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Combinatorial Withaferin A And Sulforaphane And Their Potential Roles In Breast Cancer Prevention And Therapy

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COMBINATORIAL WITHAFERIN A AND SULFORAPHANE AND THEIR POTENTIAL ROLES IN BREAST CANCER PREVENTION AND THERAPY

by

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KENDRA JEANINE ROYSTON

BIOLOGY

ABSTRACT

Cancer is a leading cause of death worldwide and is a devastating disease. Breast cancer is of particular interest because it is a major cause of cancer-related fatality in women in the United States. To date, more than 40,000 women are expected to die due to breast cancer and more than 200,000 will be diagnosed every year. Despite numerous advancements in the field, breast cancer remedies can be harsh and while life may be prolonged, the quality of life may decrease. This being said, preventive measures and less harsh treatment options are needed to help eliminate breast cancer as a life threatening disease. This project explores the roles of combinatorial dietary compounds sulforaphane (SFN) and withaferin A (WA) in an effort to elucidate the mechanisms responsible for their efficacy in breast cancer cell death. We provide preliminary evidence that the use of these two compounds in conjunction may serve as ideal candidates for further study with regard to adjuvant therapy and secondary prevention of breast cancer. Our results show that combinatorial WA and SFN causes significant decreases in breast cancer cell viability and promotes apoptosis in two breast cancer cell lines. We further show that these compounds regulate epigenetic modifiers. In addition, we report that tumor suppressor genes are modulated and the genes associated with cell cycle progression are also impeded in response to these treatments. We attribute this regulation of the tumor suppressor genes studied in this project to changes in the epigenome induced by combinatorial WA and SFN.

DEDICATION

I dedicate this dissertation to God and my family. Your numerous prayers, monetary support and faith in my ability to succeed gave me the courage and confidence to complete this chapter in my life. Through the moments of frustration and heartache, I could always count on you all for words of wisdom, encouragement and sound advice. Know that you are appreciated and loved dearly.

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Dr. Wendy Demark-Wahnefried and Dr. Karen Meneses were extremely instrumental in my professional development as a scientist. I am eternally grateful for the opportunities they provided through the Cancer Prevention and Control Training Program and the Komen Graduate Training in Disparities Research program. Finally, I greatly

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GENERAL INTRODUCTION

Cancer is a complex disease that results in uncontrolled proliferation leading to the formation of tumors [1]. Several carcinogenic changes are mediated by the dysregulation of the cell cycle and the suppression of tumor suppressor genes such as p53 and p21 [2-4]. Numerous studies have revealed that epigenetic mechanisms, such as DNA methylation and histone acetylation are responsible for regulating the expression of both p53 and p21 [5-7]. [8]. It is well known that DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) are not only key regulators of transcription and gene expression, and important in cell cycle progression, proliferation and cell survival [9, 10]. Literature has revealed that enzymes such as HDAC1 are required for cell cycle exit and cellular differentiation. In addition, HDACs have been reported to be closely associated with the cyclin dependent kinase inhibitor (CKI) p21. Several studies reveal that HDAC inhibition leads to p21 activation, thereby impeding cell cycle progression [11].

Understanding the mechanisms involved in cancer development is a key step to finding a method to eliminate the disease. With this in mind, it is important to realize that cancer varies from person to person. There are numerous types of cancers and within each type, there are different grades and levels of aggressiveness. To date, breast cancer is the second leading cause of cancer-related mortality in women in the United States. While considerable progress has been made with decreasing mortality annually, the mortality rate of cancer is still unacceptably high. We have only studied two breast

cancer types in this body of work; however, it is important to note the need for studies that address the various other cancer types.

Several studies have been launched in hopes of better understanding ways to make current chemotherapies more effective as well as preventing the acquisition of the disease through the consumption of dietary compounds, which may be responsible for epigenetic modifications to the genome. It has been discovered that cruciferous vegetables contain chemical components that showed promising results in the inhibition of cancer and its elimination as a life threatening disease [19]. Many studies place emphasis on the regulation of p53, due to its roles in the mediation of DNA damage and its mutation in most strains of cancer [2]. As mentioned, cruciferous vegetables and other dietary agents are of extreme interest due to their potential to behave as preventers of cancer and their apoptotic effects on these cells [20].

Chemo-preventive dietary compounds have the ability to inhibit DNMTs and act as histone modifiers, both of which are regulators of gene expression, in addition to altering the epigenome of cancer cells through various mechanisms [21, 22]. Epigallocatechin gallate (EGCG), a polyphenol found in green tea, and sulforaphane (SFN), derived from cruciferous vegetables, in combination with several other compounds such as the soy-bean derived genistein (GE), have been studied in depth and show positive results as potential candidates for improved ways to treat and prevent breast cancer [16, 17, 20, 23]. SFN is an isothiocyanate that shows promising results in chemoprevention and is of extreme interest due to its role in HDAC inhibition [24, 25]. Research also reveals that SFN promotes apoptosis in cancer cells and prevents the continued proliferation of breast cancer cells [26, 27]. Several studies show SFN to work

well in conjunction with other compounds, such as EGCG and GE, at increasing the efficacy of programmed cell death within colorectal cancer, prostate cancer and ovarian cancer cell lines in addition to the regulation of both DNMTs and HDACs.

Withaferin, isolated from a winter cherry that is prevalent in India, also has promising roles in cancer prevention and therapy. The plant, which this fruit is derived, has roots that have been used medicinally for years by the indigenous population due to its wound healing properties. Research shows that the steroidal lactone withaferin A (WA) causes decreased cellular proliferation and viability in colorectal and breast cancer cell lines and is an inducer of apoptosis which has increased interest in this compound as a potential chemotherapeutic agent. In addition, WA is thought to be a DNMT inhibitor [28] as well as a suppresser/ regulator of estrogen receptor alpha (ERα) [29] and NFKβ, a protein complex that controls DNA transcription, cell survival and the production of cytokines [30]. It is possible that WA has the ability to prevent the malignant behavior of tumors and lessen the occurrence of carcinogenic fatality. Withaferin A has also gained popularity due to its promise in inhibition of metastasis and ability to impede angiogenesis, a process instrumental in the formation of malignant tumors [31-33]. It is also important to note that WA is effective at promoting cell death in multiple various breast cancer cell types indiscriminant of ER status. Our lab has previously reported the synergistic effectiveness of the combination of HDAC and DNMT inhibitors in the combat against cancer. We believe that SFN's HDAC inhibitory ability in conjunction with WA's potential DNMT inhibitory abilities is a worthy area of investigation. Here in we study the combined effects of WA and SFN in the inhibition of breast cancer cell

viability while examining the mechanisms influenced by these compounds with the intention of progressing into adjuvant and chemo-prevention studies.

A NOVEL COMBINATION OF WITHAFERIN A AND SULFORAPHANE INHIBITS EPIGENETIC MACHINERY, CELLULAR VIABILITY AND INDUCES APOPTOSIS OF BREAST CANCER CELLS.

by

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ABSTRACT

With cancer often classified as a disease that has an important epigenetic component, natural compounds that have the ability to regulate the epigenome become ideal candidates for study. Humans have a complex diet, which illustrates the need to elucidate the mechanisms of interaction between these bioactive compounds in combination. The natural compounds withaferin A (WA), from the Indian winter cherry, and sulforaphane (SFN), from cruciferous vegetables, have numerous anti-cancer effects and some report their ability to regulate epigenetic processes. Our study is the first to investigate the combinatorial effects of low physiologically achievable concentrations of WA and SFN on breast cancer cell proliferation, histone deacetylase1 (HDAC1) and DNA methyltransferases (DNMTs). No adverse effects were observed on control cells at optimal concentrations. There was synergistic inhibition of cellular viability in MCF-7 cells and a greater induction of apoptosis with the combinatorial approach than with either compound administered alone in both MDA-MB-231 and MCF-7 cells. HDAC expression was down-regulated at multiple levels. Lastly, we determined the combined effects of these bioactive compounds on the pro-apoptotic *BAX* and anti-apoptotic *BCL-2* and found decreases in *BCL-2* and increases in *BAX*. Taken together, our findings demonstrate the ability of low concentrations of combinatorial WA and SFN to promote cancer cell death and regulate key epigenetic modifiers in human breast cancer cells.

Keywords: Sulforaphane, withaferin, breast cancer, HDAC1, DNMTs, chemoprevention, epigenetics.

INTRODUCTION

Epigenetics is the study of changes in gene expression caused by mechanisms other than changes in the underlying DNA sequence. Many studies have taken an epigenetic approach to cancer prevention by focusing on the modulation of the expression of key epigenetically controlled genes [1]. It is known that several cancers are characterized by an overexpression of histone deacetylases (HDACs) and DNA methyltransferases (DNMTs). Each of these epigenetic enzymes has varying roles. The inhibition and regulation of these enzymes, as well as the genes that control their expression, is at least partially responsible for decreased cell viability and regulation of tumor suppressor genes in several cancer types [2-4]. Due to the promising role of the inhibition of epigenetic modifiers in cancer cell death, chemotherapies with epigenetic targets have been FDA-approved and are being used in the clinical setting [5].

Breast cancer, one of the leading causes of death in women in the United States, has an incidence rate of more than 200,000 new cases and a mortality rate of about 40,000 women per year [6]. Numerous investigations have been launched with the intent to better understand novel approaches to enhance current chemotherapies as well as preventing the acquisition of the disease through the consumption of dietary compounds, which may be responsible for epigenetic modifications to the genome. Recently, Esmaeili reported that epigallocatechin gallate (EGCG), a component of green tea, is responsible for the reversal of chemoresistance in breast cancer cells [7]. Moreover, our studies have indicated that genistein, a soybean isoflavone, is instrumental in the reactivation of estrogen receptor alpha (ERα) in triple-negative breast cancer cells, which enhanced the efficacy of hormone therapy in these cells [8]. The regulation of DNMTs and HDACs

was shown to be an important factor in ERα conversion in these cells. In addition, sulforaphane (SFN) can be effective in the inhibition of several different cancer types in part through its ability to serve as an epigenetic modifier [9-13].

Sulforaphane (SFN) and withaferin A (WA)

SFN is an isothiocyanate found in cruciferous vegetables that has shown promising results in chemoprevention and is of high interest due to its role in HDAC inhibition [14, 15]. This dietary bioactive compound promotes apoptosis and prevents the continued proliferation of breast cancer cells through various mechanisms. For example, SFN can work well in conjunction with other compounds, thereby increasing the efficacy of programmed cell death and the regulation of epigenetic processes within many different cell lines [16, 17]. Withaferin A (WA), a withanaloid isolated from a winter cherry prevalent in India, has promising roles in cancer prevention and therapy. The plant from which this compound is derived has roots that have been used medicinally for years by the indigenous population due to its wound healing properties.

WA is a steroidal lactone that can lead to decreased cellular proliferation and viability in certain cancer cell lines, regulate inflammatory pathways, and is an inducer of apoptosis, all of which have piqued the interest in use of this compound as a potential chemotherapeutic agent [18-21]. In contrast, however, less is known about the epigenetic roles of WA, although some studies have found that it behaves as a DNMT inhibitor [22]. This compound has also received much acclaim due to its promise in the inhibitory effects of angiogenesis, which is a fundamental step in the formation of malignant tumors [23]. Thaiparambil *et al.* have shown WA to be effective in the inhibition of breast cancer invasion and metastasis through its ability to induce vimentin disassembly [18]. It may be

possible that WA has the ability to prevent the malignant behavior of tumors while lessening the incidence of carcinogenic fatality. In this study we aimed to investigate the impact of combinatorial SFN and WA on MCF-7 estrogen receptor-positive ER (+) and MDA-MB-231 ER (-) breast cancer cell proliferation in conjunction with their role in the epigenetic gene expression of DNMT1, 3A, 3B and HDAC1. The present study is the first to show changes in the expression of epigenetic modifiers using these two compounds in combination at such low concentrations.

MATERIALS AND METHODS

Cell lines

The ERα (+) MCF-7 and ERα (-) MDA-MB-231 breast cancer cells were selected for this study. MCF10A human mammary epithelial cells were used as a non-cancerous control (ATCC, Manassas, VA).

Chemicals

Withaferin A (≥ 95% pure) was purchased from Sigma-Aldrich (St. Louis, MO), R, S-sulforaphane (≥ 98% pure) was acquired from LKT Laboratories (Minneapolis, MN) and SAHA was purchased from Sigma-Aldrich (≥98% pure). Each compound was diluted in dimethyl sulfoxide (DMSO) and stored in stocks of 10 mmol/L at -20^{O} C.

Cell Culture and treatment

MCF-7 and MDA-MB-231 were both cultured using DMEM 1X media in addition to 10% total volume of fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville GA) and 1% total volume of 50X penicillin streptomycin (Corning Cellgro, Manassas VA).

MCF10A cells were cultured using DMEM F12 media in addition to 5% Donor Horse Serum, 100 µL of 20 ng/ml EGF, 50 µL of 100 ng/mL cholera endotoxin, 100 µL of 0.05 µg/mL hydrocortisone, 0.292 g of 2 mmol/L L-Glutamine and 5 mL of 100 units/mL penicillin streptomycin. Cells were maintained in a humidified environment at 5% CO_2 and 95% air at 37^{O} C. Cells were sub-cultured at approximately 90% confluency. After seeding, cells were allowed 24 h to adhere to plates after which they were treated over a one or three-day period with SFN, WA or both at the indicated concentrations. Treatments were replenished every 24 h with fresh media. DMSO was used as a vehicle control of which the maximum concentration was 1.2 µM. SFN and WA were stored as 10 mm stock solutions at -20°C.

Cell Density Assay

Approximately 200,000 cells were plated in 6-well plates. Upon the 24 h incubation period, treatments with WA and SFN were administered over a three-day period during which media was replaced accordingly. On day five after plating, cells were viewed under a microscope and images were taken at 100X or 40X magnification.

MTT Assay

Percent viability was determined by counting the number of viable cells in each well via the uptake of tetrazolium, 3-(4, 5-dimethylthiazol-2-yl)- diphenyl tetrazolium bromide (MTT) (Sigma Aldrich, St. Louis, MO). The living cells cause a dark purple color to appear due to a formazan reaction initiated by the mitochondrial enzymes of the cells. Approximately 2000 cells were seeded in triplicate and allowed to incubate for 24 h to adhere to the 96-well plates. The cells were treated over a one or three-day period as described above. On day three or day five after plating, 50 µL of MTT (1mg/mL)

dissolved from 5 g/L in PBS wash buffer was added and allowed to incubate at 37^{O} C for 3 h after which the MTT reagent was removed and DMSO was added to each well. A microplate reader (model 680, Bio-Rad) with the absorbance set to read at 595 nm was then used to obtain the values that determined % viability.

RNA isolation

RNA was extracted using the RNeasy kit from Qiagen (Valencia, CA) according to the manufacturer's instructions.

Protein extraction

RIPA Lysis Buffer from Upstate Biotechnology (Charlottesville, VA) was used to prepare protein extracts according to the manufacturer's protocol.

Quantitative real time PCR

qRT-PCR was used to determine the expression of specific genes of interest. RNA was reverse transcribed to cDNA 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 iTaq 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^{O} C, 15s; 60^{O} C, 30s; 72^{O} C, 30s). GAPDH was used as an endogenous control in order to calculate fold change using the ΔΔCq method described by Chen *et al* [17]. Primers were purchased from Integrated DNA Technologies (IDT, Coralville, IA), and sequences are listed in Table I**.**

Table I: qRT-PCR primer sequences

DNMT1 Sense: 5'-AACCTTCACCTAGCCCCAG-3'	DNMT1 Anti-sense: 5'-CTCATCCGATTTGGCTCTTCA-3'
DNMT3A Sense: 5′-TATTGATGAGCGCACAAGGC-3′	DNMT3A Anti-sense: 5′-GGGTGTTCCAGGGTAACATTGAG-3′
DNMT3B Sense: 5′-TGGTACATGGCTTTTCGATAGGA-3′	DNMT3B Anti-sense: 5′-GGCAAGTTCTCCGAGGTCTCTG-3′
HDAC1 Sense: 5′-CTGTCCGGTATTTGATGGCT-3′	HDAC1 Anti-sense 5′-CACGAACTCCACACACTTGG-3′
BAX Sense: 5'-TGG AGCTGCAGAGGATGATTG-3'	BAX Anti-sense: 5'-GAAGTTGCCGTCAGAAAACATG-3'
BCL-2 Sense: 5′-CATGCTGGGGCCGTACAG-3	BCL-2 Anti-sense: 5′-GAACCGGCACCTGCACAC-3′
GAPDH Sense: 5′-TGCACCACCAACTGCTTAGC-3′	GAPDH Anti-Sense: 5′-GGCATGGACTGTGGTCATGAG-3′
Caspase 3 Sense: 5'-TTAATAAAGGTATCCATGGAGAACACT-3'	Caspase 3 Anti-Sense: 5'-TTAGTGATAAAAA TAGAGTTCTTTTGTGAG-3'.

Primers were purchased from Integrated DNA Technologies (IDT) using the indicated primer sequences.

Annexin V apoptosis assay FACS

The induction of apoptosis in breast cancer cells via WA and SFN was quantitatively determined using flow cytometry and the Annexin V—conjugated Alexafluor 488 (Alexa488) Apoptosis Vybrant Assay Kit (Life Technologies, Carlsbad, CA). After treatment, cells were harvested using the digestive enzyme trypsin. Upon detachment,

cell pellets were collected via centrifugation. PBS wash buffer was used to wash pelleted cells twice, and after washing, cells were incubated with Alexa488 and propidium iodide (PI) for cellular staining in annexin binding buffer for 10 min in the dark at room temperature. The stained cells were analyzed by FACS by using a FACS-Caliber instrument (BD Biosciences, San Jose, CA) equipped with Cell Quest 3.3 software.

Western blot analysis

Protein expression was determined with the use of Western blotting. Protein extracts were prepared by RIPA Lysis Buffer as mentioned previously. Bradford assays were performed to determine the protein concentration (Bio-Rad Protein Assay, Bio-Rad; Hercules, CA). The protein was loaded onto a 4-15% premade Tris-HCl gel from Bio-Rad, and separated by electrophoresis at 200 V until the dye ran off the gel. Separated proteins were then transferred to nitrocellulose membrane using the Trans Turbo Blot from Bio-Rad. Membranes were then blocked in 5% dry milk in TBS solution with 1% Tween (TBST) using the Millipore SnapID (Billerica, Massachusetts). Primary antibody incubations were carried out at room temperature and membranes were washed four times with 30 mL of TBST before probing with secondary antibody for 1 h 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 antibodies.

DNMTs activity assay

After treatment with WA and SFN accordingly, nuclear extracts were prepared using the EpiQuik nuclear extraction kit from EpiGenTek (OP-0002-1). DNMTs activity was

determined via the EpiQuik DNA Methyltransferase Activity/ Inhibition Colorimetric Assay Kit (P-3009) following the manufacturer's procedures (Farmingdale, NY).

HDACs activity assay

Nuclear extracts were prepared as mentioned above, and the EpiQuik HDAC Activity/ Inhibition Colorimetric Assay Kit (P-4002) was used. The assay was performed according to the provided protocol from EpiGenTek (Farmingdale, NY).

CompuSyn

The CompuSyn version 1.0 software was downloaded from http://www.combosyn.com/ and used to determine synergism of the combinatorial WA and SFN. A combination index (CI) value greater than 1 denotes antagonism, a value below 1 indicates synergism and a value at one indicates an additive effect of the compounds being assessed [24, 37].

Statistical Analysis

Error bars represent standard error of the mean (SEM). Each assay was completed in triplicate culturing experiments with 3 or 4 technical replicates. The student's t test was used to determine significance.

RESULTS

Combinatorial WA and SFN promote cell death

As seen in Figure 1 (A-B) treatments were administered for one and three day intervals. At one day, cancer cells were unaffected by treatments; however, after three days each of the compounds administered individually was able to induce cancer cell death as indicated through decreases in cell viability with MTT analysis and increases in the induction of apoptosis but had a greater impact when used in combination. Both cell lines show these compounds to be effective in promoting cancer cell death after three

days (Figure 1, D-E). No significant effects of these compounds administered singly or in combination were observed after three days (Figure 1, C) on control MCF10A cells, indicating the relative safety of these compounds at the low concentrations that were employed. As evidenced through cell density analysis in Figure 2, both MCF-7 and MDA-MB-231 breast cancer cell lines show an increase in surface area with the incorporation of the predetermined optimal concentrations of 1.0 µM WA and 5.0 µM SFN in comparison to the MCF10A non-cancerous control cells. Moreover, the combination of administered 1.0 µM WA and 5.0 µM SFN was more effective than either of the compounds acting alone. Using CompuSyn software analysis [24] we observed a synergistic effect with our combined concentrations in the MCF-7 ER(+) cells and an additive effect with combinatorial WA and SFN in MDA-MB-231 ER(-) breast cancer cells (Table II).

Table II: CompuSyn data of MTT values indicate combinatorial synergy in MCF-7 cells

Cell Lines	SFN Dose	WA Dose	Average CI
MCF-7	5.0 µM	1.0 µM	0.715503
MDA-MB-231	5.0 µM	1.0 µM	1.000525

Using the software CompuSyn Version 1.0 by Ting Chao Chou and Nick Martin to determine combination index (CI) [24], we were able to show synergy (CI <1) with combinatorial WA and SFN treatments of MCF-7 cells and an additive effect (CI ~1) for the MDA-MB-231 breast cancer cells.

Figure 1

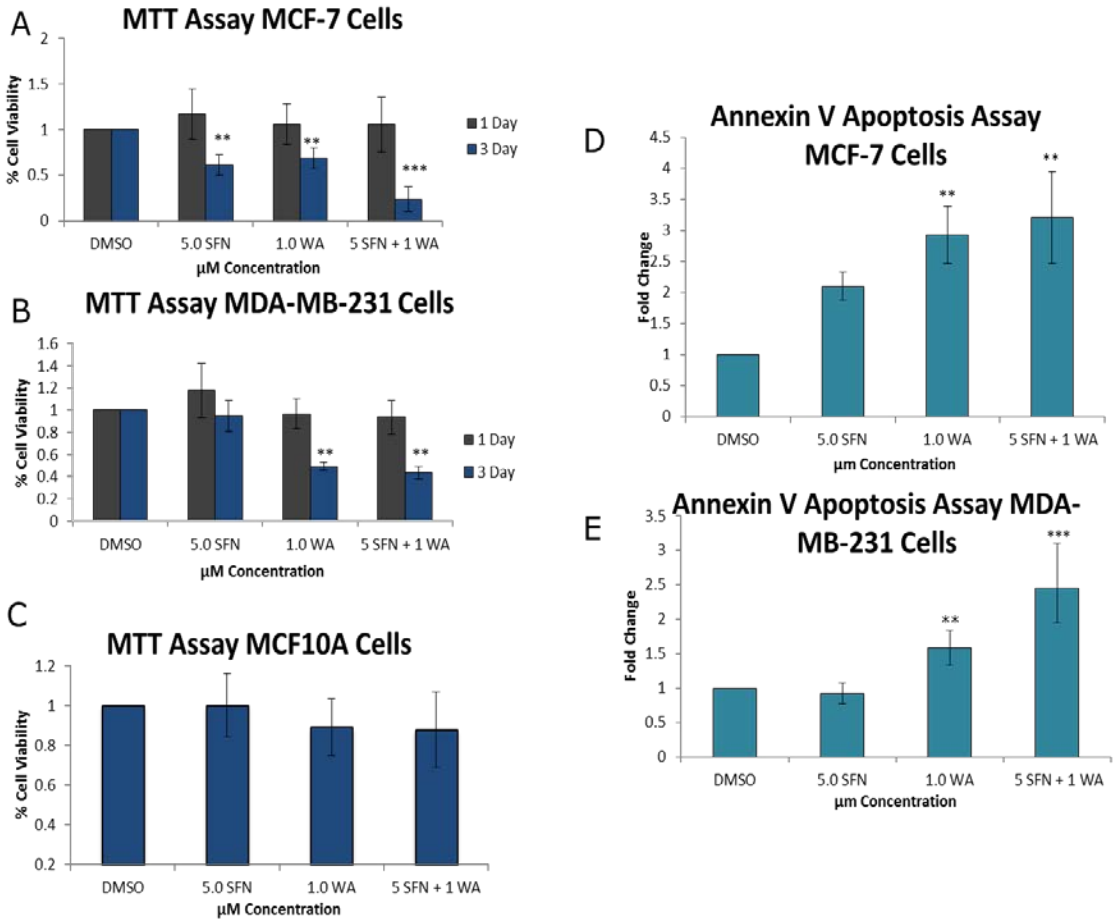

Figure 1: Combinatorial WA and SFN decreases cellular viability and promotes apoptosis in breast cancer cells. A. MTT assays of MCF-7 breast cancer cells were performed using either 5.0 µM SFN, 1.0 µM WA, or both compounds at the indicated concentrations for a period of either 1 or 3 days. B. MTT assays were performed on MDA-MB-231 cells for 1 or 3 days at the indicated concentrations.

C. MTT assay was performed on MCF10A cells for 3 days using the same concentrations mentioned previously. D. Annexin V Apoptosis assay employing FACS analysis was completed on MCF-7 cells using 5.0 µM SFN, 1.0 µM WA, or 5.0 µM SFN + 1.0 µM WA for 3 days. **E.** Apoptosis assay was completed on MDA-MB-231 breast cancer cells using 3-day treatments at the indicated concentrations of SFN, WA or both compounds ($p<0.05$*, $p<0.01$**, $p<0.001$***). The results represent 3 separately cultured experimental replicates. Numbers on the X-axis indicate compound concentrations.

Figure 2

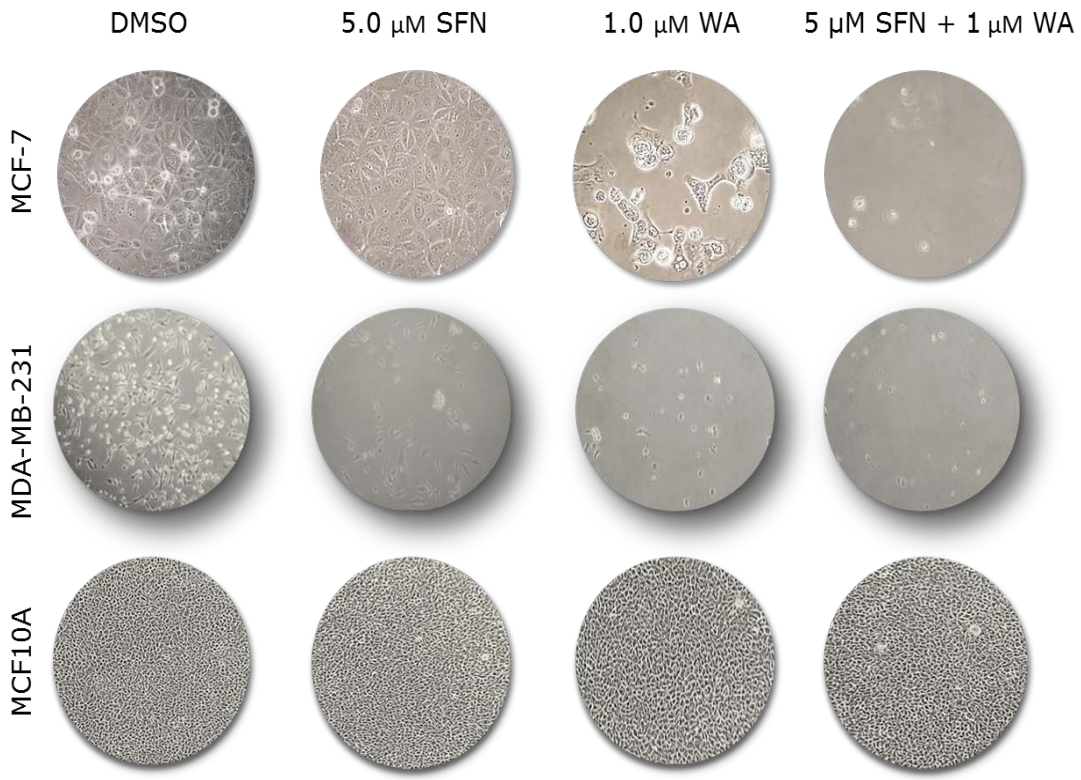

Figure 2: Combinatorial WA and SFN promote increases in surface area of breast cancer cells. MCF-7 and MDA-MB-231 breast cancer cells as well as the non-cancerous MCF10A cells were treated with predetermined optimized concentrations of 5.0 μM SFN or 1.0 μM WA singly and both compounds at the same concentrations for 3 days. Photographs were taken on the fifth day of culture.

Combinatorial WA and SFN administration decreases HDAC expression and promotes varying changes in DNMT expression

In an effort to understand some of the mechanisms underlying our observations we next sought to determine any changes in the expression of known epigenetic modifiers, DNMTs and HDACs, in the treated cells. In Figure 3 we demonstrate decreases in enzymatic activity of DNMTs in both cell lines. The combination treatment of SFN and WA in MCF-7 cells is more effective in the inhibition of DNMT activity than singly administered SFN but not WA, and in MDA-MB-231 cells the combination treatment effect was highly significant, more so than with the singly administered WA. Clearly these compounds are capable of modulating DNMTs activity in at least two commonly used cell types of breast cancer.

In an effort to examine specific DNMTs we performed quantitative real time PCR on *DNMT1, 3A and 3B* as seen in Figure 4. WA decreased *DNMT1* mRNA expression in MCF-7 cells, and this was more pronounced with the combinatorial treatments of SFN + WA (Figure 4A). In MDA-MB-231 cells the combinatorial treatment also led to a significant decrease in DNMT1 expression with varying effects on *DNMT1* expression by the compounds administered singly (Figure 4B). Due to these varying effects on *DNMT1* and the results from the DNMTs activity analysis we decided to determine if there were any changes in *DNMT3A* and *DNMT3B*. It can be noted that *DNMT3A* and *3B* mRNA expression is down-regulated in an extremely significant manner in both cell lines (Figure 4, C-F).

Figure 3

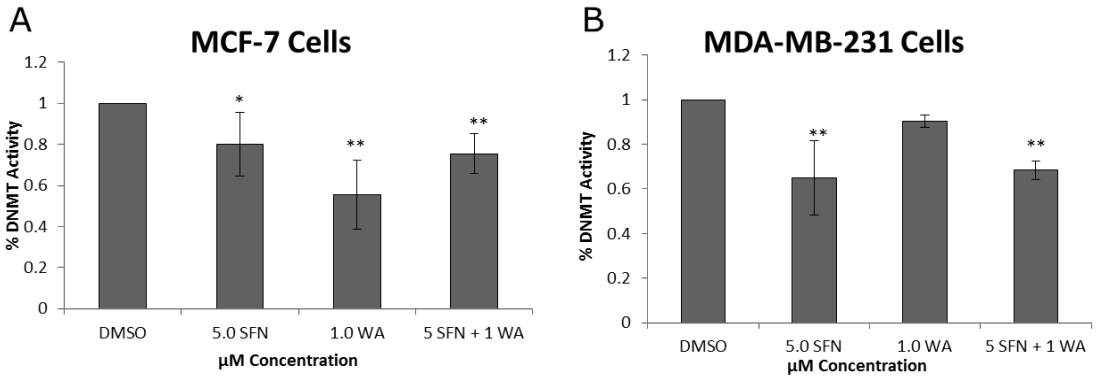

Figure 3: Combinatorial WA and SFN affect enzymatic activity of DNMTs. **A.** DNMT activity assays were conducted using nuclear extracts that were prepared after 3-day treatments with the indicated concentrations in MCF-7 cells. **B**. DNMT activity was assessed in the same way using MDA-MB-231 cells. Depicted results are the means of 4 separately cultured experiments. ($p<0.05$*, $p<0.01$**, $p<0.001$***)

Figure 4

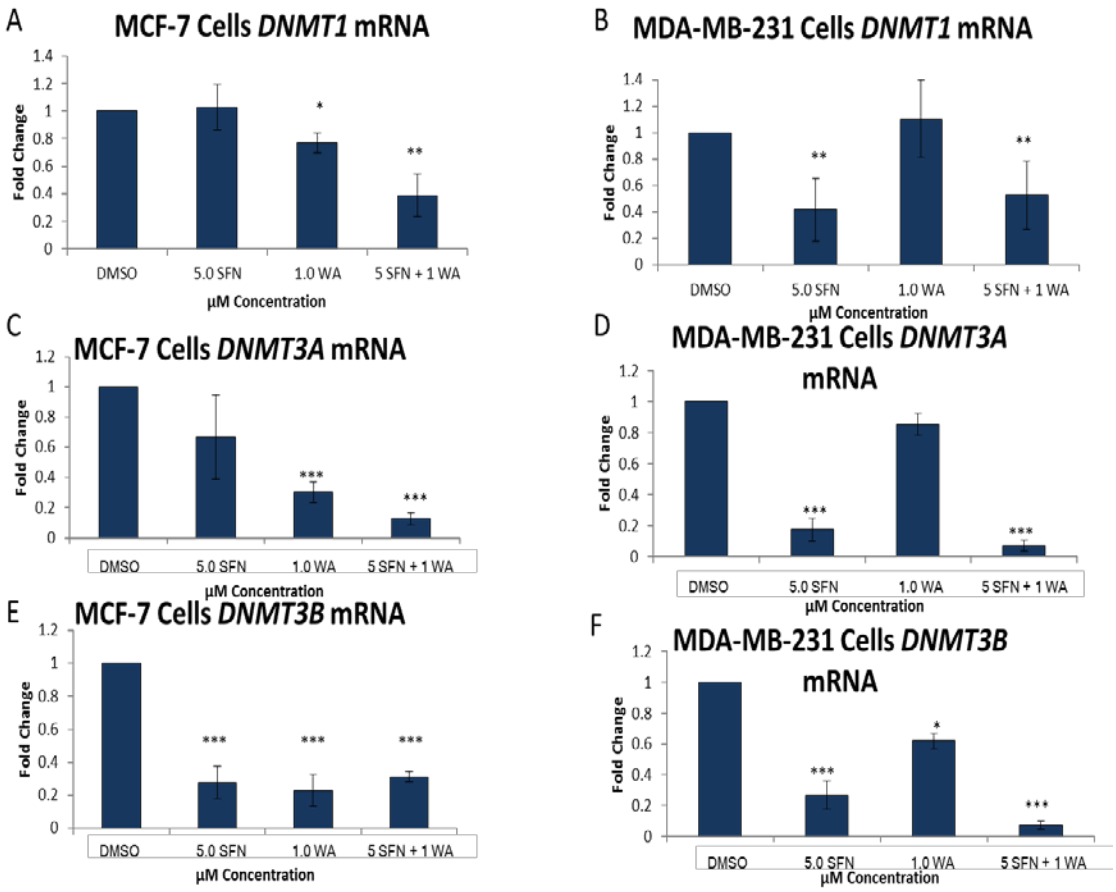

Figure 4: Combinatorial WA and SFN induce changes in the mRNA expression of *DNMTs.* A. qRT-PCR was completed using MCF-7 cells after 3 day treatments of the indicated compounds was conducted using *DNMT1* forward and reverse primers (n=3). *GAPDH* was used for comparison. B. The same was done in MDA-MB-231 cells (n=4). C. qRT-PCR was performed in MCF-7 cells using *DNMT3A* primers. (n=3) D. qRT-PCR was performed in MDA-MB-231 cells using *DNMT3A* primers (n=3). E. *DNMT3B* mRNA expression was determined in MCF-7 cells. F. *DNMT3B* mRNA expression in MDA-MB-231 cells (n=3). ($p<0.05$*, $p<0.01$**, $p<0.001$***)

We also determined the protein expression of each of these DNMTs as shown in Figure 5 and show that the SFN + WA treatments were effective in the inhibition of each DNMT1, 3A and 3B in comparison to the DMSO control. Next we sought to determine the effects of WA and SFN on HDACs and found significant decreases in HDACs activity in both cell lines with the incorporation of our compounds (Figure 6); however, the MDA-MB-231 cells do not show greater significance after combinatorial treatment (Figure 6B). A downward trend in the mRNA expression fold change of HDAC1 was observed at all tested concentrations in both cell lines (Figure 6 C-D). This was highly significant for the combinatorial treatments of SFN + WA. In Figure 7 it can be noted that Western blot analysis revealed that HDAC1 was down-regulated posttranslationally with the incorporation of the selected compounds and that this effect was most apparent in the combination treatments in both MCF-7 and MDA-MB-231 breast cancer cells.

Figure 5

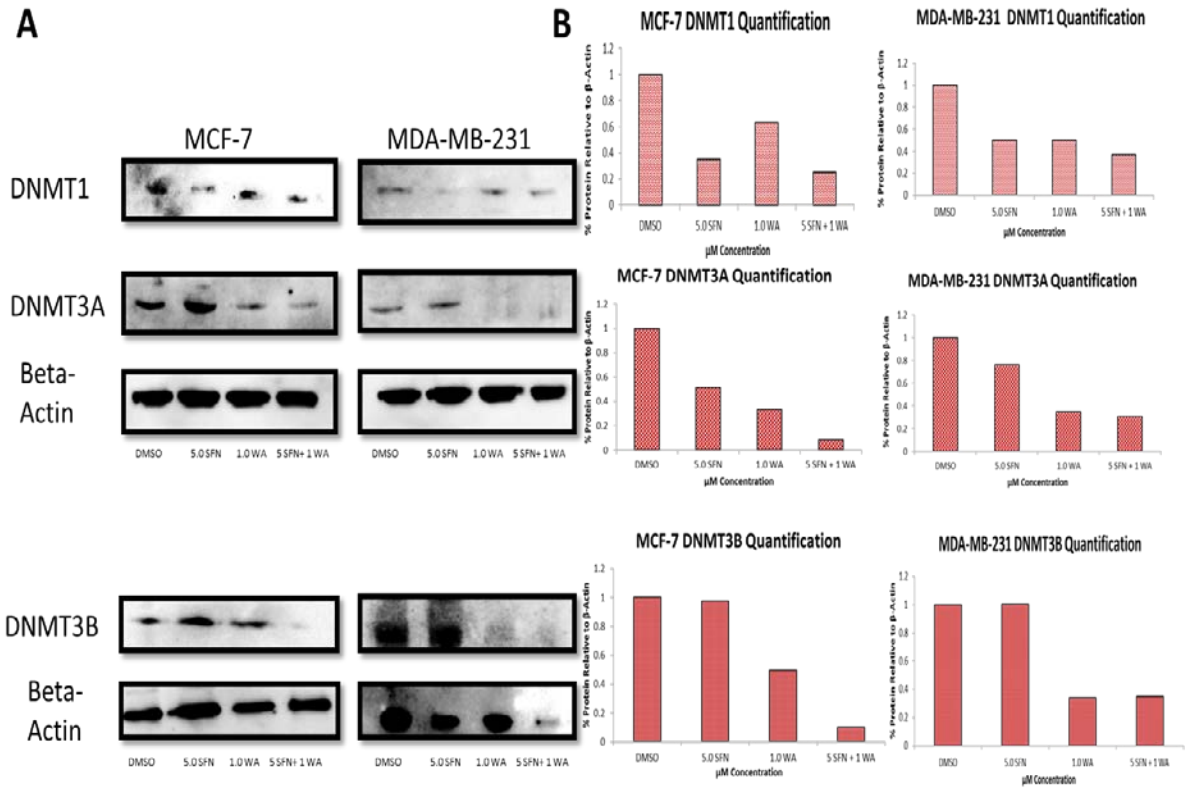

Figure 5: Combinatorial WA and SFN promote decreases in DNMT protein expression. A. Representative images of the protein expression for DNMT1, 3A and 3B in both MCF-7 and MDA-MB-231 breast cancer cells are shown. Western blots were completed after 3-day treatments of the indicated concentrations and probed with the corresponding antibodies. B. Quantification was performed using the averages of multiple blots using ImageJ software.

Figure 6

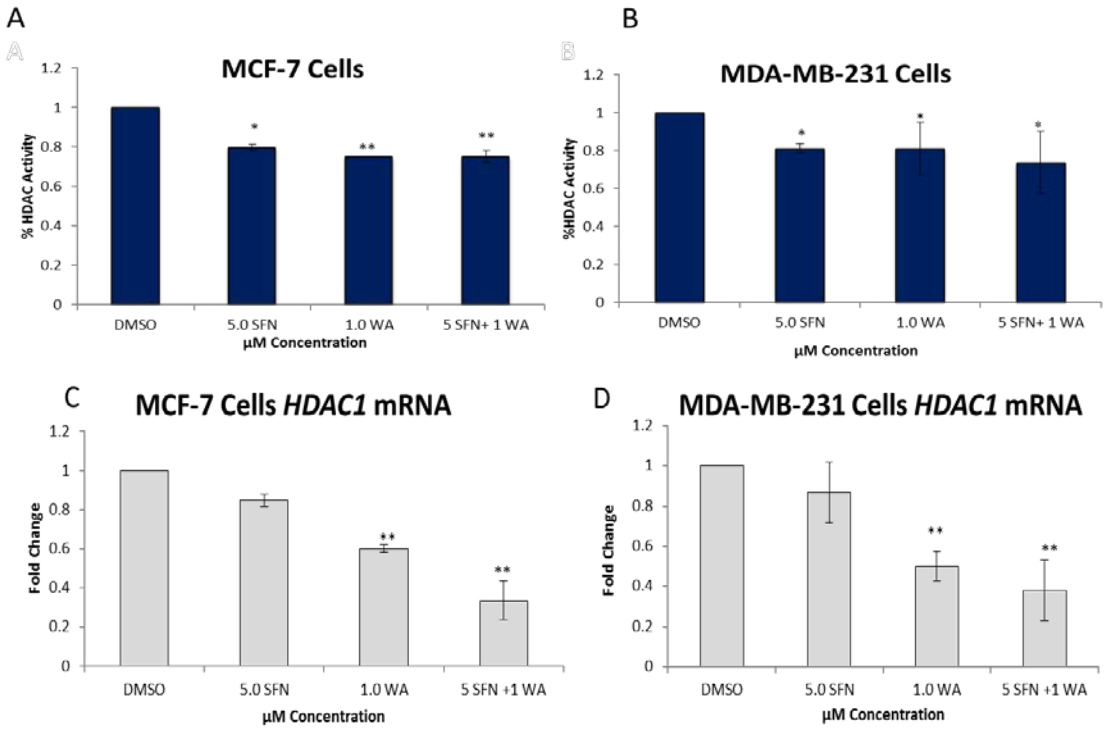

Figure 6: Combinatorial WA and SFN down-regulate HDACs activity and mRNA expression. A. HDAC activity/ inhibition assays were performed using nuclear extracts from 3-day treatments of MCF-7 cells at the indicated concentrations. B. MDA-MB-231 cell HDAC activity assays were performed using the same methodology described above. C. qRT-PCR was completed to determine the mRNA expression of *HDAC1* in MCF-7 cells. D. *HDAC1* mRNA expression is shown in MDA-MB-231 cells. The results represent the means of 3 separately cultured experimental replicates. ($p<0.05$*, $p<0.01$**, $p<0.001$***)

Figure 7

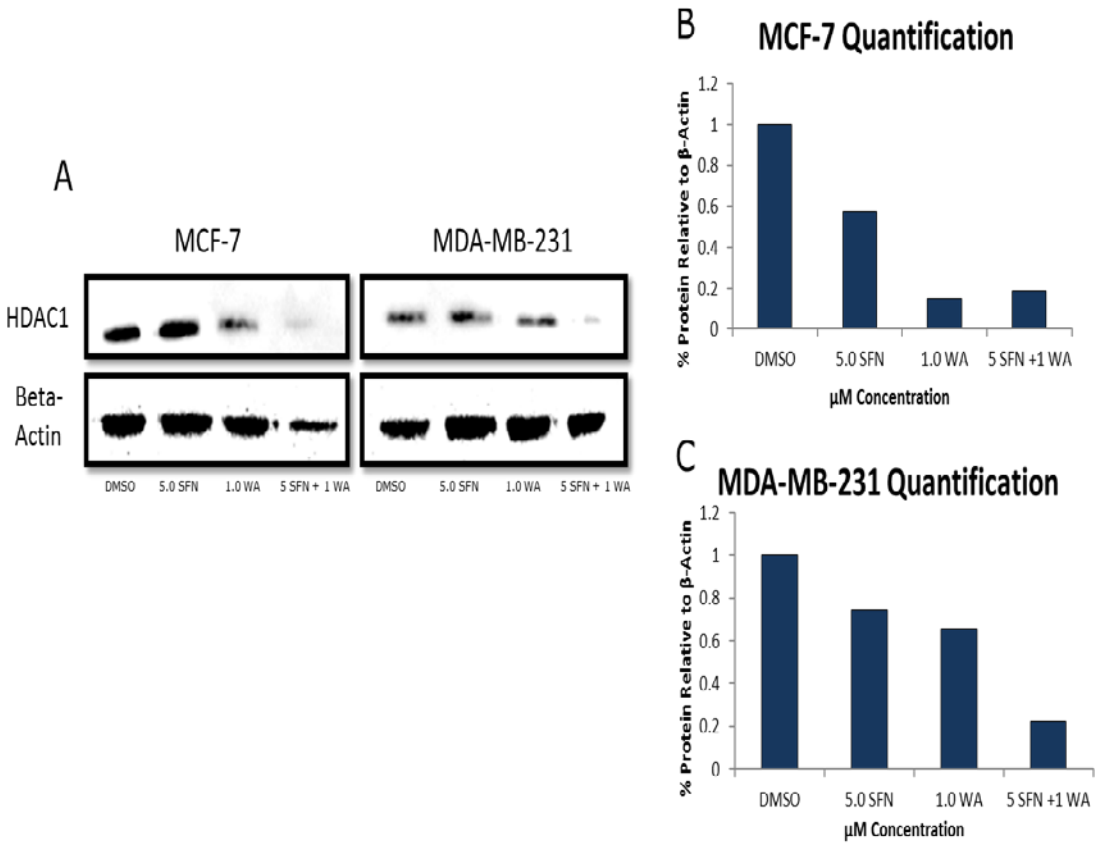

Figure 7: Combinatorial WA and SFN change the expression of HDAC1 at the protein level. A. MCF-7 cells (left) and MDA-MB-231 cells (right) were treated for 3 days at the indicated concentrations and images are representative. Protein was extracted and used to perform Western blot analysis of HDAC1. B. ImageJ was used to quantify results. The results are presented in comparison to β-actin as indicated via the bar graph for MCF-7 cells. C. HDAC1 protein quantification was completed in MDA-MB-231 cells.

Combinatorial WA and SFN induce changes in BAX and BCL-2

BAX and BCL-2 have been shown to be inversely associated with one another. Due to the ability of combinatorial WA and SFN to promote apoptosis in both MCF-7 and MDA-MB-231 cells we sought to determine the expression of both BAX and BCL-2 (Figure 8). Our results show BAX expression to be induced whereas BCL-2 expression is inhibited. The consistent downward trends found in HDAC expression in both cell lines led us to believe that combinatorial WA and SFN decrease cell viability and promote apoptosis in part through their ability to inhibit HDAC1. In Supplementary Figure 1 we demonstrate the ability of suberoylanilide hydroxamic acid (SAHA) to decrease cell proliferation in both MCF-7 and MDA-MB-231 cells as was shown with combinatorial WA and SFN (Figure 1). SAHA is an HDAC inhibitor that is clinically approved and marketed as Vorinostat for the treatment of cutaneous T-cell lymphoma (CTCL). Because this is a synthetic compound, we expected to see some reduction in viability in our noncancerous control MCF10A cells. Using MCF-7 and MDA-MB-231 cells there is a significant decrease in cellular viability beginning at 2 μM SAHA and continuing through 7 μM SAHA (Supplementary Figure 1 (SF1)). We show in Figure 9 that these compounds, in a similar fashion to SAHA, affect both apoptotic genes, *BAX* and *BCL-2*, while repressing *HDAC1*. It can be noted that while SAHA inhibits *HDAC1* expression, combinatorial WA and SFN are more effective in the MDA-MB-231 cells (Figure 9A). In contrast, SAHA is more effective than SFN + WA in the inhibition of *HDAC1* expression in MCF-7 cells (Figure 9D). Figures 9B and 9E show SFN + WA to be effective inducers of *BAX* expression to a greater degree than SAHA in MDA-MB-231 cells (Figure 9B); *BAX* expression is decreased with SAHA treatment in MCF-7 cells (Figure 9E). Figure

9C demonstrates the ability of SAHA and SFN + WA to decrease *BCL-2* expression in the ER (-) MDA-MB-231 cells. The same is shown in Figure 9F for the ER (+) MCF-7 cells.

Figure 8

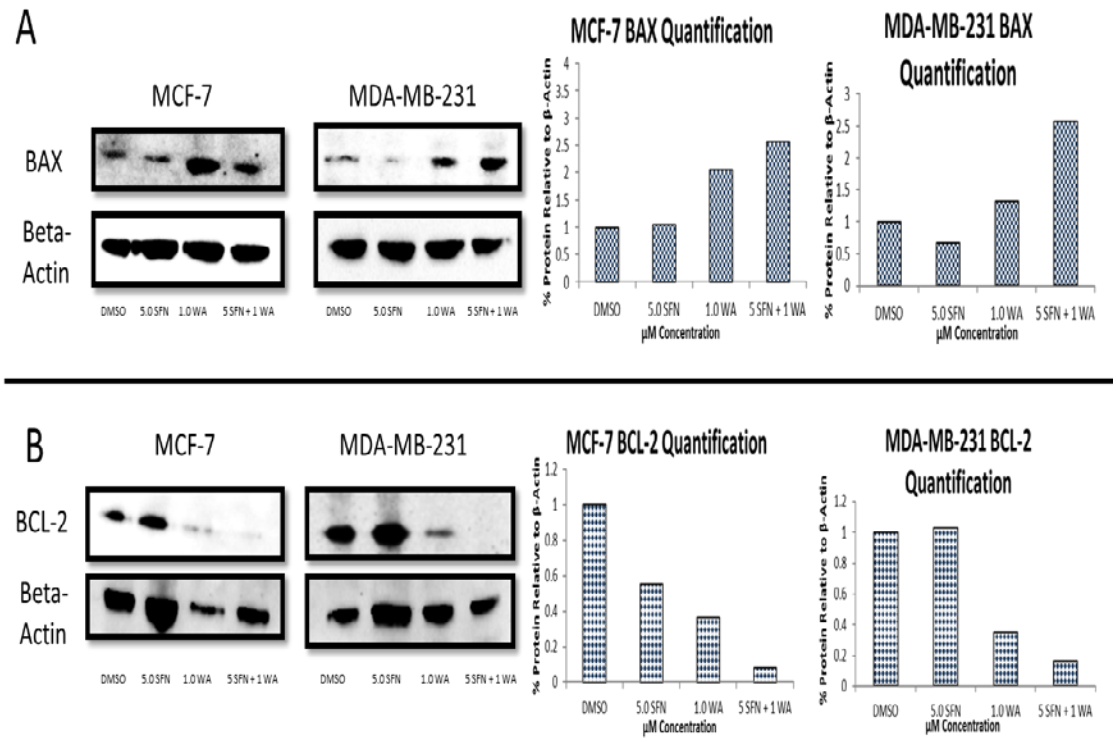

Figure 8: Combinatorial WA and SFN induce changes in the protein expression of the pro-apoptotic BAX and anti-apoptotic BCL-2. A. BAX protein expression in both MCF-7 and MDA-MB-231 cells is induced with the incorporation of the natural compounds. Image is representative and quantification is indicative of the averages of 3 different blots. **B.** BCL-2 protein expression in both MCF-7 and MDA-MB-231 is reduced with the incorporation of the natural compounds. Image

is representative and quantification is indicative of the averages of 3 different blots.

Figure 9

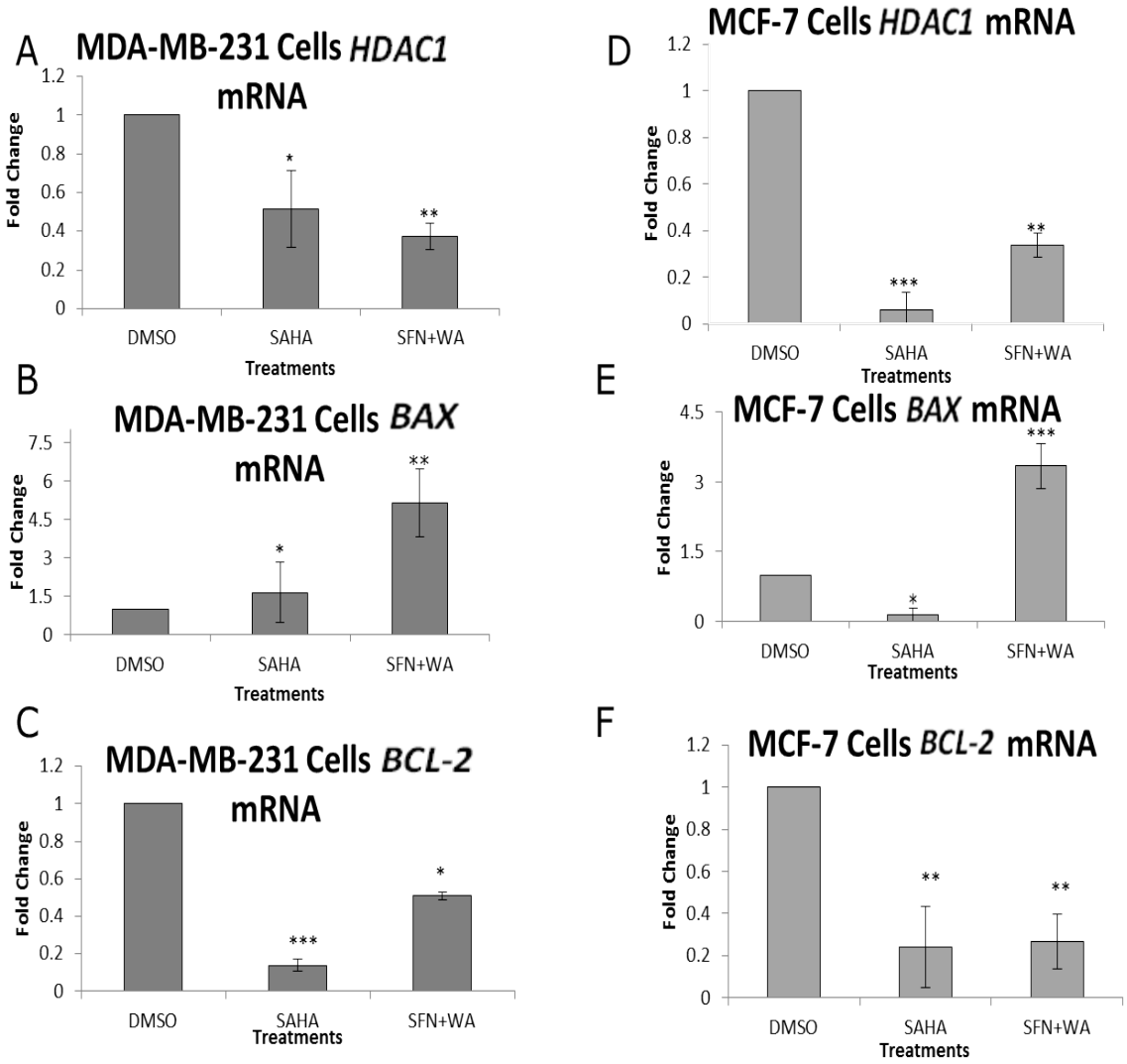

Figure 9: SAHA and combinatorial SFN+WA promote changes in apoptotic genes at the mRNA level. A. qRT-PCR was used to determine the mRNA expression of HDAC1 in MDA-MB-231 triple-negative breast cancer cells in

comparison to the 3.0 µM optimal concentration of SAHA. SFN and WA concentrations are 5.0 µM and 1.0 µM respectively. B. mRNA expression of the pro-apoptotic *BAX* is shown in MDA-MB-231 cells. C. qRT-PCR shows the expression of the anti-apoptotic *BCL-2* in MDA-MB-231 cells. D. HDAC1 mRNA expression in MCF-7 cells shows changes with the incorporation of the chosen compounds. E. mRNA expression of the pro-apoptotic gene BAX is upregulated by combinatorial SFN and WA. F. The anti-apoptotic gene *BCL-2* shows a downward progression in MCF-7 cells with the incorporation of the indicated drugs and compounds (n=3: SEM, $p<0.05$*, $p<0.01$**, $p<0.001$***).

DISCUSSION

For the first time we report the epigenetic effects of combinatorial WA and SFN in any cancer type. This study is of particular interest due to the increasing awareness of the effects of dietary compounds on epigenetic changes in cancer. We report that combinatorial WA and SFN were more effective than either compound alone in decreasing cellular viability and promoting apoptosis in both MCF-7 ER (+) and MDA-MB-231 ER (-) breast cancer cells at relatively low concentrations (Figure 1). Synergy from this unique approach using combined WA and SFN in cancer cells was detected in MCF-7 cells and we found additive effects in the MDA-MB-231 cells (Table I). Previous studies show SFN to be an effective HDAC inhibitor. Specifically, Clarke *et al*. reported SFN to be an effective inhibitor of several class I and II HDACs. In their study they compared normal prostate cells with cancerous and hyperplastic prostate cells and demonstrated a selective induction of cell cycle arrest along with selective decreases in HDAC activity using a 15 μM concentration of SFN [10]. In addition, our lab observed SFN in combination with a green tea polyphenol (epigallocatechin gallate, EGCG) and found the compounds to work well in combination at decreasing colony forming potential and increasing apoptosis in chemo-resistant ovarian cancer cells. It was hypothesized and demonstrated that regulation of human telomerase reverse transcriptase (hTERT) and BCL-2 may serve as explanations for increases in apoptosis of ovarian cancer cells with the incorporation of combined SFN and EGCG [16].

In this current study we chose a much lower concentration of SFN to study in conjunction with WA, which may also have a significant impact on HDAC activity. To date there have been very limited studies implicating WA as an epigenetic modifier, and

those that do have varying reports. Mirza and colleagues reported decreases in the transcript levels of *DNMT1, 3A* and *3B* with the incorporation of WA, and use their findings to suggest that WA may have beneficial therapeutic effects against cancer through its ability to reverse changes in the epigenome [22]. In contrast, Szarc Vel Szic *et al.* were unable to show WA induced decreases in DNMTs [25]. In our study, we show variances between the different DNMTs with respect to the mRNA and protein levels with the treatment of WA and SFN. According to Dov Greenbaum and colleagues this is quite common, and what is found at the gene level is not a direct correlation of what may be found at the protein level. Along with there being several complex mechanisms involved in converting mRNA to protein, proteins also differ drastically in their half-lives [26].

In an effort to gain clarity about what effects our chosen compounds have on breast cancer cells we sought to determine the role of WA on HDACs and DNMTs. Several studies have outlined the importance of DNMT1 and HDAC1 in tumor cell growth and development, hence the use of epigenetic inhibitors in the clinic [4, 27, 28]. One explanation for decreases in cellular viability induced by our compounds could be associated with the changes we observed in DNMTs and HDACs expression. DNMT and HDAC activity assays were conducted to assess changes in these enzymes and to gain a general understanding of the effects of SFN and WA on the overall enzymatic activity of DNMTs and HDACs in breast cancer cells. Here we report significant decreases in overall DNMT and HDAC enzymatic activity in both MCF-7 ER (+) and MDA-MB-231 ER (-) breast cancer cells with the introduction of WA and SFN. To further analyze DNMTs and HDACs we assessed key epigenetic modifiers, DNMT1, 3A, 3B and

HDAC1, and found decreases at both the mRNA and protein levels in one or both breast cancer cell lines. Our results indicate that combinatorial WA and SFN work extremely well in the inhibition of HDAC1 in both cell lines. An explanation for the less significance in the HDACs activity assay in the MDA-MB-231 cells when comparing these results (Figure 6D) to the results in Figure 6B could be attributed to the fact that the activity assay is an assessment of overall enzymatic activity and there may be other HDACs that are contributing to our findings. The same can be noted with regard to the DNMTs (Figure 3 and Figure 4) as we show with the examination of DNMT3A and 3B.

Our data demonstrate that combinatorial WA and SFN are effective in the inhibition of cell viability irrespective of ER status. Varying efficacy with respect to HDACs and DNMTs is to be expected due to the differing characteristics of each cell line. Previous studies show WA to be an inhibitor of ERα [29], while SFN is an activator [30]. As it stands, these compounds could be competing with each other with regard ER, which in turn is causing the differential effects in DNMTs and HDACs. Although there were significant differences in HDACs and DNMTs expression in these cells in response to WA and SFN, the combination of the two compounds resulted in even greater induction of apoptosis and less cell viability in both breast cancer cell lines. This implies that there are yet other factors that contribute to cell death initiated by these compounds. Several reports have shown that both WA and SFN are effective in the inhibition of pro-inflammatory cytokines, as well as the aberrant expression of epigenetic modifiers [11, 13, 31-33]. Moreover, Hahm and colleagues reported that WA-induced apoptosis was mediated through reactive oxygen species and Nagalingam *et al.* found that WA inhibited breast tumor formation *in vivo* through the activation of the ERK/RSK axis, DR5

upregulation, and elevated nuclear accumulation of Elk1 and CHOP in breast cancer [19, 34].

We assessed the pro-apoptotic gene *BAX* and the anti-apoptotic gene *BCL-2* with combinatorial WA and SFN as well as singly administered SAHA and found there to be an inverse relationship in these treated breast cancer cells (Figure 9). Where *HDAC1* was decreased with our compounds in comparison to the FDA-approved chemotherapeutic SAHA (Figure 9A, 9D) we demonstrate an induction of *BAX* (Figure 9B, 9E) and a reduction of *BCL-2* with SFN + WA (Figure 9C, 9F). Interestingly, SFN + WA induced *BAX* expression to a greater extent than SAHA in MCF-7 cells. We recognize that many mechanisms may contribute to *BAX* induction. As seen in Figure 4A, SFN + WA affect DNMT1 expression greater than either compound alone in the ER (+) MCF-7 cells. Future studies may show that the combined effect of HDACs and DNMTs may be involved in *BAX* regulation in the MCF-7 cells. Nonetheless HDAC1 was down-regulated in both ER (+) and ER (-) cell lines. This finding supports the claim that HDAC1 regulation by combinatorial WA and SFN is responsible in part for induction of apoptosis in breast cancer cells.

In 2014 Xu, Chen and colleagues reported synergistic apoptotic effects with the combination of a synthetic HDAC inhibitor and DNMT inhibitor [35]. With the varying reports on WA being a DNMT inhibitor, we found merit in studying this compound. We confirm WA to be capable of inhibiting DNMTs to an extent and this compound shows synergy in reduction of cell viability when used in conjunction with SFN, a well-documented natural HDAC inhibitor. We hypothesize that the combined efficacy of these natural compounds on breast cancer cell death can be attributed in part through their

impact on the epigenome. To begin establishing this we examined the clinically approved HDAC inhibitor SAHA and found similar trends in comparison to combinatorial WA and SFN with the natural compounds being more effective in the promotion of the pro-apoptotic gene BAX, which is promising considering the numerous side effects associated with SAHA. This further confirms that the inhibition of both HDACs and DNMTs through the use of this novel combination of compounds (SFN+WA) may serve as a less harsh treatment option or preventive measure for breast cancer upon further study.

The current study has provided a basis of support behind the rationale to study WA and SFN in more depth with regard to specific epigenetic mechanisms. Our results support the role of combinatorial WA and SFN in the regulation of HDACs and also DNMTs, which are instrumental in a number of cancer developmental processes. Studies show WA to regulate mechanisms involved in the apoptotic pathway and our findings provide a framework to begin establishing epigenetic linkage of the combined WA and SFN with HDAC1 and cell cycle progression in cancer [20, 31, 34, 36]. Future studies will focus on assessing more genes in association with epigenetic modifiers with the intent of providing a stronger association between HDAC1 and DNMTs and their regulation by combinatorial WA and SFN. In an effort to gain a better understanding of the epigenetic mechanisms involved in the changes induced by combinatorial WA and SFN, we intend to examine tumor suppressor genes that have been linked to epigenetic regulation by determining if there are any changes at the promoter region of the specified genes after treatment with these two compounds.

CONCLUSION

In summary, WA and SFN are two compounds that have been shown to be effective inhibitors of cancer cell growth; however, the literature is limited with respect to WA and its regulatory roles on key epigenetic modifiers. Prior studies have also not yet addressed the effects of either of these compounds in conjunction with one another. We report greater efficacy of these compounds in combination with regard to breast cancer cell death and down-regulation of overexpressed HDAC1, DNMT3A and 3B. We believe that further study of combinatorial WA and SFN may have translational significance through their potential to serve as ideal candidates for prevention of breast cancer-related fatality.

Author Contributions

KR and TT designed and conceived the experiments. KR, NU, and KL conducted the experiments. KR and TT analyzed the results. KR and TT contributed to reagents/materials/analysis tools. All authors reviewed and approved the final manuscript.

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Conflicts of Interest

The authors disclose that there are no conflicts of interest.

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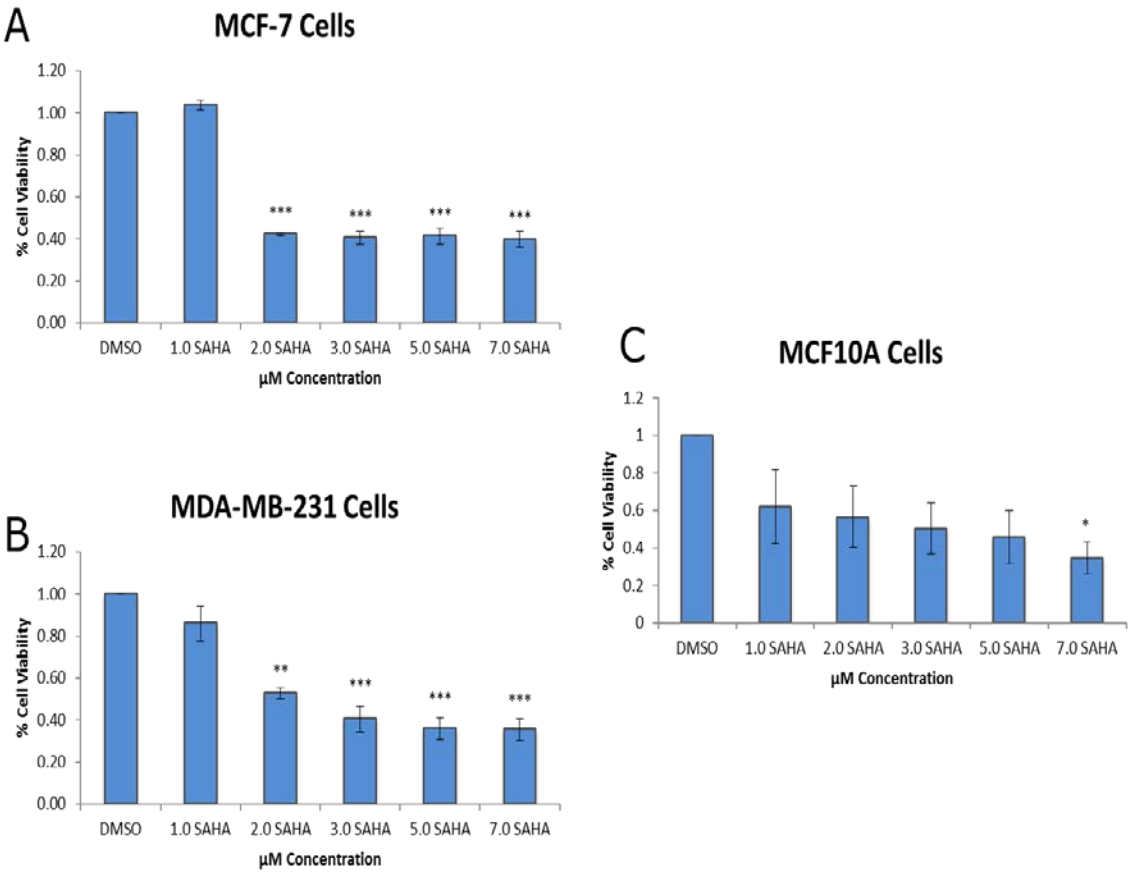
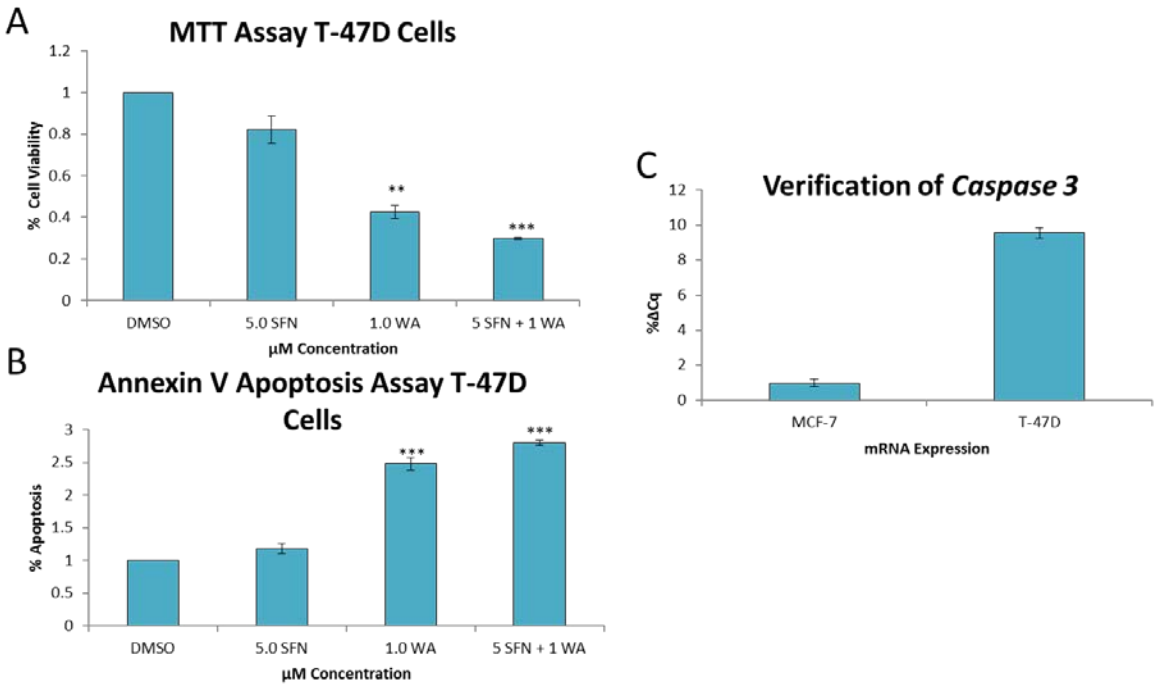
Supplementary Figure 1



Supplementary Figure 1: FDA-approved HDAC inhibitor SAHA decreases cellular viability in MCF-7 and MDA-MB-231 breast cancer cells. A. MTT assay of MCF-7 cells indicates decreases in cell viability at increasing concentrations of SAHA. B. MDA-MB-231 cells show decreases in viability at increasing concentrations of SAHA. C. The non-cancerous MCF10A cells show statistically insignificant decreases in cell viability except for the relatively high concentration of 7 µM SAHA. (n=3: SEM, $p<0.05$*, $p<0.01$**, $p<0.001$***)

Supplementary Figure 2



Supplementary Figure 2: SFN and WA promote cell death in T-47D breast cancer cells. A. MTT assay of T-47D cells indicates decreases in cell viability with the incorporation of the indicated compounds after 3 days. B. FACS analysis demonstrates an increase in apoptosis caused by combinatorial WA and SFN after 3 days. C. Cells were treated for 3 days with DMSO. qRT-PCR verifies that the caspase 3 gene is expressed in T-47D breast cancer cells (n=3: SEM, $p<0.05$*, $p<0.01$**, $p<0.001$***).

COMBINED WITHAFERIN A AND SULFORAPHANE REGULATE BREAST CANCER CELL CYCLE PROGRESSION THROUGH EPIGENETIC MECHANISMS

by

KENDRA J. ROYSTON, BIDISHA PAUL, SUSAN NOZELL, RAJANI RAJBHANDARI AND TRYGVE O. TOLLEFSBOL

Submitted to Experimental Cell Research

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.

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 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 MDA-MB-231 and MCF-7 breast cancer cells; therefore, we hypothesized that these compounds may regulate one or more tumor suppressor genes responsible for cell cycle progression. Our previous studies also found that combinatorial SFN and WA is effective in impeding overexpressed epigenetic genes and enzymes in addition to cellular proliferation. 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.

MATERIALS AND METHODS

Chemicals

R, S-sulforaphane (≥ 98% pure) was purchased from LKT Laboratories (Minneapolis, MN) and Withaferin A (≥ 95% pure) from Sigma-Aldrich (St. Louis, MO) was acquired.

Compounds were diluted in dimethyl sulfoxide (DMSO) and stored in stocks of 10 mmol/L at -20^{O} C.

Cell Culture

Cells were cultured using DMEM 1X media supplemented with 10% total volume of fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) and 0.5% total volume of 100X penicillin streptomycin purchased from Corning Cellgro (Corning, NY). After seeding, cells were allowed 24 h to adhere to plates and all cells for 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. Two breast cancer cell lines were used in this study, MCF-7 (ERα (+)) and the ERα (-) MDA-MB-231 (ATCC, Manassas, VA).

Cell Cycle Analysis

Flow cytometry cell cycle analysis was determined utilizing Propidium iodide staining. Cells were harvested 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 ºC, samples were washed twice in PBS and centrifuged at 850g. 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.

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.

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.

Protein Extraction

Protein was extracted using the TeloTAAAGG Lysis buffer purchased from Roche. Cell pellets were collected after 3 day treatments and spun 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 being spun down again for 20 min at 4 ºC. Approximately 175 µL of lysate was then transferred to a new collection tube. Samples were stored at -80 ºC and protein concentrations were later determined via Bradford assay (Bio-Rad Protein Assay, Bio-Rad; Hercules, CA).

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^{O} C, 15s; 60^{O} C, 30s; 72^{O} C, 30s). GAPDH was used as an endogenous control in order to calculate fold change using the ΔΔCq method described in our previous paper. Primers were purchased from Integrated DNA Technologies (IDT, Coralville, IA) where forward 5'CCGTCCATGCGGAAGATC-3 and reverse- 5'-GAAGACCTCCTCCTCGCACT-3 were the sequences for Cyclin D1. 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'GTCTGGTTGCTATGGTAGCTGGC-3'; - 5'-ACTCCTCGCAGATCGTCATCATCT-3' respectively.

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 antibodies.

Global Methylation Activity Assay

The MethylFlash Global DNA Methylation (5-mC) ELISA Easy Kit (Colorimetric) from EpiGenTek was used to assess overall DNA methylation activity from DNA extracts that were gathered using the methodology described in the above section.

Histone Acetyltransferase Activity/ Inhibition Assay

Histone acetyltransferase enzymatic activity was determined utilizing the Histone Acetyltransferase Activity/ Inhibition Assay from EpiGenTek and the provided protocol was followed.

Histone Methyltransferase Activity/Inhibition Assay

Histone methyltransferase activity was assessed via the HMT Activity Assay kit from EpiGenTek. Nuclear extracts were prepared as described above and the manufacturer's protocol was used to assess HMT activity.

Chromatin Immunoprecipitation (ChIP) Analyses

ChIP assays were performed as previously described [18]. Cells were grown as described above and nuclei from cross-linked cells were resuspended in Tris/EDTA. The soluble chromatin was adjusted into RIPA buffer and precleared with salmon sperm blocked protein A beads. Immunoprecipitation was performed with 5 µg of antibodies directed against trimethylated lysine 4 of histone 3 (H3K4Me3), or IgG, as described [18]. Immune complexes were absorbed with protein A beads blocked with salmon sperm DNA. After pre-clearing and before immunoprecipitation, equal amounts of sonicated DNA (10% volume of each sample) were reserved for qPCR (input) analysis. The CDKN2A promoter was probed with specific primers against the immunoprecipitated DNA by qPCR using primers sets based on known sequences, based on known mouse

sequences. Reactions for each sample were performed in triplicate using an ABI StepOnePlus Detection System and a PCR protocol comprising an initial 10-min incubation at 95˚C followed by 40 cycles of 15s at 95˚C and 1 min at 60-65˚C. The raw data were analyzed using StepOnePlus software and ΔΔCt values for each gene in each sample were determined.

Statistical Analysis

Error bars represent standard deviation (SD). Each assay was completed in triplicate culturing experiments with 3 or 4 technical replicates. The student's t test was used to determine significance where a $p<0.5$ is significant.

RESULTS

WA and SFN regulate cell cycle progression through inhibition of cell cycle genes.

Figure 1 demonstrates the effects of SFN and WA on the cell cycle in two drastically different breast cancer cell lines. Cells were treated as described previously [7] and as indicated in figure 1A, SFN and WA alone show a decrease in transition into the G2/M phase of the cell cycle, however the combination of SFN and WA arrests cells primarily at the G1 phase in MCF-7 cells. Similarly, MDA-MB-231 cells (figure 1B) show a decrease in transition into S phase with the incorporation of the combination of SFN and WA, with SFN appearing to be more effective than WA. Due to an increase in G1 arrest in both cell lines, we found merit in analyzing cell cyclin D1 (*CCND1*), cyclin D kinase 4 (*CDK4*), and *E2F*. These genes are known to have various roles in the cell cycle.

Figure 1

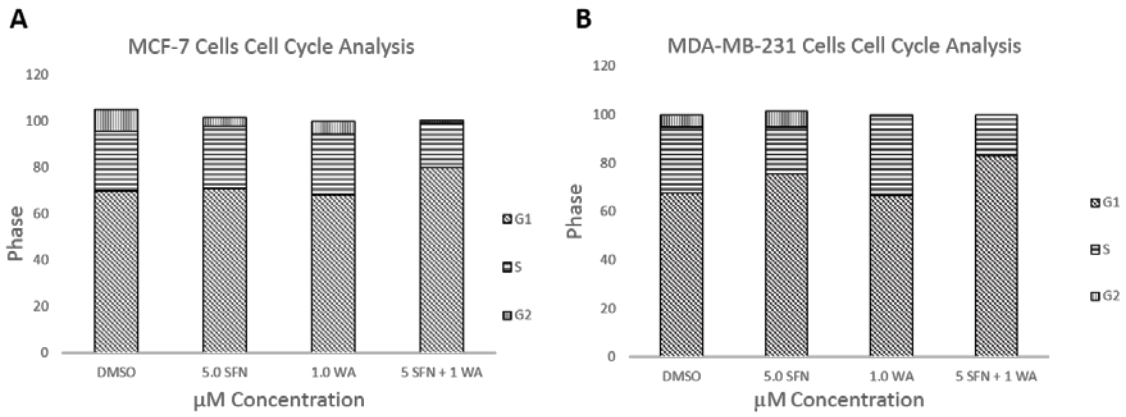


Figure 1: Combinatorial SFN and WA arrest cells most abundantly at G1: A. Combinatorial SFN and WA arrest cells at G1 phase and prevent transition into G2 in MCF-7 cells. B. InMDA-MB-231 breast cancer cells it can be noted that the incorporation of these compounds prevent transition into G2 phase (n=3).

Much of the literature suggests that CCND1 regulates cell cycle progression through its ability to promote transition from G1 to S phase. CDK4 and E2F are also closely associated with these changes in the cell cycle. Upon analyzing changes in these genes using qRT-PCR, we found that *CCND1* and *CDK4* expression was significantly decreased with the incorporation of SFN and WA alone in both MCF-7 and MDA-MB-231 breast cancer cells (Figure 2). Interestingly, the combination of these compounds was not more effective than the single dosages as we originally hypothesized. Figures 2C and 2F show a comparable increase in *E2F* in both cell lines. We further observed retinoblastoma protein. Much of the literature suggests that RB is another cell cycle regulator instrumental in the regulation of the G1 check point. We report a decrease in phosphorylated RB (pRB) upon introduction of SFN and WA in both MCF-7 and MDA-MB-231 cells with the combination being most effective as seen in figure 3.

Figure 2

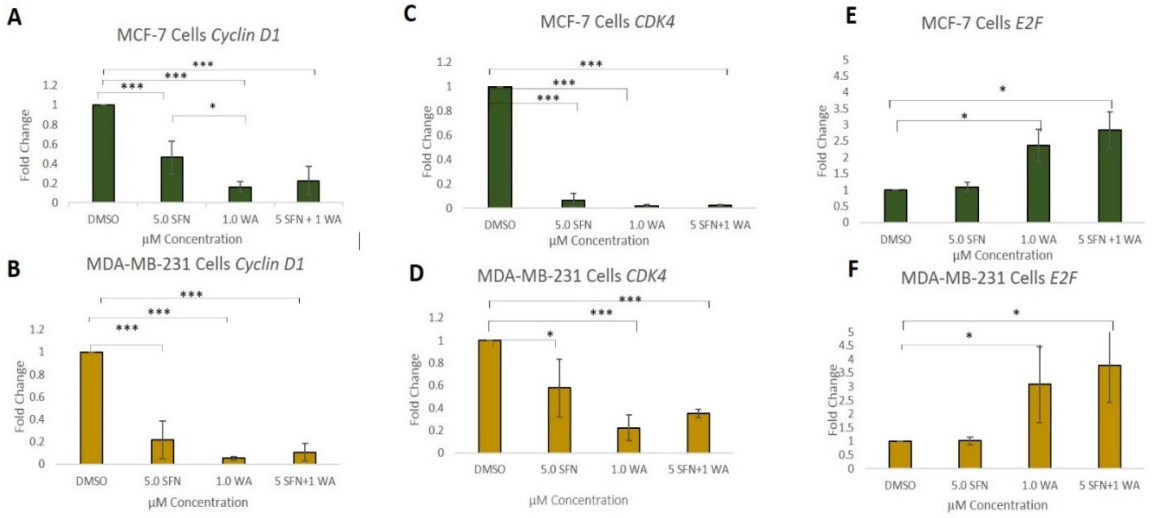


Figure 2: Cell cycle genes are regulated by WA and SFN at the mRNA level: A. Combinatorial SFN+WA down regulate cyclin D1 in an extremely significant manner but not significantly more than the single dosages in MCF-7 breast cancer cells. B. Cyclin D1 is also down-regulated in MDA-MB-231 breast cancer cells yet the combination is not significantly different from the signal dosages. C. CDK4 in MCF-7 Cells shows a downward trend however SFN+WA does not show greater significance than the single dosages. D. CDK4 in MDA-MB-231 Cells. E. E2F gene expression in MCF-7 cells is significantly upregulated in comparison to the control with the introduction of WA and the combo. F. E2F mRNA expression in MDA-MB-231 cells is significantly increased in comparison to the control (n=3; $p<0.05$*, $p<0.01$**, $p<0.001$***).

Figure 3

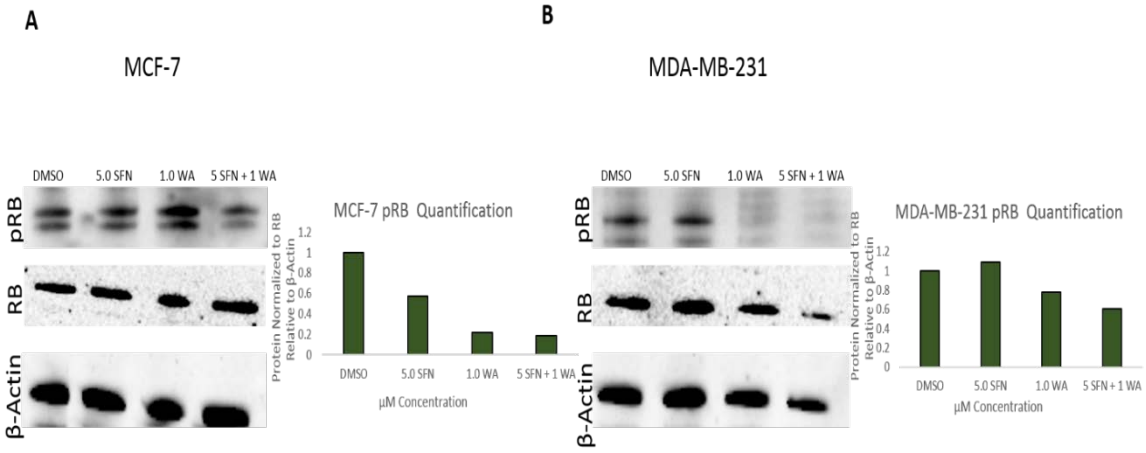


Figure 3: pRB protein is inhibited by SFN and WA: A. Combinatorial SFN and WA downregulate pRB expression in MCF-7 breast cancer cells with the combination being the most effective. Densitometry was determined using ImageJ and bar graphs represent 3 replicates relative to B-actin. B. pRB protein is downregulated by SFN + WA in MDA-MB-231. ImageJ was used to calculate densitometry (n=3; images are representative).

WA and SFN Promote changes in epigenetic regulators in MCF-7 and MDA-MB-231 cells.

Since we previously found that combinatorial SFN and WA treatments decrease HDAC1 and overall HDAC activity as well as modulate DNMTs in breast cancer cells [7], we further studied epigenetic modulators to determine if changes in the expression of these genes are partially responsible for the breast cancer cell death reported. We show decreases in HDAC2 and HDAC3 at the protein level in both cell lines upon incorporation of SFN and WA with the combination being most effective (Figure 4). In addition, the effects of the chosen nutritive compounds on histone methyltransferase activity (HMT) were analyzed. In Figure 5A it can be noted that SFN alone decreased overall HMT enzymatic activity more than WA and the combination in MCF-7 cells. In contrast, HMT activity in MDA-MB-231 cells was significantly decreased by WA and the combination treatment as seen in Figure 5B.

Figure 6 shows the effects of SFN and WA on histone acetyltransferase activity (HAT) and in accordance to previous findings in our lab [19], SFN has no significant effect on HAT activity in either cell line. In contrast, WA appears to increase HAT activity in both cell lines but only significantly in MDA-MB-231 cells. The combination is not more effective than WA alone; however, there is an upward trend in HAT activity in these cells (Figure 6B). Global methylation was determined after treatment with SFN, WA and both compounds. It is known that many different cancer types show global hypomethylation leaving the promoter region of aberrantly expressed genes and tumor suppressors to be hypermethylated [20]. We report a significant increase in global methylation with the incorporation of the combination in both MCF-7 and MDA-MB-231

cells (Figure 7). The single dosages of SFN and WA do not appear effective in reversing global hypomethlyation in the breast cancer cells studied.

Figure 4

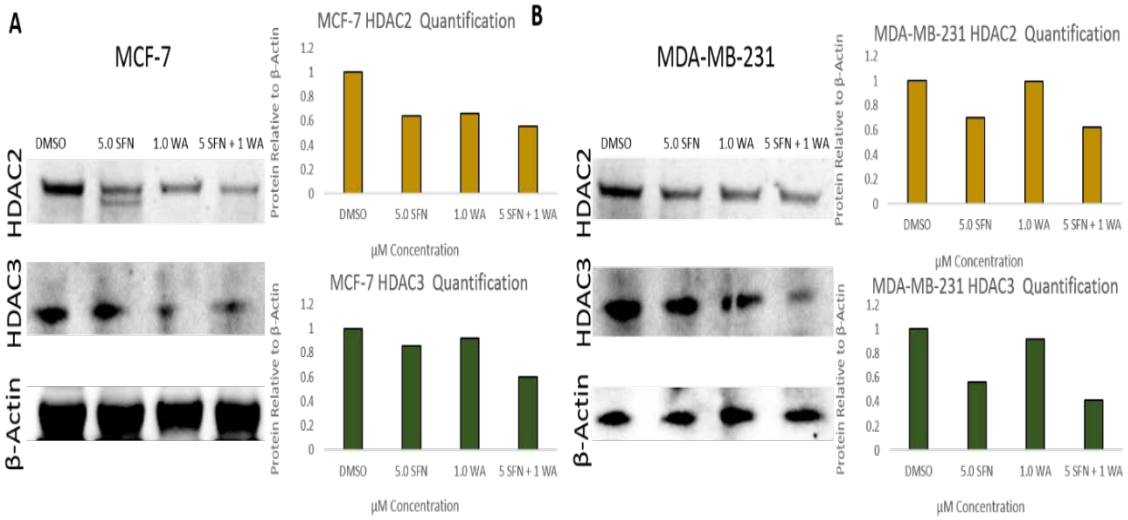


Figure 4: HDAC2 and HDAC3 protein levels are down regulated by SFN and WA: A. Combinatorial SFN and WA downregulate HDAC2 and HDAC3 expression in MCF-7 breast cancer cells with the combination appearing to be the most effective. Densitometry was determined using ImageJ and bar graphs represent 3 replicates relative to B-actin. B. HDAC2 and HDAC3 protein is downregulated by SFN + WA in MDA-MB-231. ImageJ was used to calculate densitometry (n=3; images are representative).

Figure 5

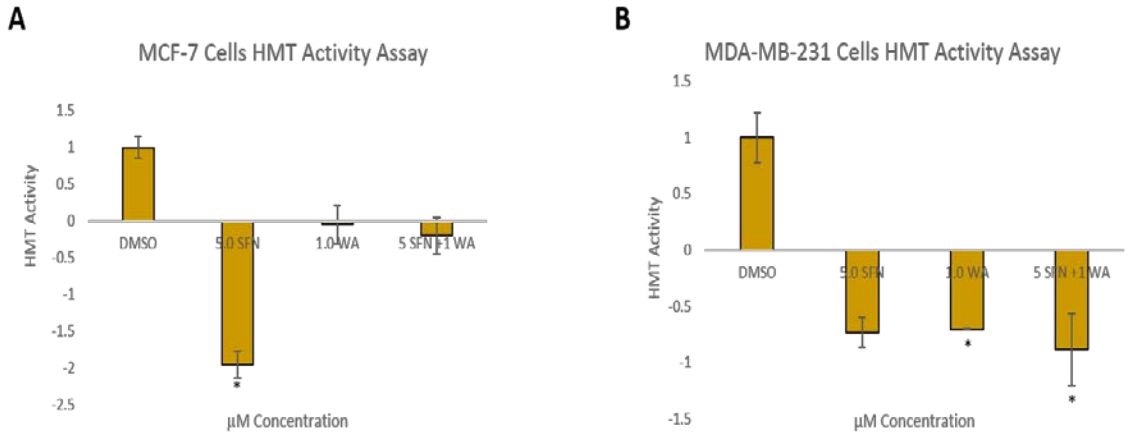


Figure 5: HMT enzymatic activity is downregulated by natural compounds: A. Combinatorial SFN and WA downregulate HMT activity with SFN being most effective in MCF-7 breast cancer cells. B. Histone methyltransferase activity is downregulated by SFN and WA in MDA-MB-231(n=3; $p<0.05$*).

Figure 6

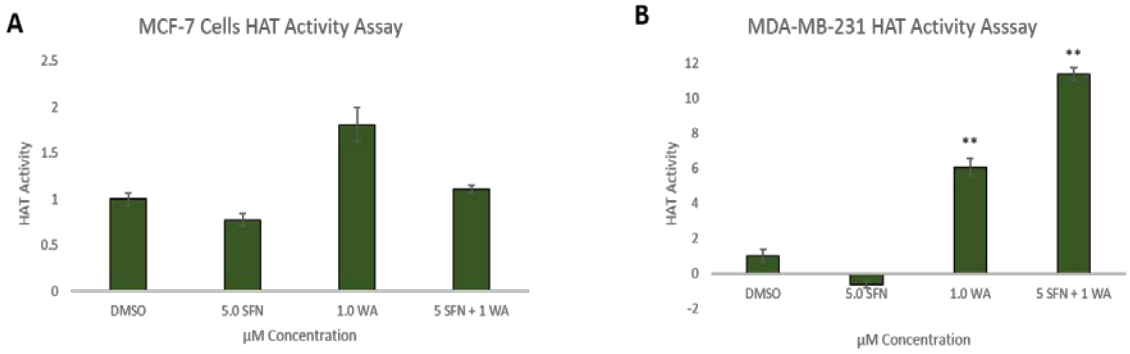


Figure 6: SFN and WA on HAT Activity in MDA-MB-231 Cells: A. Combinatorial SFN and WA show no significant change in HAT activity in MCF-7 breast cancer cells. B.

Histone acetyltransferase activity is upregulated by WA and the combo appears more effective in MDA-MB-231 but not significantly (n =3; p<0.05*, p<0.01**).

Figure 7

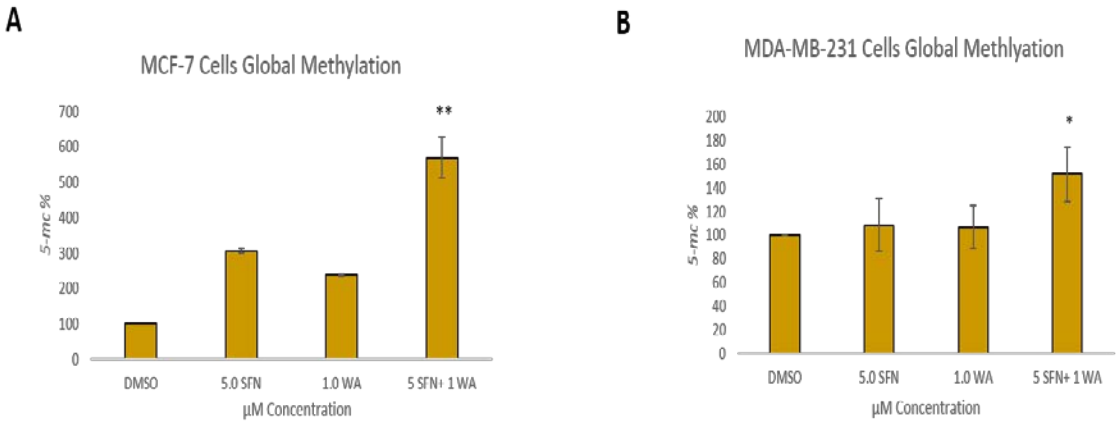


Figure 7: Global methylation is increased by combinatorial WA and SFN: A. SFN and WA alone have no significant effect on global methylation but when these two compounds are used together we report an increase in methylation in MCF-7 cells. B. Global methylation is significantly upregulated by combinatorial WA and SFN in MDA-MB-231 cells (n =3; p<0.05*, p<0.01**).

WA and SFN promote changes in p53 and p21in breast cancer cells.

Two tumor suppressors with roles in cell cycle progression are p53 and p21 of which mutated p53 has been frequently implicated in tumor cell progression [21]. Several studies also reveal p21 to be responsible for inhibiting the cell cycle [17, 22]. Upon Western blot analyses we reveal a slight increase in p53 in MCF-7 cells as well as an induction of p21 after treatment with SFN, WA and both compounds (Figure 8A). In Figure 8B we also show an increase in p21 expression with the combination being the most effective in MDA-MB-231 cells. Unlike the MCF-7 cells p53 is reduced in Figure 8B. It is important to note that MDA-MB-231 cells have a high level of mutant p53 and it serves as an oncogene instead of a tumor suppressor [21]. Because p21 was re-expressed in both cell lines upon analysis we decided to determine if this regulation was attributed to changes in histone modifications at its promoter. To assess the impact of SFN, WA or both on epigenetic modifications at the p21 promoter, we performed chromatin immunoprecipitation (ChIP) assays using antibodies specific for trimethylated lysine 4 of histone (H3K4Me3), a mark which is associated with transcriptional activation. We determined that SFN or WA alone, or in combination with WA significantly increased the levels of H3K4Me3 at the p21 promoter, indicating these dietary compounds positively modify the epigenome at the promoter region (Figure 9).

Figure 8

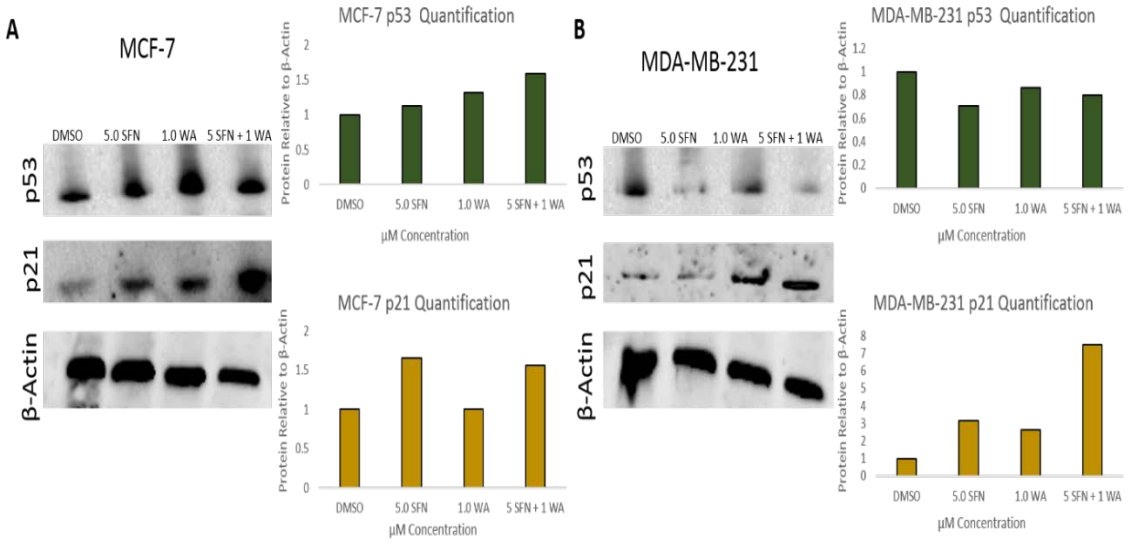


Figure 8: Tumor suppressor proteins implicated in cell cycle progression are modulated by SFN and WA: A. Combinatorial SFN and WA show no significant change p52 protein expression in MCF-7 breast cancer cells; however, p21 is upregulated with WA being more effective than the combination. B. p53 protein expression is downregulated by SFN and the combination of SFN and WA where p21 is upregulated with the combination being the most effective (n=3; images are representative and bar graphs represent the densitometry results of 3 replicates).

Figure 9

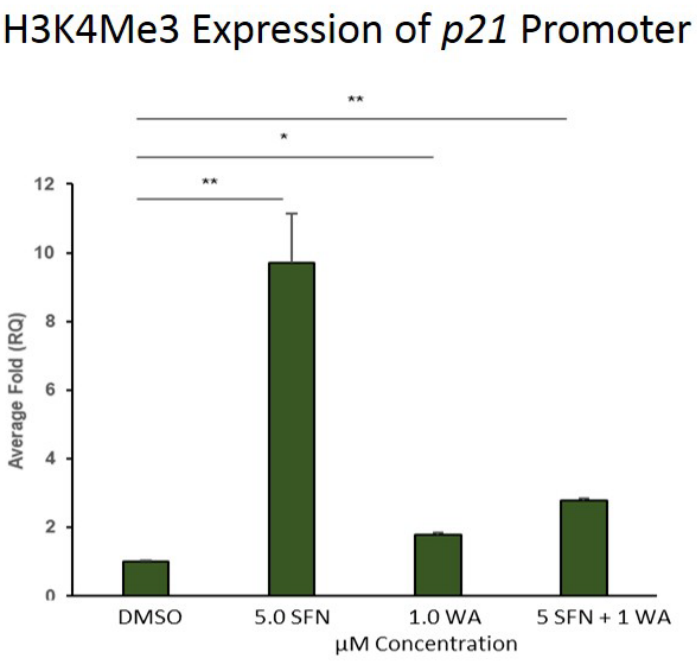


Figure 9: Activation of p21 is mediated by transcriptional activator H3K4Me3 in MDA-MB-231 cells: ChIP assay reveals a significant increase in the expression of H3K4Me3 at the p21 promoter of triple negative breast cancer cells. Values represent average +/- SEM. One exemplar shown (n=3; $p<0.05$*, $p<0.01$**).

DISCUSSION

Our previous study on the topic of combinatorial SFN and WA revealed a synergistic inhibition of breast cancer cell viability with limited effects on a noncancerous control cell line [7]. We found that both WA and SFN were capable of down-regulating epigenetic modifiers which led us to hypothesize that WA and SFN's ability to promote apoptosis and cell death in breast cancer cells is due to their epigenetic control of cell cycle progression. We therefore conducted cell cycle analysis in this study to determine which phase of the cell cycle breast cancer cells were impeded. As seen in Figure 1, our results indicate that the breast cancer cells were arrested primarily at the G1 phase of the cell cycle with treatments of WA and SFN.

Since it is known that CCND1 and CDK4 are primarily responsible for the transition from G1 into S phase, we expected to observe a decrease in these two genes in the breast cancer cells that we treated. Though the combination was not more significant in the down-regulation of CCND1 and CDK4 despite an increase in arrest at G1, we do report a decrease in pRB and an increase in E2F (Figure 2). RB is present at the promoters during quiescence, senescence and in cycling cells in which it represses G1-S genes. It could be that the repression of pRB (Figure 3) by combinatorial WA and SFN is partially responsible for the lack of greater CCND1 and CDK4 inhibition in comparison to the single dosages. A study by Stanelle et.al describes the varying roles of E2F in the cell cycle which notes that an upregulation of the E2F-1 gene drives overall E2F expression and when overexpressed has roles in apoptosis [23]. Interestingly, a review of the literature indicates that E2F, CCND1, CDK4 and pRB form a complex with p21 that is implicit in cell cycle progression [24]. Though pRB is typically associated with

negatively regulating entry into the cell cycle, there have been some studies indicating that elevated pRB loses its cell cycle inhibitory effects in cancer [25]. In addition, RB also has roles in the negative regulation of p21 [14]. If SFN and WA are working through similar mechanisms, an alternative explanation for the lack of greater inhibition of CCND1 and CDK4 in the breast cancer cells studied could be that the combination is not more effective due to an unknown mechanism having already been acted upon by the other compound. Further studies should focus on determining potential modes of action. For example, research into helicases may reveal that combinatorial WA and SFN's ability to decrease cancer cell viability and promote DNA damage is resultant from modulation of helicases thereby further inhibiting the cell cycle and promoting G1 arrest.

The tumor suppressor and cell cycle regulator p21 has been associated with impeding the cell cycle in cancer cells [26, 27]. Our study shows an increase in this tumor suppressor at the protein level independent of p53 expression. The down-regulation of pRB, as shown in Figure 3, could be at least partially responsible for the increase in expression of p21 (Figure 8). Several studies show that the suppression of HDACs and other epigenetic enzymes are associated with the status of both p53 and p21, and it has been shown that p21-dependent G1 arrest is accompanied by RB hypophosphrylation with the incorporation of a synthetic HDAC inhibitor [28]. This adds supporting evidence that the compounds used in this study promote breast cancer cell death through their ability to impede HDACs (Figure 4). Another study revealed HDAC inhibition to be capable of promoting p21 expression at both the gene and protein levels in addition to gene associated acetylation [29]. Further, Lagger and colleagues report a direct inhibition of p21 by HDAC1 in their study [30].

This study aimed to focus on H3K4Me3 since it is a methylation marker associated with transcriptional activation. As suspected, we report significant increases of H3K4Me3 at the p21 promoter in concert with increased p21 protein expression. The combination of SFN and WA, though effective, does not have greater significance in epigenetic modulatory abilities according to the genes we assessed in this research. Our previous study showed a synergistic inhibition of breast cancer cell proliferation, and while p21 activation serves as a partial explanation for decreased proliferation, H3K4Me3 at its promoter is not increased significantly more than SFN alone. We may find there to be a greater abundance of acetyl markers and changes in methyl markers associated with suppression of transcription by the combination. As mentioned, HDAC has roles in p21 suppression and it therefore remains feasible that the down-regulation of HDACs caused by these compounds is promoting acetylation of p21 leading to transcriptional activation.

HDACs and DNMTs are extremely important in the regulation of the cell cycle and the binding of transcription factors. Hypermethylation and hypoacetylation are typically associated with gene silencing [8] and many tumor suppressors and oncogenes are dysregulated through epigenetic modifiers that are instrumental in the regulation of a number of carcinogenic processes [31-35]. One hallmark of cancer is global DNA hypomethylation which promotes genome instability [36]. Our data indicate that combinatorial SFN and WA are capable of significantly increasing global methylation. Interestingly, our previous results showed DNMTs to be down regulated by SFN + WA [7] and other studies indicate that the inhibition of DNMTs results in decreased CpG methylation [37]. It is important to note that class I DNMTs are not the only

methyltransferases. In fact, EZH2 has been reported to directly control DNA methylation and an assessment of this polycomb group protein could reveal additional information that explains the decrease in hypomethylation reported in this study [38], however the ELISA used for this assessment only covers a small percentage of CpG sites so a more comprehensive analysis is needed.

The negative regulation of HDAC1, HDAC2 and HDAC3 induced by SFN and WA are responsible for the changes seen in p53 and p21 in this study. Interestingly, combinatorial SFN and WA was more effective in the reduction of HMT activity (Figure 5B) and induction of HAT activity in the triple-negative MDA-MB-231 cells (Figure 6B). The combination was also more effective in p21 activation in these cells (Figure 8B). Zupkovitz and colleagues conducted a study that confirms HDAC regulation of p21 [39] in which direct binding of HDAC1 to the p21 promoter was shown. This provides supporting evidence to our study that the down-regulation of HDACs and DNMTs reported in both this study and our previous work is linked to the epigenetic re-expression of the p21 tumor suppressor. In addition to the epigenetic modifiers examined in this report, further studies of the p21 promoter may reveal E2F to aide in the activation of p21 as reported by Gartel and colleagues [14]. The regulation of cell cycle progression and associated genes and epigenetic mechanisms via these compounds has been demonstrated in this study although there could be other modes of action for these compounds as well. An in vivo component testing the efficacy of WA and SFN in combination in a xenograft or transgenic mouse model will also be warranted in future studies.

CONCLUSION

The necessity of a better comprehension of the effects of multiple nutritive compounds on cancer progression is apparent. By understanding how modifiable factors such as diet and lifestyle promote an anti-cancerous epigenome we are steps closer to identifying ways to prevent the malignancy/ occurrence of the disease. Many advancements have been made with respect to decreasing breast cancer related mortality; yet it cannot be denied that the effects of chemo-therapy are extremely harsh and riddled with numerous side effects. In addition, hormone therapies are not a viable option for individuals with triple negative breast cancer. Our study has much chemo-preventative potential. Not only that, but we have reported previously WA and SFN to be effective at impeding breast cancer cell proliferation in both ERα (+) and triple negative breast cancer cell lines.

Author Contributions

KJR and TOT designed and conceived the experiments. KJR, BP, SN and RR performed the experiments. KJR, BP and SN analyzed the results. KJR and TT contributed to reagents/materials/analysis tools. All authors reviewed and approved the final manuscript.

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Conflicts of Interest

The authors disclose that there are no conflicts of interest.

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THE EPIGENETIC IMPACT OF CRUCIFEROUS VEGETABLES ON CANCER PREVENTION

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ABSTRACT

The answer to chemoprevention has perhaps been available to the general public since the dawn of time. The epigenetic diet is of extreme interest, for research suggests that cruciferous vegetables are not only an important source of nutrients, but perhaps a key to eliminating cancer as life threatening disease. Cruciferous vegetables such as kale, cabbage, Brussels sprouts, and broccoli sprouts contain chemical components, such as sulforaphane (SFN) and indole-3-carbinol (I3C), which have been revealed to be regulators of microRNAs (miRNAs) and inhibitors of histone deacetylases (HDACs) and DNA methyltransferases (DNMTs). The mis-regulation and overexpression of these genes are responsible for the uncontrolled cellular proliferation and viability of various types of cancer cells. The field of epigenetics and its incorporation into modern medicinal investigation is an exponentially growing field of interest and it is becoming increasingly apparent that the incorporation of an epigenetic diet may in fact be the key to chemoprevention.

Keywords: sulforaphane; indoles; glucosinolates; cruciferous; vegetables; cancer; prevention; chemo-preventive diet; epigenetics; food; broccoli; cabbage; isothiocyanates; indole-3-carbinol; medicine; heredity; HDAC; DNMT; miRNA; nutrients; apoptosis.

INTRODUCTION

The field of epigenetics has rapidly expanded since the 1940's, especially with respect to cruciferous vegetables and chemoprevention. The answer to chemoprevention has perhaps been available to the masses in the form of healthy eating throughout the course of mankind's existence. This fast growing field of study— in which investigators are quickly approaching broader comprehension of many diseases that occur through epigenetic modifications—has grown in importance over the years. Epigenetics, or the study of hereditable changes (passed from cell to cell or generation to generation) that are not related to the changes in underlying DNA sequences [1], is regulated via many processes but perhaps most notably DNA methylation, modifications of the histones and non-coding microRNA. DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) play important roles in cellular proliferation and viability [2-4]. MicroRNAs (miRNAs) are important in the inhibition of translation and the degradation of mRNA [5]. miRNAs negatively regulate many genes, and their malfunction has been linked to various pathways of cancer [6]. One issue that arises with uncontrolled proliferation is the ability of cancer cells to use these epigenetic traits in the continued growth and spread of diseased cells. The incorporation of miRNA regulators and DNMT /HDAC inhibitors as a means to promote apoptosis and prevent uncontrolled cellular proliferation through dietary consumption has proven to be a promising field of study to improve current cancer remediation [2, 7-9]. The ingestion of indoles and isothiocyanates show tremendous results on improving both hormone [10-12] and non-hormone based chemotherapies, which is another reason the epigenetic diet, or the control of epigenetic modifiers through the consumption of dietary phytochemicals, is of extreme interest.

Many studies suggest that cruciferous vegetables are not only an important source of nutrients, but important in the elimination of cancer as a life threatening disease [7, 13-15].

The idea that the consumption of an epigenetic diet can have life altering effects is remarkable and there are ongoing efforts to unravel the mysteries regarding the impact of glucosinolates, isothiocyanates and indoles, which have been reported to lead to apoptosis and cell cycle arrest in carcinogenic cells [13,16-18]. Cruciferous vegetables (CV) such as kale, cabbage, Brussels sprouts, and broccoli sprouts contain chemical components including sulforaphane (SFN) and indole-3-carbinol (I3C) which have been revealed to be potent inhibitors of HDACs and DNMTs [2-4, 9, 19]. The field of epigenetics and its incorporation into modern medicinal regimens appears to have considerable potential in health maintenance in that the incorporation of an epigenetic diet may in fact be the key to the prevention of cancer and many other diseases regulated via epigenetic modifications to the histones. Additionally, some studies have shown SFN and I3C to have an effect on estrogen receptor (ER) in breast cancer cells [10, 20]. These specific studies suggest that future research may lead to breakthroughs in understanding better means to treat breast cancer and enhance hormone based therapies through the incorporation of broccoli and other cruciferous vegetables into the human diet.

Cruciferous Vegetables (CV)

Brassicaceae, or cruciferous vegetables, have chemical components that exhibit anti-inflammatory effects [21]. These foods also drive the detoxification of certain carcinogenic enzymes and are toxic to many types of cancer cells [22]. The consumption of a typical serving of vegetables such as cabbage, broccoli, Brussels sprouts and many

others of cruciferin nature (Table 1), may significantly decrease and lessen the incidence of carcinogenic fatality [23]. In fact evidence shows that individuals who consume a diet rich in CV have lower risks of developing cancer [24]. As seen in Table 2, cruciferous vegetables contain chemical components referred to as glucosinolates which give rise to indoles, isothiocyanates, thiocyanates and cyano-epithioalkanes via mastication as a result of the release of the enzyme myrosinase [25]. Thiocyanates are compounds found in great abundance in foods such as cassava and yams and are evidenced to be inversely associated with sickle cell anemia and hypertension, diseases prevalent in the African American community [26, 27]. In fact, it has been reported that individuals who consume diets rich in yams and cassava are less likely to suffer from sickle cell anemia due to the high availability of thiocyanates and the anti-sickling effects of these foods [28]. Cyano-epithioalkanes are components that can be found in rapeseeds in which canola oil is derived [29]. Canola is used as common cooking oil and is beneficial in lowering cholesterol levels [30]. Of the glucosinolates listed in Table 2, indoles and isothiocyanates reveal promising results with respect to cancer prevention. These glucosinolates are important for they are precursors to compounds such as sulforaphane and indole-3-carbinol.

Table 1: Concentrations of glucosinolates present in a typical serving of CV

Common CV	Approx. Amount	Glucosinolates	References
Brussels Sprouts	50 g	123 mg	[53]
Broccoli	50 g	30.5 mg	[53]
Cabbage	50 g	54.5 mg	[53]
Cauliflower	50 g	31 mg	[53]

The amounts of glucosinolates in milligrams that are present in a typical serving of a few common cruciferous vegetables are summarized.

Table 2: Glucosinolates and their subcategories

Glucosinolates	Sub-Categories	References
Isothiocyanates	Sulforaphane, Erucin, Phenethyl isothiocyanate	[54]
Indoles	Indole-3-carbinol	[55]
Nitriles	Propionitrile, Succinonitrile, Crotonitrile, Cyanopyrazine	[56]
Thiocyanates	Cavernothiocyanate, 2-Thiocyanatoneopupu-keanane, 4-Thiocyanato-9-cadinene	[57]

Isothiocyanates, indoles, nitriles and thiocyanates give rise to a varied array of compounds. This table lists examples of the compounds in which glucosinolates are the precursors.

Isothiocyanates: Sulforaphane

As aforementioned, isothiocyanates are derived from glucosinolates and their exposure to myrosinase (Figure 1). Phenethyl isothiocyanate (PEITC) and sulforaphane (SFN) are two dietary isothiocyanates studied in abundance. PEITC modulates miRNA expression and protects the lungs from environmental smoke induced miRNA alterations [31]. This is important because miRNA mutations are one mechanism by which cancer can develop. The isothiocyanate SFN is found in abundance in cruciferous vegetables, more specifically, broccoli sprouts are the most prevalent and common source for this compound [3, 8, 9, 32]. SFN became the subject of considerable interest as a result of the development, by Prochaska and associates in the late 1980's, of a cell-culture system that detected the induction of anti-carcinogenic phase 2 enzymes. Upon analyzing a vast array of extracts from fruits and vegetables, Prochaska discovered that the broccoli extract had a significant amount of impact on phase 2 enzyme induction [33, 34]. According to Zhang and Tang, they were successful in isolating the liquid component that was responsible for more than 80% of inducer activity; thus began the incorporation of SFN as an anti-carcinogen in the vast array of chemoprevention investigations. SFN has several benefits and may be an effective therapy for the reduction of tumor size as well as for combating multiple pathways of cancer.

Figure 1

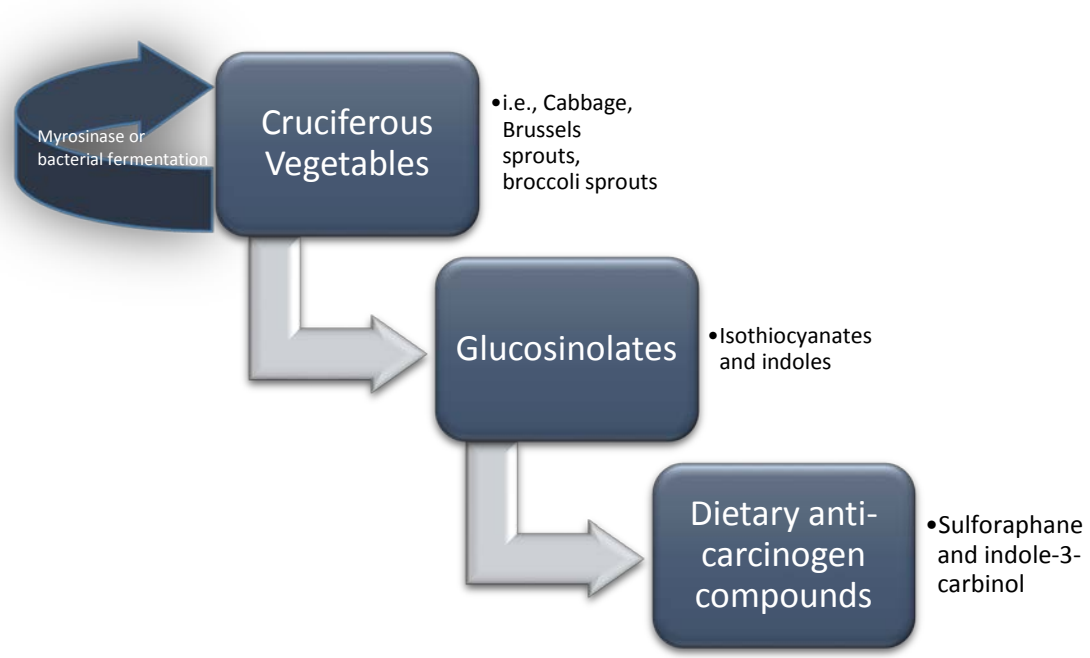

Figure 1: Steps to Anti-Carcinogen Compounds: This flow chart is a simple depiction of how compounds such as SFN and I3C are formed. Once the myrosinase enzyme is released via mastication or bacterial fermentation of cruciferous vegetables, glucosinolates are formed which give rise to isothiocyanates, indoles and other compounds.

Various studies reveal SFN to be an effective inhibitor of HDACs and an inducer of apoptosis through multiple pathways in different cancer types as well as a repressor of human telomerase reverse transcriptase (*hTERT*) gene and its protein product in breast cancer cells [35]. As an HDAC inhibitor, SFN destabilizes androgen receptor, the main signaling pathway regulated by the HDAC6 enzyme, in prostate cancer cells [36]. It is important to note that the use of the SFN HDAC inhibitor for chemoprevention renders very little effect on non-transformed cells [16]. The introduction of SFN causes the activation of caspase-3, 8 and polymerase as a result of the incorporation of the Fas ligand in the breast cancer cell line MDA-MB 231; however, in the breast cancer cell lines MDA-MB-468, MCF-7, and T47D, it is the activation of caspase-3, caspase-9, polymerase cleavage, decreased expression of Bcl-2 and the release of cytochrome-C into the cytosol that are responsible for the initiation of apoptosis [37]. Recent findings, in addition to HDAC inhibition by SFN, have indicated that SFN is an inhibitor of DNMT expression [35, 38]. Therefore, the epigenetic impact likely extends beyond changes in the chromatin of key tumor-related genes and also affects DNA methylation. DNMTs, which are prominent in most cancers, enhance and increase the methylation of DNA. Evidence from our laboratory suggests that SFN serves as a down-regulator of DNMT and is involved in the demethylation of the *hTERT* control region in the process of anti-carcinogenesis [35, 41-43]. SFN also suppresses polycomb group protein (PcG) levels in skin cancer cells which are instrumental in the methylation of histones and suppression of gene expression [39]. This isothiocyanate has also been shown to regulate miRNAs which have major roles in the regulation of genes that manipulate chemoresistance. In fact, miRNA knockdown results in not only increased apoptosis of cancer cells and

sensitivity to certain cancer therapeutics, but also the restoration of ERα in ERα-negative cell lines which has implications of improving current chemotherapies and making them more effective through the activation of estrogen hormone receptors [6, 40]. Furthermore, SFN is effective in combination with other chemopreventive compounds such as epigallocatechin-gallate [41], a green tea polyphenol which functions as an enhancement of SFN, as well as in combination with other chemotherapeutic agents making those therapies more effective [42-44]. Other sources indicate that the incorporation of SFN into the human diet may in fact help to prevent and lessen the incidence of the acquisition of breast, prostate, colon and many other cancers [2, 9, 18].

Indoles: Indole-3-carbinol

There is more than just one mechanism by which CVs negatively impact cancer progression. Indoles, another derivative of glucosinolates, are found in abundance in CVs, and indole-3-carbinol (I3C) is showing promising evidence as a cancer preventive therapeutic. I3C has been reported to be an inducer of estradiol 2-hydroxylation which influences estrogen activity, and may be one reason why it has shown positive results as a cancer therapy [45]. Further, I3C may serve as a natural antioxidant and as such is likely to intervene in cancer progression [46]. Estrogen is instrumental in the increase of tumor size in breast cancer patients, and I3C is extremely significant in this regard for it serves as a negative regulator of estrogen [10, 12]. A separate study also reveals I3C to be an inhibitor of mammary gland tumorigenesis which may be due to the increase of 2-hydroxyestrone and the decrease of 16α-hydroxyestrone [47]. This is remarkable for multiple studies reveal other byproducts of cruciferous vegetables, such as SFN, to have similar effects [48]. I3C also facilitates protection from cellular damage caused by free

radicals [46], and it is instrumental in defending against hormonal imbalances rendering it ideal as a component of prostate and breast cancer prevention in certain cases [11, 12, 49-51].

p21 and p27 are associated with cell cycle progression and Bax/BCI2 is associated with apoptosis. I3C has several effects on the expression of many genes via nuclear regulation of transcription factors which include the up-regulation of p21, p27, Bax/BCI2, CYP1A, BRCA, GADD153 and the down-regulation of the food carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) [11,12, 48-50]. PhIP is a heterocyclic amine resultant from the preparation of meats at high temperatures [48,49]. This has significance because BRCA and GADD153 are associated with carcinogen bio-activation and PhIP-DNA is associated with DNA repair [49, 50]. Additionally, the down-regulation of miRNA-21, a miRNA that is typically overexpressed in chemo-resistant pancreatic cancer cells, is induced by I3C. This down-regulation is important in the increased sensitivity and cytotoxicity of pancreatic cancer cells [52].

CONCLUSION

Many studies have contributed to the incorporation of dietary agents as forms of cancer remediation. Cruciferous vegetables are enriched with several chemical components that have tremendous negative effects on multiple pathways of cancer cells due to their anti-proliferative and anti-tumorigenic properties. The consumption of these vegetables is beneficial in the sense that they are precursors to glucosinolates which give rise to isothiocyanates such as sulforaphane and indoles such as indole-3-carbinol. Most cancers are characterized by the overexpression of HDAC and DNMT and the mis-

expression of miRNAs. Both I3C and SFN are inhibitors and regulators of these processes and the incorporation of these compounds causes cancerous cell lines to take on a healthier, and more normalized appearance. In addition, significant decreases in uncontrolled cell growth as well as increases in programed cell death are noticed with the incorporation of SFN and I3C. Many studies reveal that cruciferous vegetables are key instruments in advancing progress toward the prevention of cancer. Future studies will undoubtedly be directed toward further deciding the epigenetic events impacted by the bioactive components of cruciferous vegetables and their significance with respect to not only cancer prevention, but also many other biological processes.

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GENERAL DISCUSSION

Studies conducted in this dissertation seek to discover innovative approaches for preventing and/or treating breast cancer in the form of nutritive compounds. We have focused on two compounds in conjunction in an effort to facilitate understanding the combinatorial effects of dietary phytochemicals in the human organism with respect to the fight against cancer. No other group has sought to determine the efficacy of combinatorial WA and SFN in any disease. While the study of HDAC and DNMT inhibitors and their anti-cancer potential is not a new concept [24, 34], the use of WA as a DNMT inhibitor is relatively novel and controversy surrounds its efficacy [20, 28, 35]. WA has been studied for a number of years in relation to cancer. However, unlike SFN, its epigenetic potential is a novel area and its efficacy as an epigenetic modulator is speculative.

Our study has presented a new perspective on the use of combinatorial compounds and how SFN and WA promote cancer cell death via epigenetic mechanisms. We have explored the effects of these compounds on the CDKN1A cell cycle regulator and found that cell death mediated by p21 is under epigenetic influence mediated by these compounds. Because we have shown WA and SFN's ability to epigenetically modify the gene activity of a well-studied tumor suppressor gene, we can generate new hypotheses that delve into the impact of diet on the regulation of epigenomic aberrations leading to the development of breast cancer. We reported changes in DNA global methylation with the combination of WA and SFN being most effective in increasing

genome-wide methylation in MDA-MB-231 TNBC cells. Considering the limited effectiveness of current breast cancer remedies on TNBC, the results generated in this study are exciting. Many of our findings suggest that SFN + WA are effective in reversing unregulated growth potential through the inhibition of HDACs and DNMTs protein expression, the reactivation of the tumor suppressor protein p21 and inhibition of pRB in both ER+ and TNBC cell lines; however, these dietary compounds appeared to be more effective in modulating epigenetic mechanisms (H3K4ME3, global methylation and enzymes responsible for methylation and acetylation) in the more aggressive MDA-MB-231 cells.

Additional studies could reveal that SFN + WA will work extremely well as adjuvant therapies resulting in shorter durations and lower dosages of chemotherapy, which could inevitably lead to fewer chemo-associated side effects. Patient derived xenografted mice have enabled scientists to design and implement therapies specific to individual tumors. We could launch a study utilizing such mouse models to test the efficacy of SFN + WA in conjunction with doxorubicin chemotherapy or tamoxifen in the future. We sought to determine if combinatorial WA and SFN was more effective at promoting breast cancer cell death than either compound alone and to discover the mechanisms involved. We found synergy in the inhibition of breast cancer cell growth after combinatorial treatment and we report the modulation of cell cycle progression. One of our hypotheses was that the cell cycle was impeded by SFN + WA through epigenetic mechanisms and provided supporting evidence. We recognize that the modulation of epigenetic genes is likely not the sole contributor to the changes in cell cycle and cell death to SFN + WA's impact on the cell lines studied; however, the ability of these

compounds to increase a methyl marker associated with transcriptional activation at the *p21* promoter supports the idea that these nutritive compounds promote breast cancer cell death through epigenetic modulation.

We show that HDAC inhibition is not the only mechanism responsible for the regulation of breast cancer cell death through a comparison of SFN+WA with SAHA. Since SAHA did not have the same efficacy in BAX and BCL-2 regulation, we can conclude that SFN+WA compounds' pro-apoptotic ability is caused by more than HDAC inhibition. We hypothesized that modulation of HMT and other epigenetic modifying genes by SFN and WA have roles in cell cycle regulation in the breast cancer cell lines studied, and our study has supported this hypothesis. Both MCF-7 and MDA-MB-231 cells had a greater expression of p21 after treatment with SFN + WA independent of wild type or mutated p53 status.

Literature reveals that WA also inhibits vimentin, which has functions in cancer's metastatic ability [32]. Alternatively, studies need to be performed to test if the combination of WA and SFN further decreases or enhances the motility of breast cancer cells secondary to changes in vimentin expression *in vivo*. In addition, we may yet find that WA's ability to negatively affect angiogenesis and NFKβ via inhibition of pro-inflammatory cytokines is the primary reason this combination is so effective in breast cancer cell death [31, 36, 37]. Further study is warranted; however, we feel that we have proven the study of combinatorial SFN and WA is meritorious in the field of cancer therapy and prevention.

This study could be strengthened by determining the dependency of cellular proliferation on the epigenetic genes and enzymes studied in this body of work. Though

some studies have indicated that cellular proliferation is dependent upon class I HDACs in some cell types, we have yet to link this strictly to the compounds studied in this dissertation [38, 39]. We recognize our study has some limitations. Genome-wide association studies would have been ideal in identifying candidate genes, and while this study provides limited information, it does not diminish our findings. It will be necessary to go into more depth at the molecular level in hopes of discovering alternative pathways that may be directly altered. This will help to develop a specific explanation for the plasticity of the epigenome and why the regulation of aberrancies that result in disease occurs with the ingestion of certain dietary compounds.

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