**ABSTRACT**

**Aim:** The aim was to examine the anti-proliferative effect of *Withania somnifera* (WS) root extract in cell cultures and nude mouse xenografts of breast cancer cell line MDA-MB-231. **Methods:** WS root extract was used to treat tumor cells at concentrations up to 100 μg and for nude mouse experiments, the mice received daily WS at 300 mg/kg by oral gavage for 8 weeks. **Results:** The WS extract reduced viability of MDA-MB-231 cells by 75% and 88% after exposure of the cells to 50 and 100 μg/mL, respectively, compared to vehicle-treated controls. WS extract caused a dose-dependent increase in the percentage of cells in the sub-G1 phase compared to untreated controls by 6% and 10% after exposure to 25 and 50 μg/mL WS extract, respectively. WS extract also inhibited proliferation of xenografted MDA-MB-231 cells. The WS extract caused reductions in xenograft size by 60% compared to the untreated control after 8 weeks of treatment. Six of ten mice in the control group showed tumor metastasis to the lung, whereas there was none in the mice treated with the WS extract. At the gene level, WS caused a 75% reduction in chemokine CCL2 expression \((p < 0.05)\) in the xenografted tumors of the treated mice. **Conclusion:** WS root extract inhibited proliferation of breast cancer cells *in vitro* and *in vivo* and significantly reduced expression of the cytokine, CCL2. These results warrant further studies to assess the underlying molecular mechanism of the anti-tumor activity of the WS extract in breast cancer.

**Key words:** *Withania somnifera* extract, MDA-MB-231, breast cancer, metastasis, animal model

**Introduction**

Invasive breast cancer is considered one of the great challenges for clinicians to control and improve survival of patients. In 2013, an estimated 232,340 new cases of invasive breast cancer were diagnosed in women in the USA, along with other 64,640 cases of non-invasive breast cancer.[1] For women under 45, deadly forms of this type of breast cancer are more common in African-American women than white women, and African-American women are more likely to die of breast cancer.[2] Despite three decades of advances in treatment of breast cancer using hormone receptor modulators, aromatase inhibitors, and surgery,[3-5] mortality remains high due to tumor metastasis to the lymph nodes, liver, and lung.[6] Triple-negative breast cancer (TNBC) accounts for 10-20% of diagnosed breast cancers and is more likely to affect younger African Americans, Hispanics, and/or those with *BRCA1* mutations. TNBCs are more aggressive, difficult to treat, and more likely to spread and recur.[3] TNBCs are different from other kinds of breast cancer in that they are highly metastatic and resistant to conventional therapies, such as anticancer drugs and radiation.[2]

In a search for an agent that inhibits proliferation and invasion of TNBCs, we evaluated an extract derived from an Indian herb, *Withania somnifera* (WS), which is a nightshade medicinal plant that contains active components for the treatment of a variety of ailments, including cancer.[7-10] The use of WS root extract is practical since it contains the active compounds present in the plant. In TNBC cells, sub-cytotoxic concentrations of withaferin A, derived from WS, reduce various effectors of metastasis.[11] In the present study, we assessed the effect of the WS extract on proliferation and metastasis of MDA-MB-231 cells, derived from a TNBC, in cell cultures, and in mice.

**Methods**

**Preparation of WS extract**

Roots of WS were ground to a paste, and then extracted with 5 volumes of 70% ethanol by stirring for 2 days. The alcoholic extract was filtered, and the solvent was evaporated under a vacuum. The extract was then dried to a powder and kept in a closed container until use.[11] To avoid variations in the activity of different preparations, the sufficient extract was obtained in one batch for use throughout the experiments.
Reagents and antibodies

WS roots were purchased from a local market in the USA and dimethyl sulfoxide (DMSO) from Sigma (St. Louis, MO, USA). Antibodies (anti-chemokine CCL2, CXCL1, CXCL2, CXCL3, PARP, and GAPDH) were from Cell Signaling (Beverly, MA, USA). Human breast cancer MDA-MB-231 cell line and a normal breast cell line, MCF10A, were obtained from ATCC (Manassas, VA, USA). The HCA-II human cytokine primer kit was obtained from Real Time Primers (Elkins Park, PA, USA).

Cell culture and treatment

Breast cancer MDA-MB-231 cells were maintained in Dulbecco’s Modified Eagle’s Medium (ATCC) supplemented with 10% fetal bovine serum and penicillin/streptomycin. MCF10A cells were maintained in complete MEGM (Lonza, Houston, TX, USA). All cell cultures were incubated at 37 °C with 5% CO2 in a humidified incubator.

Assessment of cell viability

To assess the effect of the WS extract on regulation of cell viability, cells were seeded into 96-well, 6-well or 6-cm plates at densities of 10^3, 10^4 or 10^5 cells per well, respectively. For experiments requiring longer than 48 h, cell numbers were reduced by one half. Viability was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazoium assay in 96-well plates in triplicate with CellTiter (3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazoium was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazoium assay in 96-well plates in triplicate with CellTiter.

Flow cytometry

Cells treated with the WS extract were harvested and prepared for flow cytometry as described by Samuel et al., with some modifications. WS treated and untreated cells were harvested by trypsinization in 0.25% trypsin/ethylenediaminetetraacetic acid. Prior to trypsinization, floating or loose cells were harvested by gentle rocking of the culture dishes and transferring the culture medium containing the cells into centrifuge tubes. Trypsinized and detached cells were then combined and centrifuged. Cell pellets were suspended in 300 μL of phosphate-buffered saline (PBS), fixed with 700 μL of 100% ethanol with vortexing, and stored at -20 °C overnight. The fixed cells were centrifuged and stained in fluorescence-activated cell sorting staining solution (3 mg/mL RNase A, 0.4 mg/mL propidium iodide) in PBS without calcium or magnesium for 30 min at 37 °C and then filtered through a 70-μm filter and analyzed by flow cytometry (FACScalibur® Becton Dickinson or C6 Accuri® flow cytometer). Data were analyzed with CellQuest and CFlow software (BD).

Immunocytochemistry

Breast cancer MDA-MB-231 cells were seeded in 4-well plates and grown for 16 h. The cells were then treated with DMSO (vehicle) or with 25 or 50 μg/mL of WS root extract for 18 h. After treatment, the culture medium was removed, and the cells were fixed with 10% neutral buffered formalin. Xenograft tissues were placed in an automatic tissue processor, embedded in paraffin, sectioned at 5-μm thickness, and stained with hematoxylin and eosin (HE). For immunohistochemistry, the fixed cells and tissues from xenografted tumors were stained with CCL2 antibody because this cytokine is considered to be most responsible for metastasis of breast cancer. The sections were de-paraffinized in xylene and rehydrated through a series of graded ethanol (100%, 95%, and 70%) and in water for 5 min each. The sections were then washed three times for 5 min each in PBS containing 0.05% Tween 80 (pH 7.4). Antigen retrieval was achieved by heating the sections in a microwave with 0.01 mol/L sodium citrate (pH 6.0) solution and subsequently cooling down to room temperature. Endogenous peroxidase activity was blocked by incubating the sections for 30 min in 1% hydrogen peroxide in methanol. Non-specific binding was blocked by incubating the sections for 1 h with a normal horse serum (Vector Laboratories, Inc., Burlingame, CA, USA). The sections were then incubated with mouse anti-CCL2 (MCP-1, eBioscience, San Diego, CA, USA) overnight at 4 °C. On the next day, the sections were rinsed 3 times with PBS at room temperature and then further incubated with goat anti-mouse IgG-FITC (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. The fluorescence was then read using a wide-field fluorescent microscope (Olympus, Center Valley, PA, USA). Stained sections were reviewed and scored according to the intensity of staining (0, +1, +2 or +3) and for the percentage of tumor cells staining positive for CCL2 (0%, 0.1-30%, +1; 31-70%, +2; or > 70%, +3). The score of the intensity of immunostaining was multiplied by the score of percentage of cell staining to obtain the final staining index.

RNA isolation and quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated from treated and control samples with RNeasy Mini Kits (Qiagen, Valencia, CA, USA) and reversely transcribed into cDNA using Quantitect Reverse Transcriptase Kits (Qiagen) according to the manufacturer’s instructions. All primers were from SABiosciences (Valencia, CA, USA); and quantitative polymerase chain reaction (qPCR) amplification was performed using 50 ng of cDNA, 10 μL of Brilliant III Ultra-Fast SYBR Green qPCR Master Mix.
(Agilent Technologies, Santa Clara, CA, USA), and 500 nM of each primer. β-Actin was used as the internal control, and the final reactions were adjusted to a total volume of 20 µL with DNase RNase-free water (Qiagen). All qPCR amplification was performed in duplicates with a Stratagene Mx 3005P system (Agilent Technologies), and the conditions were set to initial cycle of denaturation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. The final segment involved generation of a dissociation curve. This comprised one cycle at 95 °C for 1 min, followed by 55 °C for 30 s and 95 °C for 30 s. Inclusion of a dissociation curve in each qPCR run ensured specificity of the amplicon.

Microarray analysis

To determine the effect of WS extract on expression of cytokines in MDA-MB-231 cells, cells were incubated overnight with either 50 µg/mL WS or DMSO (vehicle) as a control. The analysis was accomplished by use of HCA-II cytokine primer library II according to the manufacturer’s instructions.

Experimental mice and treatments

Athymic Nude-Foxn1nu mice at 6 weeks of age were obtained from Harlan Sprague-Dawley and housed in animal quarters at 22 °C with a 12 h light/dark cycle. Animals were given free access to water and food. These studies were approved by the Tuskegee University Institutional Animal Care and Use Committee. At 8 weeks of age, mice were injected subcutaneously with 0.2 mL of PBS containing 1.5 × 10⁶ human breast cancer MDA-MB-231 cells into the right flanks. Twenty mice that developed tumor sizes of 50-200 mm³ were divided into two equal groups. The control group received 0.2 mL of 5% DMSO orally by gavage, and the treated group received 300 mg/kg/day WS root extract dissolved in 5% DMSO orally by gavage daily for 5 days a week for 8 weeks. Tumor sizes were checked weekly in each group. Tumor dimensions in mm (length and width) were measured with vernier calipers and calculated for each tumor by using the following equation: tumor volume = (length × width²). At the end of the 8th week, mice were euthanized with CO₂. Tumors and lung tissues were collected and fixed with 10% formalin for histopathological and immunochemistry analysis.

Evaluation of lung metastasis

Two pathologists histopathologically evaluated lung metastases in untreated and treated groups after staining of sections with HE, and the results were reported independently. The number of metastatic foci was counted in each stained tissue section.

Statistical analyses

Student’s t-test was used to assess differences between values for the treated and control groups. One-way analysis of variance was used with Dunnett’s test.

Results

WS extract caused a dose-dependent reduction of viability of breast cancer MDA-MB-231 cells by 75% and 88% after treatment with 50 or 100 µg/mL WS extract, respectively, compared to vehicle-treated controls [Figure 1], but WS treatment did not affect the viability of non-cancerous epithelial mammary cells, MCF10A [Figure 2]. Moreover, compared to untreated controls, WS extract caused a concentration-dependent increase in the sub-G1 phase of the cell population, by 6% and 10% after exposure to 25 µg/mL and 50 µg/mL, respectively [Figure 3]. Furthermore, WS extract inhibited proliferation of xenografted MDA-MB-231 cells, reducing the size of xenografted tumors by 60% compared to the untreated control after 8 weeks of treatment (P < 0.05) [Figure 4]. In addition, after euthanasia, six of ten mice in the control group showed tumor metastasis to the lung, whereas none of the mice in WS-treated group developed metastasized tumor lesions in the lung [Figure 5]. This finding motivated us to explore the underlying molecular mechanism by which the WS extract inhibited tumor metastases to the lung.

Microarray analysis of gene expression of cytokines was then performed. WS suppressed expression of CCL2, CXCL1, CXCL2, CXCL3, IL1B, TGFB3, and BMP4 mRNA [Figure 6]. These inhibitory effects were confirmed by quantitative reverse transcription-polymerase chain reaction analysis [Figure 7]. WS caused a 75% reduction in CCL2 expression (P < 0.05) in the xenografted tumors of treated mice [Figure 8].

Discussion

The current study assessed the effect of an alcoholic extract of WS roots on proliferation and metastasis of...
breast cancer MDA-MB-231 cells in vitro and in nude mice, respectively. WS roots have been used in ayurvedic medicine for their anti-inflammatory, analgesic, anticancer, and anti-stress properties.[7,8] These diverse effects are attributed to the presence of active steroidal compounds that are called withanolides.[15] Our current data showed that the WS extract inhibited proliferation and metastasis of MDA-MB-231 cells in vitro and in nude mice. This inhibition was greater than that caused by withaferin A.[16] The difference in inhibition may be attributed to the fact that the whole extract contains active ingredients that have a synergistic effect against breast cancer cells.[7,17] Since MDA-MB-231 cells are “triple-negative” form estrogen-independent tumors in vivo, the anti-proliferative effect of WS is apparently estrogen-independent. The WS extract caused increases in the percentage of MDA-MB-231 cells in the sub-G1 phase, indicating that WS causes apoptosis. Withaferin A, one of the active compounds of WS, causes G (2)/M cell cycle arrest, associated with modulation of cyclin B1, p34(cdc2), and PCNA levels, decreases the levels of STAT3 and its phosphorylation at Tyr(705) and Ser(727), and alters expression levels of p53-mediated apoptotic markers-Bcl2, Bax, caspase-3, and cleaved PARP.[18]

Results of our current mouse experiments are consistent with in vitro data. The WS extract, administered orally, inhibited formation and growth of MDA-MB-231 cell xenografts in nude mice, indicating that the active ingredients of the WS extract are bioavailable after oral administration.[19] Six mice of the untreated group developed tumor metastasis to the lung, whereas none of the treated mice showed such tumor metastases. This effect may be attributed to inhibition of CCL2 in xenografted tumors after treatment with WS root extract. These results are consistent with a previous study concerning the inhibition of CCL2 in animals. Inhibition of CCL2/CCR2 signaling by anti-CCL2 antibodies blocks recruitment of inflammatory monocytes, inhibits metastasis, and prolongs the survival of tumor-bearing mice. Depletion of tumor cell-derived CCL2 also inhibits metastatic seeding. Moreover, CCL2 mediates development of cancer stem cell (CSC) phenotypes. Promotion of CSC is relevant since these cells, through self-renewal, maintain heterogeneity and give rise to metastasis of breast cancer.[21]

Figure 2: Effect of WS on the viability of non-cancerous epithelial mammary cells, MCF10A. The bars represent the mean ± standard deviation of six 72-h treatments for the vehicle and different concentrations of WS. As determined by one-way ANOVA, results of treated cells are not statistically significant compared to the DMSO-treated (control) cells. WS: Withania somnifera; ANOVA: Analysis of variance; DMSO: Dimethyl sulfoxide

Figure 3: Effect of different concentrations of WS on the cell cycle of MDA-MB231 breast cancer cells. (a) Cell cycle histograms by treatment (vehicle, WS 25 µg/ml and WS 50 µg/ml). Range gates show cell percentage in each cell cycle stage; (b) percentage of cells in cell cycle arrest by treatment. WS: Withania somnifera
Our current data are consistent with those reported by others.\[17\] A root extract of WS showed dose-dependent inhibition of tumor growth and metastatic lung nodule formation with the minimal toxicity to mice.\[17\] The extract apparently inhibited cancer metastasis through inhibition of the epithelial-mesenchymal transition (EMT). Furthermore, withaferin A treatment of MCF-10A cells inhibited EMT and in mice, reduced mammary cancer growth, effects of which were associated with reduced vimentin expression.\[22\] In the present study, the oral dose of WS extract used to inhibit tumor metastasis to the lungs was 300 mg/kg/day body weight. This dose was extrapolated from the cell culture experiments regarding the effect of WS extract on MDA-MB-230 cells. This dose was selected based on a pilot study involving a range of doses to estimate the optimal dose. In addition, the *in vitro* cytotoxic concentration, ranging between 50 and 100 μg/mL, gave us an idea about the dose. In a previous study, WS root extract inhibited lung metastasis of xenografted MDA-MB-231 cells at a dose of 8 mg/kg body weight, administered 3 times a week for 4 weeks.\[19\] This dose is 37.5 times less than the dose used in our current study. There is no obvious explanation for the difference in the two doses. Differences in the source of roots, age of roots, and extraction yield may contribute to different dose-responses when using crude plant extracts. However, the WS extract, at a dose of 150 mg/kg/day for 155 days, caused a 23% reduction in development of mammary tumors in rats administered the carcinogen, methylnitrosourea.\[23\]

In transgenic (MMTV/Neu) mice that received a diet containing the extract (750 mg/kg of diet) for 10 months, mice in the treated group (n = 35) had an average of 1.66 mammary tumors, and mice in the control group (n = 33) had 2.48, a reduction of 33%. Moreover, in treated mice, WS caused a 50% reduction in the expression of CCL2.\[24\]

WS caused *in vitro* and *in vivo* inhibition of breast cancer MDA-MB-231 cells and caused a significant reduction in expression of the cytokine, CCL2, a marker of the metastasis of breast cancer to other organs. These results warrant further studies to assess the underlying mechanisms.
Figure 8: Effect of WS on expression of CCL2 in MDA-MB-231 xenografted tumors. (a and b, ×20) Hematoxylin and eosin sections of untreated and WS treated tumors, respectively; (c and d, ×20) immunohistochemical staining of CCL2 in untreated and WS treated tumors; (e) summarized data. There was a significant reduction ($P < 0.05$) in CCL2 expression in WS-treated tumors compared to untreated tumors as determined by Student’s t-test. WS: Withania somnifera; CCL2: Chemokine (C-C motif) ligand 2

molecular mechanism of WS extract antitumor activity in the breast cancer metastasis.

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References


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