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Withaferin A and sulforaphane regulate breast cancer cell cycle progression through epigenetic mechanisms

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ABSTRACT

Little is known about the effects of combinatorial dietary compounds on the regulation of epigenetic mechanisms involved in breast cancer prevention. The human diet consists of a multitude of components, and there is a need to elucidate how certain compounds interact in collaboration. Withaferin A (WA), found in the Indian winter cherry and documented as a DNA methyltransferase (DNMT) inhibitor, and sulforaphane (SFN), a well-known histone deacetylase (HDAC) inhibitor found in cruciferous vegetables, are two epigenetic modifying compounds that have only recently been studied in conjunction. The use of DNMT and HDAC inhibitors to reverse the malignant expression of certain genes in breast cancer has shown considerable promise. Previously, we found that SFN + WA synergistically promote breast cancer cell death. Herein, we determined that these compounds inhibit cell cycle progression from S to G2 phase in MDA-MB-231 and MCF-7 breast cancer. Furthermore, we demonstrate that this unique combination of epigenetic modifying compounds down-regulates the levels of *Cyclin D1* and *CDK4*, and pRB; conversely, the levels of *E2F* mRNA and tumor suppressor p21 are increased independently of p53. We find these events coincide with an increase in unrestricted histone methylation. We propose SFN + WA-induced breast cancer cell death is attributed, in part, to epigenetic modifications that result in the modulated expression of key genes responsible for the regulation of cancer cell senescence.

1. Introduction

Many advancements have been made with regard to breast cancer treatment and prevention and an area of prevention that has gained increasing interest is alteration of the diet. It is known that cancer can be classified as an epigenetic disease, as many cancers result from environmental factors that promote carcinogenesis as a result of aberrant expression of tumor suppressor genes [1–3]. The epigenetic impact of dietary compounds on cancer is a topic of continuous emerging interest, and there is a need to elucidate the mechanisms behind how dietary compounds are effective. Over the past several years, we have found that sulforaphane (SFN), epigallocatechin gallate, resveratrol, pterostilbene, genistein and others have chemopreventive capability, and the combination of some of these compounds is more efficient than their singular use [4–6]. More recently, we have begun to study withaferin A (WA), a steroidal lactone, in conjunction with SFN [7]. Our previous results show efficacy in the use of these compounds for breast cancer cell death, thus providing merit to study their combined effects in depth. We found there to be synergy with regard to inhibition of cell viability in MCF-7 breast cancer cells. No significant cell death was demonstrated in MCF10A control cells thus indicating the safety of these treatments. We further showed induction of BAX and reduction of BCL-2 after treatment with SFN + WA in cancer cells in addition to changes in DNMTs and HDAC1 expression. The current study has been conducted in an effort to examine regulators of cell cycle progression along with the tumor suppressor genes that are known to be aberrantly expressed in multiple cancer types.

There are several genes that have been identified as potential tumor suppressors and oncogenes; to date, p53 is one of the most studied genes correlated with the inhibition or progression of breast cancer dependent upon its wild type or mutated status, respectively [8–10]. P53 activates the tumor suppressor p21, a cyclin dependent kinase

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inhibitor (CKI). Studies show that DNA damage-induced p21 expression is dependent on p53 [11,12]. Though several studies report p21 to act independently of p53 in some cases [13], it is important to note that in reference to DNA damage these two genes appear to be linked. Another tumor suppressor implicated in the regulation of cell cycle progression is retinoblastoma protein (RB); RB can induce both p53 dependent-and-independent-apoptosis upon inactivation, and is a negative regulator of p21 [14,15].

Several studies indicate that p21 is responsible for the inhibition of cell cycle progression and promotion of apoptosis in some cases [16,17]. We previously reported that combined use of WA and SFN induced apoptosis in both triple negative MDA-MB-231 and ERα positive MCF-7 breast cancer cells; therefore, we hypothesized that these compounds may regulate one or more tumor suppressor genes responsible for cell cycle progression. It is important to note that these two cell lines are considerably different. MCF-7 cells were originally derived from a 69-year-old Caucasian female. These cells are slow growing and have a wild-type p53 status [18]. On the other hand, MDA-MB-231 cells are relatively aggressive in comparison to MCF-7 cells and have a mutated p53 status [19]. Our previous studies found that combinatorial SFN and WA is effective in impeding overexpressed epigenetic genes and enzymes in addition to cellular proliferation in MCF-7 and MDA-MB-231 breast cancer cell lines. Herein, we investigated whether SFN + WA-induced epigenetic changes, i.e., acetylation and methylation, result in the activation of tumor suppressor genes that in turn inhibits cell cycle progression of two breast cancer cell lines.

2. Materials and methods

2.1. Chemicals

R, S-sulforaphane (≥ 98% pure), $C_6H_{11}NOS_2$ with a molecular weight of 177.28 g/mol, was purchased from LKT Laboratories (Minneapolis, MN). Withaferin A (≥ 95% pure), $C_{28}H_{38}O_6$, has a molecular weight of 470.606 g/mol and was acquired from Sigma-Aldrich (St. Louis, MO). Compounds were diluted in dimethyl sulfoxide (DMSO), also purchased from Sigma-Aldrich, and stored in stocks of 10 mmol/L at −20 °C.

2.2. Cell Culture

Cells were cultured using DMEM 1× media supplemented with 10% total volume of fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) and 0.5% total volume of 100× penicillin-streptomycin purchased from Corning Cellgro (Corning, NY). After seeding, cells were allowed 24 h to adhere to plates and all cells used in this study were treated with over a 3-day period with either 5.0 μM SFN, 1.0 μM WA or both. Treatments were refreshed every 24 h with fresh media. A maximum of 1.2 μM of DMSO was used as a vehicle control. The breast cancer cell lines were used in this study were MCF-7 [ERα (+)] and the ERα (-) MDA-MB-231 (ATCC, Manassas, VA).

2.3. Cell cycle analysis

Flow cytometry cell cycle analysis was determined utilizing propidium iodide staining. Cells were harvested and then washed in PBS after which they were fixed with 70% ethanol which was added drop wise while vortexing. After a 30 min fixation at 4 °C, samples were washed twice in PBS and centrifuged at 850 g. Cells were then treated with approximately 50 μL of ribonuclease A at 100 μg/mL. Cells were then sent to the campus Flow Cytometry Center at the University of Alabama at Birmingham and analyzed by measuring the forward and side scatter and pulse processing excluding cell doublets.

2.4. DNA extraction

DNA extracts were prepared using the PureYield Plasmid MiniPrep System from Promega. The manufacturer's protocol was followed accordingly, then the Nano-drop 2000 was used to assess sufficient DNA yields.

2.5. Nuclear protein extraction

Nuclear extracts were prepared using the EpiQuik nuclear extraction kit from EpiGenTek (OP-0002-1) (Farmingdale, NY) and the manufacturer's procedure was followed.

2.6. Protein extraction

Protein was extracted using the TeloTAAAGG Lysis buffer purchased from Roche. Cell pellets were collected after 3-day treatments and centrifuged at approximately 8000 RPM for 5 min. Afterwards media was removed and cells were washed twice with PBS before 200 μL of the lysis buffer was added. Samples were left to incubate on ice for 30 min before centrifugation again for 20 min at 4 °C. Approximately 175 μL of lysate was then transferred to a new collection tube. Samples were stored at −80 °C and protein concentrations were later determined via Bradford assay (Bio-Rad Protein Assay, Bio-Rad; Hercules, CA).

2.7. Quantitative RT-PCR

qRT-PCR was used to determine the mRNA expression of the cell cycle genes of interest. RNA was extracted using the Qiagen RNeasy kit (Valencia, CA) and the manufacturer's instructions were followed. cDNA was made from RNA extracts using the cDNA synthesis kit from Bio-Rad (Hercules, CA). PCR reactions were completed in triplicate using 1 μL of cDNA for each sample. Both forward and reverse primers (1 μL) for the gene of interest were used along with 5 μL of SSO SYBR green from Bio-Rad and 2 μL of nuclease free water for a total volume of 10 μL. Once samples were prepared they were placed in the CFX Connect Real Time System from Bio-Rad upon which the 3-step amplification protocol was selected. Thermal cycling was initiated at 94^{O} C for 4 min followed by 35 cycles of PCR (94 °C, 15 s; 60 °C, 30 s; 72 °C, 30 s). GAPDH was used as an endogenous control in order to calculate fold change using the ΔΔCq method as we reported previously. Primers were purchased from Integrated DNA Technologies (IDT, Coralville, IA) where forward 5′CCGTCCATGCGGAAGATC-3 and reverse 5′-GAAGAC CTCCTCCTCGCACT-3′ were the sequences for *Cyclin D1*. The *CDK4* forward primer sequence was 5′-CTT CTG CAG TCC ACA TAT GCA ACA-3′ and the reverse was 5′-CAA CTG GTC GGC TTC AGA GTT TC-3′, and finally the *E2F* forward and reverse primers were 5′-GTCTGGTTG CTATGGTAGCTGGC-3′; 5′-ACTCCTCGCAGATCGTCATCATCT-3′ respectively.

2.8. Western blot

Protein was loaded onto the Novex NuPage 4–12% premade Bis-Tris gel from Invitrogen and separated by electrophoresis at 200 V until the dye almost ran off the gel. Proteins were then transferred to nitrocellulose membrane using the Trans Turbo Blot from Bio-Rad. Membranes were then blocked in milk buffer [5% dry milk, Tris Buffered Saline (TBS) and 1% Tween (T)] using the Millipore SnapID (Billerica, Massachusetts). Primary antibody incubations were carried out at room temperature for no more than 30 min and membranes were washed four times with 30 mL of TBS + T before probing with secondary antibody for 15 min followed by four more washes. Immunoreactive bands were visualized using an enhanced chemiluminescence detection system (Bio-Rad). Santa Cruz Biotechnology (Dallas, TX) and Cell Signaling Technology (Danvers, MA) were the suppliers of the selected