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Effects of Histone Deacetylase Inhibitors on Vitamin D Activity in Human Breast Cancer Cells

Brooke Savage

University of Massachusetts Amherst

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**EFFECTS OF HISTONE DEACETYLASE INHIBITORS ON VITAMIN D ACTIVITY IN
HUMAN BREAST CANCER CELLS**

A Thesis Presented

by

BROOKE S. SAVAGE

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTERS OF SCIENCE

September 2013

Nutrition

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Approved as to style and content by:

Richard J. Wood, Chair

Zhenhua Liu, Member

Susan R. Sturgeon, Member

Nancy Cohen, Department Head
Nutrition Department

DEDICATION

I dedicate this to all breast cancer survivors and their families.

My heart goes out to those currently struggling with the disease.

ACKNOWLEDGMENTS

This thesis could not have been completed without the assistance and dedication of many people. First and foremost, I would like to thank my advisor, Dr. Richard Wood, whom has provided support and counsel throughout this entire project. I am grateful that he was able to fight his own battle with cancer, for without his inspiration and guidance this project would not have come together. I would also like to thank my committee members, Dr. Zhenhua Liu and Dr. Susan Sturgeon for their commitment and advice throughout this project. I extend my gratitude to the members of Dr. Park's lab, for their generosity in letting us use their laboratory equipment and their assistance with protocols. Finally, I would like to thank those I hold closest to me for their love, support and belief in me.

ABSTRACT

EFFECTS OF HISTONE DEACETYLASE INHIBITORS ON VITAMIN D ACTIVITY IN HUMAN BREAST CANCER CELLS

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BROOKE S. SAVAGE, B.A., HOFSTRA UNIVERSITY

M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Richard J. Wood

Breast cancer is one of the leading causes of death among cancer cases in women worldwide. Ultimately, cancer is the result of a combination of environmental and genetic factors that contribute to alterations in cellular control of proliferation, differentiation and programmed cell death. Vitamin D had been emerging as a potentially important nutrient in the prevention and treatment of cancer due to its ability to modulate proliferation, apoptosis, invasion and metastasis *in vivo* in animal cancer models and *in vitro* cell culture studies. To accomplish these cellular effects, Vitamin D exerts its biological activity via the binding of its hormonal metabolite 1,25(OH)₂D₃ to a specific, high-affinity, intracellular vitamin D receptor (VDR). Although VDR expression can be identified in mammary cancer cell lines, levels are often reduced compared to non-cancerous cells, which could limit vitamin D-induced gene expression and function in these cells. In addition, changes in chromatin structure, associated with epigenetic modifications of nuclear histone proteins, can lead to changes in gene expression, including the suppression of important “tumor suppressor” genes, thereby promoting carcinogenesis. Our study investigated the extent to which two compounds with histone deacetylase inhibitor (HDACI) activity, trichostatin A

(TSA), a well-known and potent HDACI, and sulforaphane (SFN), a bioactive food component with HDACI activity, can influence the expression of some important genes involved in vitamin D action and metabolism in cells. The genes investigated were: CYP24A1, which codes for a 24-hydroxylase that deactivates $1,25(\text{OH})_2\text{D}_3$; CYP27B1, which codes for a 1α -hydroxylase that activates $25(\text{OH})\text{D}$ to the $1,25(\text{OH})_2\text{D}_3$ hormone; VDR, the nuclear receptor transcription factor that is activated by $1,25(\text{OH})_2\text{D}_3$; and TRPV6, which codes for a calcium-specific channel implicated in breast cancer cell invasiveness.

We found that in MCF-7 breast cancer cells, $1,25(\text{OH})_2\text{D}_3$ treatment alone induced the expression of VDR mRNA (in 1 of 2 studies), CYP24A1 mRNA (in both studies) and CYP27B1 (in 1 of 1 study where measured). Surprisingly, TRPV6 mRNA expression was not evident in MCF-7 cells in either the presence or absence of $1,25(\text{OH})_2\text{D}_3$. The HDACI TSA alone increased expression of VDR mRNA, but SFN alone had no effect. Importantly, VDR mRNA was increased by co-treatment of $1,25(\text{OH})_2\text{D}_3$ and TSA compared to $1,25(\text{OH})_2\text{D}_3$ alone in Experiment 1 with a similar, but not statistically significant, trend in Experiment 2. As expected, $1,25(\text{OH})_2\text{D}_3$ markedly increased CYP24A1 mRNA. Unexpectedly, treatment with either TSA or SFN alone increased CYP24A1 mRNA, suggesting that endogenous expression of CYP24A1 may be normally suppressed in MCF-7 cells by epigenetic mechanisms involving histone acetylation status. An apparent increase in CYP24A1 mRNA following co-treatment of TSA with $1,25(\text{OH})_2\text{D}_3$ in both experiments was not statistically significant. SFN co-treatment with $1,25(\text{OH})_2\text{D}_3$ also did not cause a further increase in CYP24A1 mRNA. $1,25(\text{OH})_2\text{D}_3$ treatment increased CYP27B1

expression, but co-treatment with TSA had no additional effect. In contrast, co-treatment of SFN and $1,25(\text{OH})_2\text{D}_3$ inhibited much of the positive effects of vitamin D. TSA is already being used as a co-treatment in cancer therapy. Vorinostat, a drug with the same chemical structure as TSA, is being investigated in hundreds of clinical trials. (Marks & Breslow, 2007) Our findings support further study of the effects of the HDACI TSA in breast cancer, and suggest that this HDACI may be beneficial in augmenting vitamin D cellular responsiveness.

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CHAPTER 1

LITERATURE REVIEW

1.1 Breast Cancer

Cancer represents a group of diseases that all share the same fundamental feature, the abnormal growth of cells. The National Cancer Institute (NCI) defines cancer as a collection of diseases that results from the breakdown of regulatory mechanisms, due to damaged DNA, which govern normal cell behavior. When cell regulation is lost, cancer cells uncontrollably grow and divide, ultimately spreading throughout the body and thus interfering with the normal function of tissues and organs. (National Cancer Institute, 2013)

According to the American Cancer Society, breast cancer is the second most common cancer, to lung cancer, among women in the United States; it represents 26% of all cancer cases in women. Breast cancer forms in the breast tissue, usually the ducts and lobules, and can occur in both men and women, but male cases are rare. Although incidence rates have been decreasing over the last decade, 12% of women in the United States will develop invasive breast cancer at one point in their lifetime; and it is more likely to occur in white women than African American, Hispanic or Indian women. Estimates for 2013 show just over 232,000 new cases of breast cancer will be diagnosed among women in the US; and about 40,000 women will die of breast cancer. (American Cancer Society, 2013)

Breast cancer has emotional and physical implications on the women it affects. Treatment can be long and arduous, leading to depression, decreased

physical ability, family strain and even financial hardship. The financial burden is also felt by the society as a whole; according to the NCI, in 2006 breast cancer totaled \$14 billion in total health expenditures, and additionally \$16 billion in indirect mortality cost, such as loss of productivity from premature death. Clearly breast cancer is a far-reaching public health issue with serious implications and consequences. (National Cancer Institute, 2013)

Breast cancer is caused by an interaction between genetic and environmental factors. Risk factors include gender, age, race and genetics; there are other risk factors that have the potential to be modified, thus offering the opportunity to develop useful preventative strategies. In vivo and in vitro studies strongly suggest a relationship between certain nutrient exposure and breast cancer risk. (Vera-Ramirez et al., 2013) A review of observational studies identified that alcohol intake as well as a high-energy intake were associated with an increased risk of breast cancer, whereas low-fat and high-fiber diets reduced the risk. (Thomson, 2012) Preclinical studies support a potential role for vitamin D in breast cancer prevention and treatment, and low vitamin D intake in women has been implicated as a risk factor for breast cancer (Lee, 2011), but the need for vitamin D supplementation in breast cancer prevention remains controversial.

1.2 Vitamin D and Breast Cancer

Although the relationship between vitamin D and breast cancer remains unclear, there is increasing evidence that optimal vitamin D status does play a marginal role in reducing breast cancer risk. (Bertone-Johnson, 2009) There have

been numerous experimental studies to show that vitamin D inhibits cellular proliferation as well as induces differentiation and apoptosis in both normal and transformed breast cells. (Colston, 2008; Vera-Ramirez et al., 2013; Welsh, 2011)

Case-control studies have shown a significant and inverse association between vitamin D levels and breast cancer risk. (Abbas et al., 2008; Blackmore et al., 2008; Knight, Lesosky, Barnett, Raboud, & Vieth, 2007; Rossi et al., 2009)

Ecological and observational studies yield the strongest evidence that higher serum 25-hydroxyvitamin D levels are associated with a reduce risk of breast cancer. A meta-analysis of six observational studies concluded that there is a 50% reduction in incidence rate for breast cancer associated with an increased serum 25 hydroxyvitamin D from 24nmole/L to 78nmol/L. (Grant, 2010) Another meta-analysis looking at vitamin D intake and breast cancer risk reflected a trend towards lower risk of breast cancer with intakes of vitamin D \geq 400 IU/day. (Hong, Tian, & Zhang, 2012)

Despite this evidence, vitamin D supplementation trials have not shown convincing effects of vitamin D supplementation on primary prevention of breast cancer. The Women's Lifestyle and Health Cohort Study looked at 42,000 Swedish women and examined solar exposure and vitamin D intake to find serum levels had no significant relationship to breast cancer risk. (Kuper et al., 2009) This study concludes a need for large randomized control trials, designed to adjust for confounding variables, in order to delineate the possible relationship between serum vitamin D status and breast cancer risk. The Women's Health Initiative study supplemented over 36,000 women with 1,000 mg of calcium and 400IU of vitamin

D₃ daily for 7 years, and they did not find a strong reduction of breast cancer risk among the supplemented group. (Prentice et al., 2013) Despite those outcomes, *in vivo* and *in vitro* research is still showing vitamin D₃ to have chemo-protective effects, warranting further investigation.

1.3 Vitamin D Sources

Optimal vitamin D status is dependent upon a variety of factors. Vitamin D is obtained from both dietary sources and from cutaneous synthesis with exposure to UVB sunlight. The Recommended Dietary Allowances (RDA) for healthy adults ages 19 to 50 is 15µg (600IU) daily; this equates to a serum level of 50nM. (National Institute of Health, 2011) Natural dietary sources include oily fish, such as mackerel and salmon, but the major dietary sources are fortified dairy products and cereal grains. (Stipanuk & Holick, 2006) A cup of skim, 1%, 2% or whole milk fortified with vitamin D contains 115 to 125 IU, which amounts to about 20% of the RDA (600 IU/day). A cup of fortified breakfast cereal contains about 40 IU, about 7% of the RDA. Since it can be difficult to obtain the recommended 600 IU of vitamin D from dietary sources, vitamin D supplements are readily available.

Daily sunlight exposure is the main contributing factor, 90%, to vitamin D status, and can be sufficient to meet vitamin D requirements in certain situations. However, the time of day, season of the year, and the geographical latitude have dramatic effects on the amount of solar UVB radiation that reaches the earth's surface. In winter months, the ability to produce vitamin D in the skin is reduced because the vitamin D-producing UVB photons pass through the ozone layer at an

oblique angle and are absorbed to a greater extent by the ozone reducing surface radiation. In contrast, the relative position of the sun in spring and summer months allows for less ozone UVB absorption and therefore more UVB photons to penetrate to the earth's surface. At a latitude of 42°N (Boston), sunlight is incapable of producing vitamin D in the skin from November through February. (Stipanuk & Holick, 2006) Since our exposure to sunlight provides 90% of our vitamin D exposure, people living in more northern and southern latitudes will frequently show insufficient vitamin D levels during their respective winter months.

The influence of latitude on cutaneous vitamin D production has initiated a hypothesis as to why women in the northern hemisphere, North America and northern European countries, exhibit the highest incidence rate of breast cancer, where women in regions with relatively more sun exposure are relatively protected from breast cancer. (Suba, 2012) If this hypothesis is correct, achieving a higher vitamin D status in these women living in areas that put them at a higher risk of developing breast cancer could help to prevent the initiation and development of the disease.

1.3.1 Vitamin D Absorption, Transport, Metabolism and Biodegradation

The first step of vitamin D activation and metabolism involves the formation of vitamin D₃ in the skin from ultraviolet irradiation at 290nm – 315nm (P. H. Anderson, May, & Morris, 2003) , or vitamin D₃ (cholecalciferol) intake from eating certain animal-based foods in the diet or vitamin D₂ (ergocalciferol) derived from plant sources. Vitamin D is fat soluble and is incorporated with other lipids in

micelles in the digestive track. The vitamin D taken up by intestinal enterocytes is incorporated into chylomicrons. The chylomicron is released by the enterocyte and is drained into the lymphatic circulation for systemic delivery.

When vitamin D₃ reaches the liver from either endogenous cutaneous production or from the diet, 25-hydroxylation of vitamin D occurs with the action of the liver enzyme 25-hydroxylases (25-OHase; encoded by the gene CYP27A1) to produce 25-hydroxyvitamin D₃ (25(OH)D₃), the main storage and circulating form for vitamin D. The 25(OH)D₃ metabolite produced in the liver is attached to plasma vitamin D-binding protein (DBP)¹ to then re-enter circulation. (P. H. Anderson, May, & Morris, 2003) Once 25(OH)D₃ reaches the kidney, it can be then 1 α -hydroxylated by the enzyme 1 α -hydroxylase (1 α -OHase; encoded by the gene CYP27B1). This yields the hormonally active secosteroid 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), also called calcitriol. Circulating 1,25(OH)₂D₃ can enter cells and bind to the vitamin D receptor (VDR), which activates this transcription factor to initiate the process of transcription of vitamin D-dependent genes by binding to a vitamin D response element in the promoter region of certain genes. In addition, it is now appreciated the 1 α -hydroxylase CYP27B1 gene can also be expressed in non-renal cell types, which opens the possibility of local cellular control of 1,25(OH)₂D₃ exposure by coordinating the cellular expression of CYP27B1 to increase 1,25(OH)₂D₃ and CYP24A1 to decrease 1,25(OH)₂D₃. The possible complex paracrine and intracrine vitamin D pathways has provided an intriguing scientific basis for interpreting

¹ The 25(OH)D₃ metabolite bound to vitamin D-binding protein has a relatively long biological half-life and is stable in the blood for a significant periods of time (weeks) making it a more useful biomarker to assess overall vitamin D exposure.

possible effects of vitamin D exposure in a variety of chronic diseases, including cancer.

1,25(OH)₂D₃ is inactivated by metabolism through a C-24 oxidation pathway. This biodegradation pathway is comprised of five enzymatic steps involving 24-hydroxylase, (encoded by gene CYP24A1) which breaks down 1,25(OH)₂D₃ to a final water-soluble excretory product (calcitroic acid) that can be eliminated in the urine. Due to the presence of multiple vitamin D response elements in the promoter region of the CYP24A1 gene, the expression of CYP24A1 mRNA is highly responsive to 1,25(OH)₂D₃ activation of the VDR. The response of CYP24A1 expression in a cell can thus be used as an index of 1,25(OH)₂D₃ activity in a cell. (P. H. Anderson, May, & Morris, 2003)

1.4 Vitamin D₃: Calcemic Functions

1,25(OH)₂D₃ is an important hormone in the regulation of serum calcium homeostasis and proper bone metabolism. 1,25(OH)₂D₃ helps to maintain serum calcium homeostasis by increasing the efficiency of intestinal calcium absorption in response to a drop in serum calcium. This calcemic effect of vitamin D effect occurs indirectly. A drop in serum calcium leads to increased secretion of parathyroid hormone (PTH) from the parathyroid gland, which then increases the renal synthesis of 1,25(OH)₂D₃ by increasing CYP27B1 (1α-hydroxylase) expression. Higher circulating 1,25(OH)₂D₃ concentrations help maintain calcium homeostasis by increasing vitamin D-dependent expression of calcium transport proteins, TRPV6 and calbindin D, in the enterocyte, which promote increased calcium absorption.

This physiologic effect of $1,25(\text{OH})_2\text{D}_3$ in the intestine is complimented by the anticalcuric and bone-resorbing effect of PTH. Once serum calcium levels are increased back to normal, PTH secretion is reduced and $1,25(\text{OH})_2\text{D}_3$ synthesis decreases. Elevated $1,25(\text{OH})_2\text{D}_3$ participates in a negative feedback signal on the parathyroid glands to suppress further synthesis of the PTH precursor, and vitamin D-induced CYP24A1 expression leads to increased breakdown of the vitamin D hormone.

1.4.1 Vitamin D₃: Non-calcemic Functions

As mentioned above, the non-calcemic functions of $1,25(\text{OH})_2\text{D}_3$ may have important implications for carcinogenesis and include the regulation of cell proliferation and differentiation, hormone secretion, and immunomodulation. Vitamin D exerts most of its biological activities by binding to specific high-affinity vitamin D receptor (VDR). (Lopes et al., 2010) As the mediator of $1,25(\text{OH})_2\text{D}_3$ action, the VDR is now widely recognized to be expressed in almost every tissue in the body; including: breast, brain, pituitary, stomach, skin, pancreas, central nervous system, and immune system. (Welsh et al., 2003) The presence of VDRs in these diverse tissues suggests that these tissues are in some way vitamin D responsive, and the physiological effect of vitamin D is an active area of investigation.

Research has shown that proliferation and differentiation of cancerous cells are affected by $1,25(\text{OH})_2\text{D}_3$ via an alteration of transcription of various cell cycle and other regulatory genes. For example, in human cell lines derived from normal breast tissue and breast cancers, $1,25(\text{OH})_2\text{D}_3$ and other VDR agonists can induce

cell cycle arrest, differentiation, and apoptosis. (Welsh et al., 2003) Welsh and colleagues discovered that both HME (non-tumorigenic mammary epithelial cells) and MCF-7 (breast cancer cells) responded to increasing doses of $1,25(\text{OH})_2\text{D}_3$, which triggered cell growth arrest and apoptosis in comparison to non-treated cells. In a subsequent study, Welsh measured CYP24A1 gene expression (a marker of vitamin D status and biodegradation) in HME, MCF-7 and HKC-8 (proximal renal tubule cells), and found that after a 48 hour treatment with 100nM $1,25(\text{OH})_2\text{D}_3$, gene expression was increased by 60, 110, and 150% respectively. (Welsh, 2011)

1.5 Molecular Mechanism of Vitamin D Genomic Activity

Nuclear receptors (NR) are a class of proteins that are intracellular transcription factors that directly regulate gene expression, thereby controlling development, homeostasis and metabolism of an organism. (Nuclear Receptor Resource, 2011) The regulation of gene expression by NR usually only occur when a ligand is present. When a cognate ligand binds to its NR, it causes a conformational change in the receptor protein that activates the receptor, which can lead to an increased or decreased regulation of expression on certain genes. (Seuter, Heikkinen, & Carlberg, 2013a) The transcriptional outcome and dynamic balance of gene alterations of these nuclear receptors is determined by the ligand. The NR contains ligand-binding domains that will selectively bind lipid-soluble ligands, and DNA-binding domains that recognize and bind specific nucleotide sequences in target genes; thus inducing or repressing target gene promoters. (Welsh et al., 2003).

The VDR is a member of the nuclear receptor family of proteins that acts as ligand-dependent transcription factors to modulate expression of specific genes in a tissue-specific manner. The $1,25(\text{OH})_2\text{D}_3$ -VDR-dependent transcriptional activity is modulated through synergistic ligand-binding and dimerization with retinoic X receptor (RXR). This VDR-RXR receptor heterodimer is able to enter deep grooves in DNA and recognize vitamin D responsive elements in the promoter regions of vitamin D target genes. It is hypothesized that the $1,25(\text{OH})_2\text{D}_3$ -VDR complex induces a program of gene expression that suppress cancer cell proliferation and stimulates differentiation in the normal mammary gland. This would mean that a dysregulation of VDR-mediated gene expression would lead to altered development and possible cell transformation leading to cell mutation. (Welsh et al., 2003) An important goal of VDR research is to identify the optimization of VDR signaling in the mammary gland to protect against breast cancer.

Vitamin D exerts its biological activities by activating the VDR using a mechanism similar to that of other members of the steroid hormone superfamily. Its cellular actions are achieved by regulating the expression of the vitamin D responsive genes in the target tissues. (Chen & Deluca, 1995) There are many genes that have been shown to be responsive to $1,25(\text{OH})_2\text{D}_3$; many of them play a direct role in calcium regulation and bone formation; osteocalcin, osteopontin, PTH, the hydroxylases CYP24A1 and the calbindin genes. The bone protein osteocalcin is upregulated by $1,25(\text{OH})_2\text{D}_3$, whereas PTH is down regulated. The calbindin-D9k gene is upregulated with increased calcium absorption and increased $1,25(\text{OH})_2\text{D}_3$ expression. (Jones, Tighilet, Tran, & Huntsman, 1998)

The 24-hydroxylase gene CYP24A1 is an immediate target gene of VDR expression, it is one of the most highly regulated genes that respond to $1,25(\text{OH})_2\text{D}_3$, and $1,25(\text{OH})_2\text{D}_3$ is thought to be the sole physiological inducer of 24-hydroxylase activity. (Johnson, Chung, & Trump, 2010) Increased expression of the CYP24 enzyme initiates a negative feedback loop and leads to increased catabolism and inactivation of $1,25(\text{OH})_2\text{D}_3$. Studies have clearly demonstrated that the 24-hydroxylase gene (CYP24A1) is regulated at the transcriptional level by $1,25(\text{OH})_2\text{D}_3$. With the presence of the $1,25(\text{OH})_2\text{D}_3$ ligand, it is a sensitive marker for VDR function because its promoter region contains multiple vitamin D response elements (VDRE). (Chen & Deluca, 1995)

Shinki and colleagues (Shinki et al., 1992) investigated tumor-derived endothelial cells, TDEC, and Martigel plugs, MDEC, by exposing them to $1,25$ -dihydroxycholecalciferol and then measuring the mRNA of CYP24A1. The analysis found that there was no induced expression of the VDR signaling pathway in the tumor-derived cell study, and the normal MDEC showed the expected level of CYP24A1 expression. This shows that untreated tumor cells can epigenetically alter gene expression, and thus inhibit expression of VDR. Combating the tumor's epigenetic change and induce CYP24A1 expression could increased the benefits found with calcitriol-mediated therapies.

1.6 Epigenetic Modulation of Gene Expression

C. H. Waddington first introduced the concept of epigenetics in 1939 as a way to name the "causal interactions between genes and their products, which bring the

phenotype into being.” (Esteller, 2003) Currently the term epigenetics refers to the modification in gene expression caused by heritable, and potentially reversible, alterations in chromatin structure and/or DNA methylation without changes in the DNA sequence. (Gerhaeuser, 2012)

The DNA nucleotide sequences are the genetic instructions that are used to develop and regulate the functioning of all cell processes. Epigenetics is the study of changes in the gene expression or cellular phenotype caused by mechanisms other than changes to the underlying DNA sequence. It refers to the functionally relevant modifications to the genome that do not involve a change in the nucleotide sequence. Examples of such modifications are DNA methylation, histone protein modifications and microRNA expression. These changes remain through cell division for the remainder of the cell’s life, and may also last for multiple generations. These epigenetic changes can be inherited by daughter cells, thus having the potential to correct altered gene expression patterns that have been established as a consequence of environmental stimuli. (Gerhaeuser, 2012)

1.6.1 Histone Modification

Histone modification is defined as, post-translational modification at the N-terminal histone tails of histone proteins; acetylation and methylation are the two most recognized mechanisms. These contribute to epigenetic regulation of gene expression, genomic stability, DNA damage response and cell cycle checkpoint integrity. (Gerhaeuser, 2012) Histone acetylation and deacetylation are mediated by an interplay of enzymes that activate and deactivate the paired processes. Histone

acetylation, by histone acetyltransferase (HAT), acetylates the lysine residues to decrease compaction, in part due to the weakened charge attraction between the DNA and histone proteins; this increases gene activation and transcription. While histone deacetylase (HDAC) works to remove that acetyl group from the lysine and increase compaction and decrease transcription.

Perturbation of the balance between histone acetylation and deacetylation is considered a key factor in cancer development, and an overexpression of HDAC is a characteristic often seen in cancer cells. Reversible acetylation of histones and non-histone proteins has been shown to affect cell metabolism and other cellular processes, thus can be targeted by inhibitors of histone deacetylases, which will increase acetylation and can cause the chromatin structure to remain open and available for transcription of important anti-cancer genes, such as those involved in apoptosis; thus, limiting one of the cancer cell's self-preservation mechanisms.

1.7 Histone Deacetylase Inhibitors (HDACI)

As HDAC are associated with transcriptional repression (Christensen & Marsit, 2011), histone deacetylase inhibitors (HDACI) are a class of compounds that interfere with the function of HDAC and have been known to induce hyperacetylation, which affects gene expression by modulating the chromatin structure. (Dashwood & Ho, 2007; Davis & Ross, 2007) Numerous data have shown that a main characteristic of cancer is global hypomethylation and hypermethylation of specific DNA regions, mainly within the promoters of tumor suppressor genes. The increase in promoter DNA methylation was reported as a common mechanism

of tumor suppressor gene silencing observed in many types of cancers. (Stefanska, Karlic, Varga, Fabianowska-Majewska, & Haslberger, 2012)

A working hypothesis is that in the presence of HDAC, a cancer cells chromatin interactions are kept in a constrained state. HDACI enable the net transfer of acetyl groups to lysine tails in histones, thereby loosening the interactions with DNA and facilitating transcription factor access and gene activation. (Dashwood & Ho, 2007) Enabling increased transcription and activation of important tumor suppressor genes via HDACI, could yield promising treatment outcomes for cancer patients.

The classical HDACI that have been recognized are as follows in decreasing order of potency; hydroxamic acids, such as trichostatin A (TSA); cyclic tetrapeptides, such as trapoxin B; benzamides; electrophilic ketones; and aliphatic acid compounds, such as phenylbutyrate and valproic acid.

1.7.1 Trichostatin A (TSA)

Trichostatin A (7-(4-(dimethylamino)phenyl)-N-hydroxy-4,6-dimethyl-7-oxohepta-2,4-dienamide) is the most potent HDACI chemical agent that has been discovered thus far. It is an organic compound that was originally used as an antifungal antibiotic. It has now been discovered as a potent anti-proliferative agent from its ability to alter gene expression. (Yoshida, 2007) It has a large spectrum of epigenetic activities; promotes the expression of the apoptosis-related genes, induces cell differentiation thus acting to mature some of the de-differentiated cells

found in tumors and acts as an anti-cancer drug by slowing down cancer cell survival and progression. (Kim, Kang, Na, & Lee, 2010; Meadows, 2012)

TSA has been found to be effective at nanomolar concentrations, among a variety of human and animal cancer cells. Table 1.1 identifies experimental parameters tested in TSA oncology studies. Various human breast and prostate cancer cells show decreased protein stability as well as increased CYP24A1 expression with TSA treatments ranging from 3 to 400 nM. (Drzewiecka & Jagodzinski, 2012a; Kim, Kang, Na, & Lee, 2010; Luo et al., 2010)

1.7.2 Sulforaphane (SFN)

Sulforaphane (1-Isothiocyanato-4-methylsulfinylbutane) is a newly researched bioactive food compound that shows promising HDACI properties. SFN is predominantly found in *Brassica* vegetables; which are a genus of plants from the mustard family that include cruciferous vegetables (broccoli, cauliflower, water cress, and bok choy), cabbages and mustard greens. Sulforaphane is a molecule within the isothiocyanate group of organosulfur compounds. It is produced when the enzyme myrosinase transforms glucoraphanin into sulforaphane upon damage to the plant (such as mechanical chewing), which allows the two compounds to mix and react.

Further research into sulforaphane has shown that it exhibits similar HDACI activity as TSA, but at a lower intensity. SFN acts as a weak ligand for histone deacetylase, and has been shown to cause inhibition of cancer cell proliferation and stimulation of apoptosis. (Dashwood & Ho, 2007) SFN shows promise as a co-

cancer-treatment and especially for cancer prevention because it is so bioavailable from food sources, just 1¼-cup broccoli sprouts metabolizes to a 5µmol/L SFN level in breast tissue. (Li et al., 2010a)

Table 1.2 provides information on the experimental parameters in cancer cell research with SFN. In a human hepatoma cell line, a moderate dose of 1 to 10µM SFN had a dose dependent response of increasing mRNA expression of the CYP1A1 gene. (Anwar-Mohamed & El-Kadi, 2009) As well, a 1 to 20µM dose of SFN in human colorectal cells showed significant increases in human β-defensin-2. (Schwab et al., 2008)

1.7.3 Epigenetic Modulation of Vitamin D Mediated Genes by HDACI

There have been a variety of studies that demonstrate the VDR modulating antiproliferative effects of 1,25(OH)₂D₃ can be greatly enhanced with a combination treatment of HDACI; as illustrated in Table 1.3 with 1,25(OH)₂D₃ and TSA co-treatments. Banwell and colleagues (C. Banwell, O'Neill, Uskokovic, & Campbell, 2004) identified a class of breast cancer cells that show resistance to 1,25(OH)₂D₃, thus correlating to reduced VDR activity and increased nuclear co-receptors (NCoR) activity. NCoR will maintain chromatin in a transcriptionally repressed state; NCoRs are often overexpressed in several types of cancer. Recent research is showing that increasing VDR signaling will epigenetically block NCoR activity and thus maintain VDR gene expression. (Deeb, Trump, & Johnson, 2007) Banwell and colleagues examined the MDA-MB-231 cell line that is resistant to antiproliferative 1,25(OH)₂D₃ action. With a solo treatment of 1nM 1,25(OH)₂D₃, these cells exhibited

an altered expression ratio of 0.1 fold reduction in VDR and 1.7 fold increase in NCoR1, in comparison to the MCF-12A (non-malignant) cell line which shows normal $1,25(\text{OH})_2\text{D}_3$ responsiveness. With a co-treatment of 15nM TSA, the MDA-MB231 cell line previously non-responsive to vitamin D, showed drastic changes in antiproliferative effects; there was a 60% inhibition of colony formation, compared to no inhibition with only 1nM $1,25(\text{OH})_2\text{D}_3$ and a mere 30% inhibition with 15nM TSA. Concluding that the combination treatments of $1,25(\text{OH})_2\text{D}_3$ and TSA can significantly increase breast cancer cells reception to the antiproliferative effects of vitamin D.

Banwell and colleague's follow-up study (C. Banwell et al., 2006) looked more closely at the epigenetic changes with combination treatments of $1,25(\text{OH})_2\text{D}_3$ and TSA. She examined CYP24A1 gene expression as well as the antiproliferative genes GADD45 α and VDUP-1. She found that by increasing the dosage of $1,25(\text{OH})_2\text{D}_3$ to 100nM, and maintaining the same TSA treatment of 15nM, there was a 15 fold increase in CYP24A1 expression, and about a 2 fold increase in the other two antiproliferative genes as compared to the solo $1,25(\text{OH})_2\text{D}_3$ treatment. Similar results have also been seen with human prostate cells DU-145. The same dosages as the Banwell's study yielded a 2-fold increase in CYP24A1 expression. (Rashid et al., 2001)

1.8 Vitamin D Effects on MCF-7 Cells

Despite the research, the antiproliferative effect of vitamin D on cancer cells is still controversial. Research with breast cancer cells has shown that there are a

variety of characteristics between cell types, making the results varied and non-generalizable. Many researchers have utilized the unique properties of the MCF-7 cell when investigating vitamin D and HDACI with breast cancer. It is a mammary gland cell from a breast adenocarcinoma derived at a metastatic site, from a 69-year-old Caucasian female. (ATCC, 2013) MCF-7 have been found to have a specific calcitonin receptor and calcitonin-responsive adenylate cyclase, as well as have a specific macromolecular cytosol receptor for the vitamin D hormone, $1,25(\text{OH})_2\text{D}_3$ (Levenson & Jordan, 1997) , making them estrogen receptor positive (ER+) cells and quite responsive to $1,25(\text{OH})_2\text{D}_3$. (C. Banwell et al., 2006) The laboratory work on the systematic dissection of hormone action that lead to significant progress in breast cancer treatment was made possible by the MCF-7 cells hormone responsiveness.

An intensely studied model of cancer/vitamin D research is that increasing vitamin D status in breast cancer MCF-7 cells as protective against cancerous mutations, which lead to cancer cell proliferation and malignancy. (Colston, 2008) TSA has been researched to show an increase in vitamin D responsiveness in MCF-7 cells, but SFN, a bioactive food component, has had little research and may have protective capabilities as well. Therefore, in this study we propose to investigate the effects of $1,25(\text{OH})_2\text{D}_3$ on vitamin D dependent gene expression in MCF-7 human breast cancer cells in the presence of the HDACI, TSA and SFN.

Table 1.1 TSA Study Experimental Parameters

Cell Type	TSA Dosage	Exposure Time	Outcome Measure	Effects Compared to Control	Reference
Human Breast Cancer Cells MCF-7	3nM 30nM	48 hours	Cellular contents of PLC γ 1	Transcription and protein levels were reduced four fold.	(Drzewiecka & Jagodzinski, 2012b)
Human Breast Cancer T47D	1 μ M	1 to 3 hours	Protein stability of ER α and p300 proteins, to signify changes in acetylation	ER α and p300 protein stability increased by 30%, in a dose dependent manner.	(Kim, Kang, Na, & Lee, 2010)
Human Prostate Cancer Cells DU145 LNCaP PC3	50nM 100nM 200nM 300nM 400nM	8 hours	Changes in CYP24A1 gene expression	CYP24A1 gene expression was increased by 1.6-4.0 fold	(Luo et al., 2010)

Table 1.2 SFN Study Experimental Parameters

Cell Type	SFN Dosage	Exposure Time	Outcome Measure	Effects Compared to Control	Reference
Human Embryonic Kidney Cells HEK293	SFN 5 μ M + TSA 100ng/mL	48 hours	HDAC activity	HDAC activity was depressed: SFN + TSA (60%) > SFN (30%) > TSA (25%)	(M. C. Myzak, Tong, Dashwood, Dashwood, & Ho, 2007)
Human Prostate Cancer Cells LNCaP C4-2	10 μ M 20 μ M 40 μ M	24 hours	Levels of Androgen Receptor (AR) and Prostate Specific Antigen (PSA)	AR mRNA decreased by 40% to 80% in both cell lines. PSA protein levels decreased in both cell lines.	(Kim & Singh, 2009)
Human Hepatoma Cells HepG2	1.0 μ M 2.5 μ M 5.0 μ M 10 μ M	6 hours	CYP1A1 mRNA expression	Dose-dependent increased mRNA expression 1.0 μ M = 2.2 fold 2.5 μ M = 2.8 fold 5.0 μ M = 3.8 fold 10 μ M = 3.6 fold	(Anwar-Mohamed & El-Kadi, 2009)
Human Colorectal Cancer Cells Caco-2 HT-29 SW480	1 μ M 5 μ M 10 μ M 20 μ M	24 hours and 48 hours	Human β -defensin-2 (HBD-2) gene expression	At 24 hours there was a 1.6 fold increased in HBD-2 expression with 20 μ M SFN. At 48 hours there was a 2-fold increase.	(Schwab et al., 2008b)

Table 1.3 Modulation of Vitamin D-mediated Gene Expression by HDACI

Cell Type	HDACI Dosage	Exposure Time	Outcome Measure	Effects of Co-Treatment Compared to 1,25(OH) ₂ D ₃	Reference
	1,25(OH) ₂ D ₃ Dosage				
Human Breast Cancer cells MDA-MB-231	15 nM(TSA)	36 hours	Colony Formation	60% inhibition of colony formation	(C. Banwell, O'Neill, Uskokovic, & Campbell, 2004)
	1 nM				
Human Prostate Cancer cells DU-145	15 nM (TSA)	72 hours	Change in activity of mRNA expression: CYP24A1 and p21	CYP24A1 expression increased 2.5 fold. p21 increased 1 fold.	(Rashid et al., 2001)
	100 nM				
Human Leukemia Cells THP-1	300 nM (TSA)	150 minutes	Transcriptional response of genes HBEGF DUSP10	80% increase	(Seuter, Heikkinen, & Carlberg, 2013a)
	100 nM				
Human Breast Cancer Cells T-47D ZR-75-1 MCF-7 MDA-MB-231	15 nM (TSA)	12 hours	Changes in CYP24A1, GADD45 α and VDUP-1 gene expression	CYP24 increased 15 fold. GADD45 α increased 2.4 fold. VDUP-1 increased 2.1 fold.	(C. Banwell et al., 2006)
	100 nM				
Human Breast Cancer cells MCF-7 MDA-MB453	15 nM (TSA)	3 hours	Fold change of mRNA p18, p19, p21, p27	MCF-7 showed reduced p18 and p19 mRNA. MDA-MB453 showed reduction in p21 mRNA only.	(Malinen et al., 2008)
	10 nM				

CHAPTER 2

PURPOSE OF STUDY

2.1. Overview

Many studies have investigated the effects of trichostatin A (TSA), a potent histone deacetylase inhibitor (HDACI), on vitamin D-dependent gene responsiveness in various cancer cell lines; for example, human prostate cancer DU-145 (Rashid et al., 2001), human leukemia cells THP-1 (Seuter, Heikkinen, & Carlberg, 2013b) and multiple breast cancer cells lines MDA-MB-231, T-47D, ZR-75-1 and MCF-7 (C. M. Banwell, Singh, Stewart, Uskokovic, & Campbell, 2003; C. Banwell et al., 2006; Malinen et al., 2008). Sulforaphane (SFN) is a bioactive food component that has HDACI activity. However, there is a gap in our knowledge concerning the effects of SFN on vitamin D-mediated gene expression in breast cancer cells. This lack of knowledge is a problem because we are potentially missing an important opportunity to utilize a natural food component such as SFN as an epigenetic agent to alter breast cancer cell function, including the possibility of further enhancing vitamin D effects as an anti-cancer therapy. In addition, a stronger scientific understanding of the effects of SFN on the expression of certain vitamin D-dependent genes in breast cancer cells could encourage a new direction of research combining nutrients and bioactive food components with applications to other organs susceptible to cancer, such as the colon and prostate.

The main objective of this study is to better understand to what extent the HDACI TSA and the bioactive food component SFN can modulate vitamin D action in

the MCF-7 cell line. Our central hypothesis is that TSA and SFN will increase cellular vitamin D responsiveness, as measured by increased expression of specific vitamin D-induced genes. The hypotheses listed below are based upon the strong support in the published literature showing that there is an increased expression of vitamin D-induced genes following treatment with a HDACI.

2.2. Specific Aims and Hypotheses

- **Experiment 1: Effect of TSA on Vitamin D-Induced Gene Expression**

Specific Aim: Determine to what extent TSA treatment affects vitamin D-induced CYP24A1, TRPV6 and VDR mRNA expression in MCF-7 cells.

Hypothesis: Combination treatment of 1,25(OH)₂D₃ and TSA will increase vitamin D-responsive gene expression in MCF-7 cells to a greater extent than 1,25(OH)₂D₃ alone.

- **Experiment 2: Effect of SFN & TSA on Vitamin D-Induced Gene Expression**

Specific Aim: Determine to what extent a SFN or TSA treatment affects vitamin D-induced CYP24A1, CYP27B1, TRPV6 and VDR mRNA expression in MCF-7 cells.

Hypothesis: Combination treatment of 1,25(OH)₂D₃ and SFN or TSA will increase vitamin D-responsive gene expression in MCF-7 cells to a greater extent than 1,25(OH)₂D₃ alone.

2.3. Significance

Studies in breast cancer cell culture models and *in vivo* animal breast cancer models support an anti-cancer effect of 1,25(OH)₂D₃ treatment. However, systemic treatment with the vitamin D hormone elicits a calcemic response that can lead to the development of hypercalcemia, which limits the therapeutic window of this compound. Thus, the development of treatments that could enhance the anti-cancer effects of endogenous vitamin D by increasing intracellular availability/activity of 1,25(OH)₂D₃ could be therapeutically useful in treatment, or as a possible low-risk chemo-preventative strategy.

The contribution of these research studies is twofold; first, we characterized the influence of 1,25(OH)₂D₃ alone on genes potentially responsive to vitamin D (CYP24A1, TRPV6), or part of the a network of genes involved in vitamin D metabolism (VDR, CYP27B1, CYP24A1) in MCF-7 human breast cancer cells, and second, we determined the extent to which co-treatment with HDACI compounds, TSA and SFN, can increase vitamin-D induced gene expression.

These contributions are significant because they indicate to what extent these two HDACI influence vitamin D response. These findings could have future implications in cancer treatment and prevention, as well as encouraging further research into possible chemotherapy treatments for certain cancers utilizing HDACI.

2.4. Limitations

Cell culture models are useful for identifying possible molecular changes induced by various treatments. However, caution must be used when extrapolating the findings from these experiments performed in cell culture directly to humans. Human physiology is far more complex than that of cells grown in a dish. Thus, findings from this type of study will best be used to aid in hypothesis formation related to how these processes may function in humans, rather than as a basis for clinical recommendations and practices. Our experimental MCF-7 breast cancer cell model represents only one of the many approaches that could be utilized to study the interactions between 1,25-dihydroxyvitamin D and HDACI, and thus the translatability of our findings to other situations will need to be assessed.

CHAPTER 3

EXPERIMENTAL DESIGN AND METHODS

3.1 Experimental Design

This study investigated the effects of a 24h co-treatment of 10 nmol/L 1,25(OH)₂D₃ and histone deacetylase inhibitors (TSA 1 μmol/L or SFN 20 μmol/L) on CYP24A1, CYP27B1, TRPV6 and VDR mRNA in MCF-7 cells, a breast cancer cell culture model known to respond to vitamin D. (Levenson & Jordan, 1997)

Relative gene expression levels were determined by real time qPCR of mRNA following incubation of cells with 1,25(OH)₂D₃ alone or in combination with TSA in Experiment 1 and for Experiment 2, and with SFN in Experiment 2. TSA, SFN, and 1,25(OH)₂D₃ treatment concentration and incubation times were chosen in accordance with published literature values. (C. Banwell, O'Neill, Uskokovic, & Campbell, 2004; Malinen et al., 2008; Welsh, 2011) Based on the known role of TSA and SFN as a HDACI, we expected that vitamin D-mediated gene expression of CYP24A1, CYP27B1, TRPV6 and VDR would increase in the presence of either HDACI.

3.2 Methods

3.2.1 Cell Culture

Human breast cancer cells, MCF-7 (kindly provided by Dr. H. Xiao's Lab, Food Science Dept., UMass) were grown in 6-well culture plates to 100% confluence

(about 96 - 120 hours), using MEM growth media (89% MEM (Gibco), 10% fetal bovine serum, and 1% penicillin/streptomycin). Cells were incubated at 37°C with 5% CO₂. At the completion of the experiment, cells were harvested in Trizol and RNA was subsequently extracted, quantified and cDNA synthesized.

3.2.2 Experiment 1: Effect of TSA on Vitamin D-Induced Gene Expression

Upon reaching 100% confluence (96 hours), the MCF-7 cells were treated with various combinations of TSA and 1,25(OH)₂D₃. A total of four 6-well plates were used with four different treatments (n=6 wells/treatment; Table 3.1), yielding a total of 24 samples. The first treatment was the vehicle-treated control, which contained an equal volume of ethanol vehicle and was used as the reference treatment in the statistical analysis. The second treatment was 1µmol/L TSA (Sigma-Aldrich, St. Louis, MO) dissolved in ethanol and was used to detect the effect of the HDACI alone. The third treatment was 10nmol/L 1,25(OH)₂D₃ (Enzo Life Sciences, Plymouth Meeting, PA) dissolved in ethanol. The final treatment was a co-treatment of 10nmol/L 1,25(OH)₂D₃ and 1µmol/L TSA. The cells were incubated with the various treatments for 24 hours before being harvested. Treatment calculations are found in Appendix A.

Table 3.1 Experiment 1 Samples and Treatments

Sample Number	Treatment
1-6	Control (ethanol)
7-12	1µmol/L TSA
13-18	10nmol/L 1,25(OH) ₂ D ₃
19-24	10nmol/L 1,25(OH) ₂ D ₃ + 1µmol/L TSA

3.2.3 Experiment 2: Effect of SFN & TSA on Vitamin D-Induced Gene Expression

In the second experiment, MCF-7 cells were grown in 6-well dishes to 100% confluence and treated 120 hours after sub-culturing with one of 5 possible treatments for 24h prior to harvesting the cells. The treatment scheme is shown in Table 3.2 and is similar to Experiment 1 with the addition of a SFN (Sigma-Aldrich, St. Louis, MO ; 20 $\mu\text{mol/L}$) and SFN plus 1,25(OH)₂D₃ treatment groups. Half of the wells were treated with 10nmol/L 1,25(OH)₂D₃ (Enzo Life Sciences, Plymouth Meeting, PA). Treatment calculations are found in Appendix A.

Table 3.2 Experiment 2 Samples and Treatments

Sample Number	Treatment
1-4	Control
5 & 6	1 $\mu\text{mol/L}$ TSA
7-12	20 $\mu\text{mol/L}$ SFN
13-16	10nmol/L 1,25(OH) ₂ D ₃
17 & 18	10nmol/L 1,25(OH) ₂ D ₃ + 1 $\mu\text{mol/L}$ TSA
19-24	10nmol/L 1,25(OH) ₂ D ₃ + 20 $\mu\text{mol/L}$ SFN

3.3 Gene Expression Analysis

3.3.1 RNA Isolation and Quantification

After the 24-hour treatment period, total cellular RNA was harvested. The media was aspirated from the treatment wells, the cells were washed once with a PBS solution, and the cells were then treated with 750 μL TRIzol LS reagent (Life Technologies) and scraped from the plate and homogenized. The TRIzol-treated cell homogenate was stored at -80°C in 1.5mL plastic Eppendorf tubes until used for mRNA isolation.

To isolate the total RNA, 150 μ L chloroform reagent was added to each homogenate, mixed and centrifuged to separate the organic and aqueous phases. The aqueous layer was then removed with a pipet and transferred into a new 1.5mL Eppendorf tube, where 500 μ L isopropanol was added to precipitate the RNA. The precipitated RNA was washed once with 1 mL of 75% ethanol (made with Milli-Q water). Then, 40 μ L DEPC-treated water was added to each sample and incubated for ten minutes at 56°C in order to make the RNA soluble. RNA samples were quantified by uv/vis spectrophotometry, as follows: 2 μ L of the RNA sample in DEPC-treated water was diluted in 498 μ L Milli-Q water, and then placed in a quartz cuvette and absorbance was measured at $\lambda=260$ nm.

cDNA was prepared from the RNA using reverse transcriptase in the SuperScript III First-Strand Synthesis System and oligo dT primer (Life Technologies, Grand Island, NY) following the manufacturer's instructions. cDNA was measured at $\lambda=260$. Detailed protocol instructions and calculations are found in Appendix B.

3.3.2 Real Time PCR

For all experiments, mRNA expression was measured by Real Time PCR using a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). Reactions consisted of MilliQ water, 200ng of cDNA, 2x TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA), 20x TaqMan Gene Expression assays for GAPDH (housekeeping gene) and either CYP24A1, CYP27B1, TRPV6 or VDR (Applied Biosystems, Foster City, CA). Cycle number (C_T) measurements were

normalized (ΔC_T) to GAPDH C_T expression by subtracting the C_T of GAPDH expression from each target gene of interest (GOI) ($\Delta C_T = GOI_{C_T} - GAPDH_{C_T}$). A detailed protocol is found in Appendix B.

3.4 Statistical Analysis

Derived ΔC_T measurements (GOI mRNA corrected by GAPDH mRNA expression) were transcribed to Graph Pad Prism 5.0 tables for statistical analysis. Comparisons among treatment means were done by using the comparative C_T method. In Experiment 1 and 2, all ΔC_T data were initially checked for possible outliers using the Grubb's test in GraphPad Prism. One data point in the combined TSA and vitamin D treatment group in Experiment 1 was identified as being statistically significant and it was excluded. Statistical significance among treatment groups in each experiment was carried out on ΔC_T measurements and tested by a one-way ANOVA with Bonferroni's post-hoc test for specific individual pair-wise treatment mean comparisons. The alpha level for statistical significance was set at $p < 0.05$. For ease of interpretation and for graphical illustration, data were converted into relative fold-expression with the vehicle control treatment set to a value of 1, using the $\Delta\Delta C_T$ method, in which $\Delta\Delta C_T$ is calculated as follows: $\Delta\Delta C_T = (\Delta C_T_{\text{Experimental Treatment}} - \Delta C_T_{\text{Vehicle Control Treatment}})$ and the relative fold change in expression is calculated as $2^{-(\Delta\Delta C_T)}$. Data are presented in the text and figures as mean \pm standard error of the mean (SEM).

CHAPTER 4

RESULTS

4.1 Experiment 1: Effect of TSA on Vitamin D-Induced Gene Expression

Previous studies have found that a co-treatment of various breast cancer cell lines with $1,25(\text{OH})_2\text{D}_3$ and the histone deacetylase inhibitor (HDACI) trichostatin A (TSA) had an antiproliferative effects. (C. Banwell, O'Neill, Uskokovic, & Campbell, 2004; C. Banwell et al., 2006; Malinen et al., 2008) We hypothesized that a co-treatment of $1,25(\text{OH})_2\text{D}_3$ with the HDACI TSA would increase vitamin D-dependent CYP24A1, TRPV6 and VDR gene expression in MCF-7 cells.

4.1.1 VDR mRNA

Treatment of MCF-7 cells for 24 h with the histone deacetylase inhibitor TSA (Figure 4.1) caused a significant 11-fold increase in VDR mRNA compared to vehicle-treated control cells (11.2 ± 4.4 vs. 1.0 ± 0.3 , mean \pm SEM, $n=6$ and $n=5$, respectively). Treatment with a 10nmol/L dose $1,25(\text{OH})_2\text{D}_3$ alone had no effect (0.8 ± 0.4 , $n=6$) on VDR gene expression. VDR expression was significantly increased in cells treated with both $1,25(\text{OH})_2\text{D}_3$ and TSA (2.3 ± 0.1 , $n=5$) compared to $1,25(\text{OH})_2\text{D}_3$ treatment alone.

4.1.2 CYP24A1 mRNA

Treatment with TSA alone (Figure 4.2) increased CYP24A1 mRNA 10-fold compared to controls (10.0 ± 3.2 vs. 1.0 ± 0.7 , $n=5$ and $n=6$, respectively).

1,25(OH)₂D₃ treatment increased CYP24A1 gene expression by 80-fold (80.0 ± 15.8, n=6) compared to controls. Although there was an apparent doubling of CYP24A1 mRNA between the 1,25(OH)₂D₃ only treatment and the 1,25(OH)₂D₃ and TSA treatment (163.0 ± 18.0, n=6), this difference was not statistically significant.

4.1.3 TRPV6 mRNA

TRPV6 gene expression was only evident in very few samples in Experiment 1 negating an assessment of treatment effects on this gene in MCF-7 cells.

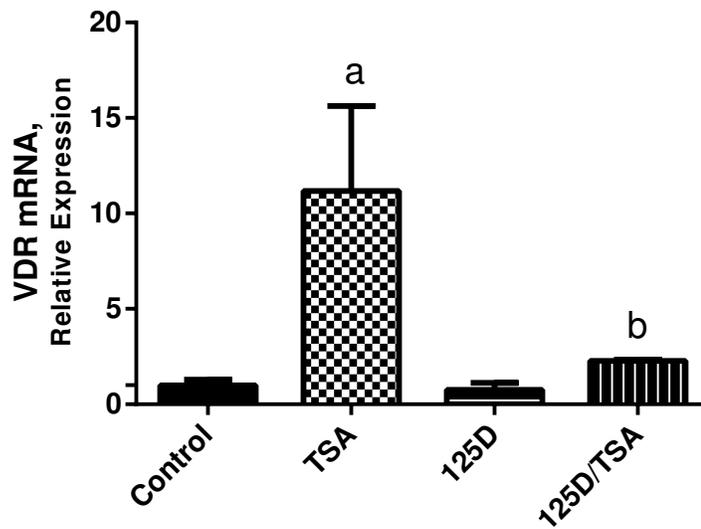


Figure 4.1 Experiment 1: Effect of TSA on VDR Gene Expression
 Mean ± SEM fold change of VDR mRNA in comparison to a control, at the value of 1. Statistical significance notation: a= p<0.05 vs. control, b= p<0.05 vitamin D compared to vitamin D & TSA.

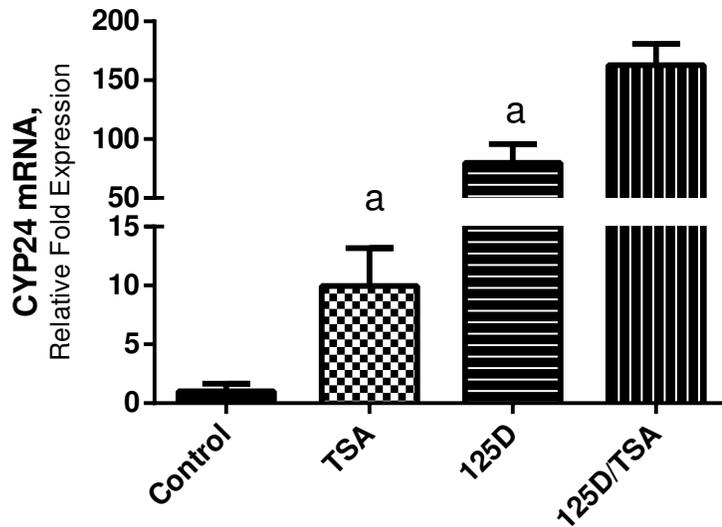


Figure 4.2 Experiment 1: Effect of TSA on CYP24A1 Gene Expression
 Mean ± SEM fold change of CYP24A1 mRNA in comparison to a control, at the value of 1. Statistical significance notation: a= p<0.05 vs. control. Note difference in scale on y-axis.

4.2 Experiment #2: Effect of SFN & TSA on Vitamin D-Induced Gene Expression

There is limited previous research on the effects of sulforaphane (SFN) on gene expression in MCF-7 breast cancer cells. Some experiments have shown that solo treatment with SFN can decrease histone deacetylase (HDAC) activity, supporting a role of SFN as a possible histone deacetylase inhibitor (HDACI). (M. Myzak, Karplus, Chung, & Dashwood, 2004) However, the effects of a co-treatment of $1,25(\text{OH})_2\text{D}_3$ and SFN in MCF-7 cells had not yet been investigated. We hypothesized that a co-treatment of $1,25(\text{OH})_2\text{D}_3$ with the HDACI, SFN, would increase vitamin D-dependent VDR, CYP24A1, CYP27B1, and TRPV6 gene expression in MCF-7 cells. We tested this hypothesis by growing MCF-7 cells to 100% confluence and treated them for 24 hours with either 10nmol/L $1,25(\text{OH})_2\text{D}_3$, 20 $\mu\text{mol/L}$ SFN or a combination treatment. We also included treatment with 1 $\mu\text{mol/L}$ TSA alone and in combination with $1,25(\text{OH})_2\text{D}_3$ to replicate the findings in Experiment 1.

4.2.1 VDR mRNA

Similar to what was observed in Experiment 1, treatment of MCF-7 cells in Experiment 2 with TSA alone (Figure 4.3) for 24h increased VDR mRNA compared to vehicle-treated controls (12.6 ± 4.2 vs. 1.0 ± 0.2 , $n=2$ and $n=4$, respectively). In contrast to the positive effect of the histone deacetylase inhibitor TSA on VDR mRNA, treatment with the histone deacetylase inhibitor SFN (20 $\mu\text{mol/L}$ for 24h) had no effect (1.1 ± 0.3 , $n=6$) on VDR mRNA compared to controls. However, in contrast to the lack of effect of $1,25(\text{OH})_2\text{D}_3$ on VDR mRNA we observed in

Experiment 1, in this experiment 10nmol/L 1,25(OH)₂D₃ had a positive effect on VDR mRNA (6.3 ± 1.8). VDR mRNA expression in the combination treatment of 1,25(OH)₂D₃ and TSA was 22.6 ± 15 (n=2), but this apparent increase in VDR mRNA was not statistically significant, which reflects the low number of samples tested and the marked variation observed in VDR mRNA between these two samples (37.6 and 7.6). Unexpectedly, we observed that VDR mRNA was lower with SFN and 1,25(OH)₂D₃ combination treatment than when 1,25(OH)₂D₃ was given alone (1.4 ± 0.3 vs 6.3 ± 15.0).

4.2.2 CYP24A1 mRNA

Compared to vehicle-treated control cells (1.0 ± 0.0, n=4), an increase in VDR mRNA was found after treatment with either TSA (14.0 ± 0.9, n=2), SFN (18.9 ± 1.6, n=2), or 1,25(OH)₂D₃ (12,746 ± 5,597) alone (Figure 4.4). The positive effect of TSA and 1,25(OH)₂D₃ on CYP24A1 mRNA is consistent with what was observed previously in Experiment 1. The combination treatment of 1,25(OH)₂D₃ with either SFN (7,603 ± 2,245) or TSA (18,393 ± 13,678, n=2), however, caused no further significant increase in CYP24A1 mRNA compared to 1,25(OH)₂D₃ alone.

4.2.3 CYP27B1 mRNA

Treatment with TSA (0.9 ± 0.1, n=4) or SFN (0.6 ± 0.0) alone had no effect on CYP27B1 expression (Figure 4.5). CYP27B1 mRNA was increased in 1,25(OH)₂D₃ treated cells (6.8 ± 5.1, n= 4) compared to controls (1.0 ± 0.0, n=4). Compared to 1,25(OH)₂D₃-treated cells, co-treatment with 1,25(OH)₂D₃ and TSA had no further

effect (6.4 ± 5.2 , $n=2$), while SFN treatment with $1,25(\text{OH})_2\text{D}_3$ (2.0 ± 0.7 , $n= 5$) significantly reduced the positive effects of $1,25(\text{OH})_2\text{D}_3$ on CYP27B1 mRNA.

4.2.4 TRPV6 mRNA

As observed in Experiment 1, TRPV6 gene expression was also only evident in very few samples in Experiment 2, negating an assessment of treatment effects on this gene in MCF-7 cells.

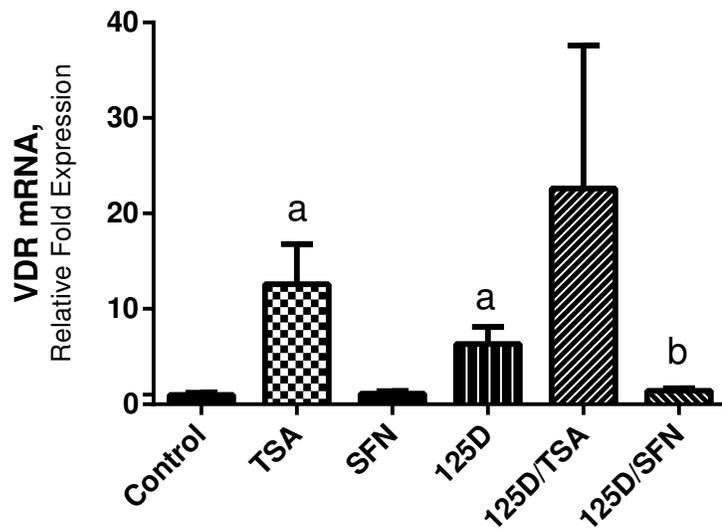


Figure 4.3 Experiment 2: Effect of SFN and TSA on VDR mRNA

Mean ± SEM fold change of VDR mRNA in comparison to vehicle-treated control, at the value of 1. Statistical significance notation: a= p<0.05 vs. control, b= p<0.05 vitamin D alone vs. vitamin D and HDACI.

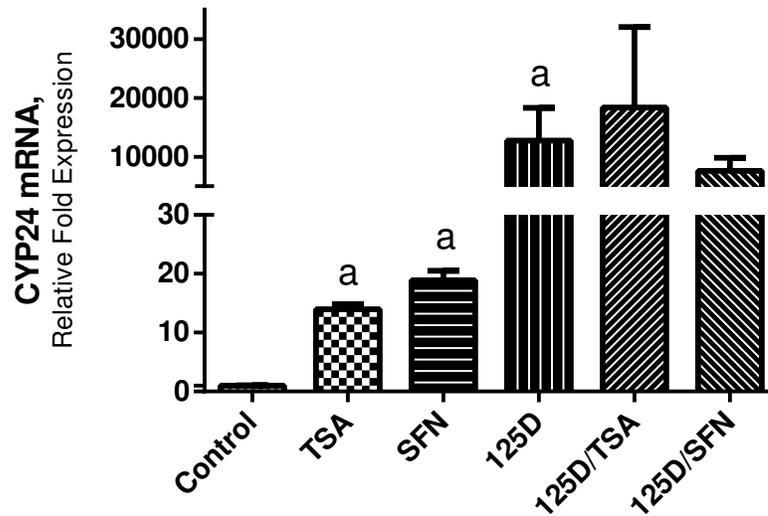


Figure 4.4 Experiment 2: Effect of SFN and TSA on CYP24A1 mRNA

Mean ± SEM fold change of CYP24A1 mRNA in comparison to a control, at the value of 1. Statistical significance notation: a= p<0.05 vs. control. Note difference in scale on y-axis.

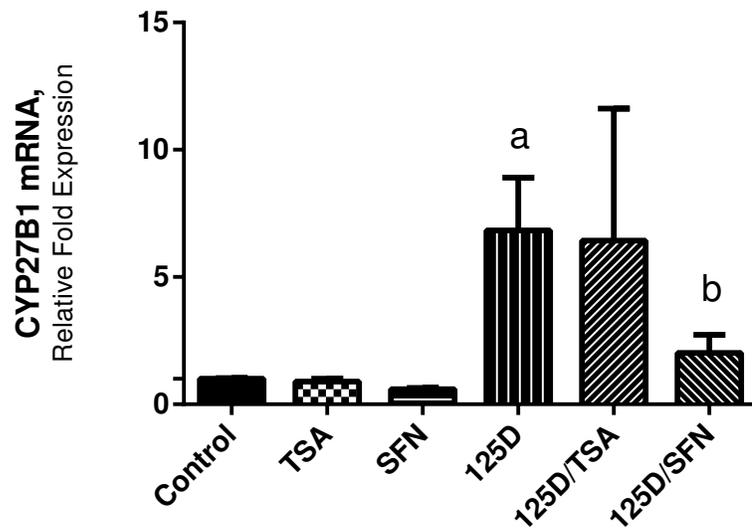


Figure 4.5 Experiment 2: Effect of SFN and TSA on CYP27B1 mRNA
 Mean ± SEM fold change of CYP27B1 mRNA in comparison to a control, at the value of 1. Statistical significance notation: a= $p < 0.05$ vs. control, b= $p < 0.05$ vs. vitamin D alone vs. vitamin D and HDACI.

CHAPTER 5

DISCUSSION

The main objective of this study was to determine the effects of histone deacetylase inhibitors (HDACI), trichostatin A (TSA) and sulforaphane (SFN) on the expression of certain genes involved in vitamin D action in MCF-7 human breast cancer cells, and to what extent co-treatment of HDACI with the active vitamin D hormone, $1,25(\text{OH})_2\text{D}_3$ affected vitamin D-dependent gene expression. Our central hypothesis was that co-treatment with $1,25(\text{OH})_2\text{D}_3$ and either TSA or SFN would increase gene expression of VDR, CYP24A1, CYP27B1 and TRPV6 in MCF-7 cells.

Histone acetylation and deacetylation are dynamic epigenetic processes due to the counteracting effects of nuclear histone acetylase and histone deacetylase enzymes, which can influence gene expression by changing the local binding of DNA to histone proteins in the nucleosome. Acetylation of histone proteins results in a less condensed chromatin structure, which can alter the interaction of DNA with proteins in the transcriptional machinery in the cell nucleus leading to an increase in gene expression. An increase in understanding about the importance of epigenetic changes in gene expression has led to increased clinical interest in evaluating compounds that can affect histone status as potential chemo-preventive or chemotherapeutic agents in cancer. Preclinical studies with HDACI in various cancer cell and animal models have been encouraging because they indicate that HDACI may have a preferential effect on cancerous versus normal cells (Dashwood & Ho, 2007) and may slow cancer cell replication. (Davis & Ross, 2007) The

mechanism underlying the effects of HDACI on the growth of cancer cells is not completely understood. Interestingly, however, studies of HDACI have found that they affect the expression of only a relatively small subset of genes. (Johnson, Chung, & Trump, 2010) Although the control of cell growth in cancer is complex, one important factor appears to be the reduced expression of “tumor suppressor” genes. (Lopes et al., 2010) Restoration of the expression of these tumor suppressor genes in cancer cells may be an important avenue in the future for cancer prevention and treatment and has spurred on clinical trials of the relative effectiveness of these potentially therapeutic compounds in various types of cancers. (Marks & Breslow, 2007) The active hormone metabolite of vitamin D is well known to have pleiotropic effects on cells, including being anti-proliferative (C. M. Banwell, Singh, Stewart, Uskokovic, & Campbell, 2003; Deeb, Trump, & Johnson, 2007) and pro-apoptotic (Swami et al., 2012) , and is currently being investigated in ongoing clinical trials in certain cancer types. The action of $1,25(\text{OH})_2\text{D}_3$ on cells is mediated by the vitamin D receptor (VDR), which is a steroid hormone intracellular transcription factor. Alteration in cellular VDR content has been shown to influence cellular $1,25(\text{OH})_2\text{D}_3$ responsiveness. (Essa et al., 2012; Matilainen, Malinen, Turunen, Carlberg, & Vaisanen, 2010) Thus, lower expression of VDR would be expected to affect vitamin D responsiveness in both normal and cancer cells. Compared to normal cells, reduced VDR expression has been found in many tumor cells and has been suspected as a possible modulator of tumor growth. (Lopes, Paredes, Costa, Ylstra, & Schmitt, 2012) For example, studies have reported a moderate down-regulation of VDR expression in breast tissue carcinomas. (M. G. Anderson, Nakane, Ruan,

Kroeger, & Wu-Wong, 2006; Lopes et al., 2010) Moreover, supportive *in vivo* evidence for a role of the VDR in cancer prevention is the observation that the induction of tumors in VDR knockout mice is greater than in control mice (Dashwood & Ho, 2007) , supporting a role for the VDR as a possible tumor suppressor gene. The normal action of the VDR in the nucleus requires the action of a nuclear histone acetylase to bring about gene transcription. (P. H. Anderson, May, & Morris, 2003) Given the above information, we hypothesized that HDACI treatment would increase vitamin D-induced gene expression in MCF-7 human breast cancer cells.

5.1 Treatment Effects on VDR Gene Expression

In both Experiment 1 and Experiment 2 we investigated the effects of TSA alone and in combination with $1,25(\text{OH})_2\text{D}_3$ on VDR mRNA. We found that TSA treatment significantly increased VDR mRNA in each of these experiments by 11- and 12.6-fold, respectively. This observation would suggest that $1,25(\text{OH})_2\text{D}_3$ - induced cell signaling should be increased in TSA-treated cells. This notion is consistent with our observations in Experiment 1 where there was a ~2-fold higher VDR mRNA expression in MCF-7 cells treated with both TSA and $1,25(\text{OH})_2\text{D}_3$ compared to $1,25(\text{OH})_2\text{D}_3$ alone. Although not statistically significant, we observed a similar trend in Experiment 2 where there was a ~3-fold higher VDR mRNA expression in cells treated with both TSA and $1,25(\text{OH})_2\text{D}_3$ compared to $1,25(\text{OH})_2\text{D}_3$ alone. Banwell and colleagues (C. Banwell, O'Neill, Uskokovic, & Campbell, 2004) had previously reported that a combination treatment of 1nmol/L

1,25(OH)₂D₃ plus 15nmol/L TSA administered for 36 hours to the relatively vitamin D-resistant breast cancer cell line MBD-MB-231 yielded only a 0.1 fold increase of VDR gene expression. (C. Banwell, O'Neill, Uskokovic, & Campbell, 2004) A study in malignant melanoma cells found that a co-treatment of 1,25(OH)₂D₃ and TSA modulated VDR mRNA expression in both 1,25(OH)₂D₃ receptive and resistant cell lines. (Essa et al., 2012) Overall, our findings and those of others suggest that the biologic actions of 1,25(OH)₂D₃ in some cancer cells may be enhanced by co-treatment with TSA or other HDACIs. This observation also suggests that epigenetic mechanisms may be at play under basal conditions to limit the expression of VDR and that TSA may be useful in other contexts where there is a need to increase VDR expression, such as in the elderly who may have lower intestinal expression of VDR that could limit cellular response to 1,25(OH)₂D₃. Rashid and coworker (Rashid et al., 2001) reported that low dose treatment with HDACIs (sodium butyrate or TSA) in combination with 1,25(OH)₂D₃ synergized to inhibit the growth of LNCaP, PC-3 and DU-145 prostate cancer cells. There has also been evidence that TSA alone exhibits anti-proliferative properties in breast cancer cells. (Drzewiecka & Jagodzinski, 2012; Tavakoli-Yaraki, Karami-Tehrani, Salimi, & Sirati-Sabet, 2013) Since our research shows a significant increase in VDR mRNA in MCF-7 cells in response to TSA, this may, at least in part, be a reason behind TSA's growth inhibitory effects. Further research with different breast cancer cells lines are needed to confirm these findings across different cancer cell types, as well as to investigate the possible benefits of combined treatment in various animal cancer models. In the context of breast cancer, it should also be noted that the expression

of estrogen receptor- β is often epigenetically silenced and leads to tamoxifen resistance. TSA treatment can increase the expression of estrogen receptor- β and restore responsiveness to tamoxifen and other anti-estrogen agents. (Pitta, Papageorgis, Charalambous, & Constantinou, 2013; Tu et al., 2012) A similar scenario in breast cancer cells with reduced VDR expression should be investigated.

Based on the demonstration that SFN has histone deacetylase inhibitor activity (Li et al., 2010b; M. Myzak, Karplus, Chung, & Dashwood, 2004; Pledgie-Tracy, Sobolewski, & Davidson, 2007) , we expected that SFN would have similar effects to TSA in MCF-7 cells. However, we found that SFN treatment alone had no effect on VDR mRNA. In addition, co-treatment of SFN and $1,25(\text{OH})_2\text{D}_3$ led to significantly lower VDR mRNA levels than with $1,25(\text{OH})_2\text{D}_3$ alone. The reason for the discrepancy in the response of VDR gene expression to these two HDACI in MCF-7 cells is unknown. The effects of SFN on vitamin D-induced gene expression have not been previously addressed in this human breast cancer cell line. *In vitro*, SFN has apoptotic properties similar to TSA (Meeran, Patel, & Tollefsbol, 2010; Pledgie-Tracy, Sobolewski, & Davidson, 2007) ; so our conflicting results suggest that SFN may not have a strong enough HDACI effect on MCF-7 cells, or perhaps our dosage and treatment time needs to be increased. Additional studies will be needed to directly assess HDACI activity of SFN compared to TSA in MCF-7 cells. Differences in bioavailability of SFN in MCF-7 cells may exist due to differences in membrane transport or metabolism of these two compounds.

Discordant findings between Experiment 1 and Experiment 2 were observed when cells were treated with $1,25(\text{OH})_2\text{D}_3$ alone. In Experiment 1, $1,25(\text{OH})_2\text{D}_3$

treatment had no effect. In contrast, in Experiment 2, 1,25(OH)₂D₃ treatment led to a significant 6-fold increase in VDR mRNA. The reason for the more robust response to 1,25(OH)₂D₃ in the second experiment is unknown. Clearly, 1,25(OH)₂D₃ was able to affect gene expression in Experiment 1 because the expression of CYP24A1, a highly vitamin D-responsive gene, was markedly increased, ruling out inactivity of the hormone.

5.2 Treatment Effects on CYP24A1 Gene Expression

Treatment with either HDACI alone caused a significant induction of CYP24A1 expression. The physiologic effect of increased CYP24A1 expression is to dampen the vitamin D signal in cells by initiating catabolism of the active hormone. Thus, the end result of HDACI treatment on vitamin D-mediated cell function needs further detailed study. Treatment of MCF-7 breast cancer cells with TSA alone on CYP24A1 expression has not been previously examined, but Luo and colleagues (Luo et al., 2010) found similar results in prostate cancer cells, where doses of TSA half as much as were used in these experiments were capable of inducing CYP24A1 gene expression. In Experiment 2, we found that SFN treatment increased CYP24A1 expression to a similar extent as TSA. These observations demonstrating a consistent effect of HDACI on CYP24A1 expression in MCF-7 cells suggest that epigenetic mechanisms affecting histone acetylation status may be causing CYP24A1 expression to be silenced in the basal state. However, we found no evidence of a significant increase in CYP24A1 expression with co-treatment of 1,25(OH)₂D₃ and HDACI.

The CYP24A1 gene is known as the strongest $1,25(\text{OH})_2\text{D}_3$ responsive gene, and is commonly used to assess changes in cellular vitamin D signaling. The ability of $1,25(\text{OH})_2\text{D}_3$ treatment to induce CYP24A1 mRNA in MCF-7 cells was consistent in both experiments, although relative vitamin D-induced CYP24A1 expression was much greater in Experiment 2. Induction of CYP24A1 by $1,25(\text{OH})_2\text{D}_3$ was expected and is in agreement with a recent study in MCF-7 cells. (Matilainen, Malinen, Turunen, Carlberg, & Vaisanen, 2010) Some researchers have found that expression of CYP24A1 in breast carcinoma cells is down regulated, (M. G. Anderson, Nakane, Ruan, Kroeger, & Wu-Wong, 2006) , but other studies conclude that expression of this gene is up regulated in cancer cells (Lopes et al., 2010). The reason for these apparent differences is not evident, but could be differences in estrogen receptor status in different breast cancer cell lines. Overall, the lack of an increase in vitamin D-induced CYP24A1 mRNA following co-treatment with $1,25(\text{OH})_2\text{D}_3$ and HDACI is contrary to our working hypothesis that HDACI would increase vitamin D-induced gene expression.

5.3 Treatment Effects on CYP27B1 Gene Expression

In Experiment 2, we investigated the effects of $1,25(\text{OH})_2\text{D}_3$ and HDACI on CYP27B1 expression. The CYP27B1 gene codes for the $1-\alpha$ hydroxylase enzyme that activates the $25(\text{OH})\text{D}$ prohormone to the active hormone $1,25(\text{OH})_2\text{D}_3$. An increase in cellular CYP27B1 expression could lead to an increase in cellular $1,25(\text{OH})_2\text{D}_3$ due to increased cellular conversion of $25(\text{OH})\text{D}$ to the active hormone. This particular possibility was not investigated in the current experiments. We found that neither

HDACI affected CYP27B1 expression in MCF-7 cells. However, 1,25(OH)₂D₃ treatment caused a significant ~7-fold increase in expression.

A previous study done with MCF-7 cell tumors in rats with exposure to 25(OH)D, found that after feeding a 5000 IU/kg high vitamin D₃ supplemented diet, the tumor cells had an increased expression of CYP27B1 mRNA, indicating that a xenograph of MCF-7 cells could still actively potentially synthesize 1,25(OH)₂D₃ in the presence of 25(OH)D. (Swami et al., 2012) This observation is important because it suggests that treatment with a non-toxic dose of vitamin D could be useful therapeutically. Research also shows that carcinoma cells will often show down-regulated expression of CYP27B1. (Lopes et al., 2010) Our results suggest that short-term 1,25(OH)₂D₃ or a vitamin D analog drug treatment could be useful to raise CYP27B1 expression to enhance the anti-cancer effects of endogenous vitamin D or vitamin D supplementation.

We found that combination treatment of TSA with 1,25(OH)₂D₃ did not further increase vitamin D-induced CYP27B1 expression. In contrast, co-treatment with both SFN and 1,25(OH)₂D₃ inhibited vitamin D-induced CYP27B1 expression. The reason for this discrepancy in the action of TSA and SFN on vitamin D-induced CYP27B1 expression is unknown. To our knowledge, the effects of HDACIs on CYP27B1 gene expression have not been previously examined. Since a reduction in CYP27B1 by SFN under in vivo conditions could have detrimental effects on vitamin D action, further study of this relationship is warranted. This is particularly true in light of the fact that SFN is a bioactive food component found in cruciferous vegetables, such as broccoli, cabbage, etc.

5.4 Treatment Effects on TRPV6 Gene Expression

In both experiments, the expression of TRPV6 mRNA was apparently absent in most samples. Recent research has concluded, however, that elevated TRPV6 expression is common in estrogen receptor negative breast cancer cells lines (Ouadid-Ahidouch, Dhennin-Duthille, Gautier, Sevestre, & Ahidouch, 2012; Peters et al., 2012) ; so one explanation for our null finding in MCF-7 cells is that they are estrogen receptor positive, and would not necessarily share similar TRPV6 gene expression characteristics with estrogen receptor negative breast cancer cells. Additional investigation of this notion is needed.

5.5 Limitations

There are a few limitations with the work presented that are important to discuss. Due to constraints with time and resources, we were only able to complete two experiments with a relatively small sample size for each. Seeing conflicting results between Experiment 1 and 2 in regards to VDR gene expression with the $1,25(\text{OH})_2\text{D}_3$ treatment, leads us to believe that additional study is needed to have a more solid understanding of HDACI effects on VDR mRNA in MCF-7 cells.

Another possible limitation in our research was our treatment dosages and how they relate to biological serum levels. Our treatment dosage of $1,25(\text{OH})_2\text{D}_3$ was 10 nmol/L, which is ~1000 times higher than the typical circulating $1,25(\text{OH})_2\text{D}_3$ concentration. However, this dose of $1,25(\text{OH})_2\text{D}_3$ is commonly used in cell culture experiments and does not represent the top of the dose-response curve

for many vitamin D-induced actions in cultured cells. The TSA dose was 1 $\mu\text{mol/L}$, which is a relatively high dose, and many cellular effects of TSA can be seen at lower doses. The SFN dose was 20 $\mu\text{mol/L}$ and is in the range used in many other cell culture studies. However, serum SFN concentration after a meal of cruciferous vegetables may be much lower.

CHAPTER 6

SUMMARY AND CONCLUSIONS

At the conclusion of our study, we have gained insight into the, as of yet, undefined effects that the histone deacetylase inhibitors TSA and SFN have on vitamin D-modulated gene expression, and their ability to work with $1,25(\text{OH})_2\text{D}_3$ to alter overall gene expression of CYP24A1, CYP27B1, and VDR in MCF-7 breast cancer cells. Since an overall trend of down-regulation in vitamin D modulated gene expression is often seen in breast cancer cells (Lopes et al., 2010), the ability of HDACI to effect epigenetic changes in cells may be important to optimize vitamin D's anti-proliferative effects in cancer cells. TSA is already being used as a co-treatment in cancer therapy. Vorinostat, which is a laboratory developed compound with the same chemical structure to TSA, is being studied in hundreds of clinical trials. (Marks & Breslow, 2007) The findings from these clinical studies are showing that TSA has HDACI effects and is capable of increasing the therapeutic effects of different anti-cancer hormone therapies. Although much more work is needed, our preliminary study of TSA in MCF-7 cells suggests that TSA may have useful properties to modulate therapeutic responses to vitamin D-based breast cancer therapies by increasing VDR gene expression. An important next step in our research would be to show that TSA increases VDR protein in MCF-7 cells and potentiates the anti-cancer effects of vitamin D hormone or vitamin D analogs.

This is one of the first studies to look at SFN as a HDACI in co-treatment with $1,25(\text{OH})_2\text{D}_3$. Our working hypothesis was based upon the findings from previous research that SFN has HDACI activity. (M. C. Myzak, Tong, Dashwood, Dashwood, &

Ho, 2007) We expected that SFN and TSA would act through a similar epigenetic mechanism and would have similar effects in MCF-7 cells. However, the apparent inhibition by SFN of vitamin D-induced VDR and CYP27B1 expression was totally unexpected. Additional study will be needed to understand why TSA and SFN had different effects on vitamin D-induced VDR and CYP27B1 expression. Despite this, cancer prevention research with SFN has been producing positive results; SFN has been seen to inhibit HDAC activity in human colon, prostate and breast cancer cells lines, as well as induce histone hyperacetylation; both of which are linked to regulate proliferation, differentiation and apoptosis in cancer cells lines. (Anwar-Mohamed & El-Kadi, 2009; Kim & Singh, 2009; Meeran, Patel, & Tollefsbol, 2010; M. C. Myzak, Tong, Dashwood, Dashwood, & Ho, 2007; Pledge-Tracy, Sobolewski, & Davidson, 2007) But our conclusions force us to examine the role that HDACI plays on cellular vitamin D metabolism in a different way. Working at a cellular level may not be the best platform for this research, examining the relationship *in vivo* may yield some new evidence.

6.1 Further Research

The results from this research have allowed us to gain additional knowledge about the interactions between $1,25(\text{OH})_2\text{D}_3$ and two HDACI, TSA and SFN *in vitro*; but there are many more interactions that should be explored to fully understand these mechanisms. It would be important to investigate the effects of circulating vitamin D, 25-hydroxyvitamin D, especially in a co-treatment with TSA and SFN in

regards to CYP27B1 gene expression to see if the inactive form of vitamin D and a HDACI can raise expression higher than we saw with the $1,25(\text{OH})_2\text{D}_3$ treatment.

It would also be important to look at the dose dependent reactions of these treatments; there is limited understanding gained from only one treatment dose. Since our SFN treatment yielded only slight changes, it would be important to raise that dose as well to identify maximum responses. However, the issue of cellular toxicity may limit raising the HDACI dose.

Continued research will increase our understanding of the interaction taking place between $1,25(\text{OH})_2\text{D}_3$, TSA and SFN; and their impact on vitamin D mediated gene expression, as well as cancer cell proliferation, differentiation and apoptosis. This knowledge could eventually aid us in the formation of new treatments and prevention strategies against the deadly disease, cancer.

APPENDIX A

TREATMENT CALCULATIONS

Vitamin D (Enzo: BML-DM200-0050)	
Stock Solution	50µg in 50µL EtOH =2.4mM (2.4×10^{-3})
Working Solution	Dilute to 1:1,000 2µL stock solution in 2,000µL (2mL) EtOH =2.4µM (2.4×10^{-6} M)
Treatment Solution	Dilute 4.2µL working solution in 1mL Media

Trichostatin A (TSA) Sigma T8552-1MG (10mM in 330µM in EtOH)	
Stock Solution	10mM concentration
Working Solution	5µL stock solution in 500 µL EtOH = 100µM concentration
Treatment Solution	10µL working solution in 1mL media

L-Sulforaphane (SFN) (Sigma: S6317)	
Stock Solution	20mM in DMSO
Treatment Solution	20µmol/L = 1µL working solution in 1mL media

APPENDIX B
LABORATORY PROTOCOLS

RNA Isolation (Make sure to use all autoclaved supplies)

1. Cell Harvest
 - a. Remove the media
 - b. Use 1mL Tri-Reagent (Trizol) per well (in 6-well plate)
 - c. Scratch the cells from the well with a cell scraper
 - d. Pipet suspension till thick and stringy, transfer to eppendorf tubes
 - e. Can then be frozen at -80°C
 - f. Keep 5 minutes at room temp or warm water bath to thaw when ready to complete separation phase
2. Phase Separation (Keep on ice between steps)
 - a. Add 150µL chloroform reagent to the homogenate
 - b. Invert vigorously for 15 seconds
 - c. Allow the mixture to sit at room temperature for 5 minutes
 - d. Centrifuge at 12,000g for 15 minutes at 4°C
3. RNA Precipitation
 - a. Transfer the aqueous phase to a fresh 1.5mL eppendorf tube
 - i. RNA is the top clear aqueous layer, white strip is DNA in the interphase and bottom pink is protein in the organic phase
 - b. Add 0.5mL of 100% isopropanol and mix gently by inversion
 - c. Keep on ice for 15 minutes
 - d. Centrifuge at 12,000 g for 20 min at 4°C

4. RNA Washing and Solubilization

- a. Remove the supernatant (all liquid above small pellet on bottom, be careful not to disturb the pellet, may appear white or as a clear gel)
- b. Add 1mL of 75% Ethanol (EtOH) (made with Milli-Q water)
- c. Kick samples at the bottom to dislodge the pellet
- d. Centrifuge at 7,500 g for 5 minutes at 4°C
- e. Remove EtOH: pipette the solution out but be careful not to lose the pellet at the bottom, and pipette the rest out with a 200µL pipette
- f. Briefly spin samples down, remove the remaining liquid with a 10µL pipette
- g. Air dry: keep samples open for several minutes, don't dry completely
- h. Add 40µL of Milli-Q water (or DEPC treated water), invert and spin down
- i. Incubate 10 minutes at 56°C to make RNA molecule spread evenly and then keep on ice for several minutes

RNA Quantification

1. Combine 498µL Milli-Q water and 2µL of RNA sample together in eppendorf tubes
2. On spectrophotometer
 - a. Turn on the machine (Allow about 10 minutes to boot up)
 - b. Press 7 = BioMethods
 - c. Press 1 = DNA Quantification
 - d. Rinse cuvette very well to remove ethanol and dry with Kim wipes

- e. Blank the spectrometer with 500 μ L Milli-Q water
 - f. Press start to test blank is at 0.000
 - g. Run samples and record A260, A280 (A320 can identify contamination) and the ratio. The A260/A280 ratio should be between 1.5-1.8
3. Enter A260 and A 280 readings into "RNA Concentration" excel spreadsheet
 4. The concentration of RNA is A₂₆₀ of 10 x μ g/ μ L because the A₂₆₀ of 40 μ g/mL RNA samples equals to 1
 5. Dilute RNA samples to 1 μ g RNA/4.75 μ L we need at least 2 μ g RNA for PCR

First Strand cDNA Synthesis

1. The following procedure is designed to convert 5 μ g of total RNA into first-strand cDNA
2. Mix and briefly centrifuge each component before each use
3. Combine the following in 0.2 or 0.5mL RNAased tube (will add up to 10 μ L)
 - a. 1 μ L Primer: 50 μ M oligo(dT)
 - b. 1 μ L 10mM dNTP mix
 - c. 8 μ L 5 μ g RNA and DEPC water (information found in Excel Spreadsheet)
 - d. Incubate at 65°C for 5 minutes, then place on ice for at least 1 minute.
(Protocol Library: Kim pre-cDNA)
4. Prepare the following cDNA Synthesis Mix, adding each component in the indicated order (listed are amounts for 1 reaction)
 - a. 2 μ L 10X RT buffer

- b. 4 μ L 25mM MgCl²
 - c. 2 μ L 0.1 M DTT
 - d. 1 μ L RNaseOUT (40 U/ μ L)
 - e. 1 μ L SuperScript III-RT (200 U/ μ L)
5. Add 10 μ L of cDNA Synthesis Mix to each RNA/ primer mixture; mix gently and collect by brief centrifuge.
 6. Incubate as follows: (Protocol Library: Kim cDNA)
 - a. 10 minute at 25°C followed by
 - b. 50 minutes at 50°C
 - c. Terminate the reaction at 85°C for 5 minutes, then chill on ice
 7. Collect the reactions by brief centrifuge. Add 1 μ L of RNase H to each tube and incubate for 20 minutes at 37°C (Protocol Library: RNaseH)
 8. cDNA synthesis reactions can be stored at -30°C to -10°C or used for PCR immediately

Real Time qPCR

1. cDNA quantification
 - a. Add 1 μ L of each cDNA sample to 499 μ L Milli-Q Water in eppendorf tubes
 - b. Use spectrophotometer to obtain A=260 and A=280
 - c. Use "DNA Quantification" excel spreadsheet
 - i. $(A_{280}/.027) \times 500 = \text{cDNA concentration}$
 - ii. $\text{cDNA concentration}/50 = \text{Total volume of sample containing } 50\mu\text{g/mL cDNA}$

- iii. Total Volume - $1\mu\text{L cDNA} = \text{Volume of Milli-Q Water to add to } 1\mu\text{L cDNA}$ to have each sample have $50\mu\text{g/mL cDNA}$
2. Correcting cDNA concentration
 - a. Add $1\mu\text{L}$ of each cDNA sample to new PCR tube
 - b. Add the amount of Milli-Q water specified in “DNA Quantification” excel spreadsheet to bring samples to the same concentration ($50\mu\text{g/mL cDNA}$)
 3. Preparation of Master Mix (in eppendorf tube): each qPCR reaction will have $20\mu\text{L}$ total
 - a. $10\mu\text{L}$ TaqMan Gene Expression Master Mix
 - i. Applied Biosystems #4369016
 - b. $1\mu\text{L}$ TaqMan Gene Expression Assay: Housekeeping-GAPDH (VIC dye)
 - i. Applied Biosystems Hs99999905_m1
 - c. $1\mu\text{L}$ TaqMan Gene Expression Assay
 - i. Target-CYP24A1 with FAM dye
 1. Applied Biosystems Hs00167999_m1
 - ii. Target-CYP27B1 with FAM dye
 1. Applied Biosystems Hs00168017_m1
 - iii. Target- TRPV6 with FAM dye
 1. Applied Biosystems Hs01114089_g1
 - iv. Target-VDR with FAM dye
 1. Applied Biosystems Hs00172113_m1
 - d. $4\mu\text{L}$ Milli-Q Water

- e. Added later to reaction plate: 4 μ L cDNA (200ng/mL total cDNA)
4. Preparation of qPCR plate
- a. Pipette 16 μ L of prepared master mix (from eppendorf tube) into each reaction well in 96 well plate
 - i. Applied Biosystems #4346906
 - b. Pipette 4 μ L of cDNA sample into each reaction well (run in triplicate)
 - c. Cover plate with 96 well plate cover
 - i. Applied Biosystems #4360954
 - d. Tear off white ends of plate cover and smooth out plate cover with a “plate cover smoother” so that it adheres to the plate
 - e. Centrifuge plate in plate spinner (make sure it’s balanced) for 30 seconds
5. StepOnePlus Real-Time PCR System
- a. Turn on machine
 - b. Place plate into machine making sure the numbers and letters are lined up
 - c. Make sure machine has fully turned on (takes 15 minutes)
 - d. Click on StepOne Software v2.0 on desktop
 - e. Click advanced step up
 - f. For tab “Experiment Properties”
 - i. Create name for experiment (experiment # and target gene)
 - ii. Make sure the following settings are clicked:
 - 1. StepOnePlus (96 wells)

2. Quantitation-comparative Ct ($\Delta\Delta C_t$)
 3. TaqMan Reagents
 4. Standard (~2 hours)
- g. For tab "PLATE-SET UP"
- i. Make two targets: One is the housekeeping gene (GAPDH), with VIC dye selected, target gene should have FAM dye selected
 - ii. Add the number of samples wells you have
 - iii. Click "Assign Targets and Samples"
 1. Assign samples to the wells that they are in, assign each triplicate as one sample, and all control wells as one sample; double check these
 2. Assign both GAPDH and target to all sample wells
 3. Select ROX dye for passive reference
- h. Click "Start Run"
- i. Make sure the machine actually starts the reaction
- i. Come back in two hours to check status of reaction, discard plate and export data to the desired place

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