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The Effects of 1,25-Dihydroxyvitamin D3, Docosahexaenoic Acid and 5-Fluorouracil on Human Breast Cancer Cells

Liv Astri Engelsen
Central Washington University

THE EFFECTS OF 1,25-DIHYDROXYVITAMIN D_3, DOCOSAHEXAENOIC ACID AND 5-FLUOROURACIL ON HUMAN BREAST CANCER CELLS

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Liv Astri Engelsen

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We hereby approve the thesis of

Liv Astri Engelsen

Candidate for the degree of Master of Science

APPROVED FOR THE GRADUATE FACULTY

May 18, 2012

Dr. Susan Hawk, Committee Chair

May 18, 2012

Dr. Kelly Pritchett

May 18, 2012

Dr. Virginia Bennett

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Dean of Graduate Studies

ABSTRACT

THE EFFECTS OF 1,25-DIHYDROXYVITAMIN D_3, DOCOSAHEXAENOIC ACID AND 5-FLUOROURACIL ON HUMAN BREAST CANCER CELLS

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It is well documented that vitamin D and DHA have antiproliferative effects on a variety of human cancers, including breast cancer. Studies have shown that a combination approach to cancer treatment is more effective than any one treatment administered alone. In this study, human mammary epithelial cells from the MCF-7 cell line were treated with 25 μM DHA, 1 μM calcitriol, and 15 μM 5-Fluorourocil alone and in multiple combinations for 72 hours. Both DHA and 5-Fluorouracil slowed growth significantly ($p < 0.05$). In contrast, vitamin D did not inhibit cell growth at 1 μM. The combination of vitamin D and DHA inhibited cell growth slightly more than DHA alone. Interestingly, DHA was just as effective as 5-Fluorouracil at inhibiting cell growth. These results suggest that DHA may be just as efficacious as 5-Fluorouracil in slowing breast cancer progression and therefore may suggest a dietary approach to breast cancer treatment with low toxicity.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

Cancer broadly describes a large number of disease types all characterized by uncontrolled cell division and regulation. Currently one in four deaths (approximately 1500 per day) occur as a result of cancer, making it the second cause of death in the United States (Jemal et al., 2010). The process of cancer development, carcinogenesis, occurs in three steps – initiation, promotion and progression. During the initiation stage, the DNA within a cell is altered. This can be a result of an environmental insult (exposure to a carcinogen) which can result in the formation of free radicals that can cause damage to many parts of the cell, including the DNA. In the promotion stage, the damaged cells begin to divide at an uncontrollable rate resulting in tumor formation. The tumor may then either remain benign and contained, or it may enter the progression stage in which metastasis occurs and the cells infect other healthy tissues of the body.

Under normal conditions, there are several mechanisms in place that keep cell division tightly regulated. Some of these controls are genetic or hormonal, often involving growth factors such as interleukins and cytokines, or chemical signals from other cells. Two types of genetic mechanisms involve genes called oncogenes and tumor-suppressor genes. While oncogenes turn on the signal for cell growth, suppressor genes turn it off (Nelms et al., 2007). An important protein involved in the normal cell cycle process is p53, which is a tumor suppressor gene that prevents cells with damaged DNA

from dividing until the DNA is repaired. Known as the "guardian" of the genome, this protein exerts its effects by upregulating the gene p21 which inhibits cyclin-dependent kinase (CDK). Once the cell cycle is disrupted by these proteins, DNA can then be repaired or the cell can be stimulated to undergo apoptosis (Ford et al., 2004). Unfortunately, p53 is suppressed in many tumors and completely inactivated in about half of all cancer cases (Levine, 1997). In fact, p53 mutations occur in breast cancer cases only about 30% of the time, which is much lower than some other cancer types. In many of the breast cancer cases in which a mutation does not occur, p53 is removed from the nucleus and compartmentalized in the cytoplasm of the cell, known as nuclear exclusion, where it is completely inactivated (Moll et al., 1992).

Apoptosis, or programmed cell death, is a process that normal cells undergo to prevent damaged cells from reproducing and is vital to eukaryotic life. The main groups of molecules that play a major role in the process of apoptosis include caspases, Bcl-2 proteins, members of the tumor necrosis factor receptor (TNF-R) superfamily, and p53 (Pardee, 2004). Apoptosis is carried out by caspases and nucleases which are activated by Bax (pro-apoptosis) and deactivated by Bcl-2 (pro-survival) proteins (Pardee, 2004). Caspases are proteases that cleave a number of proteins, which can either activate or inactivate them. These cysteine proteases exist as zymogens within the cell and must be activated under apoptotic conditions (Wang & El-Deiry, 2004). Out of the 11 caspases in humans, 7 of them are closely involved in regulating apoptosis (Reed & Green, 2011). Initiator caspases (2, 8, 9, and 10) activate effector caspases (3, 6, and 7) which induce a

cascade of activation. Once activated, effector caspases inactivate a variety of cellular proteins, ultimately killing the cell (Wang & El-Deiry, 2004).

Carcinogenesis occurs when many of these anticancer regulatory mechanisms are breached simultaneously. Researchers have identified six key physiological mutations within the cell that are required for cancer development and tumorigenesis (see Table 1). These mutations are similar in most, if not all cancer types and can arise at various times during this multi-step process (Hanahan & Weinberg, 2000).

Table 1: The six hallmark capabilities essential to tumorigenesis. (Hanahan & Weinberg, 2000)

Component	Acquired Capability	Example of Mechanism
1	Self-sufficiency in growth signals	Activate H-Ras oncogene
2	Insensitivity to antigrowth signals	Lose retinoblastoma suppressor
3	Evading apoptosis	Produce insulin-like growth factor survival factors
4	Limitless replicative potential	Turn on telomerase
5	Sustained angiogenesis	Produce VEGF inducer
6	Tissue invasion and metastasis	Inactivate E-cadherin

Breast Cancer

Breast cancer is the most frequently diagnosed type of cancer in women and is among the most lethal. An estimated 207,090 women were diagnosed with breast cancer in 2010 in the United States, which accounts for 28% of all new cancer cases in this group. Death rates from breast cancer have been steadily declining in North America since the early 1990s largely due to early detection and current treatment efforts.

However, breast cancer remains the leading cause of cancer-related death among women between the ages of 20 and 59 (Jemal et al., 2011; Jemal et al., 2010).

Over half of all breast cancer cases are in industrialized countries, and rates are nearly five times higher in Western countries than in developing countries and Japan, an observation supporting the role of environmental factors in carcinogenesis. Migrant studies have further supported that these factors play a major role in the onset of breast cancer since a woman's risk for developing breast cancer typically increases when she moves from a developing country (or from Japan) to the US or Canada (AICR/WCRF). Although epidemiological studies show correlations between cancer rates and certain dietary factors, it is challenging to label a single food or drink as a carcinogen. Relating a specific nutrient deficiency to carcinogenesis is equally difficult due to the large number of environmental factors that may play a contributing role (Nelms et al., 2007). For example, an earlier age of menarche and post-menopausal obesity are more common in Western cultures, both of which increase estrogen levels, a hormone that has been linked to the development of breast cancer (AICR/WCRF).

Characterized by estrogen-dependent growth, breast cancer progression is most often dependent on proliferation of breast cancer cells that occurs through the estrogen receptors (ER) of the cell, which are classified as steroid receptors in the nuclear receptor superfamily (Mangelsdorf et al., 1995). While the role of ERβ receptors in breast cancer is unclear, the estrogen receptor ERα is an important marker for breast cancer diagnosis and prognosis. About one third of all breast cancer cases are ERα negative which are

known to have a poorer prognosis than those that are ERα positive (Osborne, 1998; Jensen, 2004).

A tumor suppressor gene known as breast cancer gene 1 (BRCA1) plays an important role in mammary carcinogenesis. Mutations in the BRCA1 and BRCA2 genes have been linked to a poor prognosis for recovery in breast cancer cases, and complete loss of BRCA1 results in DNA replication errors that can facilitate other malignancies (Madjd et al., 2011). Genetic inheritance of a mutant BRCA1 or BRCA2 gene correlates with an estimated lifetime risk for breast cancer of roughly 78% (Brose et al., 2002).

Once a breast tumor metastasizes, quality of life and duration of survival count on the patient's sensitivity to the chemotherapy treatments. The efficacies of such treatments are typically dose-dependent, which is often limited due to the high level of side effects from radiation and chemotherapy drugs. Any supplemental and non-toxic treatment with the potential to increase tumor sensitivity to anti-cancer drugs could contribute to a favorable prognosis and treatment outcome (Bougnoux et al., 2009).

Litrerature Review

Vitamin D

Though technically a prohormone, vitamin D is well known for its important role in bone formation and calcium homeostasis (DeLuca, 2004). Although it can be absorbed through the skin from UVB radiation and then hydroxylated to the metabolically active form in the liver and kidneys, vitamin D deficiency is still prevalent in the United States (Stiff & Miller, 2009). This is partially due to the fact that between October and March,

the amount of ultraviolet radiation from the sun is inadequate in any location above 35° latitude, which includes about two-thirds of the U.S. It is logical that most adults in the U.S., especially those who live in the northern regions, have insufficient levels (21 ng/mL to 29 ng/mL) of serum vitamin D (Stiff & Miller, 2009). Although vitamin D can be consumed in the diet, it is only found naturally in seafood, mushrooms, and egg yolks, so nutritional supplementation is generally recommended (Moyad, 2009).

The Institute of Medicine (IOM) updated the recommended dietary allowances (RDA) for vitamin D in November of 2010, but recommended levels fall short of the amounts required for optimal health, according to many professionals (see Table 2). This is partially due to the fact that the IOM made the recommendations based on the appropriate levels of vitamin D necessary for bone health since evidence for the role of vitamin D in the prevention of cancer, cardiovascular disease, diabetes, and other autoimmune disorders is inconclusive (Ross et al., 2011). The RDA for vitamin D for individuals between the ages of 1 and 70 is currently 600 IU/day, which corresponds with a serum level of 20 ng/ml (50 nmol/L). Many experts believe a more appropriate recommendation for vitamin D would be in the range of 1000-2000 IU/day, especially in locations north of the equator. It is estimated that the body's daily requirement of vitamin D is around 3000-5000 IU/day (Heaney et al., 2003).

Table 2: Assessment of serum 25(OH)D levels for health. (Holick, 2009)

Deficient	**Insufficient**	**Optimal**	**Toxic**
<20 ng/ml	21-29 ng/ml	>30 ng/ml	>150-200 ng/ml

Vitamin D functions through the vitamin D receptor (VDR) of human cells. Once believed to be present only in cells involved in bone formation, VDRs are now known to be present in nearly every nucleated cell in the human body, including mammary cells. Along with their ligands, VDRs control proliferation, apoptosis and differentiation at different stages in cellular development (Welsh, 2007). VDRs belong to the nuclear receptor superfamily and provide a link between signaling molecules on the cell surface and gene transcription factors within the cell. When calcitriol, the active form of vitamin D, binds to the VDR, it forms a dimer complex with another receptor known as retinoid X receptor (RXR). This complex then binds to vitamin D response elements (VDREs) which are specific DNA sequences of target genes, allowing transcription to occur (Ma, et al., 2010; Colston & Hansen, 2001). Through this regulation of gene expression, vitamin D is able to maintain the differentiated phenotype of mammary cells and allows it to possibly play a role in the prevention and treatment of breast cancer (Welsh, 2007).

The relationship between vitamin D status and disease onset and prognosis has been a recent area of interest in all levels of research. Many studies have suggested that there is a negative relationship between vitamin D status and breast cancer risk (Abbas et al., 2008; Crew et al., 2009; Rejnmark et al., 2009) although other studies have failed to establish this relationship (Chlebowski et al., 2008; Freedman et al., 2008; McCullough et al., 2009). A case control study comparing 701 cancer cases to 724 matched controls revealed that women with high levels of serum 1,25(OH)D, and possibly $1,25(OH)_2D$, were at a low risk of developing breast cancer, and this was especially evident in women over 60 years of age (Bertone-Johnson et al., 2005). One study looking at data from

NHANES III observed no relationship between vitamin D status and cancer death, but did find that individuals with vitamin D levels of <17.8 ng/ml had a 26% higher risk of all-cause mortality (Melamed et al., 2008). Other studies have indicated that the lowest number of cancer mortalities occurred in cancer cases that were diagnosed during the summer and fall, when serum vitamin D_3 levels are typically the highest (Robsahm et al., 2004; Lim et al., 2006). A randomized placebo controlled trial in 2007 gave 1179 post-menopausal women either calcium supplementation (1400-1500 mg), calcium plus vitamin D (1100 IU), or a placebo each day and followed them for 4 years. They discovered that the rate of cancer diagnosis was significantly lower in the calcium plus vitamin D group than the calcium or control group. The findings suggest that increasing dietary intake of calcium and vitamin D decreases cancer risk in this population (Lappe et al., 2007).

Research has consistently found that vitamin D exhibits antitumor effects on breast cancer cells *in vitro*, mainly by inducing apoptosis, but also through proliferation inhibition (Deeb et al., 2007). One study showed that apoptosis is induced within 48 hours in MCF-7 cells treated with 100 nM of $1,25(OH)_2D_3$ (Welsh, 1994). Lower concentrations appear to be effective at reducing cell viability as well and MCF-7 cells exhibited growth inhibition after 96 hours of treatment at concentrations ranging from 10 to 100 nM/L $1,25(OH)_2D_3$ (Kemmis et al., 2006). A similar study compared the effects of $1,25(OH)_2D_3$ to a low calcemic vitamin D analog on MCF-7 cells. They discovered that both compounds reduced the number of viable cells in a dose and time dependent manner, and that the vitamin D analog was 50 times more effective than $1,25(OH)_2D_3$

(Simboli-Campbell et al., 1997). Researchers in Denmark discovered that vitamin D and their analogues both slowed the growth and induced apoptosis of human MCF-7 breast cancer cells. Interestingly, these effects occurred independently of caspase activation and p53, while overexpression of Bcl-2 protected the cells from apoptosis (Mathiasen et al., 1999). In another study, MCF-7 cells were pre-treated with the polyphenols resveratrol (from red wine) and genistein (from soy) to mimic the effects of estrogen and increase VDR expression prior to treatment with 100 nM calcitriol. The researchers found that both phytoestrogens increased cellular sensitivity to vitamin D, but more so with reservatrol (Wietzke & Welsh, 2003).

The MCF-7 cell line is characterized by a phenotype that is estrogen-receptor (ER) positive and therefore may be more responsive to treatment. Comparison of the effects of $1,25(OH)_2D_3$ on ER+ cells (MCF-7) and ER- cells (MDA MB 231) resulted in antiproliferative effects that occurred by different genetic mechanisms, but these effects were much stronger in the MCF-7 cells. The mechanisms by which apoptosis was induced in the MCF-7 cell line included the upregulation of p53, p21-activated kinase (PAC-1), and caspases (Swami et al., 2003). Researchers discovered that vitamin D downregulates ER levels in the MCF-7 cell line which could play an important role in its antiproliferative actions (Swami et al., 2000).

Due to the fact that high levels of vitamin D are required to obtain anti-cancer effects in vitro, few clinical trials have used quantities large enough to produce similar results out of fear of inducing hypercalcemia. Daily oral dosing of 0.5 μg to 1.5 μg induced hypercalcemia and did not improve patient prognosis in a sample of 14 subjects

with advanced prostate cancer (Osborn et al., 1995). However, several studies have established that adequate doses of calcitriol can in fact be safely administered if it is done in an intermittent manner (Ma et al., 2010). In one study, oral calcitriol was given to 15 patients with refractory malignancies on a weekly basis for 20 cycles of therapy. Weekly dosing in this manner up to 2.8 μg/kg per week was effective and resulted in minimal toxicity. A plateau was observed in calcitriol concentrations at about 0.48 μg/kg and peak serum calcitriol concentrations ranged from 3.7 to 6.0 nM (Beer et al., 2001).

DHA

Docosahexaenoic Acid (DHA) is an omega-3 polyunsaturated fatty acid (PUFA) that is not only vital to proper visual and neurological development, but has been implicated in the treatment and prevention of many diseases, including cancer (Head, 2009). Both omega-3 and omega-6 fatty acids are considered to be essential fatty acids (EFAs) and must be consumed in the diet. These fatty acids are not interchangeable in the body because humans lack the enzyme omega-3 desaturase, which is necessary to convert *n-6* to *n-3* (Simopoulos, 2006). DHA is one of the three major *n-3* FAs, the other two being eicosapentaenoic acid (EPA) and α-linolenic acid (ALA) (Hardman, 2002). The best dietary source of DHA and EPA is fatty cold water fish such as wild salmon (see Table 3). Fish oil supplements usually contain both EPA (usually the dominant fatty acid) and DHA which are extracted from krill and microalgae (Head, 2009). DHA and EPA can also be synthesized in the body from ALA, the less bioavailable plant *n-3* fatty acid. However, studies suggest the capacity to convert ALA to EPA and DHA in humans is very low. It is estimated that only about 5-10% of ALA is converted to EPA and about 2-

5% is converted to DHA in healthy adults. In addition, the conversion rate is lowered by 40-50% when combined with a diet that is relatively high in *n-6* FAs (Gerster, 1998). The most abundant *n-3* fatty acid in most Western diets, ALA is found in certain vegetable oils, green leafy vegetables, walnuts, flax and chia seeds (Hardman, 2002; Leitzmann et al., 2004).

Table 3: DHA, EPA, and ALA content of omega-3-rich foods. 1 tbsp oil = 13.6 g., 1 tbsp seeds/nuts = 28.35 g. (USDA nutrient database release 24).

Food Source (per serving)	**DHA + EPA (mg)**	**Food Source** (per tablespoon)	**ALA (mg)**
Cod	134	**Pumpkin seeds**	51
Catfish	151	**Soybean oil**	1,231
Clams	241	**Canola oil**	1,302
Shrimp	267	**Walnut oil**	1,414
Flounder	426	**Flaxseeds**	2,350
Pollock	460	**Walnuts, English**	2,574
Tuna, canned	733	**Chia seeds**	5,055
Salmon	1825	**Flaxseed oil**	7,249

It appears that the type, rather than the amount, of dietary fat consumed may be more important in the pathogenesis of breast cancer (Grammatikos et al., 1994). The ratio of dietary *n-6* to *n-3* is especially important because both fatty acids compete with each other for an enzymatic substrate needed to produce prostaglandins (Bagga et al., 2002; Culp et al., 1979). The human population evolved on a ratio of dietary *n-6* to *n-3* fatty acids close to 1, but that ratio is now about 15/1 to 16.7/1 in a typical Western diet

(Simopoulos, 2001). Omega-3 fatty acids exhibit anti-inflammatory effects by suppressing inflammatory cytokines such as interleukin 1β (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor (TNFα). *N-6* fatty acids, on the other hand, are pro-inflammatory. This observation is one reason why a lower dietary ratio of *n-6* to *n-3* is beneficial to people suffering from or at risk for diseases characterized by inflammation (Simopoulos, 2006). Some of the diseases that have been linked to diets rich in *n-6* fatty acids and low in *n-3* fatty acids include cardiovascular disease, hypertension, renal disease, diabetes, arthritis, ulcerative colitis, Crohn's disease, and cancer (Simopoulos, 1999).

Epidemiological studies have revealed a negative correlation between the intake of *n-3* fatty acids and cancer risk. Omega 3 fatty acids have clear anticarcinogenic properties in animal models and *in vitro*, while omega 6 fatty acids tend to have the opposite effect by *increasing* cancer risk (Grammatikos et al., 1994; Fay et al., 1997; Hardman, 2002; Connolly et al., 1999). Not surprisingly, epidemiologic research has revealed lower incidences of breast cancer in locations where fish makes up a larger part of the diet, and higher incidences where vegetable oils are consumed in greater quantities, such as the U.S. (Grammatikos et al., 1994). As the diets of Japanese women have become more westernized and lower in *n-3* fatty acids, death rates from breast cancer in this population have been on the rise (Lands et al.,1990).

Studies have noted a negative correlation associated with risk for other types of cancers as well, including prostate cancer. A 12-year follow-up study of nearly 50,000 men revealed that the consumption of fish more than three times per week was associated

with a reduced risk of prostate cancer. In addition, it was noted that for every 0.5 g increase of dietary marine fatty acids per day, overall risk of metastatic cancer decreased by 24% (Augustsson et al., 2003).

Assessment of the dietary intakes of 3081 breast cancer survivors participating in the Women's Healthy Eating and Living (WHEL) study revealed that women with the highest intakes of EPA and DHA from food (> 73 mg/day) had a 25% reduction in further breast cancer events as compared with the group with the lowest intakes. Higher intakes of dietary EPA and DHA also correlated with a reduced risk of all-cause mortality in a dose-dependent manner (Patterson et al.,2011). The results of another study comparing the dietary patterns of 358 breast cancer patients and 360 controls with no history of cancer found that high intakes of fatty fish were associated with reduced breast cancer risk (Kim et al., 2009).

The analysis of breast adipose tissue is an accurate and practical estimate of dietary fat intake. A case-control study carried out in France examined the fatty acid composition of breast adipose tissue from 329 patients with either breast cancer or benign breast disease and determined that *n-3* fatty acid levels were inversely associated with breast cancer risk (Maillard et al., 2002). Another study comparing 73 breast cancer patients to 74 controls found that the levels of *n-6* PUFA in the breast adipose tissue was significantly higher in the breast cancer patients as compared with the controls (Bagga et al., 2002).

Research on animal models has confirmed the protective effects of DHA as it relates to carcinogenesis, as well as its effect on breast tumor growth and metastasis. One study revealed that mice fed a diet rich in ALA from flaxseed displayed a decreased growth rate of mammary tumors and an inhibition of tumor metastasis (Chen et al., 2002). A novel study using a combined treatment of fish oil (Maxepa) and 1,25-dihydroxyvitamin D_3 on rats with DMBA-induced breast cancer revealed a significant decrease in the amount and incidence of mammary tumors as compared to effects of the compounds administered alone. Researchers found that the chemotherapeutic effects of daily oral doses of fish oil and vitamin D3 occurred as a result of reduced cell proliferation and decreased levels of inducible nitric oxide synthase (iNOS) (Chatterjee et al., 2010).

In vitro studies have revealed that omega-3 fatty acids in the form of DHA significantly increase the rate of apoptosis and differentiation in human MCF-7 breast cancer cells and inhibit their growth (Kang et al., 2010). One study evaluated some of the mechanisms behind DHA-induced apoptosis in this cell line and found that it occurs via reactive oxygen species (ROS) formation and activation of caspase 8 (Kang et al., 2010). A similar study using MCF-7 cells confirmed this effect and found that as apoptosis was induced, so was the expression of procaspase-8 and bcl-2 (Corsetto et al., 2011). While DHA has been shown to reduce the growth of MCF-7 breast cancer cells by up to 50% in a dose-dependent manner (6-30 μM), DHA at levels below 24 μM had no effect on noncancerous human mammary cell lines (Grammatikos et al., 1994).

Although the mechanisms behind the chemotherapeutic effects of DHA are not well defined, evidence suggests that one reason for the opposing effects essential fatty acids have on tumor expression is due to prostaglandin E2 (an eicosanoid), which is known to enhance tumor growth. While prostaglandin E2 originates from *n-6* fatty acids, *n-3* fatty acids inhibit its production (Norman et al., 2003). Most of the various proposed mechanisms for the anticancer effects of DHA including the reduction of cell proliferation and neoplastic transformation as well as enhanced apoptosis and antiangiogenicity are either directly or indirectly related to their inhibition of eicosanoid production from *n-6* fatty acids (Kang et al., 2010). A study using the breast cancer cell line MDA-MB-231 discovered that cell growth was stimulated by *n-6* and *n-9* PUFAs, and inhibited by *n-3* PUFAs, particularly DHA and to a lesser extent, EPA (Rose & Connolly, 1990).

5-Fluorouracil

5-Fluorouracil is an antimetabolite that induces apoptosis by targeting the enzyme thymidylate synthase (TS) which disrupts DNA synthesis within the cell. It has been shown to work synergistically with DHA to slow cell growth in colon cancer cells more effectively than either compound administered alone, and was effective in low-dose combinations (Calviello et al., 2005). A study carried out on 56 breast cancer patients revealed an association between the levels of DHA in mammary adipose tissue and the patient's response to chemotherapy treatment. After a combined treatment with vindesine, cyclophosphamide, and 5-Fluorouracil, it was reported that the patients with

the largest decrease in tumor size following treatment were the ones with elevated levels of DHA (Bougnoux et al., 1999).

The combined therapeutic effect of vitamin D and 5-Fluorouracil has also been found to produce some promising results *in vitro*. A study published in 2010 revealed that vitamin D enhanced the susceptibility of human colon cancer cells to the effects of 5-Fluorouracil by activating the calcium-sensing receptor which decreases the expression of survivin and thymidylate synthase within the cell. Since 5-Fluorouracil works by targeting thymidylate synthase, a lower expression of TS increased the efficacy of the drug (Liu et al., 2010). Another study using human MCF-7 cells sought to determine whether pretreating the cells with vitamin D and/or all-*trans*-retinoic acid (ATRA) would increase the efficacy of the cytotoxic drugs paclitaxel (Taxol) and Adriamycin. The researchers concluded that vitamin D and ATRA both increased cellular sensitivity to the chemotherapy drugs, and this occurred when administered both in combination and individually (Wang et al., 2000). In another study, researchers pretreated MCF-7 cells with 10 nM $1,25(OH)_2D_3$ before exposing them to doxorubicin and observed a synergistic effect. Interestingly, in this study $1,25(OH)_2D_3$ exhibited no effect on cell number and viability on its own (Ravid et al., 1999).

In summary, vitamin D and DHA appear to have chemotherapeutic properties that are evident both *in vitro* and *in vivo.* Few studies have evaluated the combined effects of these compounds, which was a primary objective of ours. The ability of vitamin D and/or

DHA to exhibit anticancer effects *in vitro* suggests the possibility of a promising clinical application for breast cancer patients.

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CHAPTER II

THE EFFECTS OF 1,25-DIHYDROXYVITAMIN D_3, DOCOSAHEXAENOIC ACID AND 5-FLUOROURACIL ON HUMAN BREAST CANCER CELLS

Liv A. Engelsen, Susan Hawk, Kelly Pritchett, Virginia Bennett

Central Washington University

Abstract

It is well documented that vitamin D and DHA have antiproliferative effects on a variety of human cancers, including breast cancer. Studies have shown that a combination approach to cancer treatment is more effective than any one treatment administered alone. The purpose of our research was to determine whether the combination of vitamin D and DHA would demonstrate increased growth inhibition in human MCF-7 breast cancer cells. We also sought to determine whether these compounds would increase cellular sensitivity to the antimetabolite chemotherapy drug 5-Fluorourocil, which is commonly used in the treatment of breast cancer. The ability of either vitamin D or DHA to enhance the effects of a chemotherapy agent could translate to a decreased drug dosage requirement and/or a reduction in treatment time for the patient. This would be of benefit due to the undesirable side effects patients typically experience from cytotoxic drugs. In this study, human mammary epithelial cells from the MCF-7 cell line were treated with 25 μM DHA, 1 μM calcitriol, and 15 μM 5-Fluorourocil alone and in multiple combinations for 72 hours. Both DHA and 5-Fluorouracil slowed growth significantly ($p < 0.05$). In contrast, vitamin D did not inhibit cell growth at 1 μM. The combination of vitamin D and DHA inhibited cell growth slightly more than DHA alone. Interestingly, DHA was just as effective as 5-Fluorouracil at inhibiting cell growth. These results suggest that DHA may be just as efficacious as 5-Fluorouracil in slowing breast cancer progression and therefore may suggest a dietary approach to breast cancer treatment with low toxicity.

Introduction

Although breast cancer rates have been declining over the past twenty years due to early detection and treatment efforts, it still remains the leading cause of cancer death among women (Jemal et al., 2010). Established risks for developing breast cancer include obesity, reproductive factors, alcohol consumption, physical inactivity, exogenous hormones and possibly diet (Parkin et al., 2002). It has been estimated that nutrition contributes to carcinogenesis in about one-third of the cancers in developed countries, making dietary factors one of the top preventable causes of cancer (Peto, 2001). Recent estimates further support this relationship and suggest that nutrition and lifestyle factors may contribute to onset in nearly 80% of prostate and breast cancer cases (Go et al., 2001).

The relationship between vitamin D status and disease onset and prognosis has been a recent area of interest in all levels of research. A positive association between vitamin D status and reduced breast cancer risk has been noted (Abbas et al., 2008; Crew et al., 2009; Rejnmark et al., 2009). However, some studies failed to establish this relationship (Chlebowski et al., 2008; Freedman et al., 2008; McCullough et al., 2009). One study evaluating data from NHANES III observed no relationship between vitamin D status and cancer death, but did find that individuals with vitamin D levels of <17.8 ng/ml had a 26% higher risk of all-cause mortality (Melamed et al., 2008). Other studies have indicated that the lowest number of cancer mortalities occurred in cancer cases that were diagnosed during the summer and fall, when serum vitamin D_3 levels are typically the highest (Robsahm et al., 2004; Lim et al., 2006).

Omega 3 fatty acids also have clear anticarcinogenic properties in animal models and *in vitro*, while omega 6 fatty acids tend to have the opposite effect and can increase cancer risk (Grammatikos et al., 1994; Fay et al., 1997; Hardman, 2002). Since much of the essential fatty acids consumed in the diet get incorporated directly into cell membranes, they have a significant impact on cellular control and regulation. These fatty acids are not interchangeable, and by competing with each other for the substrate necessary to produce prostaglandins they promote contrasting effects on inflammation in the body. Therefore, the ratio of essential fatty acids consumed in the diet may be a key in the prevention of cancer. Not surprisingly, epidemiologic research has revealed lower incidences of breast cancer in locations where fish (rich sources of n-3 fatty acids) comprise a larger part of the diet, and higher incidences where vegetable oils (rich sources of n-6 fatty acids) are consumed in greater quantities, such as the U.S. (Grammatikos et al., 1994).

Studies have revealed that both vitamin D and omega 3 fatty acids in the form of DHA significantly increase the rate of apoptosis in human MCF-7 breast cancer cells while inhibiting their growth (Kang et al., 2010; Deeb et al., 2007). While the exact mechanisms behind the antineoplastic effects of vitamin D and DHA are still under investigation, the results of numerous research studies show that both compounds have valuable clinical potential. Many studies have shown that the combination approach to cancer treatment is more effective than any one compound administered alone. Although many studies have been done *in vitro* to examine the individual effects of vitamin D and DHA on cancer cells, few have studied their combined therapeutic outcome.

Treatment for breast cancer typically involves the combination of chemotherapy drugs which have multiple side effects and are seldom well tolerated. Therefore, any supplemental and non-toxic treatment with the potential to increase tumor sensitivity to anti-cancer drugs may contribute to a favorable prognosis and treatment outcome (Bougnoux et al., 2009). Epidemiologic and experimental research suggests that the administration of moderate doses of vitamin D ($1,25(OH)_2D_3$) in the form of calcitriol and/or omega-3 fatty acids in the form of docosahexaenoic acid (DHA) have potential in a clinical setting. These compounds could potentially reduce the amount of chemotherapeutic drugs needed or decrease the length of chemotherapy treatment required for cancer patients. The primary objective of our research was to evaluate the efficacy of vitamin D and DHA to synergistically slow cell growth in human breast cancer cells. Another purpose was to determine if DHA and/or vitamin D could enhance the sensitivity of the MCF-7 cells to 5-Fluorouracil, a chemotherapy drug frequently used in breast cancer treatment.

Materials and Methods

Cell Culture

Human mammary carcinoma cells from the MCF-7 cell line were obtained from the American Type Institute (Rockville, MD). The MCF-7 cells were cultured in 20 ml of Dulbecco's Modified Eagle Media (DMEM) along with 10% fetal bovine serum (FBS) and 1% antibiotic (50% penicillin and 50% streptomycin). They were maintained in an incubator at 37°C with 5% CO_2 until confluent.

Chemicals and Reagents

Docosahexaenoic Acid (DHA), vitamin D (Calcitriol), and 5-Fluorouracil were obtained from Fisher Scientific (Pittsburgh, PA). These compounds were dissolved in ethanol and the stock solutions were stored in the dark at -18°C. Both DHA and $1,25(OH)_2D_3$ were further diluted with Dulbecco's Modified Eagle Media (DMEM) with 10% FBS and 1% antibiotic prior to treatment. 5- Fluorouracil was dissolved in ethanol just prior to treatment.

Treatments

A total of six trials were conducted beginning on confluent cells. Once the cells reached confluency, they were counted using trypan blue and a hemacytometer and divided into two 96-well plates at a concentration of 5,000 cells per well. Cells were incubated and allowed to adhere for 24 hours. After 24 hours, media was removed and replaced with fresh media. Treatments were then applied to the two plates with 5,000 cells per well, and they were incubated for 72 hours (see Table 1).

Table 1 : Treatments

1	0.1% EtOH (control)
2	1 μM Calcitriol
3	25 μM DHA
4	1 μM Calcitriol + 25 μM DHA
5	15 μM 5-Fluorouracil
6	1 μM Calcitriol + 15 μM 5-Fluorouracil
7	25 μM DHA + 15 μM 5-Fluorouracil
8	1 μM Calcitriol + 25 μM DHA + 15 μM 5-Fluorouracil

Cell Viability Assay

After the 72-hour incubation period, the cells were treated with a tetrazolium salt solution and measured for viability using a quantitative colorimetric assay and read on a BioTek Synergy 2 microplate reader (Winooski, VT) at 490 nm .

Statistical Analysis

Statistical analysis was carried out using SPSS version 18.0 (Chicago, IL). A one-way analysis of variance (ANOVA) was used to identify statistical significance between treatment groups as compared to control values ($p \leq 0.05$). A Bonferroni post hoc was applied to further evaluate the significant differences between treatment groups.

Results

The chemotherapeutic effects of DHA, calcitriol, and 5-Fluorouracil, alone and in various combinations, were assessed with the use of a cell viability assay following a 72-hour treatment period. Results indicate that all but two treatments significantly decreased cell viability compared to the control ($p < 0.05$). Treatment with calcitriol alone and in combination with 5-Fluorouracil did not significantly alter cell viability (Fig. 1 and 2).

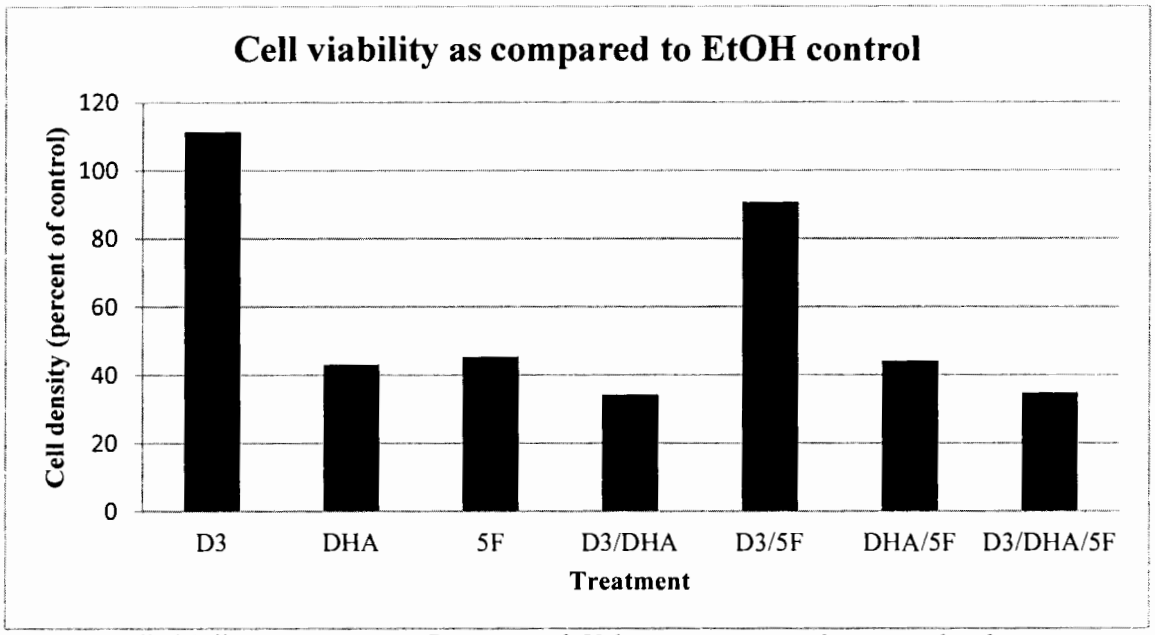

Figure 1. Cell viability as compared to EtOH control. Values are represented as mean absorbance percentages and represent the results of six trials. Bars with an asterisk are significantly different from the control ($p < 0.05$).

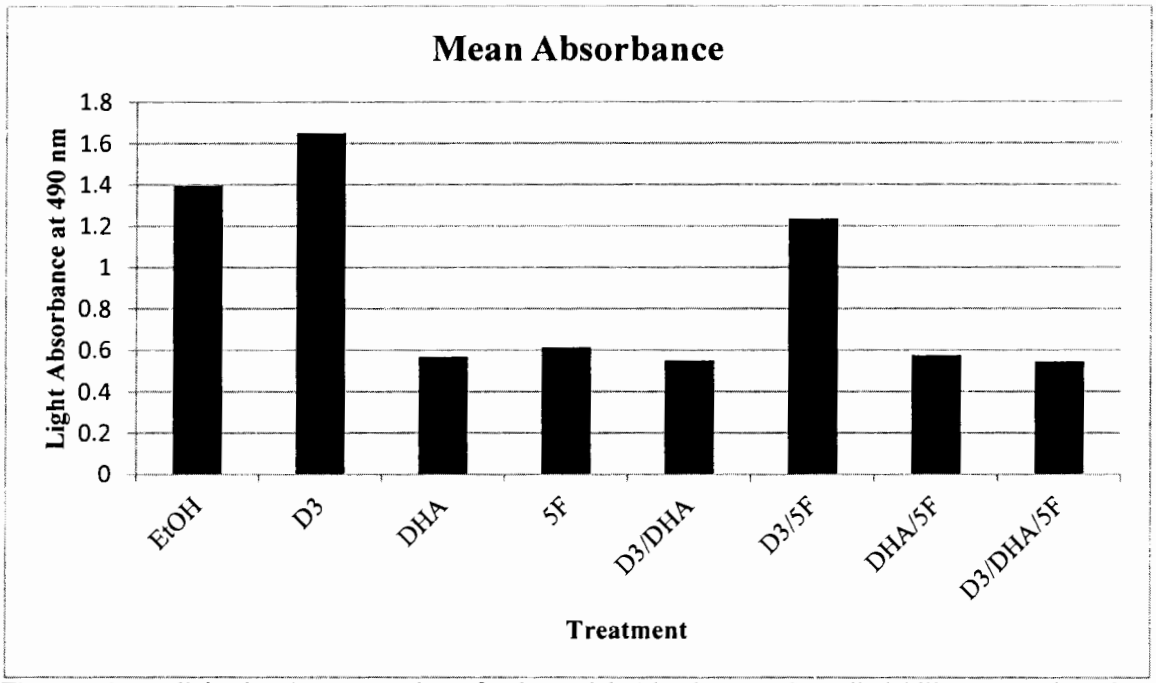

Figure 2. Mean light absorbance at 490 nm for three trials (six plates total). Cell viability was evaluated using a CCK-8 assay after a 72 hour treatment period. Higher absorbance values represent greater cell viability.

DHA

Consistent with the literature, treatment of MCF-7 cells with 25 μM DHA alone decreased cell viability by ~57% ($p < 0.05$). When combined with $1,25(OH)_2D_3$ and 5-Fluorouracil, a significant reduction in cell viability occurred after 72 hours ($p < 0.05$). DHA was as effective alone as it was when combined with other compounds. However, although not statistically significant ($p = 1.00$), vitamin D and DHA appeared to decrease cell viability slightly more than when either compound was administered alone, suggesting a possible synergistic effect. Interestingly, there was no significant difference between DHA and 5-Fluorouracil ($p = 1.00$), revealing that DHA was just as effective as 5-Fluorouracil in reducing cell growth.

Vitamin D

Treatment of MCF-7s with 1 μM $1,25(OH)_2D_3$ for 72 hours did not reveal a significant reduction in cell viability ($p = 1.00$). Although not statistically significant, cells treated with vitamin D alone actually had an increased viability as compared to the control. Overall, vitamin D displayed an 11.5% increase in cell density as compared to the control. These observations are unusual and inconsistent with similar research studies. When compared to cells treated with 5-Fluorouracil alone, those treated with the combination of 5-Fluorouracil and vitamin D displayed a significant increase in cell viability ($p < 0.05$). This indicates that the growth-inhibitory effects of 5-Fluorouracil were not sufficient to reduce the apparent increase in cell viability induced by $1,25(OH)_2D_3$.

5-Fluorouracil

At a concentration of 15 μM, 5-Fluorouracil significantly reduced cell density of MCF-7s after 72 hours of treatment ($p < 0.05$). Overall, 5-FU slowed cell growth by ~55% which is comparable to the growth inhibitory effects of DHA. 5-Fluorouracil did not appear to be any more effective when paired with vitamin D and/or DHA.

Discussion

The purpose of this study was to evaluate the chemotherapeutic effects of vitamin D ($1,25(OH)_2D_3$) and docosahexaenoic acid (DHA) on breast cancer cells both individually and in combination. We also sought to determine whether these compounds would increase sensitivity to the chemotherapeutic drug 5-Fluorouracil in the MCF-7 cell line. Our findings support the notion that DHA slows breast cancer cell growth both alone and in combination with vitamin D and 5-Fluorouracil. Interestingly, DHA is just as effective as 5-Fluorouracil in slowing cancer progression. In contrast to other research findings, $1,25(OH)_2D_3$ did not alter cell viability when administered alone.

The effects of DHA may be mediated by a number of different cellular mechanisms, many of which are not yet fully understood. Omega-3 fatty acids can inhibit the proliferation of MCF-7 cells and promote apoptosis and differentiation. In addition, DHA may suppress neoplastic formation and decrease angiogenesis in animal models (Connolly et al., 1999). These actions may be due in part to the ability of omega-3 fatty acids to interfere with the production of ecoisanoids from omega-6 fatty acids. In particular, *n-3* FAs inhibit the production of prostaglandin E_2 (PGE_2), an inflammatory

eicosanoid derived from arachidonic acid (AA) that increases estrogen production and has been shown to promote breast cancer cell growth. In addition to producing PGE_2, AA also promotes the growth of tumor cells by stimulating mitosis through the activation of protein kinase C, which is inhibited by DHA and EPA (Rose & Connolly, 1990).

To further elucidate the strong anticancer nature of DHA in the MCF-7 cell line, researchers evaluated the induction of oxidative stress and apoptosis as mediators of cell death. They observed that the removal of caspase 8 in these cells completely abolished DHA-induced cell death. Without the accumulation of reactive oxygen species (ROS), MCF-7 cells were protected from apoptosis. This suggests that caspase 8-mediated apoptosis is the primary form of cell death induced by DHA, and it is mediated by the accumulation of ROS (Kang et al., 2010).

Although the amount of DHA used in this study exceeds normal physiological levels, these findings support the notion that the protective nature of DHA may be of value for the prevention and treatment of breast cancer. DHA may be of additional benefit in reducing the risk of cachexia, a common side effect of cancer treatment that occurs as a result of changes in energy expenditure. This wasting away of lean muscle mass is not easily corrected by increasing caloric intake alone (Moses et al., 2004). Reversal of cachexia and weight stabilization has been observed with the administration of dietary EPA and DHA in cancer patients (Moses et al., 2004; Fearon et al., 2003), suggesting another therapeutic use for n-3 fatty acids.

Although we did not observe $1,25(OH)_2D_3$ exhibiting anticancer effects when administered alone, a possible synergism may exist between $1,25(OH)_2D_3$ and DHA. Studies have indicated that the chemotherapeutic effects of DHA on cancer cells are suppressed when combined with antioxidants and enhanced when combined with prooxidants (Gonzalez et al., 1991). Treating MCF-7 cells with the antioxidant α-tocopherol was effective in protecting them from DHA-induced cell death (Kang et al., 2010). Researchers also determined that $1,25(OH)_2D_3$ acts as a prooxidant in cancer cells which may explain our observation of a greater reduction in viability when cells were treated with $1,25(OH)_2D_3$ and DHA in combination (Koren et al., 2001). Further research should be conducted to evaluate the combined effects of DHA and prooxidants such as $1,25(OH)_2D_3$.

Rather than causing a reduction in cell viability, results from this study revealed that a single treatment of $1,25(OH)_2D_3$ tended to increase cell viability. One possible explanation for this is that the timecourse was inadequate to facilitate the slower kinetics of $1,25(OH)_2D_3$ *in vitro*. Previous studies have observed that apoptosis in MCF-7 cells first occurs after the third day of treatment with $1,25(OH)_2D_3$ due to intracellular transformations that must occur first (James et al., 1996). In addition, several studies grew MCF-7 cells in medium containing $1,25(OH)_2D_3$ prior to the incubation period (Narvaez & Welsh 2001; Wang et al., 2000). Thus, pretreatment with vitamin D may be necessary for the cells to become susceptible to the antiproliferative effects.

Breast cancer cells use multiple mechanisms to evade $1,25(OH)_2D_3$-mediated growth inhibition (Welsh, 2007) and several factors may have contributed to our observations. Vitamin D-mediated apoptosis in MCF-7 cells may be inhibited by the overexpression of the antiapoptotic protein Bcl-2 which is downregulated in response to $1,25(OH)_2D_3$. Researchers evaluated the effects of $1,25(OH)_2D_3$ on MCF-7 cells overexpressing Bcl-2 and found that the cells were entirely protected from vitamin D-mediated apoptosis (Mathiasen et al., 1999). The loss of VDR expression and/or changes in receptor co-regulators could also result in $1,25(OH)_2D_3$ resistance (Welsh, 2007). Additionally, the expression of some oncogenes can potentially inhibit the antiproliferative effects of vitamin D (Agadir et al., 1999).

It is well documented that both vitamin D and DHA have potential as anticancer agents in animal and cell culture models, but their clinical potential is still under investigation. Most studies first observe the antineoplastic effects of vitamin D with doses of 1 nm *in vitro,* a concentration that can be achieved in a clinical setting with intermittent oral dosing (Beer & Myrthue, 2004). Potential for the clinical application of DHA is also very promising. Burns et al. found that the maximum non-toxic dose of omega-3 fatty acids administered in a clinical setting was 0.3 g/kg per day or up to 21 g/day for a 70 kg patient. Results from similar studies have indicated that only small amounts of n-3 fatty acids are needed to significantly influence cytokine production (Hardman, 2004).

In conclusion, our research supports a protective role of DHA on breast cancer progression, one that is comparable to that of 5-Fluorouracil. It has been well established that vitamin D has similar effects. In addition to their possible chemopreventive role, both compounds could potentially enhance the therapeutic effects of traditional breast cancer treatments in a clinical setting. Our data indicates the possibility of a synergism between DHA and $1,25(OH)_2D_3$, yet additional studies are warranted to further evaluate this effect. In addition, clinical trials are necessary to evaluate the safety and efficacy of high intermittent dosing of these compounds as they apply to patient therapy.

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