**THE EFFECT OF ALL-TRANS RETINOIC ACID AND FATTY ACIDS ON
MCF-7 BREAST CANCER CELL PROGRESSION**

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ABSTRACT

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Vitamin A metabolites and retinoids may slow the progression of breast cancer and elicit anti-neoplastic properties similar to those of omega-3 fatty acids. Studies using animal models show a decrease in the incidence, growth and metastasis of mammary tumors in the presence of specific fatty acids. This effect is also seen with use of retinoids, specifically all-trans retinoic acid (AtRA). Thus, fatty acids may also alter retinoid homeostasis in mammary carcinoma cells (MCF-7s). The potential for inter/co dependency among fatty acids and retinoids is considerable, and here it has been hypothesized that a decrease in cancer progression will occur in the presence of both compounds. MCF-7's were seeded in a 48 well plate at 5,000 cells per well. After 24 hr, cells were treated with either 1 μ M AtRA alone, fatty acids alone, or AtRA + fatty acids. Fatty acid treatments (Linoleic, and Linolenic) were administered at 2.5 μ M concentrations. Each fatty acid treatment was also combined with 1 μ M AtRA to determine if there is a synergistic effect on slowing cell growth. Both culture media and treatments were changed at 24 hour intervals over a 3 day trial. When compared to the controls, cells treated with 1 μ M AtRA or 2.5 μ M Linolenic acid both inhibited cell growth. Interestingly, when combined with Linolenic acid, AtRA treatment resulted in a significant (nearly 50%) additional growth inhibition when compared to treatment with AtRA alone. Our results suggest that AtRA and Linolenic acid have a inter/co dependency that significantly inhibits breast cancer cell growth in vitro by 73.4 % compared to control, and 49.7% compared to AtRA alone over 72 hours. We conclude

that AtRA and linolenic acid have a combined effect in breast cancer cell proliferation in-vitro and their role in dietary prevention warrants further investigation.

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Chapter 1

INTRODUCTION

All-trans retinoic acid (AtRA), the intracellular derivative of vitamin A, has been shown to slow the progression of human mammary carcinoma cells (MCF-7) in vitro (Danforth 2004, Cho et al. 2007). Of perhaps equal importance is the role by which fatty acids contribute to tumor growth and stability. With both AtRA and intracellular fatty acid involvement with the nuclear hormone family of receptors, the possibility of synergism is substantial. Interestingly, altered nuclear receptor activity is known to increase carcinogenesis (Chamras et al. 2002). In either situation, both retinoids and fatty acids are directly involved with the cells ability to undergo apoptosis (Okamoto et al. 2000)

AtRA is responsible for cell growth, differentiation, and apoptosis (Liu et al. 1996). This control is mediated via hormone receptors in the nucleus. MCF-7 cells have shown diminished hormone receptor efficiency which results in a decreased availability of AtRA. The result is a reduced amount of a ligand which promotes apoptosis via genetic transcription (Budhu 2002). The steroid-thyroid receptor super-family includes the retinoic acid receptor (RAR and RXR), which also includes receptors for estrogen and vitamin D (Tighe 2004). Recently, it has been shown that many carcinogenic cell types are missing retinoic acid receptors. Because retinoic acid seems to induce apoptosis, an accumulation of receptor deficient cells could be unresponsive to normal retinoic acid levels and therefore not undergo normal cellular death required for optimal health of the organism (Merino 2003). There are many pathways involved in this process and include those involving fatty acids.

Fatty acids have been under extreme scrutiny for their involvement in breast cancer incidence and progression. Both the protective effects of omega-3 fatty acids (linolenic), and the enhancing effects of omega-6 fatty acids (linoleic) have been demonstrated in the literature (Cohen et al. 1986, Chajès et al. 1995). While there is yet to be substantial evidence to directly link linoleic acid to breast cancer in vivo, animal studies, which have shown diminished breast cancer progression as a consequence of linolenic acid treatment (Reyes et al. 2004). Epidemiological studies also link fish oil, a classic source of omega-3, intake with lower breast cancer incidence (Rose 1999). Peroxisome proliferator-activated receptors (PpAR), members of the nuclear hormone signaling family are directly involved with breast cancer tumorogenesis (Thoennes et al. 2000). Interestingly, Linolenic acid acts as an agonist to this receptor and therefore has yielded increased apoptosis in MCF-7 cells (Menendez et al. 2001). Using this acid presents a viable option to creating new and effective cancer treatments and establishing preventative lifestyle behavior choices.

Separate investigations have confirmed the use of both AtRA and Linolenic acid to slow breast cancer proliferation in vitro by establishing a higher rate of apoptosis (Liu et al. 1996, Budhu 1996). Here, we combine the two treatments to investigate the synergism. We show that physiological treatments of both AtRA and linolenic acid further increase apoptosis when compared to each treatment alone. We believe the explanation is found on the surface of the nucleus where nuclear transporters are changed dramatically in a carcinogenic cell. The PpARY receptor and RXR receptor act together as a dimer and have a unique response element (PpARE) (Kim 2003). Up-regulation of this genetic activity seems to slow cancer proliferation, and is a viable explanation for our

observation of AtRA and linolenic acid as cancer treatments in vitro. Clearly, the functional significance of these findings will require further investigation.

LITERATURE REVIEW

2.1 Breast Cancer

Breast cancer is the second leading cause of death from cancer among American women (Reyes et al. 2004). It is estimated that 217,440 new cases of this cancer was diagnosed, and over 40,000 deaths occurred in 2005 alone (Reyes et al. 2004). Age is one of the strongest risk factors in breast cancer. In women under the age of 30, breast cancer is very uncommon. The incidence rate begins to increase between ages 35 and 39 years and does not plateau until age 80 (Reyes et al. 2004). Breast cancer incidence is also influenced by race. In the United States, breast cancer risk is slightly higher in whites than in African Americans, although the incidence of early-onset cancers is higher in African Americans (Kuhajda 2000). Incidence rates are markedly lower in other racial and ethnic groups (Korde et al. 2004). The most common types of breast cancer occur in the lining of the ducts or in the lobules of the breast. Atypical ductal hyperplasia results in an overactive growth of cells lining the breast ducts, while lobular carcinoma results in uncontrolled growth of lobular cells.

On average, women have a 10% to 14% chance of having breast cancer by the time they reach 90 years of age (Reyes et al. 2004). Women at increased risk may have a strong family history of breast cancer, a known or suspected genetic predisposition for the disease, a history of radiation therapy, a previous problem of the breast, and a 5-year Gail model risk of 1.67% or greater (Murff et al. 2004).

There are two types of models used in breast cancer risk assessment--those that estimate the risk of breast cancer over time and those that estimate the risk of a mutation in one of the *BRCA* genes. These models are the Gail and Claus models, respectively. The

current version of the Gail model estimates the 5-year and lifetime risks of breast cancer by incorporating risk factors such as age, age of menarche, and age of 1st pregnancy. A 5-year risk of 1.67% is considered elevated risk (Korde et al. 2004). The Gail model has proven to be very limited as it fails to incorporate paternal family history and ovarian cancer family history (Murff et al. 2004). In contrast, the Claus model is useful because it bases risk on family history of breast and ovarian cancers on both the maternal and paternal lineage. These two models compliment each other well, and together help women to define their risk of developing breast cancer. Defining risk can also prepare both physicians and patients for effective treatment. The only true shortcoming of these models is the continuing evidence that breast cancer is simply a mutation on the genome.

Breast cancer treatments vary depending on the advancement of the cancer, and the patient's response to each treatment. The best opportunity to reduce mortality is through early detection (Maggiora et al. 2004). Generally, the location and size of the tumor in the breast and the extent of the disease in the armpit and the rest of the body will determine treatment options. Breast cancer progresses in stages. Stages 0-II are termed early stages, where the severity is a small tumor 1-2 inches in diameter with little or no lymph node invasion. Stage III is a progression towards the complete metastatic invasion known as type IV. The final stage is the most severe and advanced because of the extensive tissue invasion and malignancy (Maggiora et al. 2004). To develop a treatment plan to fit each patient's needs, the doctor also considers the woman's age and general health, as well as her feelings about the treatment options. The primary method of treatment is surgery. Few patients elect to bypass this option, as it is considered necessary to stop the cancer progression. The lumpectomy procedure removes a tumor from the breast, while a

mastectomy removes a portion of, or the entire breast. The need of the mastectomy is based on the progression of the cancer, and the literal “spread” of the cancer. This depends on the number of tumors in the area, as well as the condition of the local lymphnodes (Wiseman 2004).

The second step in treating cancer is radiation therapy, which uses x-rays and other radioactive materials to kill cancer cells. The radiation disrupts cell growth by destroying DNA and cell membranes (Serrano et al. 2004). This can be performed externally or internally depending on the growth of the cancer, and is effective when the cancer can be targeted in a specific area of the body.

The third step is chemotherapy, which is the use of chemical agents to either destroy dividing cells or stop their division. Chemotherapy can be localized, but in general is a systemic treatment. Gleevec, a pharmaceutical agent, inhibits cancer cell growth by inducing apoptosis. It binds to abnormal proteins in cancer cells, blocking uncontrolled cell growth. Because it binds only to these abnormal proteins, Gleevec does not show the high levels of toxicity exhibited by other chemotherapy drugs (Abou-Jawde et al. 2003).

The fourth and final treatment step is the use of hormone therapy. Hormone therapy can be used throughout the entire treatment process; however it is only applicable for certain types of breast cancer. In 1998, the Food and Drug Administration approved Nolvadex (tamoxifen citrate), which interferes with the activity of estrogen to reduce the incidence of breast cancer in women at high risk for developing the disease (Merino et al. 2003). It is used as an additional type of therapy in conjunction with other steps in fighting the cancer (Abou-Jawde et al. 2003). The anti-proliferative effects of Tamoxifen

have been attributed to the inhibition of protein kinase C and its calmodulin binding (Abou-Jawde et al. 2003). The activity of these proteins is directly connected to the known breast cancer susceptibility genes.

Figure 2.1.1. Image of Chromosome 17, and Chromosome 13, with location of BRCA1 and BRCA2 respectively (National Library of Medicine, NCBI, www.ncbi.nlm.nih.gov).

Two breast cancer susceptibility genes have been identified. *BRCA1* was found on chromosome 17 and *BRCA2* was found on chromosome 13 (Serrano et al. 2003). When either gene has a mutation there is an increased risk of developing breast cancer and/or ovarian cancer. The genes were discovered in 1994, and until recently researchers were not able to identify their function (Abou-Jawde et al. 2003). A yeast study revealed that they repair radiation-induced “gaps” in double-stranded DNA. A mutant form of these genes causes these “gaps” to progress into tumor growth (Abou-Jawde et al. 2003). Human epidermal growth factor receptor 2 (HER2) along with the p53 gene have also

been shown to be causative factors when their mutations lead to breast cancer (Abou-Jawde et al. 2003).

Lifestyle choices are a very important area of interest when determining possible sources of cancer development. Dietary influences have been heavily explored as of late, and have produced many promising links between diet and cancer. Vitamin A (retinoids) has been shown to play a major role in the progression of breast cancer cells in vitro (Merino et al. 2003). This important vitamin can change its role on the molecular level when not readily converted to its active form (retinoic acid) and leads to unnatural control over the human genome yielding cancerous growth. Scientists currently believe that the link between fatty acids and breast cancer is also substantial. Saturated fatty acids have been termed carcinogenic for some time, and omega 6 fatty acids seem to be following that trend and have been linked to breast cancer incidence (Thoennes et al. 1999).

On the contrary, antioxidant-rich foods protect normal cellular growth processes. Polyphenols, vitamin C, and Vitamin E have been touted as the great cancer preventors. While studies show these factors to be greatly protective against oxidative and free radical damage, there is little evidence to support these facts on the molecular and genomic level for breast cancer (Mezzetti 2001). While scientists may not argue against having a vegetable rich diet to help prevent cancer, the conclusive evidence for such has yet to be uncovered.

Diet is not the only lifestyle choice influencing cancer risk. Epidemiologic data regarding physical activity suggest a 20% to 30% reduction in the risk of breast cancer in women who are physically active compared with those who are inactive (Wiseman 2004). In addition, moderate to heavy alcohol intake (more than 45 g of alcohol, or three

drinks, per day) has been shown to increase the risk of cancer, in a dose-response relationship (Wiseman 2004). High alcohol consumption causes the liver to be less effective at controlling estrogen levels in the body. This results in increased estrogen exposure to cells, and an increase in the chance of cancerous cell growth.

High levels of hormones, particularly estrogens, have been shown to be associated with increased breast cancer occurrences (McClelland 2004). Another theory claims that breast cancer progresses over time, and only becomes a clinical disease later in life (Wiseman 2004). The decisions women make regarding different hormone therapies influence breast cancer risk. These decisions include the use of oral contraceptives, excessive exercise, and hormone replacement therapy. The increased exposure of breast tissue to estrogen during these treatments has a direct link as a risk factor. The use of hormone replacement therapy for the difficulties of menopause has also been shown to increase cancer susceptibility from 5-40% (Wiseman 2004). Oral contraceptive use has been controversial about its potential increase in breast cancer risk. However, the most recent data suggests that oral contraceptive use is not associated with increased risk of breast cancer (Korde 2004).

2.2 Retinoic Acid and Breast Cancer

The vitamin A metabolite retinoic acid (RA) and its derivatives are effective chemotherapeutic agents in the treatment of a number of types of cancer, including tumors of lung, breast, head and neck, and blood (Sun et al 1997). The historical and most significant finding to date has been the use of retinoids to treat acute promyelocytic leukemia (APL) (Jimenez-Lara et al. 2004). Along with chemotherapy, the prognosis for

the disease has improved rapidly. In animal models, retinoids have antitumor activity in cancer xenografts and induced regression of mammary carcinoma (Lehmann et al. 1991). In vitro, RA and its derivatives inhibit proliferation of a variety of tumor types including lung, breast, leukemia, and rhabdomyosarcoma (Lehmann et al. 1991). Retinoids exert their function on tumor growth in three ways: increased cellular differentiation, decreased cell proliferation, and induction of apoptosis (Merino et al. 2004). Retinoids are known to inhibit hormone dependent, but not hormone independent mammary carcinoma cells (Liu et al. 1996). Thus, retinoids are currently being used as a treatment for many epithelial cancers (Liu et al. 1996).

Retinoic acid receptors (RAR) and RA in cancer therapy was initiated in the 1970's when RA treatment was shown to disrupt anchorage-dependent growth in a few different cell lines (Lehmann et al. 1991). Recently, it has been shown that many carcinogenic cell types are missing retinoic acid receptors. Because retinoic acid seems to induce apoptosis, an accumulation of receptor deficient cells could be unresponsive to normal retinoic acid levels and therefore not undergo normal cellular death required for optimal health of the organism (Liu et al. 1996). In the nucleus, the RARs mediate the effects of RA on gene expression. The RARs are members of a large family of ligand-dependent transcription factors that include steroid, thyroid hormone, and vitamin D receptors (Bischoff et al. 1998). Numerous synthetic retinoids with receptor selective activities have been characterized. RARs have functional domains for RA and DNA binding, dimerization with other factors, and transcriptional activation. The DNA binding domain contains two zinc finger motifs. RARs interact with cognate response elements in the promoters of many genes. RARs bind DNA as heterodimers with retinoid X receptors

(RXRs). RXRs bind 9-*cis* RA and possess homology to other members of the superfamily (Mangelsdorf et al. 1995)

Figure 2.2.1. RXR binding partners

RARs have 6 domains named A-F. Domains A and B appear to be ligand independent transcription domains. Domain C is the DNA-binding domain, and D is a things domain specific for nuclear translocation and/or co-repression. Domain E seems to

be for both dimerization and ligand binding. The F region is unknown to date (Sommer et al. 2002). When examining the role of RAR and its own genetic transcription, there appears to be a promoter problem in tumorigenic cells (Sun et al. 1997). There are three genes coding for RAR, but the problematic gene is RAR-beta. The gene has two promoters, one of which (P2) is highly down regulated. The transcription of this gene is controlled by a RARE, and thus RAR. It is a unique feedback mechanism which becomes disrupted in mammary carcinoma cells (Sommer et al. 2002).

Acyclo-retinoic acid was investigated for its possible role in activating the retinoic acid response element and slowing proliferation of immortal human mammary carcinoma cells (MCF-7) cells in culture. This retinoid is structurally and functionally similar to Lycopene (Leblanc et al. 1995). The result of the study showed a 100-fold decrease in RARE activity when compared to retinoic acid alone. Lycopene apparently does not show a direct affect on MCF-7 breast cancer cells, and does not act in place of retinoic acid (Ben-dor et al. 2002). MCF-7 cells treated with lycopene did not undergo apoptosis; however, they did show reduced activation of AP-1 transcription factor, IGF-1 receptor signaling, and cell cycle progression. These results indicate an interference in cell cycle pathways by lycopene, which could be the reason for its prescribed affect of reducing cancer risk (Ben-dor et al. 2002).

2.3 Fatty Acids and Breast Cancer

In 1982 the National Academy of Sciences suggested that breast cancer was related to consumption of a high fat diet (Kushi et al. 2002). Studies now show that it is also the type of fat consumed, not just the amount of fat contributing to the rise in breast

cancer incidence. For example, Indian women have a high incidence of breast cancer in spite of a diet rich with fruits and vegetables (Kushi et al. 2002). In India, most foods are prepared with n-6 polyunsaturated rich vegetable oils (Kachhap et al. 1999). Case-control studies in Greece, Spain, and Italy have shown that consumption of olive oils (monounsaturated fat) reduce the incidence of breast cancer (Kushi et al. 2002). This protective effect may be due in part to its phenolic compounds that offer protection of lipids from oxidation (Gerber 1997). Another example of a specific fatty acid is Conjugated linoleic acid (CLA). This fatty acid is found in meat and dairy products. It can inhibit carcinogenic rat mammary tumor formation (Durgam 1997). The final lipid of interest is the omega-3 fatty acid. This fatty acid is considered anti-inflammatory, and has protective properties against breast cancer (Chajès et al. 1995).

Although not all experts agree, women who consume foods rich in omega-3 fatty acids routinely and over long periods of time are less likely to develop breast cancer than those that consume them sparsely (Wayne et al. 2004). Breast cancer mortality also seems significantly less for those who consume large quantities of omega-3 fatty acids. This is particularly true among women who substitute fish for meat in their diet. The balance between omega-3 and omega-6 fatty acids suggests an important role in the development and growth of breast cancer (Simopoulos 2002). Further research is still needed to understand the affect that omega-3 fatty acids may have on the prevention or treatment of breast cancer. Researchers speculate that omega-3 fatty acids may be more effective in combination with other nutrients (vitamin E, vitamin C, beta-carotene) in the treatment and/or prevention of breast cancer (Rose et al. 1999).

Studies on the effects of dietary fat on breast cancer have been carried out using several rodent experimental models. They consistently show that mice and rats are less

likely to develop mammary tumors when they are fed low-fat as compared to high-fat diets (Cohen et al. 1986). Experimental evidence shows that dietary fat exerts its effect most commonly during the promotion phase of developing mammary tumors. However, the type of fatty acid is paramount in relation to breast cancer. For example, a diet providing a high proportion of n-6 polyunsaturated fatty acids (PUFA) greatly enhances tumor promotion (Simopoulos 2002). In contrast, diets high in linolenic acid found in fish oil inhibited the progression of mammary carcinogenesis (Rose et al. 1999). Studies on the influence of diets rich in monosaturated fatty acids such as olive oil, are inconclusive but at this time there are several results which give them a protective antitumor effect. More recently, it has been described that conjugated linoleic acid has also inhibitory effects on breast cancer (Durgam 1997).

The precise molecular mechanisms by which dietary fat might influence cancer development are potentially numerous as dietary fatty acids can modulate gene expression resulting in changes in metabolism, cell differentiation, and proliferation. Further, the cellular response to fat depends on the type of fatty acids, cell-specific metabolism and cell-specific transcription factors and nuclear receptors. The effects of dietary fat on tumor development may be indirect as a modulation of hormonal or growth factor mediated pathways. In addition, the oxidative stress due to generation of reactive oxygen species from fatty acids may contribute to the promotional effect of dietary fat by lipid peroxidation and damage of DNA and protein. Further, the metabolism of dietary fatty acids via the cyclooxygenase or lipoxygenase pathways may alter gene expression through the regulation of G proteins involved in signal transduction cascades that target the nuclear receptors.

2.4 Fatty Acids

Dietary fats, more specifically, fatty acids have become increasingly scrutinized over the last few decades. At the center of this scrutiny are the omega 3 and omega 6 fatty acids, with saturated and trans fatty acids following close behind. Omega 3 (GLA, DHA) fatty acids possess anti-inflammatory, antiarrhythmic, and antithrombotic properties. Omega 6 fatty acids (Linoleic) are proinflammatory and prothrombotic (Thoennes et al. 1999). Saturated fats and trans fats have been shown to increase LDLs, and subsequently have lead to an American population plagued by high cholesterol and heart disease (Kushi et al. 2002). Some scientists would argue that people simply eat a poor ratio of these different fats. Americans have a high ratio of omega-6 fatty acids to omega-3 fatty acids in their diets. Simopoulos suggests that humans evolved with a ratio close to one, and the distance from that ratio of the western diet is a link to increased levels of mortality and morbidity (2002). The deviation from the evolutionary ratio seems to be a historical indicator for fatalities resulting from cardiovascular disease and cancer (Simopoulos 2002).

Fatty acids are an integral part of every physiological system in the human body. They are responsible for absorption of many essential nutrients such as vitamins A, D, and K. When examining fatty acids it is important to differentiate between dietary fatty acids and fatty acids actively involved in metabolic processes. Dietary fatty acids are typically defined by the food one eats. For example, mono-unsaturated fats originate from olives, saturated fats originate from animals, omega-3 fats originate from fish, and of course trans fats are a product of partial hydrogenation. Fats are commonly defined metabolically. For example, the omega-3 fatty acid alpha linolenic acid (ALA) is a precursor to eicosapentaenoic acid (EPA), which in turn is a precursor for

docosahexaenoic acid (DHA). Linoleic acid (LA), an omega-6 fatty acid, is the metabolic precursor to arachidonic acid (AA) (Rose et al. 1999). These fats are known as essential fatty acids (EFA), as the body does not synthesize them. Essential fatty acids belong to the class of fatty acids called polyunsaturated fatty acids (PUFAs). They have many functions among which are, stimulate hair and skin growth, maintain bone health, regulate metabolism, and maintain the capability to reproduce (Kachhap et al. 2000). They serve in forming body structures, maintaining immune system function, protecting vision, creating cell membranes, and producing eicosanoids (Thoennes et al. 1999). Eicosanoids, which include prostaglandins, are regulators of temperature, immune response, blood clotting, blood pressure, and inflammatory response.

2.4.1 Omega 3 Fatty Acids

Omega 3 fatty acids have been directly linked to the health of a human being. The omega-3 fatty acids are effective in the treatment of cardiovascular disease, arthritic disorders, and diabetes mellitus (Rose et al. 1999). The dietary omega-3 fatty acids that may impact cancer include eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA). These fats are found primarily in fatty fish such as salmon and tuna (Rose et al. 1999).). Another rich source of omega-3 fatty acids is seaweed, and is the only true plant source of EPA and DHA. The other omega-3 fatty acid is ALA and is readily found in flaxseed and dark leafy green vegetables. The body can enzymatically convert ALA to EPA. All three fatty acids are paramount to human health (Simopoulos 2002).

Approximately 1 g of EPA acid DHA consumed per day orally is recommended for cardio protection. Higher dosages of omega-3 fatty acids are required to reduce

elevated triglyceride levels (2 to 4 g per day) and to reduce the stiffness in the number of joints in patients with rheumatoid arthritis (at least 3 g per day)(Rose et al. 1999).).

Small decreases in blood pressure can occur with higher dosages of omega 3 fatty acids (Rose et al. 1999).).

2.4.2 Omega 6 Fatty Acids

Omega 6 fatty acids are derived from a variety of sources. Most omega 6 fatty acids are consumed in the human diet, and are found in vegetable oils as linoleic acid. Linoleic acid is converted to gamma-linolenic acid (GLA), and then further broken down to arachidonic acid (AA). Arachidonic acid can also be consumed directly from meat. Gamma-linolenic acid can be part of a diet containing specific plant oils including: evening primrose oil (EPO), borage oil, and black currant seed oil (Kachhap et al. 2000).

Excess amounts of LA and AA are unhealthy because they can promote inflammation, which can potentially lead to heart disease and cancer (Reyes et al. 2004). In contrast, GLA seems to reduce inflammation (Cohen et al. 1986). When GLA is taken as a supplement, it is not converted to AA. Rather, it is converted to dihomogamma-linolenic acid (DGLA). Dihomogamma-linolenic acid competes with AA and prevents the normal inflammatory effects that AA would have caused the body. Dihomogamma-linolenic acid also becomes involved with prostaglandins, which reduce inflammation. Important dietary concerns are considered for this process to take place. Adequate amounts of magnesium, zinc, and vitamins B3, B6, and C help to promote the conversion of GLA to DGLA (Reyes et al. 2004).

2.4.3 Monounsaturated Fatty Acids

Monounsaturated fatty acids have functionally specific roles in the human body. These fatty acids are an important source of energy for the body, and are also important constituents of cell membranes. Monounsaturated fatty acids regulate hormone response, nutrient uptake, and waste removal. They are also very important as oils of the skin (Gerber 1997).

Monounsaturated fatty acids (MUFAs) have a single double carbon bond found with a cis configuration. These fatty acids cannot closely associate with neighboring fatty acids due to this double bond and consequently act as a liquid oil at room temperature. Monounsaturated fatty acids can be synthesized in the body, and are normally completed by a double bond insertion between the ninth and tenth carbons of a saturated fatty acid. This reaction creates either oleic acid or palmitoleic acid, depending on the original saturated fat. The common fat, oleic acid, is created using stearic acid. Palmitoleic acid is created from a palmitic acid chain. Together, these two fatty acids can be found in a variety of foods. Oleic acid is present in almost all animal fats and vegetable oils. It is the most common fatty acid in olive oil, canola oil, and sunflower oil. Palmitoleic acid is found in coconut and palm oils (Gerber 1997).

2.4.4 Conjugated Linoleic Acid

Conjugated Linoleic Acid (CLA) has been shown to be a very effective treatment in preventing the proliferation of breast cancer cells in vitro (Maggiora et al. 2004).

Conjugated Linoleic Acid is a mixture of positional and geometric isomers of linoleic acid. It is found in ruminant foods such as meat and dairy products (0.6 – 5.6 mg CLA/g fat), and has proven to be an effective inhibitor of carcinogenic rat mammary tumors (Durgam 1999). It is unique due to its presence in animal food sources, and its anticancer efficacy is expressed at concentrations close to human consumption levels.

CLA has been shown to increase metabolic rate, decrease body fat, increase lean muscle, lowers cholesterol and reduces insulin resistance. CLA is a slightly altered form of LA, an omega-6 fatty acid important to human health. Recently, it has been widely produced as a supplement which claims cancer prevention. It remains to be extensively tested and/or completely understood (Chujo et al. 2003).

2.4.5 Trans Fatty Acids

On November 12, 1999, the Food and Drug Administration announced that it would include the trans fatty acid content of foods on standard food labels. Metabolic studies reveal that trans fats have adverse effects on blood lipid levels increasing LDL cholesterol while decreasing HDL cholesterol. This combined effect on the ratio of LDL to HDL cholesterol is double that of saturated fatty acids (Mensink 2008). Trans fats have also been associated with an increased risk of coronary heart disease in epidemiologic studies (Willett 2007).

Unsaturated fatty acids are naturally found in a cis configuration. Trans fatty acids have the hydrogen atoms on opposite sides. These trans double bonds can occur naturally in ruminants, and will show low levels in many meat and dairy products. Trans unsaturated fatty acids are produced artificially by introducing heat to liquid vegetable

oils in the presence of metal catalysts and hydrogen (Katan 2009).

2.5 Vitamin A

Vitamin A is a generic term for a large number of related compounds. The alcohol form is named retinol, while the aldehyde is termed retinal. These are commonly referred to as pre-formed vitamin A. Retinoic acid is formed in the body from retinal and is actively involved in molecular processes. These three compounds as well as others similar in form and function are collectively known as retinoids. Another group of compounds known as carotenoids have been termed provitamin A. Beta-carotene is found in this category, and is found in a variety of sources in the environment (Prakash et al. 2000).

Vitamin A is found naturally and occurs in many forms from a variety of both plant and animal sources. Retinol, the free form, is not found in foods. Carotenoids are found specifically in plants. Leafy green, and brightly colored are the most significant sources of carotenoids (Prakash et al. 2000). Retinyl palmitate is the major source of retinol found in animals. It is a storage form which acts as a precursor for retinol. In those foods where retinol activity comes mainly from provitamin A carotenoids, the carotenoid content and the retinol activity equivalents are presented (Thurnham et al. 2003). The USDA food composition database provides the content of several different carotenoids, including lycopene, lutein and zeaxanthin.

Vitamin A exists as provitamin B carotene found in foods such as milk, eggs, and many fruits and vegetables. It is absorbed in the intestine and travels to the liver as a retinyl ester. It is stored in the liver, or its ester is cleaved and it travels through the body

bound to retinol binding protein (RBP). Retinoic acid is transported bound to albumin. Once retinol enters the cell it binds to cellular retinol binding protein (CRBP) and from there can be oxidized into retinal and retinoic acid by retinaldehyde dehydrogenase (Lei 2003). It has been shown that genes induced by retinoic acid transcription factors control most of the targets involved in the metabolic activities of retinoids. It is a unique feedback system, which shows the dependency of the organism on these processes (Lei 2003).

Retinol from dietary sources (vitamin A) is metabolized intracellularly and results in its active versions: all-*trans* retinoic acid (tRA) and 9-*cis* retinoic acid (9-*cis* RA). Retinoid functions are exerted through their binding with their receptors. The receptors are termed retinoic acid receptors (RAR) and retinoic x receptors (RXR) (Ben-dor et al. 2002). The difference between the two is RARs have a high affinity for all-trans-retinoic acid, while the RXRs tend to bind with cis forms of retinoic acid. The receptors act as a dimer, and then (bound to their ligand) are imported into the nucleus as transcription factors where they bind to the retinoic acid responsive element (RARE) (Ben-dor et al. 2002). While RAR bound to trans-RA seems to have an antiproliferative effect on MCF-7 cells in culture, RXR does not show any effect on cell growth. Cis-RA and RXR have not been shown to result in the same level of cancer suppression as RAR and trans-RA (Liu et al. 1996).

Figure 2.5.1 Retinol conversions (a) and binding sites (b) intracellularly

Vitamin A deficiency among children in developing nations is the leading preventable cause of blindness (Semba et al. 2004). The most common diseases associated directly with vitamin A deficiency is of the eye. Night blindness from impaired 11- cis retinal production is common (Semba et al. 2004). Also a condition of the conjunctiva called Bitot's spots can be observed during a deficiency (Semba et al. 2004). The most severe form of vitamin A deficiency in the eyes is known as xerophthalmia. This condition leads to dry eyes, corneal ulcers, scarring, and eventually total blindness (Semba et al. 2004). Vitamin A deficiency is also considered an acquired immunodeficiency disease as described by Semba. Inadequate vitamin A intake has also

been shown to lead to increased incidence of infectious disease. Interestingly, vitamin A supplementation in developing countries decreases deaths in children from diarrhea and measles (Thurnham et al. 2003).

The RDA for vitamin A was revised by the Food and Nutrition Board (FNB) of the Institute of Medicine in 2001. Vitamin A has an RDA of 900mcg/day for men and 700mcg/day for women. The latest RDA is based on the amount needed to ensure an adequate store of vitamin A in the body. This store is a sufficient amount to support normal vision, immune function, maturation, growth, and gene expression (Semba et al. 2004).

Vitamin A is an integral part of the signal transduction required for vision. As light passes through the lens of the eye, it is received by the retina located at the rear of the eye. The light is then converted to a signal, which the brain translates into vision. At the epithelial surface of the retina, retinol is esterified to form a retinyl ester. These esters are then isomerized following hydrolysis and become 11-*cis* retinol. Following oxidation this compound becomes 11-*cis* retinal. The importance of 11-*cis* retinal is its ability to bind to opsin and form rhodopsin. Rhodopsin is found on rod cells of the eye, which are important in night vision. 11-*cis* retinal isomerizes to all-*trans* retinal, which results in the signal cascade responsible for vision.

Vitamin A is necessary for the normal and healthy activities of the human immune system. Better known as retinol, vitamin A and its metabolites are required to maintain the correct function of the mucosal cells (epithelial cells) found in the urinary, digestive, and reproductive tracts. Retinol and retinoic acid (RA) have an integral role in the development and differentiation of lymphocytes, and AtRA is required by T-lymphocytes

for proper regulation of the immune system.

Both vitamin A excess and deficiency are known to cause birth defects. Retinol and retinoic acid (RA) are essential for embryonic development (Christian 2003). During fetal development, RA functions in limb development and formation of the heart, eyes, and ears (Pavia 1999). Additionally, RA has been found to regulate expression of the gene for growth hormone (Christian 2003)

Red blood cells, like all blood cells, are derived from precursor cells called stem cells. These stem cells are dependent on retinoids for normal differentiation into red blood cells. Additionally, vitamin A appears to facilitate the mobilization of iron from storage sites to the developing red blood cell for incorporation into hemoglobin, the oxygen carrier in red blood cells (Christian 2003).

Delivery of RA to its receptor within the cell has been a topic of interest recently, and it has yet to be fully understood. The current theory on RA delivery is a combination of passive diffusion and the action of a carrier protein. Cellular retinoic acid binding protein II (CRABP II) has been uncovered as that carrier protein. Upon binding to its ligand (RA) it undergoes a massive conformational change in the cytosol near the nucleus. The change causes a rapid recruitment into the nucleus where RA is delivered to its receptor RAR or RXR. The collision-mediated process leaves the binding protein empty and is then immediately exported from the nucleus back to the cytosol where it awaits another ligand. Recent research has concluded that the CRABP II is a necessary and exciting pathway by which retinoic acid induces growth arrest in mammary carcinoma cells (Budhu et al. 2002). Over expression of CRABP II in MCF-7 cells showed a significant decrease in cell proliferation, while silencing the protein allowed the

cells to proliferate as if there was no RA in the treatment.

2.6 Nuclear Hormone Receptors

Nuclear hormone receptors may be responsible for the combined effect of Vitamin A and Fatty Acid on carcinogenesis. More specifically, the peroxisome proliferator-activated receptors (PPARs) are hypothesized to play a role in the decreased cell proliferation. (Moller et al. 2003). The PPARs are ligand-activated transcription factors of the nuclear hormone receptor superfamily. They share a high degree of structural homology with all members of the superfamily, particularly in the DNA-binding domain and ligand- and cofactor-binding domain. Many cellular and systemic roles have been attributed to these receptors, reaching far beyond the stimulation of peroxisome proliferation in rodents after which they were initially named. Peroxisome proliferator-activated receptors exhibit broad, isotype-specific tissue expression patterns. PPAR α is expressed at high levels in organs with significant catabolism of fatty acids. PPAR β/δ has the broadest expression pattern, and the levels of expression in certain tissues depend on the extent of cell proliferation and differentiation. PPAR γ is expressed as two isoforms, of which PPAR γ 2 is found at high levels in the adipose tissues, whereas PPAR γ 1 has a broader expression pattern.

Figure 2.6.1: PPAR alpha and gamma pathways.

Transcriptional regulation by PPARs requires heterodimerization with the retinoid X receptor (RXR) (Vu-Dac et al. 1998). When activated by a ligand, the dimer modulates transcription via binding to a specific DNA sequence element called a peroxisome proliferator response element (PPRE) in the promoter region of target genes (Kersten et al., 1999). A wide variety of natural or synthetic compounds have been identified as PPAR ligands. Among the synthetic ligands, the lipid-lowering drugs, fibrates, and the insulin sensitizers, thiazolidinediones, are PPAR α and PPAR γ agonists, respectively, which underscores the important role of PPARs as therapeutic anti-cancer targets (Staels et al., 1998). Transcriptional control by PPAR/RXR heterodimers also requires interaction with coregulator complexes (Kersten et al., 2001). Thus, selective action of PPARs in vivo results from the interplay at a given time point between expression levels of each of the three PPAR and RXR isotypes, affinity for a specific promoter PPRE, and ligand and cofactor availabilities.

Consistent with its distribution in tissues with high catabolic rates of fatty acids and high peroxisomal activity, the major role of PPAR α is the regulation of energy homeostasis (Lefebvre et al. 2006). In the liver especially, PPAR α activates fatty acid catabolism, stimulates gluconeogenesis and ketone body synthesis, and is involved in the control of lipoprotein assembly (Staels et al. 1995). PPAR α also stimulates heme synthesis and cholesterol catabolism (Staels et al. 1995). Furthermore, it attenuates inflammatory responses and participates in the control of amino acid metabolism and urea synthesis (Devchand et al. 1999). Increased fatty acid oxidation by activated PPAR α lowers circulating triglyceride levels, liver and muscle steatosis, and reduces adiposity, which improves insulin sensitivity (Guerre-Millo et al. 2000; Chou et al. 2002). Not surprisingly, fibrate drugs such as gemfibrozil, clofibrate, and fenofibrate that are widely used to treat hypertriglyceridemia are activators of PPAR α . In addition, PPAR α agonists have demonstrated significant anti-inflammatory activities that seem to play a role in their protective actions within the cardiovascular system (Berger et al. 2005).

2.7 Nutrient Delivery Systems

The delivery of AtRA has not been easily performed to date. The goal of establishing therapeutic levels of AtRA in tissue has also met some barriers. These barriers include issues of compound stability, and also tissue safety.

While beneficial as an anti-cancer agent, the use of AtRA is not without complications. AtRA is a water insoluble, toxic agent with limited bioavailability (Freemantle et al. 2003). Pharmacological levels can cause retinoic acid syndrome and neurotoxicity, particularly in children (Takitani et al. 2006). In addition, drug resistance has been reported in cases of sustained ATRA treatment requiring the use of additional

cytotoxic chemotherapy (Freemantle et al. 2003). Although liposomal formulations were developed a number of years ago in an effort to address these issues, they have not progressed past the clinical trial stage (Freemantle et al. 2003). Despite this, the potential benefits of associating ATRA with a lipid-based carrier are many. Not only do lipid-drug formulations address solubility issues, they also decrease toxicity and potentially avoid triggering ATRA resistance, thereby minimizing the need for additional chemotherapy (Freemantle et al. 2003).

In conjunction with this study, our research team was involved in exploring possible applications of the above named Liposomes. Liposomal delivery offers many specific stability advantages of AtRA, and we were able to produce results in vitro supporting the possibility of further usage. The application model stemmed from using dairy based fatty acids to produce liposomes that delivered AtRA. Appendix D has the full results. Liposome use is a viable option for future delivery of AtRA and could be engineered with specific fatty acids as well. The search for a viable delivery option from liposomes is currently underway, and should yield realistic applications in the future to treat carcinomas.

Chapter 3.0

MATERIALS AND METHODS

3.1 Materials

MCF-7 cells were obtained from the American Type Institute. AtRA was obtained from Sigma Aldrich (St. Louis MO). Fatty Acids and other chemicals were obtained from Fisher Scientific (Pittsburgh, PA).

3.2 Cell Experiments

MCF-7 cells were cultured at 37 °C, 5% CO₂, in Dulbecco's Modified Eagle Media (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotic. FBS and the antibiotic (50:50, penicillin and streptomycin) added to the media, sterile filtered using a bottle top vacuum filter. 75cm² cell culture flasks were carefully monitored for confluence and were passed upon a 75% confluency. Cells were grown consistently in 20 ml of media until confluent. MCF-7 cells did not require a media change to ensure confluency. The cells were considered "stock" cells which were transferred to growth plates for growth assays.

3.3 Cell Counting

Cells collected via trypsin in a 15 ml Falcon tube. The tube spun at 1800 RPM for 5 minutes in a centrifuge to collect pellet. The cells were resuspended by light vortexing in 1 ml of fresh media. Sample (5 ul) of cells was counted on a hemacytometer (Figure 3.1) to determine amount of cells in the 1 ml. 48 well plates seeded for cell proliferation assays, and each well contained 5000 cells as determined by a hemacytometer. Cells adhered for 24 hours before treatment.

3.4 Cell Treatments

AtRA treatments began with a growth curve. Cells seeded in 48 well growth plates for 24 hours until confluent. Media removed, new media (void of FBS) containing treatments added. Stepwise increases in AtRA (in DMSO) concentration (.5 – 10 μM) were used, and cells analyzed after both 24 and 48-hour growth periods. 1 μM AtRA chosen to pair with fatty acid treatments. AtRA extinction treatments also examined at time zero, vs daily treatments over 72 hours.

Fatty Acids (Linolenic, Linoleic, and Palmitic Acid) were used to treat the MCF-7 cells over 24 and 48 hours. Later, 1 μM of AtRA was combined with 2.5 μM of each fatty acid. Growth carefully monitored over 72 hours with identical wells receiving either a single treatment at time zero, or receiving multiple treatments (1 every 24 hours). DMSO was controlled at 1% by volume. Each designated time period ended with media removal from each plate and placed in a – 20 degree C freezer for later analysis.

Figure 3.1: View of hemacytometer grid (10x) and cells. Cells are counted in squares and determine the amount of cells in solution following calculation.

3.5 Cell proliferation assay

Cell proliferation measured by fluorescence using the Cyquant Cell Proliferation Assay Kit (Invitrogen CO. Carlsbad, CA). Each plate removed from freezer and thawed.

Nano-pure water (19 ml), lysis buffer (1 ml), and a florescent dye (50 ul) combined and added to each well in 200 uL aliquots. 30 minutes of incubation was required before the first reading and emission was stable for up to 60 minutes.

3.6 Cell proliferation quantification

A Typhoon Variable Mode Imager (General Electric Health Care) read the florescent stained cellular nucleic acids through a flourecein filter at 520 nm following a green laser excitation of the dye. Following the scan, the resulting image was manipulated and evaluated by the Imagequant TL software (Amersham Biosciences). Each well evaluated by color absorption then measured against control wells to give cell proliferation comparisons.

Chapter 4

RESULTS

MCF-7 cell growth was limited by media containing AtRA. A growth curve analysis of MCF-7 cells in the presence of AtRA in varying concentrations was produced

to investigate its efficacy in decreasing cell proliferation.. Figure 4.1 shows the decrease in growth with each concentration as compared to media not supplemented with AtRA (control). 5uM AtRA concentration showed the largest decrease, the 1 uM concentration was chosen for further analysis. 1 uM decreased cell growth by 32.9% after 24 hours.

Figure 4.1: Molecular Probes Cyquant Kit: 5000 cells were seeded per well and allowed to adhere overnight before treatments were given 24 hours later in serum free media. Proliferation was significantly altered with the use of ATRA. Columns C-F are 0.5uM – 5.0uM AtRA in media respectively. (Images produced on a Typhoon Scanner, and manipulated with Imagequant software. (Image is of actual size)

Figure 4.2: Cell Proliferation Assay. Standard curve for cell growth under varying amounts of AtRA delivered in vitro. Ideal treatment was found to be 1 uM which was not greater than 50% cell growth reduction. (Numbers taken from Figure 1 to produce graph)

Linolenic acid decreased MCF-7 cell proliferation in vitro. Linolenic acid delivered to MCF-7 cells via media over 72 hours decreased cell growth by 26.5%. Linoleic acid was used as a control to investigate fatty acids delivered in media. Linoleic did show a slight decrease in cell proliferation (10.1%), it was not consistent and was not considered significant.

AtRA combined with Linolenic acid decreased MCF-7 cell proliferation greater than AtRA alone. Over 72 hours of refeeding treatments, 1 uM AtRA combined with 2.5 uM Linolenic acid decreased cell proliferation by 73.4%. This was 49.7% less than AtRA alone, and 87.4% less than Linolenic acid alone. Figure 4.3 shows the percent growth of the control.

Figure 4.3: Cell Proliferation Assay: Data presented as percent of control. AtRA (1uM) along with AtRA + Linolenic acid produced significant growth differences over 72 hours. The combined affect was 49.7% greater then AtRA alone, and was significantly different.

Figure 4.4: Molecular Probes Cyquant Kit: 5000 cells were seeded per well and allowed to adhere overnight before treatments were given 24 hours later in serum free media. Proliferation was significantly altered with the use of ATRA (1uM) and the combination of ATRA (1uM) with Linolenic Acid (2.5uM). Treatments were only affective with daily dosage as opposed to a single dosage on day 1. Columns C and F illustrate this difference. (Images produced on a Typhoon Scanner, and manipulated with Imagequant software. (Image is of actual size)

4.1 AtRA and Linolenic Acid Combined Treatment Results

The combined affect of treatments was only seen after 72 hours of re-feeding, and cells given only 1 treatment on day 1 did not show the same results. Figure 4.5 shows the relationship of single treatments vs. re-feeding daily treatments.

Figure 4.5: Series 1 is cell proliferation following single treatment at time zero, vs series 2 which is treatments every 24 hours.

4.2 Statistical Analysis

Statistical significance ($p < .05$) was found when comparing AtRA+ linolenic acid, and linoleic acid treatments with an ANOVA. Daily AtRA feeding was statistically different ($p < .05$) from all of the other treatments accept AtRA combined with Linolenic Acid, and the Linolenic acid treatment alone. AtRA combined with Linoleic acid was significantly different ($p < .05$) from AtRA + linolenic Acid, and linolenic acid alone. Daily feedings were all statistically different ($p < .05$) from single feedings (at time zero) except for single AtRA feedings on day 1.

Chapter 5

DISCUSSION

All-trans retinoic acid has historically been evaluated and applied in chemoprevention of epithelial cancers. Linolenic acid has also been explored for possible application in chemoprevention. Here, we have explored the synergism of these two compounds through an in-vitro application in a specific cell line. We have also hypothesized the connection with these compounds to be found on the nuclear level in the form of transcription factor specific receptors: Peroxisome-proliferator activated receptor gamma (PPAR γ), and Retinoic Acid receptor (RXR). MCF-7 cells are an appropriate choice when exploring RXR and PPAR do to their previous use in these applications. Other epithelial cell lines do not produce the same quantity of receptors and have not shown the same promising results. Unique expression patterns of PPAR γ relative to RXR in certain cell lines led us to hypothesize that applying a known ligand for each receptor would significantly affect the progression of MCF-7 cell growth in vitro. Our hypothesis was supported by current research where up-regulation of the PPAR γ was noted with increased ligand activity of RXR (Nicol et al. 2004).

A growing body of evidence indicates PPAR γ is involved in both breast cancer development and progression. PPAR γ (+/-) mice had almost three-fold increased incidence of mammary adenocarcinomas and decreased survival rate when compared to PPAR γ (+/+) littermates (Nicol et al. 2004). Several reports have demonstrated that treating animals with PPAR γ ligands prior to chemical induction of mammary tumors is protective against tumor development (Nicol et al. 2004). It appears that expression and transactivation of PPAR γ is protective against breast tumor formation particularly when

activated by PPAR γ ligands. Our data demonstrates that a particular ligand (Linolenic Acid) seems effective at transactivating PPAR γ in normal mammary epithelia.

Once a breast tumor has formed, PPAR γ appears to have multiple effects. One study observed that distinct PPAR γ ligands induce apoptosis (Nicol et al. 2004). Conversely, a recent report by Saez et al. (2004) found that when mice expressing a constitutively active form of PPAR γ in the mammary gland were crossed with mice prone to mammary gland cancer, bigenic animals develop tumors that express higher levels of markers of malignancy. The authors conclude that once an initiating event takes place, increased PPAR γ signaling serves as a tumor promoter in the mammary gland of these experimental animals. Collectively, these data suggest that the physiological consequence of PPAR γ activation is dependent on many factors including the stage of development of the specific breast cancer cell.

Our demonstration that an individual PPAR γ ligand (Linolenic Acid) showed promising results follows the trend previously demonstrated in literature. We also demonstrated the use of a RXR ligand to slow the progression of MCF-7 cell growth in vitro. Interestingly, further decrease in progression was observed with the co-treatment of both ligands. The heterodimeric relationship of RXR and PPAR γ has been shown to have its own response element PPARE which was shown to be up-regulated by ligands of either receptor independent of each other. We have shown here that there is also promise of increased transcription through increased availability of both ligands.

It can be concluded that individual breast cancer cell types are likely to respond to PPAR γ ligands and RXR ligands in unique physiological ways and our data suggests that,

in part, variant cellular responses are the result of selective PPAR γ transactivation. Our data demonstrates that individual PPAR γ ligands combined with individual RXR ligands can selectively activate response element activity within a single cancer cell type. These data suggest that the effectiveness of PPAR γ and RXR as a target for chemotherapeutic treatment will greatly depend on the cell that is treated which opens the possibility of utilizing PPAR γ and RXR for targeted gene therapy.

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Appendix A

Cyquant Cell Proliferation Protocol

1. On day of experiment, decide number of wells to be assayed. This will depend on number of treatments and number of plates. (For example, look to image below.)
2. Obtain a plastic (preferably opaque) bottle, and aluminum foil.
3. Dilute the stock 20x Lysis Buffer Solution to 1X, so add 1ml of buffer to 19ml of Nuclease free DI water. (note this is enough for 100 assays at 200ul each)
4. Next make a 400 fold dilution with the Green Dye solution by adding 50ul of dye to the 1x lysis buffer. Cover with foil. Now we are ready.
5. Use this solution within 2 hours of creation, and keep covered with foil for light protection.

Cell culture prior to day of assay

6. Obtain a 96 well, or 48 well plate.
7. Seed the wells with 5000 cells per well as obtained from a hemacytometer count. (Hemacytometer protocol is available and separate)
8. Grow the cells with treatments (as described later) for specified durations.
9. Blot the media from the wells, and freeze immediately at -70°C for each time period.

Day of assay protocol

10. On day of assay, obtain plate from freezer and let thaw, then add 200ul of the working solution to each well to be assayed.
11. Let mix and incubate for 5min, and cover the plate with foil to protect from light.
12. Then use microplate reader to measure fluorescence with settings at 480nm excitation and 520nm emission maxima.

Standard Curve Creation

1. Obtain 96 or 48 well plate.
2. Use hemacytometer to count cells from a confluent flask.
3. Seed wells with 500, 1000, 2000, 5000, and 10000 cells per well
4. Let cells adhere and incubate for 24 hours, then freeze.
5. Run assay as described above, and pray for a linear relationship.
6. Plate will be seeded as shown below.

Appendix B

Hemacytometer Protocol

Obtain 1 flask/plate of cells, and aspirate off media. Add 5 cc of Trypsin and place in incubator for 7-10 minutes.

Add 15 cc of new media to trypsinized cells in flask, and mix via pipette.

Aliquot 10 cc of cells + media into a new 15 cc conical tube, and repeat for other 10 cc left in flask.

Take 2 15 cc conical tubes containing 10 cc of cells + media and place in centrifuge directly across from each other for balance. Set to spin at 1800 rpm for 5 minutes. Set acceleration at setting 2, and deceleration at setting 9 at 18 degrees Celsius. (This is for Eppendorf centrifuge)

Take 15 cc tubes with visible cell pellets back to sterile environment to aspirate off media, and re-suspend cell pellet in 1 cc of new media. (Do this using a transfer pipette)

With new transfer pipette, take small sample of cells plus media and slowly release onto hemacytometer. (The slide should have a coverslip on at this point, and is designed to disperse evenly due to small “channel” in glass)

Place on upright (not inverted) microscope and view on the 5X objective lens to see cells. Optimal viewing found on 10 x objective lens.

To count cells, first find center square with a 5x5 square profile. Count each corner square, and pick one more square in middle. (Note, this tactic only works if cells appear to be evenly distributed across slide... which they should!) Later the number is multiplied by 5 to account for other squares.

Number will most likely be between 50-150. Then use the following equation:

$$(\text{cell \# counted}) \times 5 \times 10^4 \times 1\text{ml} = \# \text{ of cells in suspension}$$

$$\text{Ex: } 57 \text{ cells} \times 5 \times 10^4 \times 1\text{ml} = 2.85 \times 10^6$$

Next, decide how much to aliquot for each well on a 96 well plate.

Ex: let's assume we want 5,000 cells per well.

$$.05 \times 10^6 / 2.85 \times 10^6 = .018\text{ml or.... } 18\mu\text{l per well.}$$

Max well volume should not exceed a ceiling, Of 270 ul. Therefore, the media warmed before adding cells must be less than 252 ul in this case for each well.

Appendix C

Complete Experiment Results

The experiment concluded much more data then reported in the results section of Chapter 4. The results below are day to day results that were used for final analysis. As reported earlier, the results are described by either a single treatment on day one, or daily treatments over 72 hours. Each treatment is indicated as well.

	Day 1 treatment	Daily Treatment
Cell Growth Treatment: Blank		
Day 1	18963751.5	18963751.5
Day 2	21281242.5	21281242.5
Day 3	27134548	27134548

Cell Growth (DMSO Control)

Day 1	19276753.8	19276753.8
Day 2	20773928.3	21777477.7
Day 3	30964674.7	29658439.4

Cell Growth (ATRA 1uM)

Day 1	16084480	16084480
Day 2	19283732.7	15140571
Day 3	26206974.6	9057623.09

Cell Growth (Linolenic 2.5 uM)

Day 1	18397367.7	18397367.7
Day 2	19290527	17852295.3
Day 3	25327786.1	19938678.6

Cell Growth (Linoleic 2.5 μ M)

Day 1

19306580.7

19306580.7

Day 2

21089190.7

20329843.7

Day 3

26684736

24402016.1

Cell Growth (ATRA 1 μ M + Linolenic 2.5 μ M)

Day 1

13996046.2

13996046.2

Day 2

19264504.3

12188983

Day 3

23443646.3

4499775.95

Appendix D

Liposome Delivery System Results

The research conducted here was in collaboration with the Dairy Products Technology Center at Cal Poly, San Luis Obispo during 2006-2007. Callaborators included Dr. Susan Hawk, Dr. Rafeal Jimenez, David Brown, and Jessica Yee. Other technicians included in the study were Selena Tinga, and Diana Rios.

The study yielded promising results, and would be an excellent starting point for future research. The project used a SuperCritical Fluid Extraction method to isolate milk lipids. These lipids were then manipulated through High Pressure Homogenization to creat Liposomes. The liposomes were also created with All-trans retinoic Acid as a nutrient to be delivered in vitro. Results were as follows:

By Treatment

Treatment	Cell Growth (24hrs)	CONTROL
Imidazole	21981333.5	21281242.5
Ethanol	20781992.5	21281242.5
2.5 uM ATRA	15140571	21281242.5
SE (SFE emulsiflex)	19701002.5	21281242.5
SAE (SFE+ATRA+emulsiflex)	14740598	21281242.5
SS (SFE sonicated)	20822992.5	21281242.5
SAS (SFE+ATRA+sonicated)	14999579	21281242.5
BE (buttermilk powder + emulsiflex)	20788888.5	21281242.5
BAE (Buttermilk powder + ATRA + emulsiflex)	21001982.5	21281242.5
BS (Buttermilk powder + sonicated)	19997654.5	21281242.5
BAS (Buttermilk powder + ATRA +sonicated)	17140987	21281242.5

Control	21281242.5	21281242.5
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By method

Control	21281242.5	21281242.5
Imidazole	21981333.5	21281242.5
Ethanol	20781992.5	21281242.5
2.5 uM ATRA	15140571	21281242.5
SAE (SFE+ATRA+emulsiflex)	14740598	21281242.5
SAS (SFE+ATRA+sonicated)	14999579	21281242.5
BAE (Buttermilk powder + ATRA + emulsiflex)	21001982.5	21281242.5
BAS (Buttermilk powder + ATRA +sonicated)	17140987	21281242.5
SE (SFE emulsiflex)	19701002.5	21281242.5
SS (SFE sonicated)	20822992.5	21281242.5
BE (buttermilk powder + emulsiflex)	20788888.5	21281242.5
BS (Buttermilk powder + sonicated)	19997654.5	21281242.5

MCF-7 Cell Growth Liposome (ATRA Grouped)

MCF-7 Cell Growth Liposomes (By Method)

Appendix E

Minitab Output

General Linear Model: Count versus Treatment, Application

Factor	Type	Levels	Values
Treatment	fixed	7	Atra, Atra + Leic, ATra +Lenic, control, dmso control, Linoleic, Linolenic
Application	fixed	2	Daily, Single

Analysis of Variance for Count, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	6	1.12085E+15	1.12085E+15	1.86808E+14	30.92	0.000
Application	1	8.82998E+14	8.82998E+14	8.82998E+14	146.16	0.000
Treatment*Application	6	4.85275E+14	4.85275E+14	8.08792E+13	13.39	0.000
Error	28	1.69160E+14	1.69160E+14	6.04142E+12		
Total	41	2.65828E+15				

S = 2457931 R-Sq = 93.64% R-Sq(adj) = 90.68%

Tukey 95.0% Simultaneous Confidence Intervals

Response Variable Count

All Pairwise Comparisons among Levels of Treatment*Application

Treatment = Atra

Application = Daily subtracted from:

Treatment	Application	Lower	Center	Upper
Atra	Single	9798481	17149352	24500222
Atra + Leic	Daily	10675594	18026465	25377333
Atra + Leic	Single	10306840	17657711	25008582
ATRa +Lenic	Daily	-11908718	-4557847	2793024
ATRa +Lenic	Single	7035153	14386023	21736894
control	Daily	8981153	16332024	23682895
control	Single	12470955	19821826	27172696
dms0 control	Daily	9249946	16600816	23951687
dms0 control	Single	14556181	21907052	29257922
Linoleic	Daily	3993522	11344393	18695264
Linoleic	Single	10276242	17627113	24977984
Linolenic	Daily	-4469815	2881056	10231926
Linolenic	Single	8919292	16270163	23621034

Treatment = Atra
Application = Single subtracted from:

Treatment	Application	-----+-----+-----+-----+-----
Atra + Leic	Daily	(--*--)
Atra + Leic	Single	(--*--)
ATRa +Lenic	Daily	(---*---)
ATRa +Lenic	Single	(---*--)
control	Daily	(---*--)
control	Single	(--*---)
dms0 control	Daily	(---*--)
dms0 control	Single	(--*---)
Linoleic	Daily	(---*---)
Linoleic	Single	(--*---)
Linolenic	Daily	(---*---)
Linolenic	Single	(---*--)

-2.0E+07 0 20000000 40000000

Treatment = ATRa +Lenic

Application = Daily subtracted from:

Treatment	Application	Lower	Center	Upper
ATRa +Lenic	Single	11593000	18943870	26294741
control	Daily	13539001	20889871	28240742
control	Single	17028802	24379673	31730544
dms0 control	Daily	13807793	21158663	28509534
dms0 control	Single	19114028	26464899	33815769
Linoleic	Daily	8551369	15902240	23253111
Linoleic	Single	14834089	22184960	29535831
Linolenic	Daily	88032	7438903	14789773
Linolenic	Single	13477139	20828010	28178881

Treatment = ATRa +Lenic

Application = Single subtracted from:

Treatment	Application	Lower	Center	Upper
control	Daily	-5404870	1946001	9296872
control	Single	-1915068	5435802	12786673
dms0 control	Daily	-5136078	2214793	9565664
dms0 control	Single	170158	7521028	14871899
Linoleic	Daily	-10392501	-3041630	4309241
Linoleic	Single	-4109781	3241090	10591960
Linolenic	Daily	-18855838	-11504968	-4154097
Linolenic	Single	-5466731	1884140	9235010

Treatment = control

Application = Daily subtracted from:

Treatment	Application	Lower	Center	Upper
control	Single	-3861069	3489802	10840672
dms0 control	Daily	-7082079	268792	7619663

Treatment	Application	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
Atra	Single	17149352	2006892	8.545	0.0000
Atra + Leic	Daily	18026465	2006892	8.982	0.0000
Atra + Leic	Single	17657711	2006892	8.799	0.0000
ATRa +Lenic	Daily	-4557847	2006892	-2.271	0.5865
ATRa +Lenic	Single	14386023	2006892	7.168	0.0000
control	Daily	16332024	2006892	8.138	0.0000
control	Single	19821826	2006892	9.877	0.0000
dms0 control	Daily	16600816	2006892	8.272	0.0000
dms0 control	Single	21907052	2006892	10.916	0.0000
Linoleic	Daily	11344393	2006892	5.653	0.0004
Linoleic	Single	17627113	2006892	8.783	0.0000
Linolenic	Daily	2881056	2006892	1.436	0.9686

Linolenic	Single	16270163	2006892	8.107	0.0000
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Treatment = Atra
Application = Single subtracted from:

Treatment	Application	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
Atra + Leic	Daily	877113	2006892	0.44	1.0000
Atra + Leic	Single	508359	2006892	0.25	1.0000
ATRa +Lenic	Daily	-21707199	2006892	-10.82	0.0000
ATRa +Lenic	Single	-2763328	2006892	-1.38	0.9773
control	Daily	-817327	2006892	-0.41	1.0000
control	Single	2672474	2006892	1.33	0.9826
dms0 control	Daily	-548535	2006892	-0.27	1.0000
dms0 control	Single	4757700	2006892	2.37	0.5224
Linoleic	Daily	-5804959	2006892	-2.89	0.2386
Linoleic	Single	477761	2006892	0.24	1.0000
Linolenic	Daily	-14268296	2006892	-7.11	0.0000
Linolenic	Single	-879189	2006892	-0.44	1.0000

Treatment = Atra + Leic
Application = Daily subtracted from:

Treatment	Application	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
Atra + Leic	Single	-368754	2006892	-0.18	1.0000
ATRa +Lenic	Daily	-22584312	2006892	-11.25	0.0000
ATRa +Lenic	Single	-3640441	2006892	-1.81	0.8521
control	Daily	-1694440	2006892	-0.84	0.9998
control	Single	1795361	2006892	0.89	0.9996
dms0 control	Daily	-1425648	2006892	-0.71	1.0000
dms0 control	Single	3880587	2006892	1.93	0.7923
Linoleic	Daily	-6682072	2006892	-3.33	0.1027
Linoleic	Single	-399352	2006892	-0.20	1.0000
Linolenic	Daily	-15145409	2006892	-7.55	0.0000
Linolenic	Single	-1756302	2006892	-0.88	0.9997

Treatment = Atra + Leic
Application = Single subtracted from:

Treatment	Application	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
ATRa +Lenic	Daily	-22215558	2006892	-11.07	0.0000
ATRa +Lenic	Single	-3271688	2006892	-1.63	0.9227

control	Daily	-1325687	2006892	-0.66	1.0000
control	Single	2164115	2006892	1.08	0.9973
dms0 control	Daily	-1056895	2006892	-0.53	1.0000
dms0 control	Single	4249341	2006892	2.12	0.6846
Linoleic	Daily	-6313318	2006892	-3.15	0.1489
Linoleic	Single	-30598	2006892	-0.02	1.0000
Linolenic	Daily	-14776655	2006892	-7.36	0.0000
Linolenic	Single	-1387548	2006892	-0.69	1.0000

Treatment = ATRa +Lenic

Application = Daily subtracted from:

Treatment	Application	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
ATRa +Lenic	Single	18943870	2006892	9.439	0.0000
control	Daily	20889871	2006892	10.409	0.0000
control	Single	24379673	2006892	12.148	0.0000
dms0 control	Daily	21158663	2006892	10.543	0.0000
dms0 control	Single	26464899	2006892	13.187	0.0000
Linoleic	Daily	15902240	2006892	7.924	0.0000
Linoleic	Single	22184960	2006892	11.054	0.0000
Linolenic	Daily	7438903	2006892	3.707	0.0451
Linolenic	Single	20828010	2006892	10.378	0.0000

Treatment = ATRa +Lenic

Application = Single subtracted from:

Treatment	Application	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
control	Daily	1946001	2006892	0.970	0.9991
control	Single	5435802	2006892	2.709	0.3246
dms0 control	Daily	2214793	2006892	1.104	0.9967
dms0 control	Single	7521028	2006892	3.748	0.0410
Linoleic	Daily	-3041630	2006892	-1.516	0.9531
Linoleic	Single	3241090	2006892	1.615	0.9274
Linolenic	Daily	-11504968	2006892	-5.733	0.0003
Linolenic	Single	1884140	2006892	0.939	0.9993

Treatment = control

Application = Daily subtracted from:

Treatment	Application	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
control	Single	3489802	2006892	1.739	0.8842
dms0 control	Daily	268792	2006892	0.134	1.0000
dms0 control	Single	5575027	2006892	2.778	0.2901
Linoleic	Daily	-4987631	2006892	-2.485	0.4508
Linoleic	Single	1295089	2006892	0.645	1.0000
Linolenic	Daily	-13450969	2006892	-6.702	0.0001
Linolenic	Single	-61861	2006892	-0.031	1.0000

Treatment = control

Application = Single subtracted from:

Treatment	Application	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
dms0 control	Daily	-3221009	2006892	-1.605	0.9303
dms0 control	Single	2085226	2006892	1.039	0.9981
Linoleic	Daily	-8477433	2006892	-4.224	0.0132

Linoleic	Single	-2194713	2006892	-1.094	0.9969
Linolenic	Daily	-16940770	2006892	-8.441	0.0000
Linolenic	Single	-3551663	2006892	-1.770	0.8715

Treatment = dmso control
Application = Daily subtracted from:

Treatment	Application	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
dmso control	Single	5306235	2006892	2.644	0.3588
Linoleic	Daily	-5256423	2006892	-2.619	0.3725
Linoleic	Single	1026297	2006892	0.511	1.0000
Linolenic	Daily	-13719761	2006892	-6.836	0.0001
Linolenic	Single	-330653	2006892	-0.165	1.0000

Treatment = dmso control
Application = Single subtracted from:

Treatment	Application	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
Linoleic	Daily	-10562659	2006892	-5.263	0.0010
Linoleic	Single	-4279939	2006892	-2.133	0.6750
Linolenic	Daily	-19025996	2006892	-9.480	0.0000
Linolenic	Single	-5636889	2006892	-2.809	0.2755

Treatment = Linoleic
Application = Daily subtracted from:

Treatment	Application	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
Linoleic	Single	6282720	2006892	3.131	0.1534
Linolenic	Daily	-8463338	2006892	-4.217	0.0134
Linolenic	Single	4925770	2006892	2.454	0.4698

Treatment = Linoleic
Application = Single subtracted from:

Treatment	Application	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
Linolenic	Daily	-14746057	2006892	-7.348	0.0000
Linolenic	Single	-1356950	2006892	-0.676	1.0000

Treatment = Linolenic
Application = Daily subtracted from:

Treatment	Application	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
Linolenic	Single	13389107	2006892	6.672	0.0001