdhury A, Davidson J, Whitehead S. Phytoestrogens and prevention of breast cancer: the contentious debate. *World J Clin Oncol.* 5:705-712(2014).
- Byler S, Goldgar S, Heerboth S, Leary M, Housman G, Moulton K, Sarkar S. Genetic and epigenetic aspects of breast cancer progression and therapy. *Anticancer Res.* 34:1074-1078(2014).

- Cai J., Liu M., Wang Z, Ju Y. Apoptosis induced by dioscin in Hela cells. *Biol Pharm Bull.* 25:193-196(2002).
- Chaffer CL, Brennan JP, Slavin JL, Blick T, Thompson EW, Williams ED. Mesenchymal-to epithelial transition facilitates bladder cancer metastasis: role of fibroblast growth factor receptor-2. *Can Res.* 66:11271-11278(2006).
- Chen J, Li H, Zhang X, Xiong C. Dioscin-induced apoptosis of human LNCaP prostate carcinoma cells through activation of caspase-3 and modulation of bcl-2 protein family. *J Huzhong Univ Sci Technol [Med Sci]*, 34:125-130(2014).
- Chou, Jonathan, Jeffrey H. Lin, Audrey Brenot, Jung-whan Kim, Sylvain Provot, Zena Werb. GATA3 suppresses metastasis and modulates the tumour microenvironment by regulating microRNA-29b expression. *Nature Cell Biol.* 15:201-213 (2013).
- Chu IM, Michalowski AM, Hoenerhoff M, Szauter KM, Luger D, Sato M, Flanders K, Oshima A, Csiszar K, Green JE. GATA3 inhibits lysyl oxidase-mediated metastases of human basal triple-negative breast cancer cells. *Oncogene*, 31:2017-2027(2012).
- Chu IM, Lai WC, Aprelikova O, EITouny LH, Kourous-Mehr H, Green JE. Expression of GATA3 in MDA-MB-231 triple-negative breast cancer cells induces a growth inhibitory response to TGF β . *PLoS One*, 8:e61125 (2013).
- Di Costanzo A, Del Gaudio N, Migliaccio A, Altucci L. Epigenetic drugs against cancer: an evolving landscape. *Arch Toxicol.* 88:1651-1668(2014).
- Dydensborg AB, Rose AA, Wilson BJ, Grote D, Paquet M, Giguere V, Siegel PM, Bouchard M. GATA3 inhibits breast cancer growth and pulmonary breast cancer metastasis. *Oncogene*, 28:2634-2642(2009).
- Eckes B, Colucci-Guyon E, Smola H, Nodder S, Babinet C, Krieg T, Martin P. Impaired wound healing in embryonic and adult mice lacking vimentin. *J Cell Sci.* 113(Pt 13), 2455-62 (2000).

- Gao L-L, Li F-R, Yang M-F, Zhou X-J, Si Y-H, Jiang W-J, Zheng T-T. Paris chinensis dioscin induces G2/M cell cycle arrest and apoptosis in human gastric cancer SGC-7901 cells. *World J Gastroenterol.* 17(39), 4389-95 (2011).
- Gilles C, Polette M, Mestdagt M, Nawrocki-Raby B, Ruggeri P, Birembaut P, Foidart JM. Transactivation of vimentin by β -catenin in human breast cancer cells. *Can Res.* 63:2658-2664(2003).
- Grant CM, Kyprianou N. Epithelial mesenchymal transition (EMT) in prostate growth and tumor progression. *Transl Androl Urol.* 211(2013).
- Harvey AL. Natural products in drug discovery. *Drug Discov Today*, 13 (19-20): 894-901(2008).
- Holmes MJ, Turner J, Fox A, Chisholm O, Crossley M, Chong B. hHOG2, a novel zinc finger protein, binds the co-repressor mCtBP2 and modulates GATA-mediated activation. *J Biol Chem.* 274, 23491-23498(1999).
- Hsieh M-J, Tsai T-L, Hsieh Y-S, Wang C-J, Chiou H-L. Dioscin-induced autophagy mitigates cell apoptosis through modulation of PI3K/Akt and ERK and JNK signaling pathways in human lung cancer cell lines. *Arch. Toxicol.* 87:1927-1937(2013).
- Khan S, Aumsuwan P, Khan IA, Walker LA, Dasmahapatra AK. Epigenetic events associated with breast cancer and their prevention by dietary components targeting the epigenome. *Chem Res Toxicol.* 2525:61-73(2012).
- Korsching E, Packeisen J, Liedtke C, Hungermann D, Wulfing P, van Diest PJ, Brandt B, Boecker W, Buerger H. The origin of vimentin expression in invasive breast cancer: epithelial-mesenchymal transition, myoepithelial histogenesis or histogenesis from progenitor cells with bilinear differentiation potential? *J Pathol.* 206:451-457(2005).
- Kouros-Mehr H, Slorach EM, Sternlicht MD, Werb Z. GATA3 maintains the differentiation of the luminal cell fate in the mammary gland. *Cell* 127:1041-1055(2006).
- Kouros-Mehr, H., Bechis SK, Slorach EM, Littlepage LE, Egeblad M, Ewald AJ, Pai SY, Ho IC,

- Werb Z. GATA-3 links tumor differentiation and dissemination in a luminal breast cancer model. *Cancer cell* 13:141-152 (2008).
- Li M, Han X, Yu B. Synthesis of monomethylated dioscin derivatives and their antitumor activities. *Carbohydr. Res.* 338:117-121(2003).
- Liu M-J, Wang Z, Ju Y, Zhou J-B, Wang Y, Wong R.N-S. The mitotic-arresting and apoptosis-inducing effects of diosgenyl saponins on human leukemia cell lines. *Biol Pharm Bull.* 27:1059-1065(2004).
- Lu J, Steeg PS, Price JE, Krishnamurthy S, Mani SA, Reuben J, Cristofanilli M, Dontu G, Bidaut L, Valero V, Hortobagyi GN, Yu D. Breast cancer metastasis: challenges and opportunities. *Can. Res. Treat.* 69:4951-4953(2009).
- Mallini P, Lennard T, Kirby J, Meeson A. Epithelial-to-mesenchymal transition: what is the impact on breast cancer stem cells and drug resistance. *Cancer Treat Rev.* 40:341-348(2014).
- Man S, Gao W, Zhang Y, Huang L, Liu C. Chemical study and medical application of saponins as anti-cancer agents. *Fitoterapia* 81:703-714(2010).
- Manuylov NL, Smagulova FO, Tevosian SG. *Fog2* expression in mice leads to premature mammary gland involution and reduced *Esr1* gene expression. *Oncogene*, 26:5204-5213 (2007).
- Marks PW. Decitabine for acute myeloid leukemia. *Expert Rev Anticancer Ther.* 12:299-305 (2012).
- McAnuff MA, Harding WW, Omoruyi FO, Jacobs H, Morrison FY, Asemota HN.
Hypoglycemic effects of steroidal sapogenins isolated from Jamaican bitter yam, *Dioscorea polygonoides*. *Food Chem Toxicol.* 43:1667-1672(2005).
- Onder TT, Gupta PB, Mani SA, Yang J, Lander ES, Weinberg RA. Loss of E-cadherin promotes metastasis via multiple down stream transcriptional pathways. *Cancer Res.* 68:3645-

3654(2008)

Satelli A, Li S. Vimentin in cancer and its potential as a molecular target for cancer therapy. *Cell Mol Life Sci.* 68:3033-3046(2011).

Scudiero DA, Shoemaker RH, Paull KD, Monks A, Tierney S , et al.: Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. *Cancer Res.* 48:4827-4833(1988).

Scully OJ, Bay BH, Yip G, Yu Y. Breast cancer metastasis. *Can. Genom. Proteom.* 9:311-320(2012).

Sun Z, Asmann YW, Kalari KR, Bot B, Eckel-Passow J.E., Baker TR, Carr J.M. Khrebtukova I, Lou S, Zhang L, Schroth GP, Perez EA, Thompson EA. Integrated analysis of gene expression, CpG island methylation, and gene copy number in breast cancer cells by deep sequencing. *PLoS One* 6:e17490(2011).

Svensson EC, Tufts RL, Polk CE, Leiden JM. Molecular cloning of FOG-2: a modulator of transcription factor GATA-4 in cardiomyocytes. *Proc Nat Acad Sci USA*, 96:956-961(1999).

Tevosian SG, Deconinck AE, Tanaka M, Schinke M, Litovsky SH, Izumo S, Fujiwara Y, Orkin SH. FOG-2, a cofactor for GATA transcription factors, is essential for heart morphogenesis and development of coronary vessels from epicardium, *Cell*, 101:729-739(2000).

Thiery JP. Epithelial-mesenchymal transitions in tumor progression. *Nat Rev Cancer* 2:442-454(2002)

Tong Q, Qing Y, Wu Y, Hu X, Jiang L, Wu X. Dioscin inhibits colon tumor growth and tumor angiogenesis through regulating VEGFR2 and AKT/MAPK signaling pathways. *Toxicol Appl Pharmacol.* 281:166-173(2014)

Wang Y, He QY, Chiu JF. Dioscin induced activation of p38 MAPK and JNK via mitochondrial

- pathway in HL-60 cell line. *Eur J Pharmacol.* 735:52-58(2014).
- Wang Z, Zhou J, Ju Y, Zhang H, Liu M, Li X. Effects of two saponins extracted from the *Polygonatum zanlanscianense* pamp on human leukemia (HL-60) cells. *Biol Phar Bull.* 24:159-162(2001).
- Xu T, Zhang S, Zheng L, Yin L, Xu L, Peng J. A 90-day subchronic toxicological assessment of dioscin, a natural steroid saponin, in Sprague-Dawley rats. *Food Chem. Toxicol.* 50:1279-1287(2012).
- Yan W, Cao QJ, Arenas RB, Bentley B, Shao R. GATA3 inhibits breast cancer metastasis through the reversal of epithelial-mesenchymal transition. *J Biol Chem.* 285:14042-14051(2010).
- Yang J, Weinberg RA. Epithelial-mesenchymal transition: at the cross roads of development and tumor metastasis. *Dev Cell.* 14:818-829(2008).
- Yilmaz M, Christofori G. EMT, the cytoskeleton, and cancer cell invasion. *Cancer Metastasis Rev.* 28:15-33(2009).
- Yoon NK, Maresh EL, Shen D, Elshimali Y, Apple S, Horvath S, Mah V, Bose S, Chia D, Chang HR, Goodglick L. Higher levels of GATA3 predict better survival in women with breast cancer. *Hum Pathol.* 41:1794-1801(2010).
- Yousef EM, Tahir MR, St-Pierre Y, Gaboury LA. MMP-9 expression varies according to molecular subtypes of breast cancer. *BMC Cancer,* 14: 609(2014).
- Zhang C, Peng J, Wu S, Jin Y, Xia F, Wang C, Liu K, Sun H, Liu M. Dioscin promotes osteoblastic proliferation and differentiation via Lrp5 and ER pathway in mouse and human osteoblast-like cell lines. *J Biomed Sci* 21: 30. Doi. 10.1186/1423-0127-21-30. (2014).
- Zhao X, Ren H, Gao S, Hao J. Effects of dioscin on apoptosis in pancreatic cancer MiaPaCa-2 cells and its mechanism. *Zhonghua Zhong Liu Za Zhi.* 36:5-109(2014).

Zhiyu W, Yue C, Neng W, Mei WD, Wei LY, Feng H, Gang SJ, De PY, Yuan GX, Jian-Ping C.

Dioscin induces cancer cell apoptosis through elevated oxidative stress mediated by down-regulation of peroxiredoxins. *Cancer Biol Ther.* 13:138-147(2012).

Table 1: IC₅₀ of DS as determined in human cancer cell lines.

Cell lines	Duration of exposure	Calculated IC ₅₀	Probable mechanism(s)	Ref
HeLa	48 h	4.4µM	Apoptosis	Cai et al. (2002)
Human gastric cancer cell SGC-7901	24 h	13.77±0.18 mg/mL	G2/M phase arrest	Gao et al. (2011)
	48 h	8.73 ±0.41 mg/mL		
	72 h	3.62± 0.29 mg/mL		
Human Leukemia HL60	96 h (4 days)	7.5 µM		Wang et al. (2001)
	24 h	7.6±0.12 µM	Apoptosis	Wang et al. (2014)
Human prostate carcinoma cell LNCaP	12h	2.86 µM	Apoptosis	Chen et al. (2014)
	24h	2.19 µM		
	48 h	2.03 µM		
Human esophageal cancer cell KYSE 510	24 h	5.4 µM	Apoptosis	Zhiyu et al.(2012)
Human breast cancer cell MCF-7 (non-invasive)	24h	13.70 µM	Modification of epigenome	Aumsuwan et al. (present study)
	72 h	3.85µM		
Human breast cancer cell MDA-MB-231 (invasive)	24h	6.77 µM	Modification of epigenome	Aumsuwan et al. (present study)

Figure 1

Figure 1: Chemical Structure of Dioscin

Figure 2

Figure 2: Effect of DS on MCF-7 and MDA-MB-231 cell viability in vitro. Cell viability was determined by XTT method (Scudiero et al., 1988). Each data point is the mean $\pm$ SEM of 6-8 observations.

Figure 3

Figure 3: Effect of DS and 5-aza on GATA3 mRNA expression in MCF-7 and MDA-MB-231 cells in vitro. Each bar is the mean $\pm$ SEM of six-eight independent observations. $p < 0.05$ is considered as significant. Bar head with asterisks (*) indicate that the data are significantly different from the corresponding controls.

Figure 4

Figure 4.1

Figure 4.2.

Figure 4.3

Figure 4: GATA3 protein expression analysis by immunoblotting (Figure 4.1 and 4.2) and by immunocytochemistry (Figure 4.3).

Figure 4.1. A representative picture showing GATA3 protein expression in MCF-7 and MDA-MB-231 cells from immunoblotting experiment using anti-GATA3 antibody. The upper panel shows a representative analysis of cell lysates (constant amount of protein) from MCF-7 and MDA-MB-231 cells for immunoblotting using anti-GATA3 antibody. The histogram in lower panel (mean of two observations) indicates that GATA3 protein expression was ~ 4-fold higher in MCF-7 cells compared to MDA-MB-231 cells

Figure 4.2. Effects of DS and 5-aza on GATA3 protein expression in MDA-MB-231 cells by immunoblotting using GATA3 antibody. Upper panel shows a representative photograph of an immunoblotting analysis showing GATA3 and β -actin proteins in the cell lysates (constant amount of protein) detected by GATA3 and β -actin antibodies, respectively. β -actin was used as loading control. Histogram in lower panel represents the quantitative analysis of GATA3 protein by immunoblotting. Both DS (1.15-5.75 μ M) and 5-aza (1 μ M) were able to induce GATA 3 protein expression in MDA-MB-231 cells compared to control. Each bar is the mean $\pm$ SEM of three independent experiments. Bar head with asterisks (*) represent the data are significantly different ($p < 0.05$) from controls (no DS).

Figure 4.3. Representative figures (from several different fields of three independent experiments) showing immunocytochemical analysis of GATA3 proteins in MCF-7 and MDA-MB-231 cells with and without DS treatment. Florescence was examined by a Zeiss LSM 510 META confocal microscope. Red dots in the nucleus (blue) or cytoplasm indicate the GATA binding protein 3 positive regions (GATA3) in the cells which are comparatively higher in control MCF-7 cells than control MDA-MB-231 cells. Moreover, MCF-7 cells showed cuboidal-like epithelial phenotype and MDA-MB-231 cells represented spindle-like phenotype. After DS treatment, MDA-MB-231 cells showed coexistence of both spindle-like and cuboidal phenotypes.

Figure 5

Figure 5: Effect of DS on *DNMT1*, *DNMT3A*, and *DNMT3B* mRNA content of MCF-7 and MDA-MB-231 cells. Each bar is the mean $\pm$ SEM of six-eight independent observations. $p < 0.05$ is considered as significant. Bar head with asterisks (*) indicate that the data are significantly different from the corresponding controls. Fig.5.1= *DNMT1*; Fig. 5.2=*DNMT3A*; Fig. 5.3= *DNMT3B*.

Figure 6

Figure 6: Effect of DS on *TET1*, *TET2*, and *TET3* mRNA content of MCF-7 and MDA-MB-231 cells and TET2 protein of MDA-MB-231 cells. Each bar is the mean $\pm$ SEM of six-eight independent observations. $p < 0.05$ is considered as significant. Bar head with asterisks (*) indicate that the data are significantly different from the corresponding controls. Fig.6.1= *TET1*; Fig. 6.2=*TET2*; Fig. 6.3= *TET3*; Fig. 6.4 = A representative immunoblotting experiment of TET2 protein expression in MDA-MB-231 cells; the bar diagram is the mean $\pm$ SEM of four observations.

Figure 7

Figure 7.1

Figure 7.2

Figure 7: DS inhibits MDA-MB-231 cell migration as determined by wound-healing assay.

Figure 7.1: The extent of wound healing was observed microscopically and photographs of each well were taken under a phase contrast microscope with 4X magnification at 0, 12, 18 and 24 h of treatment. Data were analyzed by using Wimasis Image Analysis software (IBIDI, Verona, WI). The concentrations of DS (1.15-5.76 μM) are mentioned on the top of the figure and the time of exposure in h are presented on the left side of the figure.

Figure 7.2: DS inhibits MDA-MB-231 cell migration in time-dependent manner. The extent of wound-healing was determined by Wimasis Image analysis software (IBIDI, Verona, WI). Each bar is the mean $\pm$ SEM of three independent experiments. Bar head with asterisks (*) indicate the data are significantly different from the controls (no DS) at the corresponding time points.

Figure 8

Figure 8: Effect of DS on cell migration (Figure 8.1 and 8.2) and invasion (8.3).

Figure 8.1: Representative figure comparing the cell migrating abilities of MCF-7 and MDA-MB-231 cells in vitro as determined by cell migration assay. It was observed that significantly more MDA-MB-231 cells were attained migration than MCF-7 cells. The data are mean $\pm$ SEM of 17 observations. Bar head with asterisk (*) indicate the data are significantly different from the MCF-7 cells.

Figure 8.2: DS significantly reduced cell migration both in MCF-7 and MDA-MB-231 cells. Data are expressed as percent of control considering control number as 100%. Bar head with asterisk (*) indicate that the data are significantly different ($p < 0.05$) from the corresponding

controls. It was observed that DS at 5.76 μM concentration significantly reduces cellular migration both in MCF-7 and MDA-MB-231 cells.

Figure 8.3: DS significantly reduced cell invasion both in MCF-7 and MDA-MB-231 cells. Data are expressed as percent of control considering control number as 100%. Bar head with asterisk (*) indicate that the data are significantly different ($p < 0.05$) from the corresponding controls. It was observed that DS at 5.76 μM concentration significantly reduces cellular invasion both in MCF-7 and MDA-MB-231 cells.

Figure 9

Figure 9: Effect of DS on *ZFMP2*, *E-cad*, *VIM* and *MMP9* mRNA contents of MCF-7 and MDA-MB-231 cells. Each bar is the mean $\pm$ SEM of six-eight independent observations. $p < 0.05$ is considered as significant. Bar head with asterisks (*) indicate that the data are significantly different from the corresponding controls. Fig.9.1=*ZFMP2*(*FOG2*); Fig. 9.2=*E-cad* (*E-cadherin*); Fig. 9.3=*VIM* (*vimentin*) and 9.4=*MMP9* (*Matrix metalloproteinase 9*).

Figure 10

Figure 10: A simplified schematic mechanisms of action of DS in MDA-MB-231 cell. TET and GATA3 expressions are elevated in response to a low concentration of DS, which control normal proliferation, differentiation, wound healing, invasion, and migration. (---) indicates either direct or indirect effects by DS.