Vitamin B_6 Decreases Proliferation and DNA Synthesis in Human Mammary Carcinoma Cell Lines *In Vitro*

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Thesis Submitted to the Faculty of the Virginia Polytechnic Institute and State University
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE
In
Human Nutrition, Foods and Exercise

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April 4, 2000
Blacksburg, Virginia

Keywords: Breast Cancer, Vitamin B_6, Pyridoxal, Mammary, Estrogen, Steroid Hormone

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ABSTRACT

The growth of many breast cancers is stimulated by the action of the hormone estrogen. Hormonal therapy used to treat these estrogen-dependent breast cancers acts by interfering with the action of estrogen. Current treatments, such as tamoxifen, are not consistently useful due to development of resistance to these drugs. Tamoxifen treatment can also lead to the development of other gynecological cancers, therefore the discovery of novel treatment options for breast cancer is critical. Vitamin B_6 is well documented for its role as a modulator of steroid hormones. Pyridoxal phosphate (PLP), the active form of Vitamin B_6, may interfere with the action of the estrogen receptor (ER) by blocking the hormone-binding and/or DNA-binding site of the ER. The objective of this study was to examine the effects of Vitamin B_6 supplementation on cell proliferation and estrogen-dependent gene expression in breast cancer cells. To accomplish this, estrogen-dependent (MCF-7 and T-47D) and estrogen-independent (BT-20) breast cancer cells were grown in medium supplemented with 0,100, or 300 µM pyridoxal (PL) in the absence or presence of 0.01µM estradiol. Cell counts and [^{3}H]-thymidine incorporation into DNA were assessed in all cell lines. The expression of pS2, an estrogen-sensitive gene, was performed using RNA extracted from MCF-7 cells. PL supplementation was found to significantly decrease total cell numbers and DNA synthesis in both the estrogen-dependent (ER+) and –independent (ER-) breast cancer cells, but did not alter the expression of pS2. These results indicate that PL significantly impairs growth of breast cancer cells and may be exerting its effects via a steroid-independent mechanism.

Acknowledgements

I would like to thank, first and foremost, Dr. Barbara A. Davis. She has been the most amazing mentor that I could have had throughout this program of study. She introduced me to the world of critical, independent thinking; something that will be a benefit to my life far after graduate school is over. She has encouraged me and pushed me to levels of learning that I never would have dreamed of reaching on my own. Not only has she been a great academic advisor, but she has also been a joy to work with. Regardless of the situation, she has always been full of patience and encouragement, and that has no doubt been the key factor in my accomplishments here. I also need to thank my family, Dad, Mom, Patty, and Mark for their encouragement and support. From a young age, they were the ones that taught me to work diligently, to persevere through difficulty, and to believe that I could pursue and achieve any goal that I set. Kathy Reynolds and Janet Rhinehart have not only been great lab technicians to work with, but "life-savers" on many occasions, and our office staff, Sherry Saville and Sherry Terry always made sure that my graduate career ran as smoothly as possible. Finally, I need to thank Matthew T. Shenk, who has been so loving and patient with me through the many ups and downs of this journey.

Table of Contents

List of Figures

Chapter 1: Introduction

The high prevalence of breast cancer in the United States together with the lack of effective treatments for women suffering from this disease, make elucidation of new treatment options imperative. Vitamin B_6 (B_6) has been shown to play a role in the modulation of steroid hormones. For example, B_6 deficiency causes an increase in the ability of the prostate (Symes et al., 1984) and uterus (Bunce and Vessal, 1987) to take up testosterone and estrogen, respectively. Increased levels of intracellular B_6 have also been shown to effectively alter transcription of genes that are induced by steroid receptors, including glucocorticoid- (Allgood et al., 1990), androgen-, progesterone-, and estrogen- (Allgood et al., 1992) regulated genes.

Researchers suggest that B_6 is an inhibitor of cancer cell growth. Pyridoxine-supplementation has been reported to kill hepatoma cells (DiSorbo and Litwack, 1993) in culture, and pyridoxal treatment has been effective in controlling the growth of melanomas (Maksymowych et al., 1993) both *in vitro* and in animal models. However, much information is still lacking with regard to the relationship between B_6 and cancer growth. Studies need to be performed to determine which types of cancer are affected by B_6, the doses of B_6 supplementation that are the most effective, and the mechanisms by which B_6 exerts its effect in these cells. Data is also lacking as to whether B_6 is acting as a modulator of steroid hormone action to alter cell growth in steroid hormone-sensitive tissues, or more importantly, steroid hormone-sensitive cancers.

To further clarify the relationship between B_6 and cancer growth, the project described here was designed to determine whether vitamin B_6 supplementation could regulate the growth of and/or steroid hormone-induced gene expression of breast cancer cells *in vitro*. Results from this study provide important information for determining whether vitamin B_6 has the potential for use as an adjuvant treatment and/or preventative agent for breast cancer.

Chapter 2: Literature Review

Breast Cancer

Background

According to the National Cancer Institute, breast cancer is the most prevalent form of cancer today. Breast cancer is the leading cause of cancer death in women aged 40-55, and the second leading cause of cancer death in women of other age groups. According to the American Cancer Society, at least 175,000 new cases of invasive breast cancer will be diagnosed this year, and approximately 43,000 women will lose their lives to this disease (Ries et al., 1999).

Breast cancer cells can be divided into two major groups. Seventy percent of breast cancer cells stain positively for the estrogen receptor (ER+), and their growth is sensitive to the presence of estrogen. Breast cancer cells that do not contain ER are considered estrogen receptor negative (ER-) and their growth is insensitive to estrogen. Only 7% of normal mammary cells are ER+ (Ferguson, 1998), strongly indicating that the ER is involved in the proliferation of many breast cancers.

Estrogen (E) and the ER play an important role in the growth and development of the mammary gland. Although there have been alternative pathways elucidated, the primary mode of E action is through the ER. The ER has four main functions, hormone binding, dimerization, DNA binding, and activation of gene expression through transcription (Katzenellenbogen et al., 1993). The mechanism by which E regulates gene expression is as follows: 1) E binds to the ER, causing the release of a heat shock protein and the phosphorylation of the activation functional domain, 2) dimerization, or the joining of two E-ER complexes, 3) the E-ER dimer binds to the Estrogen Response Element (ERE) on the DNA, and 4) transcription is activated (Ferguson et al., 1998). There are six functional domains (A-F) of the ER (see Figure 2.1), two of which (C and E) are common to most steroid hormone receptors. Domain C is the DNA binding site of the ER, and domain E is the hormone binding and dimerization site of the ER (Ferguson et al., 1998). There are two transcriptional activation functional domains (AF) on the ER, AF-1 and AF-2. AF-2 is found within the hormone-binding site (domain E) and is completely E-dependent

for its action (Parker et al., 1998). AF-1 is found within domain A/B of the ER, and is the location where heat shock protein displacement and phosphorylation occur. The action of AF-1 is not solely dependent on the binding of estrogen, but is thought to enhance the action of AF-2 when E is bound (Ferguson et al., 1998; Parker et al., 1998) There are additional factors that interact with ER to regulate its function. Like the ER, the action of these transcription factors, RIP160, ERAP 160, and $TAF_{II}30$ are dependent on estrogen (Ferguson et al., 1998).

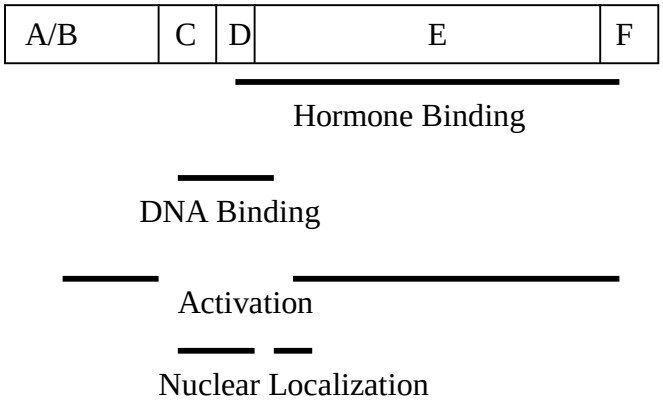

Figure 2-1: Functional Domains of the Estrogen Receptor

There is much evidence that E affects the proliferation of ER+ breast cancers through the action of the ER. It has been shown that ER directly stimulates the transcription of many genes whose functions may ultimately lead to the growth of ER+ breast cancers. The action of the E-ER complex has been demonstrated to enhance the transcription of the progesterone receptor (PR), pS2, pLIV-1, TGF-α (Ferguson et al., 1998), and the protooncogenes, *c-fos* and *c-myc (*van der Burg et al., 1989).

To decide whether a patient is an appropriate candidate for hormonal therapy, it is important to determine whether the ER regulates cancer proliferation in that patient. Some breast cancers that stain positively for ER are not responsive to hormonal therapy because the ER, although present in large amounts, is not fully functional as the regulator of proliferation (Ferguson et al., 1998). Researchers have found that a more accurate marker of hormonal responsiveness in breast cancers is the expression of pS2. The pS2 gene, originally identified in MCF-7 cells, is located on chromosome 21q in humans. It contains three exons, totaling 490 bp,

and two introns, totaling 3.87 kb. The precursor pS2 has 84 amino-acid residues (9 kDa), and is cleaved to the secreted protein size of 60 amino acids (7 kDa) (Nikolic-Vokosavljevic et al., 1998). The exact role of pS2 is not known, but it is thought to have a similar function to insulin –like growth factor (Racca et al., 1995) and a sequence homology similar to porcine pancreatic spasmolytic polypeptide (El-Tanani and Green, 1995). The expression of pS2 is known to be regulated through the action of the ER, and, unlike other E-induced genes found in ER+ cancers, is unaffected by other steroid hormones (El-Tanani and Green, 1995). Since pS2 expression is modulated via the ER, monitoring its expression in response to an experimental treatment is an excellent way to determine if the treatment is exerting its effects through the ER. These characteristics of pS2 expression make it an excellent marker for assessing the potential of breast cancer cells to be responsive to hormonal therapies.

Current Treatments

Tamoxifen

Because estrogens are well documented as having a role in the growth and development of breast cancers, anti-estrogens are widely used in the treatment of breast cancer. One of the most common forms of hormonal therapy for ER+ breast cancers is tamoxifen (TAM). TAM is a non-steroidal drug that interferes with the action of estrogen. A pure anti-estrogen is a compound that competes with E for binding to the ER and inactivates the estrogen-responsive genes (Coradini et al., 1994). The anti-estrogen binds to the ER, preventing E from binding. The antiestrogen –ER complex cannot stimulate gene expression and DNA synthesis, and (in theory) the estrogen-dependent cancer fails to grow. TAM is not a pure antiestrogen, because it has both agonist and antagonist effects on the ER (Coradini et al., 1994). The partial agonist activity of TAM leads to TAM-resistance of breast cancers after an average of 10-12 months of treatment (Osborne et al., 1995). TAM has also been shown to stimulate the growth of gynecological cancers, such as endometrial cancer, because of its estrogenic properties (Jordan and Morrow, 1994). Because of the complications with TAM treatment, researchers have studied the potential use of pure antiestrogens for the treatment of ER+ breast cancer (Coombes et al., 1993). The search for the ideal anti-estrogen, which will antagonize the action of estrogen in target tissues without negating the positive effects of estrogen in other tissues, is currently underway.

Steroidal Antiestrogens

Investigations have been made into the use of pure antiestrogens, such as ICI 182,780 and Ly 117018 for the treatment of ER+ breast cancers. The results are conflicting; some studies indicate that these antiestrogens have strong abilities to suppress estrogen-modulated gene expression (Osborne et al., 1995), but other studies demonstrate that the anti-proliferative effect of these antiestrogens is only possible at concentrations of the compound 100 times that of the estradiol concentration present (Coradini et al., 1994). There is no evidence of a consistently useful long-term treatment for ER+ breast cancers.

Vitamin B_6

Background

Vitamin B_6 (B_6) is a water-soluble vitamin that exists as several vitamers, primarily as pyridoxine, pyridoxamine, pyridoxal, and their phosphorylated derivatives (Okada et al., 1998). Rich sources of B_6 include highly fortified cereals, beef liver, organ meats, and highly fortified soy-based substitutes (FNB, 1998). According to the 1995 Continuing Survey of Food Intakes by Individuals (CFSII), most of the B_6 consumed by the United States population comes from fortified, ready-to-eat cereals; white potatoes and other starchy vegetables; non-citrus fruits; and mixed foods whose main ingredients are fish, meat, or poultry.

Pyridoxal phosphate (PLP) is the active form of B_6, and all known functions of the vitamin are attributed to this form. PLP is a cofactor for many enzymes, whose functions include amino acid metabolism, heme biosynthesis, and muscle function. PLP catalyzes reactions such as transamination, decarboxylation, transulfhydration and desulfhydration, cleavage, synthesis, and racemization of amino acids. PLP is also involved in glycogen metabolism, lipid metabolism, gluconeogenesis, immune function, and modulation of steroid hormone action (Tully et al., 1994).

Modulator of Steroid Hormone Action

The steroid hormones include glucocorticoid, mineralocorticoids, estrogen, progesterone, and androgen, and are involved in the regulation of growth, development, reproduction, and

metabolism (Tully et al., 1994; Allgood and Cidlowski, 1991). Each hormone exerts its effect in a target tissue through an interaction with its specific steroid hormone receptor. Generally, following steroid-hormone binding to its receptor, the activated steroid hormone complex is translocated to the nucleus of the cell. The activated receptor-hormone complex then interacts with a specific hormone response element located on the DNA. These hormone response elements are DNA regulatory sites, generally located in the promoter regions of certain hormone-responsive genes. The binding of the steroid hormone-receptor complex to the hormone response element modulates the action of hormone-responsive genes by either enhancing or repressing the transcription of those genes (Tully et al., 1994; Litwack et al., 1991).

In 1978, Cake and colleagues demonstrated that PLP inhibited the binding of glucocorticoid to the glucocorticoid receptor *in vitro*. Subsequent studies have shown that a decrease in tissue levels of PLP results in the accumulation of steroid hormones in those tissues. For example, increased uptake of estrogen in the uterus, liver, and hypothalamus has been demonstrated in B_6 deficient rats (Holley et al., 1983; Bowden et al., 1986; Bunce et al., 1987; Bender et al., 1989).

Additional studies have provided insight into the mechanism behind the modulation of steroid hormones by PLP. There is evidence to support a direct interaction between PLP and steroid receptors. One study investigated the effect of PLP on the proteolytic cleavage of the glucocorticoid receptor. Glucocorticoid receptors are easily digested by trypsin, which cleaves the peptide bonds between the carboxyl end of a lysine residue and its neighboring amino acid residue (Allgood and Cidlowski, 1991). When trypsin was added to a preparation of PLP and glucocorticoid receptor, it was unable to cleave the glucocorticoid receptor (Allgood and Cidlowski, 1991). This suggests that B_6 binds to the glucocorticoid receptor in the vicinity of a lysine residue. PLP functions as a coenzyme by forming a Schiff base between its carbonyl group and the amino group of lysine residues of certain enzymes. Steroid hormone receptors have several lysine residues surrounding the hormone-binding site (Katzenellenbogen et al., 1997). It is thought that PLP may bind to the lysine residue(s) of the steroid hormone receptors, therefore blocking the binding of the steroid hormone to the receptor.

PLP has been shown to inhibit the translocation of estrogen into the nucleus. Several studies using [^{3}H]-estradiol demonstrated that the estrogen ratio of the nucleus: cytosol was significantly lower in female rats fed a B_6-supplemented diet compared with those fed a diet deficient in B_6 (Bowden et al., 1986). Nuclear retention of testosterone into the prostate of male rats maintained on a B_6-deficient diet was significantly greater compared with animals consuming a B_6 adequate diet (Symes et al., 1984).

PLP also inhibits the binding of the activated steroid hormone-receptor complex to DNA. *In vitro*, PLP has been shown to inhibit the binding of progesterone, estrogen, androgen, and glucocorticoid receptors to their respective response element (Nishigori and Toft, 1980; Cake et al., 1978). The glucocorticoid receptor has been the most thoroughly studied of the steroid hormones affected by PLP. This receptor has two zinc fingers that interact with the glucocorticoid response element. It is believed that PLP binds to lysine residues on these zinc fingers, thereby inhibiting the steroid hormone receptor from binding to the DNA (Litwack et al., 1991).

Because PLP can interfere with the binding of steroid hormone to an inactivated receptor, nuclear translocation of an activated hormone-receptor complex, or DNA-binding of an activated hormone-receptor complex, it is conceivable that PLP can modulate the expression of genes controlled by the steroid hormones. PLP interaction with the steroid hormone receptors has, in fact, been shown to modulate gene expression (Allgood et al., 1990 and 1992). HeLa S3 cells were transfected with plasmids containing either glucocorticoid responsive or insensitive promoters and treated with PLP. PLP-treatment of the transfected cells was shown to decrease the activity of the reporter genes of those plasmids with responsive promoters, while producing no change in the reporter gene activity of those plasmids containing an glucocorticoid-insensitive promoter (Allgood et al., 1990). Further demonstration of the effect of PLP on the expression of steroid hormone-regulated genes was shown using T-47D, HeLa, and E8.2 cells transfected with a reporter gene sensitive to steroid hormones. These cells are known to contain endogenous progesterone/estrogen, estrogen, and androgen receptors, respectively. The actions of the progesterone, estrogen, and androgen receptors on the expression of the reporter gene were

depressed when the cells were supplemented with PL. The activity of the reporter gene increased when the cells were cultured with 4-deoxypyridoxine, a B_6 antagonist that caused a mild B_6 deficiency in the cells (Allgood et al., 1992).

Because the function of steroid hormones are essential to fundamental cellular processes, and PL has been shown to inhibit the action of the steroid hormones at many levels, studies have been conducted to examine the effects of PL on cell growth. Litwack et al., (1991) demonstrated that B_6 is capable of killing glioma cells, kidney cells, B16 mouse melanoma cells, and human melanoma cells (both metastastatic and non-metastastatic). DiSorbo and colleagues (1982) demonstrated that B_6 can kill hepatoma cells in culture, and Maksymowych et al.(1993) showed that B_6 can kill melanoma cells both in culture and *in vivo* in mice.

Immune Function

B_6 deficiency has been shown to have many detrimental effects on the health of an individual, such as decreased humoral and cell-mediated immunity. Lymphocytes from mice fed a B_6 -deficient diet have decreased proliferative and cytotoxic ability. Humans with poor B_6 status display decreased antibody production, total lymphocytes, and circulating interleukin-2 (Rall and Meydani, 1993). These results suggest that B_6 is important for normal immune function. Therefore, deficiency of this vitamin may leave an individual susceptible to the development of diseases, including cancer. The relationship between B_6 and cancer is further supported in a study showing that many breast cancer patients have low PLP levels and immunocompetence. Normalization of these parameters occurred with remission of their disease (Potera et al., 1977).

There is evidence that vitamin B_6 is a modulator of steroid hormone action, an inhibitor of certain types of cancer cell growth, and a potentiator of immune function. There is still much data lacking regarding the mechanism(s) by which B_6 exerts it effect on cancer cell growth, and whether it is occurring through modulation of steroid hormone action. This study was conducted to examine the effect of B_6 supplementation on the growth of ER+ and ER- breast cancer cells and to determine whether it would affect estrogen-regulated gene expression in breast cancer cells. Data from this study will further clarify the role of vitamin B_6 in cancer cell growth as

well as the role of this vitamin in estrogen metabolism. Clarification of these roles may lead to the potential use of vitamin B_6 or B_6 analogs for the prevention and/or treatment of breast cancer.

Chapter 3

Pyridoxal Supplementation Decreases Cell Proliferation and DNA Synthesis in Estrogen-Dependent and –Independent Mammary Carcinoma Cell Lines.

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Running Title: Breast Cancer Proliferation and Pyridoxal Supplementation.

Key Words: Breast Cancer, Vitamin B_6, Pyridoxal, Mammary, Estrogen, Steroid Hormone

ABSTRACT

The growth of many breast cancers is stimulated by the action of the hormone estrogen. Hormonal therapy used to treat these estrogen-dependent breast cancers acts by interfering with the action of estrogen. Current treatments, such as tamoxifen, are not consistently useful due to the development of resistance to these drugs. Tamoxifen treatment may also lead to the development of other gynecological cancers, therefore the discovery of novel treatment options for breast cancer is critical. Vitamin B_6 is well documented in its role as a modulator of steroid hormones. Pyridoxal phosphate (PLP), the active form of Vitamin B_6, may interfere with the action of the estrogen receptor (ER) by blocking the hormone-binding and/or DNA-binding site of the ER. The objective of this study was to examine the effects of Vitamin B_6 supplementation on cell proliferation and estrogen-dependent gene expression in breast cancer cells. To accomplish this, estrogen-dependent (MCF-7 and T-47D) and estrogen-independent (BT-20) breast cancer cells were grown in medium supplemented with 0,100, or 300 µM supplemental pyridoxal (PL) in the absence or presence of 0.01µM estradiol. Cell counts and [^{3}H]-thymidine incorporation into DNA were assessed in all cell lines. The expression of pS2, an estrogen-sensitive gene, was performed using RNA extracted from MCF-7 cells. PL supplementation was found to significantly decrease total cell numbers and DNA synthesis in both the estrogen-dependent (ER+) and –independent (ER-) breast cancer cells, but did not alter the expression of pS2. These results indicate that PL significantly impairs growth of breast cancer cells and may be exerting its effects via a steroid-independent mechanism.

Introduction

Breast Cancer

According to the National Cancer Institute, breast cancer is the leading cause of cancer death in women aged 40-55, and the second leading cause of cancer death in women of other age groups. A majority of breast cancers express the estrogen receptor (ER+); therefore their growth is sensitive to the presence of estrogen (Ferguson et al., 1998). The action of the ER directly influences the transcription of many genes whose functions may ultimately lead to the

proliferation of ER+ breast cancers, such as the progesterone receptor (PR), pS2, pLIV-1, TGF-α (Ferguson et al., 1998), and the protooncogenes, *c-fos* and *c-myc* (van der Berg et al., 1989).

Current Treatments

One of the most common forms of hormonal therapy for ER+ breast cancers is tamoxifen (TAM), a non-steroidal drug that interferes with the action of estrogen. The partial agonist activity of TAM, however, leads to TAM-resistance of breast cancers after an average of 10-12 months of treatment (Osborne et al., 1995; Johnston, 1997) and the stimulation of other gynecological cancers, such as endometrial cancer (Jordan and Morrow, 1994). Because of the complications with tamoxifen, the search for the ideal anti-estrogen, which will antagonize the action of estrogen in target tissues without negating the positive effects of estrogen in other tissues, is critical.

Several nutrients have been reported to alter the growth of breast cancer cells *in vitro*. Tocotrienols, the form of Vitamin E found largely in palm oils, has been shown to inhibit the growth of breast cancer cells, regardless of estrogen-receptor status (Nesaretnam et al., 1998), both alone and in combination with tamoxifen (Guthrie et al., 1997). Vitamin D and its analogues have also been reported to inhibit growth, induce apoptosis, and down-regulate ER expression in breast cancer cells *in vitro* (Fife et al., 1997; James et al., 1981). Clearly, nutrients may be important modulators of breast cancer cell growth, and further study of their mechanism(s) of action may identify new targets against breast cancer cell growth and, in turn, could have a significant impact on the treatment and/or prevention of this disease.

Vitamin B_6

Vitamin B_6 (B_6) is a water-soluble vitamin that exists as several vitamers. Pyridoxal phosphate (PLP) is the active vitamer, functioning as a cofactor for many enzymes and as a modulator of steroid hormone action (Tully et al., 1994). Cake and colleagues (1978) first demonstrated that PLP inhibited the binding of glucocorticoid to the glucocorticoid receptor *in vitro*. Subsequent studies have shown that a decrease in tissue levels of PLP results in increased uptake of estrogen in the uterus, liver, and hypothalamus (Holley et al., 1983; Bowden et al., 1986; Bunce et al., 1987; Bender et al., 1989). Evidence suggests that PLP may bind to the

lysine residue(s) located within the binding domains of steroid hormone receptors, therefore blocking the binding of the steroid hormone to the receptor and/or the activated steroid hormone/receptor complex from binding to the DNA (Cake et al., 1978; Katzenellenbogen et al., 1997).

The steroid hormones are involved in the regulation of growth, development, reproduction, and metabolism (Tully et al., 1994; Allgood and Cidlowski, 1991). Each hormone exerts its effect in target tissues through interaction with specific steroid hormone receptors. The binding of the steroid hormone-receptor complex to the hormone response element on DNA modulates the action of hormone-responsive genes by either enhancing or repressing the transcription of those genes (Tully et al., 1994; Litwack et al., 1991). PLP interaction with the steroid hormone receptors has, in fact, been shown to modulate glucocorticoid-(Allgood et al., 1990), estrogen-, androgen-, and progesterone-regulated gene expression (Allgood et al., 1992) and cell growth.

Litwack et al. (1991) demonstrated that treatment with 5 mM pyridoxine (PN) reduced glioma and kidney cell numbers, and inhibited the growth of hepatoma cells. DiSorbo et al., (1982) reported that B_6 can kill hepatoma cells at 10mM concentrations in culture, and Maksymowych et al., (1993) showed that PL can kill melanoma cells both in culture and *in vivo* in mice at concentrations ranging from 1.5-2 mM. It is clear from previous research that B_6 can inhibit cancer cell growth at pharmacological doses. The fact that dietary B_6 supplementation and deficiency have been shown to modulate steroid hormones *in vivo* suggests that lower concentrations of this vitamin may affect the growth of hormone-sensitive cells (Bunce and Vessal, 1987; Bowden et al., 1986; Bender et al., 1989). The objective of the present study was to determine whether supplementation with lower doses of B_6 than those previously utilized could affect the growth of steroid-sensitive breast cancer cells, and to determine whether PL supplementation at those concentrations could influence steroid hormone action.

Materials and Methods

Cell Lines and Culture Conditions

ER+ [MCF-7 (HTB-22), T-47D (HTB-133)] and ER- [BT-20 (HTB-19)] cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained according to the recommendations of the supplier in Complete Medium [Minimal Essential Medium (MEM, GIBCO BRL, Gaithersburg, MD) with Earle's balanced salt solution, supplemented with 10% fetal bovine serum (FBS, Biofluids Inc, Rockville, MD), 1.0 mM sodium bicarbonate (GIBCO), 0.1 mM non-essential amino acids (GIBCO), 1.5 g/L sodium bicarbonate (GIBCO), and 0.01 mg/mL bovine insulin (Sigma, St. Louis MO)]. Cells were cultured in T-25cm^2 flasks and maintained in a 5% CO_2 37°C incubator, with medium renewal every 24 (MCF-7) or 48 h (T-47D and BT-20). For experiments, cells were maintained in Experimental Medium [phenol red-free medium (Gibco) containing 5% dextran-coated charcoal-stripped fetal bovine serum (DCC-FBS, Biofluids Inc) and supplements as above].

Counting Experiments

Cells (3-4 $x10^4$ cells/well) in log phase growth were seeded in triplicate into six well plates, allowed to attach for 24 h in Complete Medium, then maintained for 9 d in Experimental Medium supplemented with 0, 100, or 300 µM PL (Sigma) in the absence or presence of 0.01 µM 17β-estradiol (Sigma). Following incubation, cells were removed with 0.05%-trypsin-0.02% EDTA, resuspended in serum-free MEM, and counted in duplicate in the presence of Trypan Blue Dye (Gibco) using a hemocytometer.

DNA Synthesis Experiments

The effect of PL on DNA synthesis was determined by the measurement of ^{3}[H]-thymidine incorporation into cellular DNA. MCF-7, T47D, and BT-20 cells were cultured and seeded as described for counting experiments. Following culture in Experimental Media for 9 d, [^{3}H]-thymidine (1µCi/ml, 85 Ci/mol) was added to each well, incubated for 3 h in 5% CO_2 at 37ºC, and washed twice with serum-free, phenol red-free medium to remove unincorporated [^{3}H]-thymidine. DNA was precipitated with 5% trichloracetic acid, and solubilized in 0.1N NaOH. Radioactivity was counted in BCS liquid scintillation cocktail (Beckman, Schaumberg,

IL) and 40% acetic acid using a Beckman 6500 LSC counter. Additional experiments were performed to assess the time course of PL effects on DNA synthesis by harvesting cells every 24 h for 9 d.

Analysis of pS2 expression in MCF-7 Cells

cDNA isolation and Probe Synthesis: pS2 cDNA was isolated and purified from an E.coli plasmid vector (pBR322, ATCC) using the boiling mini-prep protocol of Holmes and Quigley (1981), restriction digest with PstI, electrophoresis on a 1% agarose gel (Ultrapure, Gibco), and isolation of a 352 bp pS2 band with the Qiaquick Gel Extraction Kit (Quiagen, Santa Clarita, CA). The purified pS2 insert was radiolabeled with ^{32}P-dCTP (DuPont NEN, Boston, MA) using the RadPrime DNA Labeling System (Gibco). A GAPDH cDNA was also labeled with ^{32}P-dCTP using the same system.

Isolation of RNA and Northern Blot Analysis: MCF-7 cells in log phase growth were seeded into T-25 cm^2 flasks (2 x 10^5 cells/flask), allowed to attach for 24 h in Complete Medium, then maintained in Experimental Medium supplemented with 0, 100, or 300 µM PL and 0 or 0.01 µM 17-β-estradiol for 6 d. Cells were harvested and total RNA extracted using Tri-Reagent (Molecular Research Center Inc, Cincinnati, OH), according to the manufacturer's recommended protocol. Concentration and purity of the RNA was determined using spectrophotometry (GeneQuant, PharmaciaBiotech, Cambridge, England) at absorbances of 260 and 280 nm. Total RNA (15µg) was electrophoresed on a 1% agarose gel (Ultrapure, Gibco) containing 0.66M formaldehyde, transferred in SSPE via capillary action to a membrane (GeneScreen, DuPont-NEN, Boston, MA), and immobilized by UV cross-linking (GeneLinker, BioRad). Equal loading of wells was confirmed by visualization of ethidium bromide stain on a UV transilluminator. The membrane was then hybridized with the ^{32}P-dCTP-labelled pS2 in hybridization solution (1% BSA-fraction V, 1 mM EDTA, 0.5 M SDS) at 65ºC overnight. The membrane was washed in Solution B (1 mM EDTA, 40 mM $NaHPO_4$, pH 7.4) and exposed to film (Fuji, RX, Fisher Scientific). The membrane was then re-hybridized with ^{32}P-dCTP-labelled GAPDH. Band intensity of pS2 was quantified using an AlphaImager Image Analysis System (Alpha Innotech, San Leandro, CA). Data are expressed as a ratio of pS2/GAPDH band intensity.

Statistics

Data were analyzed by unpaired, two-tailed *t*-tests to determine whether differences existed between groups. Significance was established at $p \leq 0.05$.

Results

Pyridoxal treatment of ER+ and ER- cells reduces growth in the presence and absence of estrogen.

Supplementation of MCF-7 cells with 100 and 300μM pyridoxal (PL) resulted in lower cell counts compared to unsupplemented controls in both the absence (Figure A-1) (44% and 93%, respectively, $p<0.01$) and presence (Figure A-1) (38% and 98% reductions, respectively, $p<0.01$) of 0.01μM 17-β-estradiol. A reduction in cell counts was also observed for T47-D cells supplemented with 100 or 300μM PL in both the absence (26% and 72%, respectively, $p<0.01$) and presence (42% and 88% reductions, respectively, $p<0.01$) of estradiol. Supplementation of BT-20 cells with either 100 or 300μM PL also resulted in decreased cell numbers (Figure A-1) when compared with unsupplemented cells in both the absence (85% and 99%, respectively, $p<0.01$) and presence (81% and 99% reductions, respectively, $p<0.01$) of estradiol.

Pyridoxal supplementation results in decreased DNA synthesis of ER+ and ER- mammary carcinoma cell lines.

MCF-7 cells supplemented with 100 or 300μM PL incorporated significantly less [^{3}H]-thymidine when compared with unsupplemented controls in both the absence (30% and 96%, respectively, $p<0.01$) and presence (40% and 99% reductions, respectively, $p<0.01$) of 0.01μM 17-β-estradiol (Figure A-2). Similar decreases in [^{3}H]-thymidine incorporation were noted for T47-D cells supplemented with 100 and 300μM PL in both the absence (60% and 88%, respectively, $p<0.01$) and presence (32% and 82% reductions, respectively, $p<0.01$) of estradiol. Supplementation of BT-20 cells with 100 or 300μM PL resulted in reduced ability of the cells to incorporate [^{3}H]-thymidine into cellular DNA in both the absence (90% and 99%, respectively, $p<0.01$) and presence (92% and 99% reductions, respectively, $p<0.01$) of estradiol (Figure A-2). Time Course experiments with T-47D cells demonstrated that PL-induced inhibition in DNA synthesis occurred within 24 hours of supplementation and continued throughout the 9 d experiment (Figure A-3).

Pyridoxal supplementation did not alter pS2 expression in MCF-7 mammary carcinoma cell line.

Northern analysis of total RNA from MCF-7 cells maintained in Experimental Media for 6 d revealed no significant differences in the expression of pS2 in response to PL supplementation at either concentration (Figure A-4).

Discussion

The relationship between vitamin B_6 and steroid hormone action is well documented. Allgood and colleagues (1990) showed that concentrations of PN ranging from 1-5 mM caused dose-dependent decreases in glucocorticoid-induced gene expression in transfected HeLa cells. When cells transfected with a glucocorticoid-responsive chloramphenicol acetyltransferase (CAT) reporter plasmid were treated with dexamethasone, the resulting increase in CAT activity could be suppressed with the addition of PN. Holley et al., (1983) Bowden et al., (1986) Bunce and Vessal (1987), and Bender et al., (1989) demonstrated that B_6 deficiency resulted in increased tissue uptake and retention of steroid hormones when compared with B_6 supplementation.

Previous data also demonstrate that vitamin B_6 supplementation results in growth inhibition and killing of several types of cancer cells. Maksymowych et al., (1993) demonstrated that 1.5-2 mM PL resulted in the killing of human melanoma cells within two to four days of the onset of supplementation *in vitro*. Similar killing in the same cells following treatment with cortexolone, a synthetic glucocorticoid receptor antagonist, indicated that at those high doses, PL could be affecting cell growth and viability through glucocorticoid-dependent mechanisms. However, in the same study, these researchers showed that 1.5 mM PL killed the melanoma cells, while RU486 (a specific glucocorticoid receptor antagonist) did not have adverse effects on the growth of the melanoma cells. These data suggest that PL could have been killing the melanoma cells via both steroid-dependent and -independent mechanisms. In another study, DiSorbo and Litwack (1982) demonstrated that supplementation with 5 mM PN for 4 d inhibited the growth of human hepatoma and glioma cells, and resulted in the killing of human kidney cells, but had no effect on the growth of human MCF-7 cells. Schultz et al., (1989) incubated cells with concentrations of PN and PL for up to 20 d, and reported that DU-145 human prostate cancer cells were killed in the presence of 2.5 mM PN or 0.5 mM PL, CAKI-1 human renal carcinoma cells were killed in the presence of 2.5 mM PN and growth inhibited in the presence of 0.25 mM PL, H238 mouse fibrosarcoma cells were killed in a dose-dependent manner with 0.5 and 2.5 mM PL supplementation, and human MCF-7 cells were killed in a dose-dependent manner after being cultured in either 0.25 mM or 0.5 mM PL for 20 d.

In these same MCF-7 cells, 2.5 and 5.0 mM PN was shown to have a growth inhibitory effect after 10 d of incubation. Thus, the growth inhibitory effects of vitamin B_6 supplementation appears to be dependent on and specific to the vitamer and concentration used, as well as the length of exposure time.

In the present study, we investigated the effect of PL supplementation on breast cancer cell growth at lower concentrations than previously reported for many cell types. Our cell counts indicated that these doses (100-300 µM) resulted in a growth inhibitory effect after 9 d, but did not induce killing in any of the breast cancer cells tested. This is in agreement with previous data reported by Schultz et al. (1989) in MCF-7 cells. In their experiments, killing began by d 15, but was not seen at d 9. Longer incubation of cells with lower doses of PL used in this study may have induced cell death and is currently being studied in our lab.

Previous studies did not look at the effect of estrogen in the pyridoxal-induced growth inhibition or killing in steroid-sensitive cancers, so we supplemented both estrogen-dependent (MCF-7, T-47D) and –independent (BT-20) breast cancer cells with PL in the presence and absence of estrogen. Our data suggest that the growth inhibitory mechanism may be steroid-independent since similar growth inhibition was observed in all breast cancer cell lines tested (i.e., both ER+ and ER-). Decreases in growth and DNA synthesis did not differ in the presence or absence of estrogen, or between estrogen-dependent and –independent cell lines. These data suggest that PL is exerting its effects in the breast cancer cells in an estrogen-independent manner.

Results from the Northern analysis demonstrate that PL supplementation did not affect expression of pS2, regardless of the presence or absence of estrogen. The expression of pS2 is regulated by estrogen (El-Tanani and Green, 1995; Detre et al., 1999), and breast cancer treatments that inhibit cancer growth via the estrogen receptor are shown to decrease the expression of pS2 in those cells (Motomura et al., 1997). As has been previously demonstrated, we found that estrogen supplementation induced the expression of pS2 in MCF-7 cells. This induction was not altered by either 100 or 300 µM PL supplementation. Our data show no

regulation of pS2 in the PL-supplemented cells, providing further evidence that the action of PL in breast cancer cells is probably modulated in a steroid-independent manner.

DNA synthesis expressed per μg DNA (data not shown) indicate that PL is causing a decrease in DNA synthesis that cannot simply be explained by the PL-induced decrease in total cell numbers. This suggests that PL is exerting its influence via a mechanism that affects the synthesis of DNA in a steroid-independent manner. PL has been shown to inhibit the action of DNA polymerase (Modak, 1976) at concentrations of 0.5 mM or greater by forming a Schiff base with a free amino acid group present at the active site of the DNA polymerase. Data from that study suggest a specific affinity between PLP and the polymerase protein. PL has also been shown to inhibit the action of thymidylate synthase (Rao, 1998) in a bacterial strain by formation of a thiohemiacetal between the sulfhydryl group of the thymidylate synthase and the carbonyl group of PLP. It is conceivable that PL-supplementation at the concentrations used in our experiment could be affecting the growth and DNA synthesis of both ER+ and ER- breast cancers through several different mechanisms involving either Schiff base formation or the formation of other bonds between PLP and enzymes within the cells.

Data from this study present important information regarding the mechanisms of B_6 action in breast cancer cells. Further research needs to be performed to elucidate the exact mechanisms by which the growth inhibition is occurring in these cells, but it appears that there is a definite potential for vitamin B_6 and/or drugs that mimic its effects to be used as a treatment and/or preventative agent for breast cancer.

Chapter 4: Conclusion

Pyridoxal (PL) supplementation of breast cancer cells *in vitro* significantly reduced total cell numbers and DNA synthesis. Previous data in human melanoma (Maksymowych, 1993) and hepatoma (DiSorbo and Litwack, 1982) cells demonstrated that vitamin B_6 can inhibit growth of and induce cell death. In marked contrast to the killing actions of the high concentrations of PL previously reported by those studies, lower concentrations (100-300 μM) used in our study, indicate that the action of PL is inhibiting the growth of the cells, but not inducing killing. The PL-induced decreases observed in the total cell numbers and DNA synthesis did not differ among experimental groups grown in the absence or presence of estrogen nor between estrogen-dependent or –independent cells lines, suggesting that B_6 is modulating the breast cancer cells in a manner that is not steroid-dependent. Results from the Northern analysis further support this as PL supplementation did not affect expression of pS2 in cells, regardless of the presence or absence of estrogen. The expression of pS2 is regulated by estrogen (El-Tanani and Green, 1995; Racca, 1995), and breast cancer treatments that inhibit cancer growth via the estrogen receptor are shown to decrease the expression of pS2 in those cells (Coradini, 1994; Detre, 1999). Our data show no regulation of pS2 in the PL-supplemented cells, providing further evidence that the action of vitamin B_6 in breast cancer cells is modulated in a steroid-independent manner.

A variety of mechanisms could be responsible for the action of PL in these cells. A commonly reported action of vitamin B_6 is its inhibition of RNA polymerase (Oka, 1993). Our Northern assay refutes this mechanism in MCF-7 cells, because the expression of pS2 and GAPDH remained unaffected by PL supplementation. Pyridoxal phosphate (PLP) has been shown to affect the synthesis of DNA in cells through a number of ways. Studies have reported that PLP can decrease the binding affinity of certain transcription factors, which ultimately leads to a decrease in the expression of specific genes (Oka, 1997). PLP has also been reported to inhibit the action of DNA polymerase (Modak, 1976) and thymidylate synthase (Rao, 1998) in cells. Both enzymes are directly responsible for the synthesis of DNA in cells, so it is possible that the action of PL in our breast cancer cells was due to the modulation of those enzymes. The

expression of many genes is altered by PLP. Decreases in PLP concentration in cells have led to increases in the transcription of albumin (Oka et al., 1997), GPIIb (Chang et al., 1999), cytosolic aspartate aminotransferase, apolipoprotein A-1, phenylalanine hydroxylase, glyceraldehyde-3-phosphate dehydrogenase, and β-actin (Natori and Oka, 1997). These studies provide much evidence that PLP is a potent modulator of gene expression, and it is conceivable that PLP is affecting the transcription of genes that are eventually responsible for the synthesis of DNA in breast cancers or in the progression of breast cancer cells through the cell cycle. We are aware that the effects of PLP on gene expression occur in a tissue- and gene-specific manner (Oka et al., 1993), so it is imperative to perform further research elucidating the effect of PLP on specific gene expression in breast cancer cells.

Chapter 5: Future Research

The findings of this study have opened the door to a number of future research opportunities. In order to determine the physiological relevance of this discovery, it is necessary to ascertain whether the levels of PL needed to obtain this anti-proliferative action can be reached *in vivo* with PL supplementation, and whether the same results actually occur in an animal model. MCF-7 xenografts, in which MCF-7 cells are implanted into a mouse model, could be useful in determining whether PL supplementation can inhibit tumorigenesis and/or tumor growth *in vivo*. These studies are justified based on previously reported xenograft studies describing the benefit of dietary PL supplementation on growth of melanoma cells *in vivo* (Maksymowych et al., 1993).

Studies need to be conducted to determine the mechanism(s) by which PL is exerting its effect in these breast cancer cells. If this can be elucidated, then there is the potential for the development of a drug that can mimic the action of PL in breast cancers at more easily reached and more potent physiological doses. Most researchers tend to think that PLP acts by forming a Schiff base between its aldehyde group and the lysine residues of the molecule it acts upon, but at least some research has indicated that it is possible for PL to act through a non-Schiff base interaction. Studies, such as x-ray crystallography could be performed to determine the types of interactions PL is having within the cells. Cell cycle analyses could be done to determine what part of the cell cycle PL is affecting, and whether differential expression of genes that control cell cycle progression is occurring in response to PL supplementation. Finally, it is important to determine whether gene expression is being affected by PL supplementation, and whether this ultimately affects the growth and proliferation of breast cancer cells both *in vitro* and *in vivo*.

Breast cancer is a growing epidemic in this country, and it is important to discover novel treatment options for the women suffering from this disease. The findings of this study provide us with exciting and novel information about the potential for a vitamin to play a role in elucidating a new therapeutic and/or preventative agent for breast cancer.

Appendix A: Figures and Tables

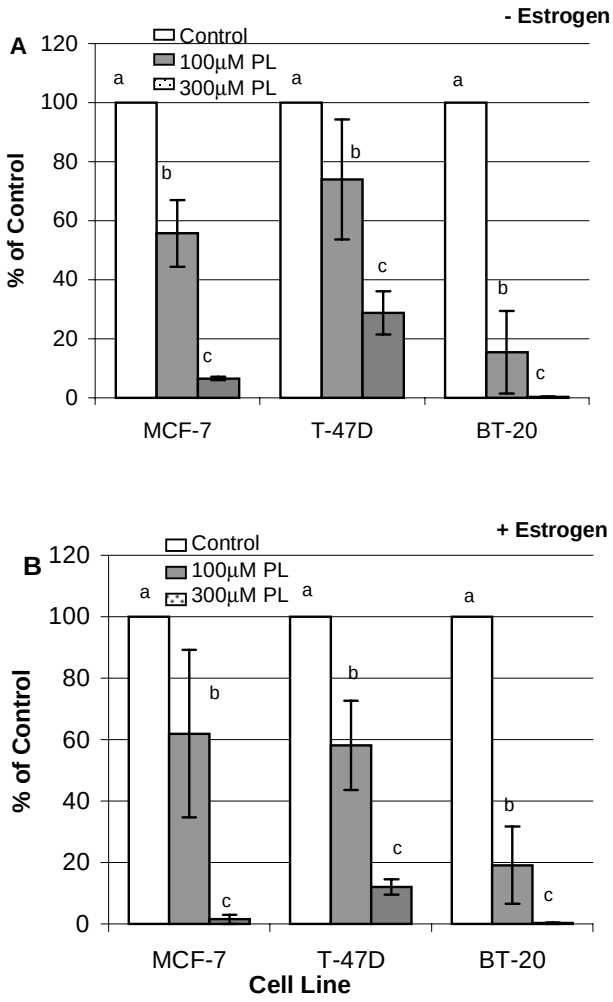


Figure A-1. Influence of pyridoxal (PL) supplementation on total cell numbers in estrogen-dependent (MCF-7, T-47D) and –independent (BT-20) breast cancer cells. Monolayers of cells were incubated in triplicate with 0, 100, or 300µM PL-supplementation for 9 d and counted in the presence of trypan blue dye. Data are the mean ± SD of three experiments. Differing letters indicate significant differences ($p<0.05$) within the same cell line.

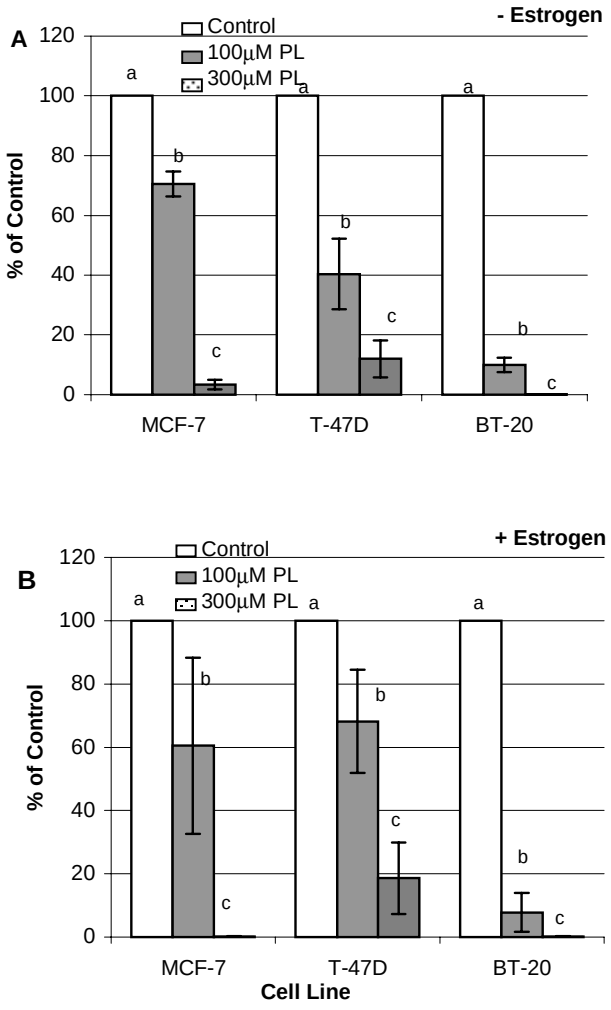


Figure A-2. Influence of pyridoxal (PL) supplementation on the incorporation of [^{3}H]-thymidine into cellular DNA by estrogen-dependent (MCF-7, T-47D) and –independent (BT-20) cells. Monolayers of cells were incubated in triplicate with 0, 100, or 300μM PL-supplementation for 9 d. Following incubation, [^{3}H]-thymidine (1μCi/ml, 85 Ci/mol) was added to each well, incubated for 3 h, precipitated with 5% TCA, and solubilized in 0.1 N NaOH. Radioactivity was counted in BSC liquid scintillation cocktail and 40% acetic acid using a Beckman 6500 LSC. Data are the mean ± SD of three experiments expressed as a percent of control cells incubated in experimental medium. Differing letters indicate significant differences ($p<0.05$) within the same cell line.

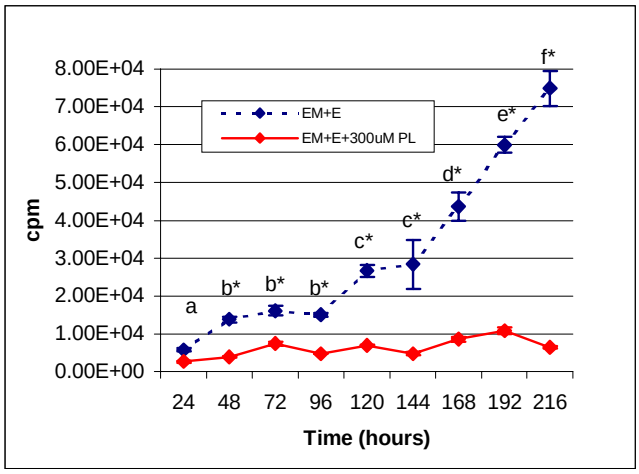
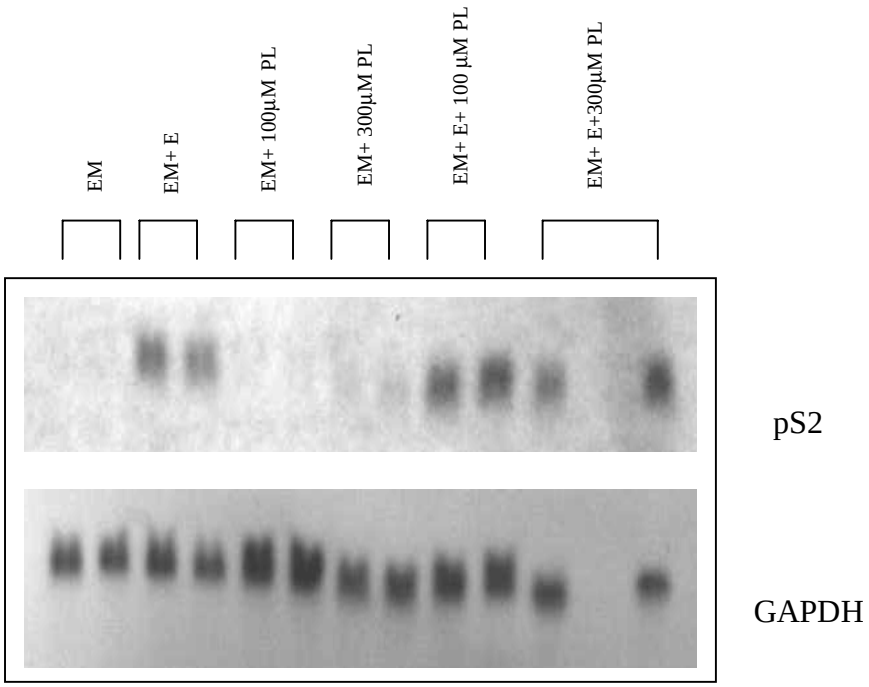


Figure A-3. Time course representation of pyridoxal (PL) supplementation on the incorporation of [^{3}H]-thymidine into cellular DNA by estrogen-dependent T-47D cells. Monolayers of cells were incubated in triplicate in 0.01 μM 17-β estradiol (E) and 300 μM PL-supplementation. [^{3}H]-thymidine incorporation was assessed as described in Figure 2, every 24 h for 9 d. Each data point represents the mean ± SD. Differing letters indicate significant differences ($p<0.05$) between time points within the EM+E treatment group; asterisks indicate significant differences ($p<0.05$) between treatments groups at the same time point. No significant differences were observed among time points within the EM+E+300 μM PL treatment group.



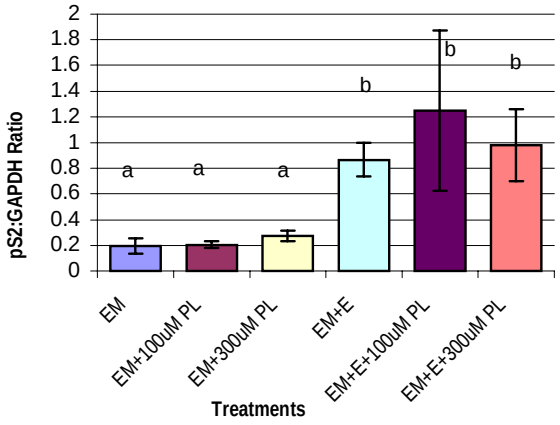


Figure A-4. Effect of PL supplementation on pS2 and GAPDH expression in estrogen-dependent MCF-7 breast cancer cells. Monolayers of cells were incubated in triplicate with 0, 100, or 300 µM PL-supplementation for 6 d. Total RNA was extracted with TRI-reagent, electrophoresed on a 1% agarose gel, and hybridized with ^{32}P-dCTP-labelled pS2 or GAPDH. Data were analyzed using a densitometer and expressed as a ratio of pS2:GAPDH. Differing letters indicate significant differences ($p<0.05$).

Table 1. Raw Data From Counting Experiments.

- Estrogen		Control	100 μM PL	300 μM PL
Cell Line	exp. #	cell #	cell #	cell #
MCF-7	1	3.42E+04	2.38E+04	6.48E+02
	2	6.1E+03	4.6E+03	1.9E+02
	3	2.4E+03	1.6E+03	1.3E+02
T-47D	1	1.20E+05	4.40E+04	8.60E+03
	2	8.40E+03	2.60E+03	8.20E+02
	3	2.80E+03	1.50E+03	5.30E+02
BT-20	1	1.35E+05	1.02E+04	2.02E+02
	2	1.44E+05	1.45E+04	2.02E+02
	3	4.1E+04	5.1E+03	4.3E+01
+Estrogen				
MCF-7	1	5.64E+05	3.29E+05	6.34E+02
	2	8.1E+04	7.27E+04	1.15E+02
	3	5.1E+04	1.73E+04	1.87E+02
T-47D	1	1.30E+06	9.28E+05	2.01E+05
	2	1.60E+05	8.08E+04	1.46E+04
	3	4.50E+04	3.72E+04	1.40E+04
BT-20	1	1.03E+05	1.08E+03	1.93E+02
	2	1.34E+05	1.27E+04	3.52E+02
	3	4.2E+04	5.40E+03	4.13E+01

Table 2. Raw Data From [^{3}H]-thymidine Incorporation Experiments.

- Estrogen		Control	100 μM PL	300 μM PL
Cell Line	exp. #	cell #	cell #	cell #
MCF-7	1	3.42E+04	2.38E+04	6.48E+02
	2	6.1E+03	4.6E+03	1.9E+02
	3	2.4E+03	1.6E+03	1.3E+02
T-47D	1	1.20E+05	4.40E+04	8.60E+03
	2	8.40E+03	2.60E+03	8.20E+02
	3	2.80E+03	1.50E+03	5.30E+02
BT-20	1	1.35E+05	1.02E+04	2.02E+02
	2	1.44E+05	1.45E+04	2.02E+02
	3	4.1E+04	5.1E+03	4.3E+01
+Estrogen				
MCF-7	1	5.64E+05	3.29E+05	6.34E+02
	2	8.1E+04	7.27E+04	1.15E+02
	3	5.1E+04	1.73E+04	1.87E+02
T-47D	1	1.30E+06	9.28E+05	2.01E+05
	2	1.60E+05	8.08E+04	1.46E+04
	3	4.50E+04	3.72E+04	1.40E+04
BT-20	1	1.03E+05	1.08E+03	1.93E+02
	2	1.34E+05	1.27E+04	3.52E+02
	3	4.2E+04	5.40E+03	4.13E+01

Appendix B: Materials and Methods

Cell lines and culture conditions

Estrogen receptor-positive [ER+, MCF-7 (HTB-22), T-47D (HTB-133)] and –negative BT-20 (HTB-19) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). All cells were maintained in T-25 cm^2 flasks at 37°C with 5% CO_2 according to the recommendations of the supplier in 5 ml Minimal Essential Medium (MEM, GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, Biofluids Inc, Rockville, MD), 1.0 mM sodium bicarbonate (GIBCO), 0.1 mM non-essential amino acids (GIBCO), 1.5 g/L sodium bicarbonate (GIBCO), and 0.01 mg/ml bovine insulin (Sigma, St. Louis, MO), fed every 24 (MCF-7) or 48 h (T-47D and BT-20), and passaged when they reached 90% confluence as observed with an inverted microscope using phase contrast. For experiments, cells were maintained in experimental medium [EM, phenol red-free medium (GIBCO) containing 5% dextran-coated charcoal-stripped fetal bovine serum (DCC-FBS) and supplements as above].

Counting experiments

Cells were seeded into six well plates (3-4 x 10^4 cells/well), allowed to attach for 24 h in culture medium, and then maintained for 9 d in 2 ml EM supplemented with 0, 100, or 300 μM PL (Sigma) in the absence or presence of 0.01 μM 17 β-estradiol (Sigma). Cells were incubated with 0.75 ml 0.05%-trypsin-0.02% EDTA for 5 min, until cells began to detach. Culture medium (1.5 ml) was then added to the well to inactivate the trypsin; the cell suspension was removed to a 15-ml conical tube and centrifuged at 400 rpm for 5 min, and the supernatant was aspirated. Cells were resuspended in an appropriate amount of serum-free MEM to yield similar numbers for cell counting, and diluted 1:1 with Trypan Blue Dye (TPB, GIBCO) (50 μL cell suspension and 50 μL Trypan Blue). Approximately 10 μL of the cell/TPB suspension was loaded into each chamber of a hemocytometer, and cells in the middle and four corner squares were counted. Cell counts from the 2 chambers were averaged and divided by 5 to provide the average cell/mm; this number was then multiplied by 20,000 (dilution factor) to render the average cell/ml, which was then multiplied by the amount of MEM that the sample was resuspended in to give the total cells per sample.

DNA synthesis experiments

The effect of PL on cell proliferation was determined by the measurement of [^{3}H]-thymidine incorporation into cellular DNA. Cells were cultured and seeded as described for counting experiments. Following culture in experimental media for 9 d, [^{3}H]-thymidine was added to each well (1 μCi/ml, 85 Ci/mol), and the cells were incubated for 3 h in 5% CO_2 at 37°C. Cells in each well were washed with 1 ml serum-free, phenol red-free medium to remove unincorporated [^{3}H]-thymidine. DNA was precipitated with 1 ml 5% trichloracetic acid, washed again with serum-free, phenol red-free medium, and solubilized with 1mL 0.1 N NaOH. An aliquot (500 μL) from each well was placed in individual liquid scintillation vials, 3.5 ml of BCS liquid scintillation cocktail (Beckman, Schaumberg, IL) and 200 μL of 40% acetic acid were added to each vial, and radioactivity was counted using a Beckman 6500 LSC counter. To determine when supplemental PL exerted its effect on DNA synthesis, time course experiments were performed. Experimental procedure was as described above, and performed every 24 h for 9d.

Analysis of pS2 expression in MCF-7 cells

RNA extraction and Northern analysis

Cells cultured as described above were seeded in T-25cm^2 flasks at a density of 2x10^5 cells/flask, and cultured in 5 ml of EM supplemented with either 0, 100 or 300 μM PL and with or without 0.01 μM 17-β-estradiol for 7 d. To harvest the cells for RNA extraction, 2ml of TRI-reagent (Molecular Research Center Inc, Cincinnati, OH) was added to each flask, and a rubber policeman was used to collect the cells to one corner of the flask. The viscous cell lysate was removed to a polypropylene tube with a serological pipette, and homogenized at a setting of 70 for approximately 30 seconds. An additional 1 ml of TRI-reagent was added, and the cell homogenate was vortexed and stored at –80ºC until extraction.

RNA was extracted with TRI-reagent according the protocol provided by the manufacturer. Chloroform (0.6 ml) was added to each tube, and the cells were incubated at room temperature for 15 min and then centrifuged at 4ºC for 20 min at 11,100 r.c.f. The aqueous

layers were then transferred to fresh polypropylene tubes, 1.5 ml of ice-cold isopropanol was added to each tube, and the RNA was precipitated at room temperature for 10 min. The precipitate was centrifuged at 11,100 r.c.f. for 15 min at 4ºC. The supernatant was carefully poured off, and the RNA precipitate was washed with 3 ml of ice-cold 75% ethanol. Each tube was vortexed and centrifuged at 10,000 r.c.f. for 7 min at 4ºC. Again, the supernatant was poured off, and the tubes were inverted against a test tube rack and allowed to air dry (~ 5 min). A sterile cotton swab was used to remove the last traces of ethanol from the tube, and the RNA was solubilized in 50 µl of RNAse-free water, removed to a fresh 1.5 ml microtube, and stored at –80ºC until RNA quantification.

The concentration and purity of each sample was determined using a spectrophotometer (GeneQuant, PharmaciaBiotech, Cambridge, England) at absorbances of 260 and 280 nm. Solubilized RNA (1µl) was added to 499 ml of TE in a quartz cuvette, and absorbance and concentration (µg/µl) of each sample were recorded. Calculations were made to determine the volume of each sample required for 15 µg of RNA to be loaded into each well of the Northern assay.

RNA (15 µg) from each sample were placed in 500 µl tubes, and incubated with 15 µl of loading dye at 65ºC for 15 min. Following incubation, samples were immediately placed on ice, and 1 µl of ethidium bromide (1 mg/ml) was added to each. Samples were loaded into wells of a 1 % agarose gel [2.5 mg agarose, 212.5 ml nanopure water, 25 ml of 10x MOPS (42 g/L NaCl, 4.1 g/L Na Acetate, 20 mL 0.5M EDTA, pH 7.0), (Ultrapure, Gibco)] containing 0.66 M 37% formaldehyde (14 ml) electrophoresed at 150 v for 2 h in 1x MOPS buffer (160 ml of 10x MOPS and 1440 ml nanopure water).

Following electrophoresis, equal loading was verified by viewing the gel using UV illumination (Alpha Imager, Alpha Innotech, San Leandro, CA). The gel was then soaked in nanopure water for approximately 10 min, with the water changed every 3-5 min. Meanwhile, an apparatus was constructed for the transfer of the gel to a membrane. An 8x11 Pyrex dish was inverted and placed in a 9x13 Pyrex dish to be used as the gel support. Whatman filter paper was

draped over the small Pyrex dish, to extend underneath it, wet with 10 x SSPE (87 g/L NaCl, 13.8 g/L $NaHPO_4$, 3.7 g/L EDTA, pH 7.4), straightened, and bubbles removed with the side of a polypropylene tube. The upper left corner of the gel was sliced off with a scalpel, and the gel was inverted and placed on the filter paper. A membrane (GeneScreen, DuPont-NEN, Boston, MA) was cut the same size as the gel, pre-wet for 1 minute in nanopure water, placed atop the gel, and trimmed. Strips of parafilm were placed from the edges of the gel to the to the edge of the large Pyrex dish to ensure that wicking would occur only through the gel, and 2 pieces of filter paper were placed atop the membrane and parafilm. A stack of paper towels (~6 inches) was then placed atop the filter paper, and a weight was added (400 ml water in a 500 ml bottle). Additional 10x SSPE (500 ml) was poured into the large Pyrex dish, and the RNA was transferred overnight. The next day, the weight, paper towel, and filter paper were removed from the membrane and gel, and discarded. The membrane and gel were inverted, and a pencil was used to mark the well locations on the membrane. The membrane was rinsed in nanopure water to remove excess salt, placed on plastic wrap, and the RNA was immobilized using UV cross-linking (GeneLinker, BioRad). The membrane was then wrapped in plastic wrap, and stored in a zippered plastic bag at 4ºC until hybridized with a probe. Transfer of RNA was verified by viewing the gel under UV light in the AlphaImager.

cDNA isolation and probe synthesis

E. coli transfected with a plasmid vector (pBR322) containing pS2 cDNA was purchased from ATCC, and cultured according to ATCC recommendations in 3 ml Luria-Bertani (LB) medium with 20 μg/ml tetracycline in a 13 ml culture tube for 24 h in a 37°C shaking waterbath.

Plasmids were isolated following the boiling mini-prep protocol of Holmes and Quigley (1981). Following overnight incubation, medium containing the E. coli cells (1.5 ml) were transferred to a 1.5-ml microfuge tube, and centrifuged for 30 sec, supernatant was removed, and the previous step was repeated with the remaining 1.5 ml of culture. Cells were lysed in 550 μL of STET buffer (8% sucrose, 5% Triton X-100, 50mM EDTA, 50mM Tris-HCL, pH 8.0) with 25 μL lysozyme (10mg/mL) in a boiling water bath 40 sec and then immediately centrifuged for 10 min at 4°C. The gelatinous pellet was removed with the wooden end of a sterile cotton swab,

discarded, and 600 µL isopropanol were added to the sample. The sample was centrifuged again for 10 min at 4°C, supernatant was removed, the pellet was re-dissolved in 100 µL TE and 7 µL 5 M NaCl, and reprecipitated in 250 µL 95% EtOH in a 4°C centrifuge for 10 min. The final pellet was air-dried and re-dissolved in 50 µL TE. The pellet was diluted with an additional 100µL TE, 10µL RNAse A (1mg/mL in nanopure water) were added to remove any RNA contamination, and the sample was incubated for 30 min at 37°C. Proteinase K (4 µl 20mg/ml in TE, pH 8.0) was added to remove the RNAse A, and the sample was incubated for 30 min at 37°C. Tris-buffered phenol (85 µL) and $CHCl_3$: Isoamyl (85 µL, 24:1v:v) were added, and the sample was vortexed and centrifuged for 10 min to remove the Proteinase K. The aqueous layer was removed to a separate microtube, 175µL $CHCl_3$ was added, and the sample was vortexed and centrifuged again for 10 min. The aqueous layer was removed to a new microtube, and the cDNA was precipitated with 10 µL of 5M NaCl and 300µL OF 100% EtOH, and incubated at -20°C for 30 min. The sample was centrifuged for 10 min, the supernatant discarded, and the sample was resuspended in 150 µL TE. The sample was again reprecipitated with 300µL EtOH, incubated at -20°C for 30 min, centrifuged for 10 min, and the supernatant removed. The sample was pulse centrifuged, and the last traces of EtOH removed with a gel-loading tip. The sample was air-dried for 5-10 min, resuspended in 50 µL sterile nanopure water, and stored at 4°C until use.

To determine which DNA samples contained the pS2 insert, a restriction digest analytical electrophoresis was performed. A 1% agarose gel was used (2.5 g agarose, 222.5 ml nanopure water, 25 ml 10x TBE), and a cut and uncut plasmid from each sample was run on the gel. The lanes containing the cut plasmids consisted of 5 µL of the isolated plasmid DNA, 2µL of Pst 1 restriction enzyme (10 µg/µL, Promega, Madison, WI), 2µL restriction buffer (10x, Promega), and 11 µL nanopure water. The uncut plasmid lanes contained 2µL plasmid and 20 µL nanopure water. Both uncut and cut samples were incubated with 4µL loading dye for 1 h at 37°C. A DNA ladder was run next to the samples (2 µL DNA ladder, 2 µL loading dye, and 8 µL nanopure water). The samples were loaded onto the gel and electrophoresed for 2 h at 100 v.

Of 8 samples electrophoresed, samples 2 and 3 contained the pS2 plasmid and were used for further isolation and probe synthesis.

Cut and uncut samples were prepared as described above, electrophoresed on a 1% agarose gel (0.75 g agarose, 7.5 ml 10x TBE, and 67.5 ml nanopure water) for 1.5 h at 75 v, followed by 33 min at 100 v. The gel was then stained with 1μg/ml ethidium bromide for 1 min, and viewed using UV illumination (AlphaImager). Bands from each of the cut samples coinciding with the 352 bp band on the DNA ladder was excised from the underside of the gel. The pS2 cDNA was extracted from the gel using QIAquick Gel Extraction Kit (QIAgen, Santa Clarita, CA). A 1.5 ml microtube was weighed before and after adding the agarose gel slice. The total weight of each sample was calculated by subtracting the weight of the empty tube from the weight of the agarose-containing tube. Buffer QG1 was added each sample (300 μl buffer:100mg gel), and the samples were incubated for 10 min at 50°C (and vortexed every 2-3 min). Isopropanol (100μl isopropanol:100mg gel) was added to each sample, mixed, and centrifuged in a QIAquick column for 1 min. The flow-through was discarded, and the column was washed with 0.75 ml of Buffer PE. The flow-through from the wash was discarded, and the column was centrifuged again at 10,000 x g for 1 min, to remove any residual traces of EtOH from the Buffer PE. The cDNA was eluted from each sample by adding 30 μl of sterile nanopure water to the columns and centrifuged for 1 min (allowing to sit for 1 min prior to centrifugation to aid in solubility). cDNA from each sample was stored at 4°C until use.

Probes were synthesized for Northern Analyses using a RadPrime DNA Labeling System (Gibco). cDNA was quantitated using spectrophotometry (GeneQuant) , and 25ng of DNA was diluted in TE to a total volume of 21 μl. The sample was heated for 5 min in a boiling water bath and immediately placed on ice. On ice, 3 μl of dNTP mix (10 μl each of dATP, dGTP, and dTTP), 20 μl 2.5x Random Primers Solution, and 5 μl (~50 μCi) of [^{32}P]-dCTP (3000 Ci/mmol, 10mCi/ml) were added, and the cDNA solution was mixed briefly with a pipette. (All procedures after, and including, the addition of radioactivity were performed behind a plexiglass shield on the bench designated for use of radioactive materials). Klenow fragment (1 μl) was added, and the solution was incubated at 37°C for 30 min to allow DNA synthesis. Stop buffer

(5 µl) was added, along with 45 µl of TE, and centrifuged at 2000 rpm for 2 min through a 1 cc Sephadex spin column [Sephadex G-50 (Pharmacia Biotech), 50 mM EDTA, pH 8.0]. An aliquot (1µl) of the eluate was counted on a kimwipe in the Beckman LSC. The probe (100µl) was added to the pre-hybridized membrane (1% BSA-fraction V, 1 mM EDTA, 0.5 M SDS), and the membrane was hybridized to the probe overnight at 65ºC. The membrane was then washed with solution B three times (1 mM EDTA, 40 mM $NaHPO_4$, pH 7.4), wrapped in plastic wrap, exposed to x-ray film (Fuji RX, Fisher Scientific) at –80ºC for 3-5 d. The film was then developed, and the pS2 and GAPDH bands were quantitated with a densitometer (AlphaImager). Bands were expressed as pS2:GAPDH ratio.

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Vita

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