

Dietary vitamin B₆ supplementation promotes the growth of 7,12-dimethylbenz(a)anthracene-induced mammary carcinoma in Sprague Dawley rats.

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[Under the supervision of Barbara A. Davis]

(ABSTRACT)

In vitro data from our laboratory demonstrate that vitamin B₆ (B₆) supplementation of estrogen receptor – positive and – negative breast cancer cells is growth inhibitory. Others have reported that dietary B₆ supplementation resulted in increased fibrosarcoma pyridoxal phosphate (PLP) concentrations and a significant inverse relationship between tumor PLP concentration and tumor volume in mice. This suggests that, in contrast to data reported for normal cells, tumor cells are capable of accumulating supplemental B₆. In the current study, we investigated the effects of dietary B₆ supplementation on 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary carcinoma in rats. Specifically, we aimed to identify the effect of pyridoxine (PN) supplementation on tumor growth and vitamin uptake by tumor cells. To accomplish this, 50 d old female Sprague Dawley rats were gavaged with 15 mg DMBA and fed a diet containing either 7, 350, or 1050 mg PN-HCl/kg diet, which is the equivalent of 1, 50, or 150x the National Research Council's B₆ requirement for rats, respectively. These levels of PN have previously been shown to produce no overt signs of toxicity in rats. Throughout the experiment, the percent of rats with tumors and the average number of tumors per rat remained similar between groups. Mammary tumor growth rates were significantly increased in response to dietary B₆ supplementation ($P < 0.05$). Liver PLP and pyridoxal (PL) concentrations did not differ between dietary treatment groups. Plasma PL and PLP concentrations were significantly higher in the group fed the 150x diet compared with the 1x diet ($P < 0.001$, $P < 0.05$). Mammary tissue PL concentrations of the 150x group were significantly higher ($P < 0.05$) than the 1x group, but no differences were observed in mammary PLP concentrations. Similarly to mammary tissue, no differences between groups were observed in tumor PLP concentration. However, tumor PL concentrations in both the 50x and 150x dietary treatment groups were significantly higher than those from the rats fed the 1x diet ($P <$

0.002). These data demonstrate that previously reported inhibitory effects of supplemental B₆ on breast cancer growth *in vitro* do not occur in response to dietary supplementation at 50 or 150 times the B₆ requirement *in vivo*. In fact, dietary B₆ at 150x the requirement may actually promote mammary tumor growth. In light of these results, investigation of the effects of supplemental B₆ on cancer growth in humans is warranted. Supported by American Cancer Society Grant # IRG-99-225-01.

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Chapter 1: Introduction

As breast cancer is a serious disease that claims the lives of thousands of people every year, it is clear that new and more effective treatments are needed. The effectiveness of vitamin B₆ (B₆) deficiency in the inhibition of tumor growth in animal models has long been observed (Mihich et al.; 1959, Tryfiates et al.,1978; Fortmeyer et al., 1988). Presumably this is attributable to the essential role B₆ plays in one carbon metabolism and nucleic acid synthesis (Leklem, 2000; Coombs, 1998). However, due to the many vital functions B₆ serves, a deficiency of this vitamin is detrimental to many normal physiological processes in the human body. Conversely, the effects of moderate supplemental doses of B₆ are not as harmful. The Tolerable Upper Intake Level (UL) set for B₆ is 100 mg/d, and the No-Observed-Adverse-Effect Level is set at 200 mg/d, these are approximately 75 and 150 times higher than the Recommended Dietary Allowance (RDA), respectively (National Academy of Sciences, 2000). Interestingly, B₆ in pharmacological doses has been shown to inhibit the growth of a variety of cancer cells *in vitro*, including human breast cancer cells. In addition, dietary B₆ supplementation has proven effective in inhibiting the growth of fibrosarcoma tumors in mice (Gridley et al., 1987). This effect of supplemental B₆ on breast cancer growth has not, until now, been studied *in vivo*. Also, the mechanism by which supplemental B₆ acts to inhibit tumor cell growth has not been determined but may involve an accumulation of B₆ in the malignant cells. Therefore, the purpose of this research was to determine the consequences of dietary B₆ supplementation on 7, 12- dimethylbenz(a)anthracene (DMBA)-induced mammary carcinoma growth *in vivo*. Previously reported data describe an inverse relationship between tumor B₆ accumulation and tumor growth. We hypothesized that mammary tumor cells would accumulate B₆ in response to dietary supplementation of this vitamin and subsequently inhibit tumor growth. Specifically, we conducted a study to determine whether dietary supplementation with B₆ would modulate DMBA-induced mammary tumor growth in virgin female Sprague-Dawley rats. Secondly, we aimed to determine whether mammary tumor cells are capable of

accumulating pyridoxal or pyridoxal phosphate in response to dietary supplementation of vitamin B₆.

Chapter 2: Literature Review

Breast Cancer

Background

Although mortality rates declined considerably between 1990 and 1997, breast cancer remains the second leading cause of death among women (American Cancer Society, 2001). The American Cancer Society estimates that over 40,000 deaths will be attributed to breast cancer this year, despite recent advances in the detection and management of the disease. The breast is the leading site of new cancer cases for females, with over 192,000 new invasive cases estimated to be diagnosed in the year 2001. Unavoidable breast cancer risk factors include gender, aging, family or personal history of breast cancer, race, and long menstrual history. A woman's risk is also increased with recent oral contraceptive use, not having children, having the first child after the age of 30, hormone replacement therapy, alcohol consumption, and obesity, especially after menopause (American Cancer Society, 2001).

Current Treatments

As the biology of breast cancer is slowly unveiled, treatments are becoming increasingly tailored to the disease. Currently, treatments that target the growth factors and enzymes involved in the progression of breast cancer are being investigated.

Tamoxifen

Hormonal therapy is of importance in the treatment of breast cancer because the growth of the majority of breast cancers is responsive to estrogen (E) (Ferguson et al., 1998). Breast cancers that are estrogen responsive have estrogen receptors (ER) and are referred to as estrogen receptor-positive (ER+); those that do not have ER are termed estrogen receptor-negative (ER-). Estrogen exerts its actions by binding to the ER to produce an activated E-ER complex. This complex then binds estrogen response elements (ERE) on the DNA of estrogen-responsive genes and activates their

transcription ultimately leading to the growth of ER+ breast cancers (Ferguson et al., 1998).

A commonly used drug for the treatment and prevention of ER+ breast cancer is the non-steroidal antiestrogen, tamoxifen. Tamoxifen effectively competes with estrogen for ER binding. The tamoxifen- ER complex will not bind the ERE on the DNA, and therefore cannot initiate transcription, resulting in poor cell growth. Unfortunately, after an average of 10-12 months of treatment with tamoxifen, resistance often develops, and the drug is no longer effective (Osborne et al., 1995). Tamoxifen treatment has also been shown to increase the risk of other gynecological cancers, and cataracts (Jordan and Morrow, 1994; Paganini-Hill & Clark, 2000).

Herceptin

Thirty percent of all breast cancers have been found to overexpress the HER2 (c-erbB-2) protein (Schaller et al., 1999). Overexpression of HER2 is associated with high tumor proliferation rates, ER- cancers and an overall poor prognosis. HER2 overexpression may also confer resistance to the chemotherapeutic drugs tamoxifen and methotrexate. HER2 is a transmembrane growth factor receptor with an extracellular ligand binding domain, a single transmembrane domain and an internal domain with tyrosine kinase activity. When the HER2 receptor receives a signal, tyrosine kinase is activated and catalyzes several reactions regulating cell cycle progression and cell to cell contact (Miller and Sledge, 1999). It is through these reactions that apoptosis is down regulated and proliferation stimulated (Schaller et al., 1999).

A humanized mouse antiHER2 monoclonal antibody, herceptin (also known as trastuzumab), has been developed for the treatment of HER2 over-expressing breast cancers. The binding of herceptin to overexpressing cells causes a down regulation of the HER2 receptor, inhibition of cell growth, and reduced production of vascular endothelial growth factor (Sliwkowski et al., 1999). In addition to the inhibition of growth seen when herceptin binds to these cells, immune cells attack and kill the herceptin-bound tumor cells. Herceptin appears to be the most effective when administered in addition to other chemotherapeutic agents such as doxorubicin and

paclitaxel, because it enhances the cytotoxic effect of these other chemotherapeutic agents (Sliwkowski et al., 1999).

Micronutrients

Over the past several years research has linked cancer and diet. Although it appears that a diet low in fat and high in fruits and vegetables is beneficial in the prevention of cancer, it is not yet known which factors within these foods are responsible. The effect of several micronutrients in the prevention and treatment of breast cancer has been and is currently under investigation. Micronutrients that show promise in this area include vitamins A, E, C, B₆, and selenium.

Retinoic acid, a vitamer of vitamin A, has been shown to decrease the effect of the growth factor IGF-1 on ER+ breast cancers, consequently, decreasing the proliferation of these cells (Favoni et al., 1998). Research also indicates that retinoic acid induces apoptosis in these cells (Toma et al., 1998). Vitamin A treatment also decreases HER2 protein expression (Grunt et al., 1998). As toxicity is a problem with consuming high doses of vitamin A, analogues have been developed and are currently being tested in clinical trials.

Selenium, carotenoids, and vitamins E and C have not yet proven to be as efficacious as vitamin A, but more research is needed to determine their cancer inhibitory potential (Ruffin and Rock, 2001). Vitamin D has proven effective in chemoprevention and treatment of breast cancer (Guyton et al., 2001). However, therapeutic doses of vitamin D have unwanted side effects, including alterations in calcium homeostasis, that limit its use in cancer therapy. Consequently, the development and testing of vitamin D analogues is currently underway and show beneficial therapeutic profiles with limited effects on calcium metabolism. *In vitro* research involving the treatment of breast cancer cells with vitamin B₆ has exhibited positive results (Shultz et al., 1989; Davis and Cowing, 2000); however, more research needs to be conducted in order to determine its efficacy in the treatment of breast cancer *in vivo*.

Just as there are many chemotherapeutic agents available to treat breast cancer, there are also as many unwanted side effects. It is essential that research continue in this

area to develop new and improved treatments that increase life expectancy and improve quality of life for all breast cancer patients.

Vitamin B₆

Background

Vitamin B₆ (B₆) is a water-soluble vitamin that primarily exists as 6 vitamers, pyridoxal (PL), pyridoxamine (PM), pyridoxine (PN) and their respective 5' phosphorylated derivatives PLP, PMP, and PNP (Figure 2.1). B₆ is stable under acidic conditions, but degrades in neutral or alkaline environments or under white light. PN is the most stable form of the vitamin and is found mainly in foods of plant origin. Due to its stability, pyridoxine hydrochloride (PN-HCl) is often used in the fortification of foods and in vitamin supplements. PL and PM are the predominant forms of the vitamin in animal products. Meats, whole grains, vegetables, and nuts are all good sources of B₆ (Coombs, 1998). However, the 1995 Continuing Survey of Food Intakes by Individuals (CSFII) indicated that fortified, ready-to-eat mixed cereals were the highest contributor to the B₆ intake of both men and women providing 10.8 and 13.7 % of total B₆ intake, respectively (National Academy of Sciences, 1999).

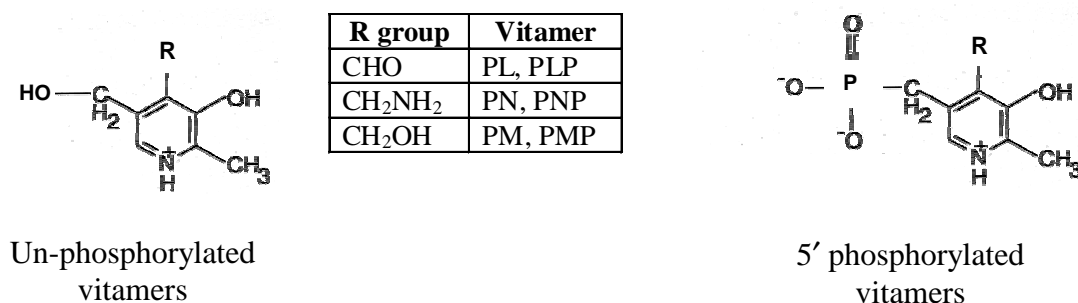


Figure 2.1. B₆ vitamer structures. PL, pyridoxal; PLP, pyridoxal phosphate; PN, pyridoxine; PNP, pyridoxine phosphate; PM, pyridoxamine; PMP, pyridoxamine phosphate.

Function

Acting via the formation of Schiff base linkages with various proteins, PLP is an important coenzyme for over 100 biological reactions. A Schiff base is formed when the aldehyde group of the PLP molecule condenses with the ϵ -amino group of a lysine residue on an enzyme or substrate (Figure 2.2). Accordingly, PLP is considered to be the metabolically active vitamer of B₆. Several enzymatic reactions in the metabolism of amino acids require PLP as a coenzyme; these include decarboxylations, transaminations, trans- and de-sulfhydration, cleavage, synthesis, and racemization (Groff, 1995). PLP is also involved in glycogen and lipid metabolism, heme biosynthesis, steroid hormone modulation, and immune and nervous system function (Leklem, 2000). A novel role for B₆ in the modulation of gene expression has been proposed by Oka et al., (1995 a, b) who show generalized enhancement of RNA transcripts in liver during B₆ deficiency.

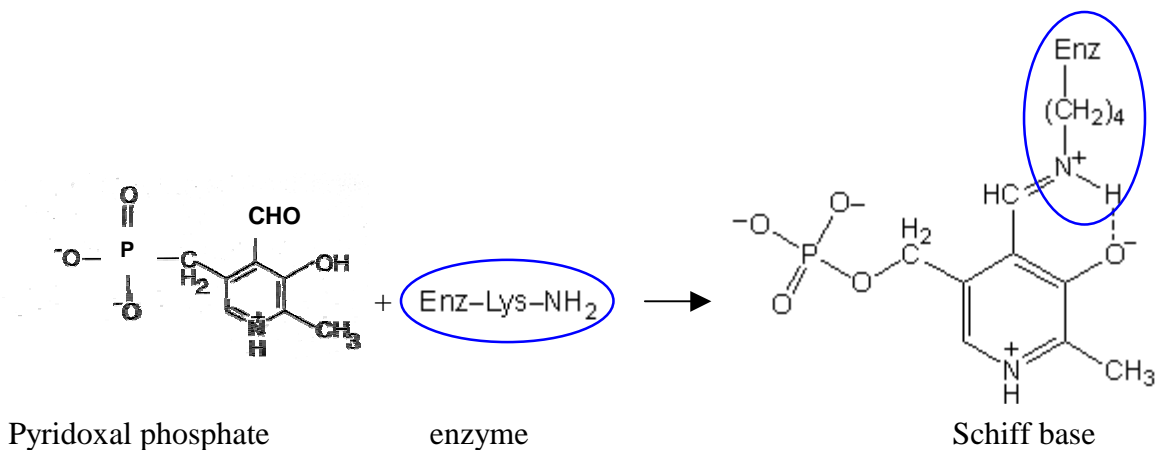


Figure 2.2. Formation of a Schiff base between pyridoxal phosphate and a lysine residue on an enzyme

Absorption and Metabolism

Absorption of the non-phosphorylated vitamers occurs by non-saturable passive diffusion. Therefore, the phosphorylated forms of B₆ must be hydrolyzed by intestinal alkaline phosphatases prior to absorption, which occurs mainly in the jejunum. Most of

the absorbed vitamers enter the portal circulation, and are taken up by the liver. In the liver, PL, PM, and PN are phosphorylated by PL kinase to form PLP, PMP, and PNP, respectively. PMP and PNP are then converted to PLP via a flavin mononucleotide (FMN) -dependent PNP/PMP oxidase (Figure 2.3). The limiting enzyme in the pathway is considered to be the PNP/PMP oxidase, due to its dependency on riboflavin (Leklem, 2000). While PL kinase is present in all tissues, PNP/PMP oxidase activity is highest in the liver, brain and kidney. Consequently, the liver is the main site of B₆ vitamers interconversion. PLP, the primary vitamers released from the liver, circulates in the blood bound via Schiff base linkage to albumin. The binding of PLP to various proteins, such as albumin, serves as protection against hydrolysis.

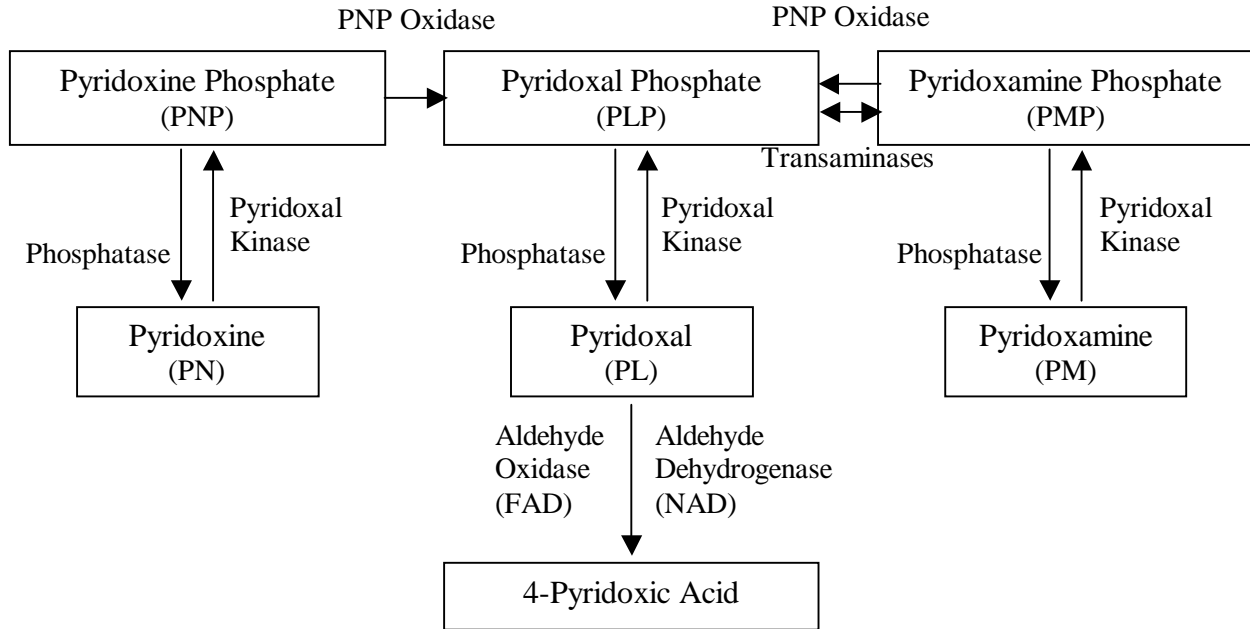


Figure 2.3. Metabolic interconversions of B₆ vitamers. FAD = flavin adenine dinucleotide, NAD = nicotinamide adenine dinucleotide.

Intracellular Vitamin B₆ Regulation

Once PLP reaches its target tissue, it is dephosphorylated, typically, this occurs via membrane-bound alkaline phosphatases (Leklem, 2000). The fate of PL taken into the cell is dependent on the need for B₆ within the cell or the status of B₆ in the cell. It

can either be re-phosphorylated intracellularly to PLP by PL kinase, or it is irreversibly oxidized to 4-pyridoxic acid, the principal urinary metabolite of B₆, by either a nicotinic adenine dinucleotide (NAD)-dependent dehydrogenase or a flavin adenine dinucleotide (FAD)-dependent aldehyde oxidase (Leklem, 2000). Newly generated PLP binds to proteins in the cell by forming Schiff bases. Phosphorylation of PL and the binding of PLP to intracellular proteins “traps” this vitamin in the cell. An example of this binding occurs in the muscle tissue; 80% to 90% of the body’s B₆ is bound to glycogen phosphorylase in the muscle (Coombs, 1998). Upon saturation of PLP binding proteins within the cell, PLP is converted by intracellular phosphatases to PL, thereby limiting the accumulation of PLP in the cell. In the case of the liver, when the proteins capable of binding PLP are saturated, PLP is rapidly hydrolyzed and the release of non-phosphorylated vitamers and 4-pyridoxic acid occurs. Control of intracellular PLP is also thought to occur via a negative feedback loop mechanism. An increasing concentration of PLP within the cell causes the inhibition of PNP/PMP oxidase in both the liver and the intestine, thus decreasing the synthesis of this active vitamer (Allgood and Cidlowski, 1991).

Schaeffer and colleagues (1995) have illustrated the regulation of B₆ in normal rat tissue. B₆ vitamer concentrations were measured in the tissues of rats fed diets consisting of 1, 10, 100, 175, or 250 times the National Research Council (NRC) recommended level of PN for 10 weeks. Significant increases were seen in plasma 4-pyridoxic acid and PL, erythrocyte PL and PLP, and all urinary B₆ vitamers with increasing PN-HCl supplementation. However, no significant differences were observed in the B₆ vitamer concentrations of the brain, liver, kidney, or muscle of the rats fasted overnight. These data indicate that normal tissues are capable of stringently controlling intracellular B₆ concentrations.

Contrary to the regulatory phenomenon observed in normal tissue, Shultz and colleagues (1989) report the accumulation of PL and PLP in cultured tumor cells supplemented with PN or PL. The murine fibrosarcoma cells, H238, were supplemented with PN or PL ranging in concentration from 0.05 mM to 5.0 mM. Intracellular PL and PLP concentrations were analyzed following a 24 h supplementation period. Shultz reported that intracellular PL and PLP levels were significantly increased by about 46%

and 28% respectively. Also, Gridley and colleagues (1987) report that mice implanted with H238 fibrosarcoma cells and fed up to 74.3 times their B₆ requirement produced tumors in which PLP concentration increased significantly with increasing dietary supplementation. Additionally, preliminary *in vitro* data from our lab indicate that human breast cancer cells grown in media supplemented with PL are also capable of accumulating concentrations of B₆ in response to supplementation. However, until now no data have existed regarding whether mammary tumors are capable of regulating vitamin B₆ concentrations *in vivo*.

Vitamin B₆ Requirements of Humans

The current B₆ RDA for males and females between the ages of 19 and 50 is 1.3 mg/d (National Academy of Sciences, 1999). For men and women ages 51 and older, the RDA is increased to 1.7 and 1.5 mg/d, respectively. The higher recommendation for people over the age of 51 is to counter possible age-associated changes in absorption and metabolism of the vitamin. B₆ deficiency is relatively uncommon in humans due to its abundance in a variety of foods. However, research has shown that plasma PLP is reduced in patients with advanced breast cancer (Potera, 1977). Also, oral contraceptive users and HIV-infected individuals may have inadequate B₆ status. As B₆ is a water-soluble vitamin, a low toxicity is expected. High intakes of B₆ from food sources have not been associated with any adverse effects. However, high supplemental doses of B₆ can produce toxic effects in humans. Intakes of greater than 500 mg/day for prolonged periods have been associated with adverse effects such as sensory neuropathy and dermatologic skin lesions (Cohen and Bendich, 1986).

Therapeutic doses from B₆ of 25 to 500 mg/day have been recommended for the treatment of depression, premenstrual syndrome, morning sickness, hypertension, and carpal tunnel syndrome. Although physiological and biochemical mechanisms exist to support the use of supplementation in these conditions there is little evidence of efficacy (Bender, 1999).

Vitamin B₆ Requirements of the Rat

The NRC (1995) estimates the B₆ requirement for maintenance, growth and reproduction of rats to be 6 mg PN-HCl/kg diet. Signs of B₆ deficiency in the rat include scaling dermatitis on the tail, paws, face, and ears, microcytic anemia, hyperexcitability, and convulsions (NRC, 1995); B₆ toxicity in rats produces neurological symptoms such as gait ataxia (Krinke et al., 1985; Windebank et al., 1985). For example, intraperitoneal injections of 600 mg PN/kg body weight administered twice daily for a period of 1 to 3 d produced peripheral ataxia followed by severe paralysis and extreme body weight loss in male Sprague-Dawley rats within 2 wk of administration (Krinke et al., 1985). Windebank and colleagues (1985) administered daily intraperitoneal injections of 200 mg PN-HCl/kg body weight for 4 wk followed by 4 additional weeks of 400 mg PN-HCl/kg to male Sprague-Dawley rats. At 6 wk, rats developed gait ataxia and a small number of rats were unable to walk. Symptoms diminished by the end of a 12 wk recovery period. However, other studies have found that concentrations up to 250 times the requirement have not produced signs of toxicity. Schaeffer and colleagues (1989, 1995) fed 12-wk-old female Long-Evans rats diets containing 1, 10, 100, 175, 200, and 250 times the NRC recommended level of PN-HCl for 6 or 10 wk, and did not observe any overt signs of B₆ toxicity.

Vitamin B₆ and Cancer

***In Vitro* Studies**

DiSorbo and Litwack (1982) examined the effect of B₆ supplementation on the growth of rat hepatoma (Fu5-5), human kidney (293-31), rat glial (C₆), and human mammary (MCF-7) tumor cells. The primary hypothesis of these researchers was that PN supplementation would cause an intracellular shortage of ATP as a result of the increased phosphorylation of PN. An ATP shortage would have an overall negative effect on the metabolism of the cell, and result in inhibited cell growth or death. A second theory put forth was that PL or PLP inhibited DNA polymerase. Consequently, DNA synthesis and cell growth would be inhibited. Supplementation with 5 mM PN for 4 d severely retarded growth of each of the cell lines tested except the human mammary

cell line. The authors hypothesized that PLP is the vitamer causing growth inhibition by interfering with glycolytic enzymes and polymerases, and that the lack of effect observed in the mammary cells was due to a low concentration of PNP/PMP oxidase in these cells. In theory, low oxidase activity in these cells would result in ineffective conversion of the supplemented PN into PLP, the proposed growth inhibiting vitamer. As previously mentioned, PNP/PMP oxidase activity has been found to be the highest in the liver, brain, and kidney. Other cell types obtain their required PLP from the circulating plasma pool. Contrary to their original hypothesis, DiSorbo and Litwack found an unexplained increase in the intracellular levels of ATP.

In another study by DiSorbo and colleagues, (1985) B₆ supplementation of cancer cells in culture was examined. These researchers reported that 4 d of 0.5 mM PL or 5.0 mM PN supplementation produced an 80% inhibition in growth of cultured B16 melanoma cells. As PLP has been shown to inhibit a number of glycolytic enzymes and both RNA and DNA polymerases (Martial et al., 1975; Modak, 1976), these researchers investigated the effect of 0.5 mM PL supplementation on uptake and incorporation of labeled glucose, thymidine, thymine, uridine, and leucine by the B16 cells. Although, uptake of glucose, thymidine, thymine, and uridine was inhibited, incorporation of the labeled nucleotide precursors was not affected. Based on their results, these authors hypothesized that B₆ acted on the membrane to inhibit transport of these compounds into the cell possibly by inhibiting membrane bound ATPase activity. Results also suggest that, in these cells, PL is a more efficient growth inhibitor than PN, which could be due to a low oxidase activity in the B16 cells.

Additionally, Disorbo and Nathanson (1983) examined the effects of B₆ supplementation on human malignant melanoma (NEL) cells in culture. NEL cells cultured in medium supplemented with 0.5 mM PL for 3 d resulted in a 90% inhibition of growth compared to control cells. Similarly to results previously found by this group in studies of B16 cells (1985), uptake but not incorporation of uridine, glucose, and thymidine was inhibited with 0.5 mM PL supplementation. However, if the incubation period was extended, incorporation of uridine was also inhibited. To determine if the supplemental PL, or the PLP formed intracellularly as a result of the supplementation, was responsible for the observed growth inhibition, cells were supplemented with 0.5

mM PL and a B₆ antagonist, 4-deoxypyridoxine. 4-deoxypyridoxine competes with PL for pyridoxal kinase. Cells supplemented with PL alone took up and incorporated 19% less labeled uridine than did the controls. The addition of 4-deoxypyridoxine to the PL supplemented media produced a 19% inhibition of uptake and a 6% inhibition of incorporation. The authors concluded that PL was responsible for the decreased uptake of the labeled uridine, and that PLP was probably responsible for the decrease of uridine incorporation into RNA. However, the mechanisms by which these B₆ vitamers interfered with normal cell metabolism were not investigated in this study.

Contrary to the results obtained by DiSorbo and Litwack (1982), Shultz and colleagues (1989) reported a significant decrease in the growth of the human mammary tumor cell line, MCF-7, in response to 5.0 mM PN supplementation. Media was supplemented with a range of concentrations of PN or PL. Supplementation of the MCF-7 media with 2.5 mM and 5.0 mM PN for 20 d resulted in 85% and 94% cell mortality, respectively. Supplementation of 0.5, 2.5, and 5.0 mM PL resulted in 100% cell mortality. The different conclusions obtained from these two studies can be attributed to the number of d of supplementation. The MCF-7 growth curve published in Shultz et al. (1989) shows only slight growth inhibition by 5.0 mM PN in the first 7 d of supplementation. This is in agreement with results obtained by DiSorbo and Litwack. In the same study, Shultz et al. also examined the effects of supplemental B₆ on human prostate (Du-145) and renal (CAKI-1) tumor cells as well as murine fibrosarcoma (H238). These cell lines also showed significant cell mortality when supplemented with 2.5 mM and 5.0 mM PN or 0.05 mM to 5.0 mM PL. However, supplementation with 5 μ M PL did not affect the growth of any of the cell types. In addition to supplementing cells with PL, these researchers conducted an incorporation experiment similar to those reported by Disorbo et al. (1983, 1985). Shultz and colleagues found that each cell line except the H238 cells incorporated significantly less labeled leucine, uridine, and thymidine when supplemented with 0.5 mM PL than the controls. In response to 0.5 mM PL supplementation the H238 cell line was found to have incorporated less thymidine, but not leucine or uridine. PL supplementation of 5 μ M did not produce any significant differences in incorporation. These researchers also used 4-deoxypyridoxine to determine if the reduction in labeled thymidine in the H238 cells was due to PLP

inhibition of intracellular enzymes. Using 0.5 mM PL alone resulted in a 29% reduction in labeled thymidine incorporation, however, when 0.5 mM PL was used in conjunction with 4-deoxypyridoxine the inhibition of incorporation was diminished to only 18%. The researchers speculate that the growth inhibition seen may be a product of PLP inhibiting intracellular enzymes through formation of a Schiff base.

In agreement with the effects of PL supplementation on cancer growth described above, Davis and Cowing (2000) witnessed dramatic reductions in MCF-7 cell number in response to PL supplementation of 0.1 mM and 0.3 mM for 9 d. The hypothesis here was that PL would inhibit cell growth via modulation of steroid hormone action based on previous data showing that B₆ was involved in steroid hormone modulation. Specifically, they theorized that PLP would form a Schiff base with the ER on either the ligand binding domain or on the DNA binding domain, and this would lead to a down regulation of cell growth. Davis and Cowing utilized 3 human mammary cancer cell lines, MCF-7 (ER+), T47-D (ER+), and BT20 (ER-), to test their hypothesis. Supplementation of the T47-D cells with 0.1 mM PL and 0.3 mM PL decreased the total cell number by 26% and 72%, respectively, in the absence of estrogen, and by 42% and 88% in the presence of estrogen. Similar reductions in cell number were also observed in the MCF-7 cell line. In theory, PL supplementation should not have effected BT20 cell proliferation if its growth-inhibiting mechanism involved interfering with estrogen action. However, a significant decrease in total cell number was observed: 0.1 mM PL and 0.3 mM PL supplementation reduced cell number by 85% and 98% respectively, compared to vehicle treated controls. These data suggest that PL acted to inhibit tumor growth via an estrogen-independent mechanism. Further investigation by Davis and Cowing supports this conclusion. pS2 is an estrogen-induced gene found in MCF-7 cells. Theoretically, if the activated ER is not able to bind DNA, expression of this gene will be altered. The expression of pS2 was not altered by PL supplementation, further strengthening the argument that this vitamin affects growth through an estrogen-independent mechanism.

Animal Studies

In all of the *in vitro* studies that document a relationship between B₆ supplementation and inhibition of tumor cell growth, the effective vitamin concentration

is higher than what is probably achievable *in vivo*. However, the stability of vitamins and other nutrients in a cell culture environment could also account for the relatively high concentrations required to elicit a response. This is especially important to consider in the case of B₆, in that, as previously mentioned, B₆ is relatively unstable in neutral or alkaline environments, as would exist in cell culture. In support of this rationale are studies that have shown beneficial effects of B₆ supplementation on cancer growth *in vivo*. Most of these studies have concentrated on carcinoma of the skin in mice.

DiSorbo and colleagues (1985) studied the effect of B₆ on implanted B16 melanoma cells in male BDF1 mice. Two different experiments were conducted to examine prevention versus treatment effects of B₆ on melanoma growth. The first examined the effect of PL pretreatment on tumor growth. Mice were injected subcutaneously with 0.5 mg PL/kg body weight twice per wk for 2 wk prior to and 3 wk following the B16 cell injection. This protocol produced a 62% decrease in tumor growth. The second experiment allowed for a 2 wk tumor development period prior to PL treatment. Administration of 0.5 mg PL/kg body weight injected directly into the tumor daily for 6 d produced a decrease in tumor growth of 39%. Other than hyperexcitability of the mice, no toxicity signs were observed. Although no mechanisms were studied in this experiment, the researchers hypothesized that, based on their *in vitro* studies, the vitamers may generate its effect by acting on the cell membrane.

Gridley and colleagues (1987) fed male BALB/c mice diets containing 0.2x (B₆ deficient), 1.2x (control), 7.7x, and 74.3x the estimated mouse requirement of PN, for 4 to 11 wk. After 4 wk of dietary treatment, the mice received an injection of H238 fibrosarcoma cells. At the end of wk 11, tumor volume was the smallest in the mice fed a B₆ deficient diet, and the largest in the mice fed the control diet, followed by the 7.7x diet and then the 74.3x diet. Liver and tumor PLP concentrations were analyzed. Liver PLP concentrations increased with level of PN in the diet but remained constant within each group throughout the study. The concentration of PLP in the tumors of the B₆ deficient mice was significantly lower than that of the B₆ sufficient mice. However, tumor volume was inversely related to tumor PLP concentration in the mice consuming 1.2x, 7.7x, and 74.3x their requirement. No B₆ toxicity symptoms were observed. Immune

system enhancement was also observed and thought to be a possible factor in the inhibition of tumor growth.

Gebhard and colleagues (1990) treated athymic male nude mice with a diet containing either 4.1 mg PN/kg diet (control) or 61.6 mg PN/kg diet for 10 wk. At 4 wk, the mice were injected with human melanoma cells (M21-HPB). No difference was observed in either tumor incidence or tumor volume between the 2 groups. Gebhard et al. suggest that the tumor growth inhibition reported in other studies may be T-lymphocyte dependent, and that inhibition was not observed in this study because athymic mice were used.

In another study, mammary tumor cells (Ma 21 224) were implanted into inbred CBA/Bln mice. Sixteen days later mice were dosed with 0.2 mg vitamin B-complex/kg body weight daily for 3 wk with doses of 1.0 mg/kg body weight given on days 2, 8, and 15. Except that B₆ was included, no information was given regarding the amount of the vitamins used. The administration of B-complex reduced the number of metastases in the mice to 30% of the controls, but no conclusions were made as to a possible mechanism of action of the B-complex (von Ardenne and Chaplain, 1971).

Fortmeyer and colleagues (1988) report that human breast and lung cancers passaged into athymic mice grew more rapidly in mice fed adequate amounts of B₆ compared to those in mice fed a diet deficient in B₆. In both types of cancer the mean tumors weights 28 d after transplantation were significantly lower in the B₆ deficient groups. The combined use of a diet deficient in B₆ and a B₆ antagonist produced an even more pronounced inhibition of tumor growth. Fortmeyer et al. did not offer any possible explanation for their results; however, they did suggest that clinicians should aim to restrict B₆ in cancer patients. Given the many functions of B₆ in cell proliferation (e.g. protein and nucleic acid synthesis), a deficiency of this vitamin would appear to be detrimental to all types of cells in the body. Numerous publications (Rall and Meydani, 1993; Ha et al., 1994) have documented the negative consequences of inadequate B₆ status on normal immune function. Since a previous report (Gebhard et al., 1990) suggests that T cell mediated immunity may be an important component of B₆ mediated inhibition of carcinoma growth, restriction of this vitamin for the treatment of cancer is not logical.

Human Studies

Potera and colleagues (1977) determined the plasma PLP and urinary 4-pyridoxic acid concentrations of breast cancer patients. Women with early breast cancer had normal levels of plasma PLP. However, women with local recurrent and systemic metastases had significantly lower than normal plasma PLP levels ($P < 0.01$ and $P < 0.001$, respectively). All three groups of women had normal urinary 4-pyridoxic acid levels, therefore ruling out B₆ deficiency as a cause of the reduced plasma PLP. This may suggest PLP uptake by rapidly proliferating cancer cells. Similarly, Chabner et al. (1970) found a reduced plasma PLP level and also impaired immune function associated with Hodgkin's disease that returned to normal with remission of the disease. Although it is known that B₆ is essential for normal immune function, the significance of reduced plasma PLP in cancer patients has not yet been elucidated.

Mammary Gland Biology

Mammary Glands of the Rat

The rat has six pairs of mammary glands located subcutaneously in the mammary fat pad. These glands are distributed along the milk lines, which are located on either side of the ventral midline. The milk lines extend along the body in a cranial-caudal direction from the axillary space to the inguinal region (van Zwieten, 1984). Three pairs of mammary glands are located in the thoracic area and three are located in the abdominal region. The three thoracic pairs are referred to as the cervical, cranial thoracic, and caudal thoracic glands, and the three abdominal pairs are termed abdominal, cranial inguinal, and caudal inguinal (van Zwieten, 1984). The nipples are medially located with respect to each gland (Russo et al., 1989).

At birth, each gland is comprised of one or two main lactiferous ducts that emanate from the nipple; these ducts are described as the primary ducts (Russo et al., 1989). The primary ducts branch into four or five secondary ducts, which in turn

dichotomously branch into the tertiary and then quaternary ducts (Russo et al., 1989). For the ease of description, the mammary gland is divided longitudinally into thirds, and these thirds are termed zones A, B, and C (Russo et al., 1989). Zone A is located proximally to the nipple and contains the primary ducts. These ducts are generally straight and broad, with no lateral branching within zone A. Occasionally small buds arise laterally; these buds are termed lateral buds (LB) (Russo et al., 1989). Zone B is comprised of the intermediate area. In this zone the number of LB increase and secondary ducts arise from the dichotomous branching of the primary ducts and tertiary ducts from the secondary and so on. Zone C encompasses the distal most third of the gland. The ducts terminate in this zone, ending in club shaped bulbous structures termed terminal end buds (TEB) (Russo et al., 1989). TEB occur in variable numbers in each of the mammary glands, as do the other structures of the gland (Russo et al., 1989). TEB are the most abundant and the largest in the thoracic glands, with the greatest difference seen in the caudal thoracic gland (Russo and Russo, 1987; Russo et al., 1989).

Development of the mammary gland of the rat is a constant and dynamic, hormone-dependent process. During the first few weeks of postnatal life, the surface area of the mammary glands increase in proportion to that of the body surface. However, during the fourth week and continuing until the 13th to 16th week of life, increases in the surface area of the mammary gland begin to surpass that of the body (van Zwieten, 1984). The average area of a mammary gland increases from an average 4.8 mm² at 5 d of age to 26.5 mm² at 21 d and 553.15 mm² at 55 d of age (Russo et al., 1989). This period of rapid growth, between 21 and 55 d, can be impaired by removal of the ovaries (Russo et al., 1989). With increasing age, the number of LB in zones A and B increase and the LB split to become alveolar buds (AB). Many TEBs also split into ABs, which in turn become type 1 lobules, or clusters of ABs around a small duct (Russo et al., 1989; van Zwieten, 1984). Pregnancy and lactation will cause these lobules to mature into type 3 lobules, which will regress to type 2 upon the cessation of lactation. The TEBs that do not form ABs become finger like structures called terminal ducts (TD). As long as the rat remains a virgin these TDs will not progress further (Russo et al., 1989). By 55 days of age the number of TEB in zones A and B, but not C has diminished greatly.

The Human Mammary Gland

Postnatally, the human mammary gland progresses through 2 phases, development and differentiation. During childhood, the mammary gland is a simple structure that grows at a rate relative to that of the body. As puberty approaches the stroma and glandular tissue shows signs of growth. At this time, the primary and secondary ducts of the gland lengthen and divide. These ducts end in club like structures equivalent to the TEB of the rat. In a process similar to that of the rat the TEB develop into TD, or split into AB, which in turn can form lobules of type 1. In the human these lobules are referred to as terminal ductal lobular units (TDLU) and are the functional unit of the breast. Formation of the lobules typically occurs 1-2 years following the onset of menstruation, although the stages of human mammary gland development do not correlate well with age. The breast of a non-pregnant woman can form lobules of type 2. These lobules arise from AB sprouting from the type 1 lobules. The AB of type 2 lobules are referred to as ductules. In the human, full development and differentiation of the mammary gland only occur with pregnancy (Russo et al., 1990).

Human vs. Rat Mammary Glands

Although there are a number of similarities between the human and the rat mammary gland, there are also a few key differences. While the development of the rat mammary gland is correlated to age, this is apparently not the case for the human gland. Normal breast tissue from women of various ages and reproductive histories has been examined and no correlation has been found between age and gland development. The mammary gland of the rat appears to develop directionally from the nipple to the dorsal portion of the gland, whereas the development of the human mammary gland appears to have no obvious topographical order. Another difference between the two species relates to the tissue surrounding the gland. The mammary gland of the rat grows into the surrounding fat tissue, the mammary fat pad, while this type of growth is rarely seen in humans (Russo et al., 1990). Nevertheless, the chemically-induced rat mammary tumor model described below is widely accepted, and has been utilized in the development of many of the currently used breast cancer therapies.

7,12-dimethylbenz(a)anthracene-induced Mammary Carcinoma Rat Model

Various carcinogenic polycyclic hydrocarbons are known to induce a diversity of cancers in the rat. The location of the cancer appears to be dependent on the route of administration of the carcinogen. The first hydrocarbon discovered to initiate mammary cancer via oral administration was 2-actylaminofluorene (Wilson, 1941). Huggins and colleagues (1961) developed a rat model for the study of mammary carcinoma utilizing the polycyclic hydrocarbon 7,12-dimethylbenz(a)anthracene (DMBA).

Research has shown that the administration of DMBA induces carcinoma that initiates primarily in the epithelium of the TEB during its transition to ABs or TDs (Haslam and Bern, 1977; Russo and Russo, 1980). This conclusion was reached in part through the observation that the induction of carcinomas by DMBA is age dependent. DMBA administered to rats between the ages of 21 days to 55 days produces the largest number of mammary carcinomas. It is between these ages that the number of TEB declines and the number of AB increases. The administration of DMBA to rats of older ages with more differentiated mammary structure produces a larger number of benign lesions. Carcinomas appear to be produced when the TEB differentiate into intraductal proliferations (IP) rather than into AB or TD. When the IP become confluent they are known as carcinomas (Russo et al., 1990). Figure 2.3 illustrates the process of mammary carcinoma induction in the rat.

Research has also shown that DMBA produces a greater number of tumors in the thoracic mammary glands than in the abdominal glands (Torgersen, 1975). And as previously noted, the thoracic glands are composed of a significantly larger number of TEB than the abdominal glands (Russo and Russo, 1987; Russo et al., 1989). The process of mammary carcinoma induction in humans is not as clearly known as the process is in the rat. Figure 2.4 demonstrates the pathway that is thought to occur.

The DMBA-induced mammary carcinoma rat model was selected for use in this research project. The DMBA model is a well-accepted, and widely used model due to its availability and suitability to study mammary cancer. Specifically, the tumors produced by DMBA are comparable to human mammary tumors in terms of their overall biological behavior, for example they have a long relative tumor latency, and similar histotypes, and endocrine responsiveness (Russo et al., 1990).

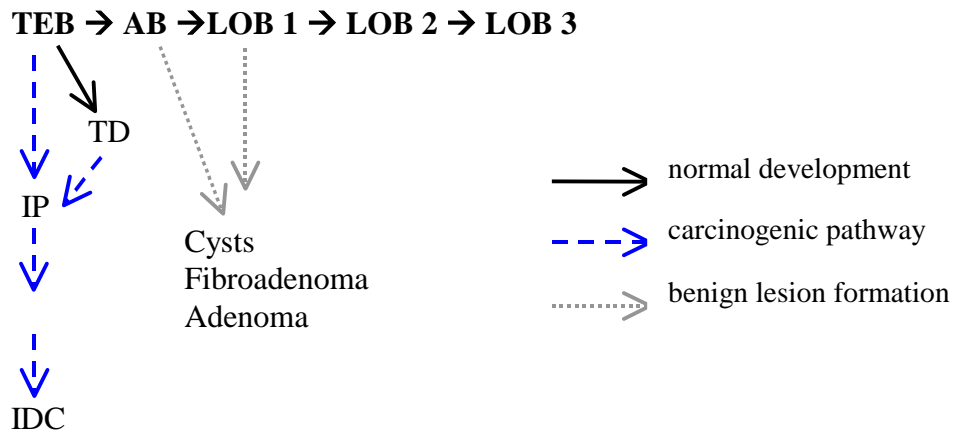


Figure 2.3. Pathogenetic pathway of rat mammary carcinogenesis*

TEB = terminal end bud, AB = aveolar bud, TD = terminal duct, LOB = lobule, IP = intraductal proliferation, DCIS = ductal carcinoma in situ, IDC = invasive duct carcinoma

*Adapted from Russo et al., 1990

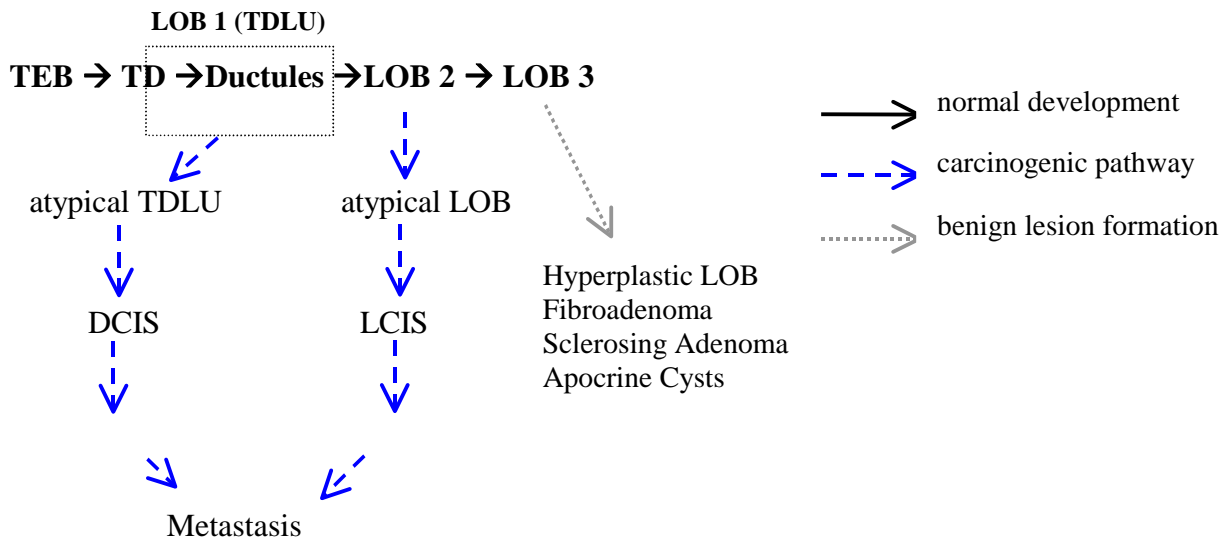


Figure 2.4. Pathogenetic Pathway of Human Breast Cancer*

TEB = terminal end bud, TDLU = terminal ductal lobular unit, AB = aveolar bud, TD = terminal duct, LOB = lobule, IP = intraductal proliferation, DCIS = ductal carcinoma in situ, IDC = invasive duct carcinoma, LCIS = lobular carcinoma in situ, LIC = lobular invasive carcinoma

*Adapted from Russo et al., 1990

Chapter 3: Dietary vitamin B₆ supplementation promotes the growth of 7,12-dimethylbenz(a)anthracene-induced mammary carcinoma in Sprague Dawley rats.

ABSTRACT

In vitro data from our laboratory demonstrate that vitamin B₆ supplementation of estrogen receptor – positive and – negative breast cancer cells is growth inhibitory. Others have reported that dietary vitamin B₆ supplementation resulted in increased fibrosarcoma pyridoxal phosphate (PLP) concentrations and a significant inverse relationship between tumor PLP concentration and tumor volume in mice. This suggests that, in contrast to data reported for normal cells, tumor cells are capable of accumulating supplemental vitamin B₆. In the current study, we investigated the effects of dietary vitamin B₆ supplementation on 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary carcinoma in rats. Our objective was to identify the effect of pyridoxine (PN) supplementation on tumor growth and vitamin B₆ uptake by tumor cells. To accomplish this, 50-d-old female Sprague Dawley rats were gavaged with 15 mg DMBA and fed a diet containing either 7, 350, or 1050 mg PN-HCl/kg diet, the equivalent of 1, 50, or 150x the National Research Council's estimated vitamin B₆ requirement for rats, respectively. These levels of PN have previously been shown to produce no overt signs of toxicity in rats. Throughout the experiment, the percentage of rats with tumors and the average number of tumors per rat remained similar between groups. Mammary tumor growth rates were significantly increased in response to dietary vitamin B₆ supplementation ($P < 0.05$). Liver PLP and pyridoxal (PL) concentrations did not differ between dietary treatment groups. Plasma PL and PLP concentrations were significantly higher in the group fed the 150x diet compared with the 1x diet ($P < 0.001$, $P < 0.05$). Mammary tissue PL concentrations of the 150x group were significantly higher ($P < 0.05$) than the 1x group, but no differences were observed in mammary PLP concentrations. Similarly to mammary tissue, no differences between groups were observed in tumor PLP concentration. However, tumor PL concentrations in both the 50x and 150x dietary treatment groups were significantly higher than those from the rats fed the 1x diet ($P < 0.002$). These data demonstrate that previously reported inhibitory effects of supplemental vitamin B₆ on breast cancer growth *in vitro* do not occur in response to

dietary supplementation at 50 or 150 times the vitamin B₆ requirement *in vivo*. In fact, dietary vitamin B₆ at 150 times the requirement actually promoted mammary tumor growth. In light of these results, investigation of the effects of supplemental vitamin B₆ on cancer growth in humans is warranted.

Introduction

The effectiveness of vitamin B₆ (B₆) deficiency on the inhibition of tumor growth in animal models has been reported (Mihich et al., 1959; Tryfiates et al., 1978; Fortmeyer et al., 1988). However, due to the many vital functions B₆ serves, a deficiency of this vitamin is also detrimental to many normal physiological processes and therefore is not a viable therapeutic option to inhibit cancer growth. Conversely, the effects of moderate supplemental doses of B₆ do not appear to be harmful. The Tolerable Upper Intake Level (UL) set for B₆ is 100 mg/d, and the No-Observed-Adverse-Effect Level is set at 200 mg/d, these are approximately 75 and 150 times higher than the Recommended Dietary Allowance (RDA), respectively (National Academy of Sciences, 2000).

Interestingly, evidence shows that the growth of cultured murine melanoma (B16), fibrosarcoma (H238), hepatoma (Fu5-5), glial (C₆), human renal (CAKI-1, 293-31), prostate (DU-145), skin (NEL), and mammary (MCF-7, T47-D, and BT20) cancer cells is inhibited by supplementation with high concentrations of B₆ (Disorbo & Litwack, 1982; Disorbo et al., 1985; Shultz et al. 1989; Disorbo & Nathanson; 1983, Davis & Cowing, 2000). The inhibition of murine melanoma, and fibrosarcoma growth has also been reproduced *in vivo* with either B₆ injections directly into the tumors or through dietary supplementation (Disorbo et al., 1985; Gridley et al., 1987). Although the mechanism of action of B₆ on growth inhibition has not yet been elucidated, Shultz et al. (1989) reported the accumulation of PLP and PL in murine fibrosarcoma cells supplemented with B₆. Additionally, Gridley et al. (1987) found an inverse correlation between fibrosarcoma PLP concentration and tumor volume in mice fed diets supplemented with B₆. Also, unpublished data from our lab show that human breast cancer cells grown in media supplemented with B₆ are capable of accumulating PLP. As normal tissues are capable of stringently controlling intracellular concentrations of B₆, the

accumulation seen in cancer cells may create a cytotoxic environment, thereby preventing tumor growth. This distinct characteristic of malignancy could potentially be exploited for therapeutic benefit.

Disturbances in B₆ metabolism have also been reported in women with breast cancer. Potera et al. (1977) found that, in the absence of B₆ deficiency as assessed by measurement of urinary 4-pyridoxic acid excretion, women with advanced breast cancer had lower plasma PLP levels than women with early or no signs of breast cancer.

The effect of B₆ supplementation on mammary carcinoma growth and tumor cell B₆ accumulation has not previously been assessed *in vivo*. Therefore, it was the aim of this study to examine whether dietary B₆ supplementation would inhibit the growth of chemically-induced mammary carcinoma in rats. Potential growth modulation properties of supplemental B₆ on mammary carcinoma are of interest due to recently published *in vitro* data, low toxicity of this compound at relatively high doses, and its economic viability as a breast cancer treatment.

Materials and Methods

Animals, diets, and experimental design. Female Sprague Dawley rats (n = 95, Harlan, Indianapolis, IN) 43 - 45 d old, weighing 110 – 153 g were randomly divided into 3 dietary treatment groups. At 50 d of age each rat received 15 mg of 7, 12 - dimethylbenz(a)anthracene (DMBA, ICN Biomedicals, Inc., Costa Mesa, CA) suspended in 1 mL of sesame oil (Sigma, St. Louis, MO) by oral gavage. Rats were housed individually in stainless steel mesh bottom cages in a light (12h/d) and temperature-controlled (24°C) barrier facility. All rats were then fed a purified AIN-93M (Reeves et al., 1993) rodent diet containing 7 mg PN-HCl/kg diet for 7 d before and after DMBA administration (Dyets, Inc., Bethlehem, PA). Animal care and use was approved by the Virginia Tech Animal Care Committee.

At 58 d of age, rats were started on their previously assigned diets. The 1x diet (AIN-93M, Dyets, Inc.) contained 7 mg PN-HCl/kg diet, or the estimated rat requirement for growth and reproduction (NRC, 1995). The 50x and 150x diets contained 350 and 1050 mg PN-HCl/kg diet, or 50 and 150 times the B₆ NRC recommendation,

respectively. PN-HCl replaced the equivalent amount of cornstarch in the 50x and 150x diets. Rats were given free access to food and water.

The diets were continued for 17 wk; food intake and body weight were recorded weekly throughout the experiment. General health was monitored for signs of B₆ toxicity. Moribund rats were killed prior to the end of the experiment.

Upon termination of the experiment, rats were fasted overnight, anesthetized using isoflurane (Abbott Laboratories, North Chicago, IL), and killed by exsanguination via cardiac puncture. Necropsy included a brief examination of the internal cavity, removal of all mammary tumors, at least one unaffected mammary gland, and liver.

Assessment of mammary tumors. Beginning 4 wk after DMBA administration, rats were palpated twice per wk to identify mammary tumors; the time of appearance and location of each tumor were recorded. Tumor latency period was determined as the number of d after DMBA administration until appearance of the first tumor in each rat. On a weekly basis each tumor was measured with vernier calipers; the largest diameter (length) and its perpendicular counterpart (width) were recorded. Relative tumor volume was then calculated using the formula $[(\text{width})^2 \text{ length}]/2$.

In the first 3 wk after DMBA administration, before the appearance of any tumors, 10 moribund rats died or were euthanized and are not included in the final analyses. Due to overwhelming tumor burden, several rats (1x, n = 7; 50x, n = 9; 150x, n = 10) were killed prior to the termination of the study. Therefore, tumor assessment data are presented at wk 13 when all rats were alive and at wk 18 post DMBA administration when the study was terminated.

Vitamin B₆ analysis. PLP and PL concentrations in liver, mammary tissue, tumor, and plasma were determined using an ion-paired, reversed-phase HPLC method modified from Ubbink et al. (1985), and Mahuren & Coburn (1990). Briefly, tissues and plasma were homogenized in 5% and 10% TCA, respectively, followed by extraction with diethyl ether and dichloromethane. Aliquots of 100 or 50 μL (for analysis of PLP or PL, respectively) of the extracted samples were injected onto a Zorbax ODS 4.5 mm x 25 cm analytical column (MAC-MOD Analytical, Chadds Ford, PA). The mobile phase,

0.05 M potassium dihydrogen phosphate with 8 % acetonitrile (v/v), pH 2.9, was run at 1.1 mL/min. A solution of 4 % sodium hydroxide (w/v) was used for post column alkalisation to enhance fluorescence; the flow rate was 0.1 mL/min (Perkin Elmer Series 10 Liquid Chromatograph). The fluorescence detector (Waters 470, Scanning Fluorescence Detector, Waters Corporation, Milford, MA) was set at excitation and emission wavelengths of 367 nm and 478 nm, respectively. Chromatographic data were processed using Millennium³² version 3.20 software (Waters Corporation).

Statistical analyses. Data are presented as means \pm SEM. Data were analyzed by analysis of variance (ANOVA) followed by the Tukey – Kramer multiple comparisons test. Repeated measures ANOVA was used to determine differences between groups over time. Pearson’s correlations were computed to identify significant correlations existing between variables. Results were considered statistically significant at $P \leq 0.05$. Statistical analyses were performed using the statistical software package SAS (SAS Institute, Cary, NC).

Results

Body weight and food intake. Body weight remained similar between groups throughout the study with the exception of wk 16 and 18 post DMBA administration, at which points the 1x group had a mean body weight significantly greater than the 150x group ($P < 0.05$, **Figure 3.1**). Food intake also remained similar throughout the dietary treatment period although significant differences occurred between the 50x and 150x, 50x and both 1x and 150x, and 50x and 150x at 2, 10, and 11 wk post DMBA administration, respectively ($P < 0.05$).

Tumor assessment. Tumor latency period was similar between groups with an average of 64.3 ± 4.0 d, 66.4 ± 4.0 d, and 66.2 ± 4.1 d in the 1x, 50x, and 150x groups, respectively. Tumor incidence and the average number of tumors per rat in each group did not differ significantly (**Tables 3.1 and 3.2**).

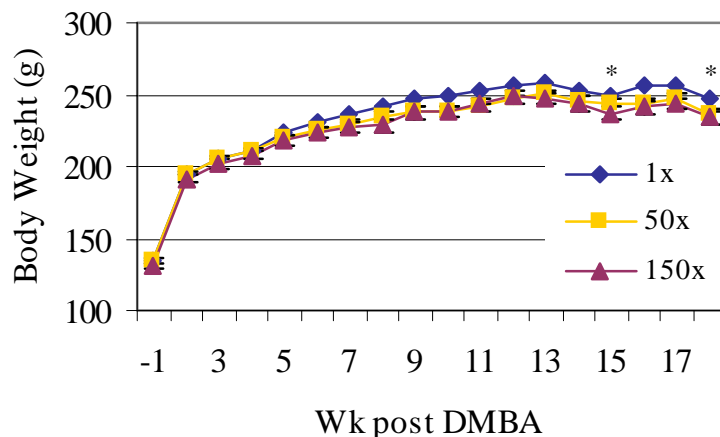


Figure 3.1. Growth of female Sprague Dawley rats treated with 7, 12-dimethylbenz(a)anthracene (DMBA) and fed diets containing either 7 (1x), 350 (50x), or 1050 (150x) mg PN-HCl/kg diet. Asterisk indicates a significant difference between the 1x and 150x groups ($P < 0.05$).

Table 3. 1

Tumor incidence, number of tumors/rat, total tumor volume/rat, and average tumor volume/rat at 13 wk post 7, 12-dimethylbenz(a)anthracene administration¹

Diet ²	n	Tumor incidence (%)	Number of tumors/rat	Total tumor volume/rat (cm ³)	Average tumor volume/rat (cm ³)
1x	26	73.1	1.7 ± 0.3	2.6 ± 0.9	0.8 ± 0.3
50x	31	78.1	1.9 ± 0.4	3.1 ± 0.9	1.3 ± 0.4
150x	28	75.0	1.9 ± 0.4	7.4 ± 3.2	2.0 ± 0.6

¹Values are means ± SEM. No significant differences were observed. ²Diets varied in concentration of PN-HCl, 1x, 7 mg/kg diet; 50x, 350 mg/kg diet; 150x, 1050 mg/kg diet.

Table 3.2
Tumor incidence, latency period, number of tumors/rat, total tumor volume/rat, and average tumor volume/rat of rats remaining at 18 wk post 7, 12-dimethylbenz(a)anthracene administration¹

Diet ²	n	Tumor incidence	Number of tumors/rat	Total tumor volume/rat	Average tumor volume/rat
		(%)		(cm ³)	(cm ³)
1x	19	84.2	1.8 ± 0.3	0.6 ± 0.2	0.3 ± 0.1
50x	22	82.6	1.4 ± 0.2	0.5 ± 0.2	0.3 ± 0.1
150x	18	72.2	1.7 ± 0.3	1.3 ± 0.5	0.6 ± 0.2

¹Values are means ± SEM. No significant differences were observed. ²Diets varied in concentration of PN-HCl, 1x, 7 mg/kg diet; 50x, 350 mg/kg diet; 150x, 1050 mg/kg diet.

Despite the occurrence of larger tumors in the 150x group than the 1x or 50x groups, total tumor volume/rat was not found to be statistically different between dietary treatment groups (**Tables 3.1 and 3.2**). Total tumor volume/rat at 13 wk ranged from 0 to 15.7, 0 to 18.5, and 0 to 80.0 cm³ and at 18 wk 0 to 2.6, 0 to 2.4, and 0 to 6.4 cm³ for the 1x, 50x, and 150x groups respectively, thus marked animal variability contributed to lack of statistically significant differences between groups. However, the growth rate of tumors was significantly effected by dietary treatment ($P < 0.05$, **Figure 3.3**). **Figure 3.2** clearly demonstrates that tumor burden in the moribund rats euthanized prior to the termination of the study was greater than that of the rats remaining until wk 18.

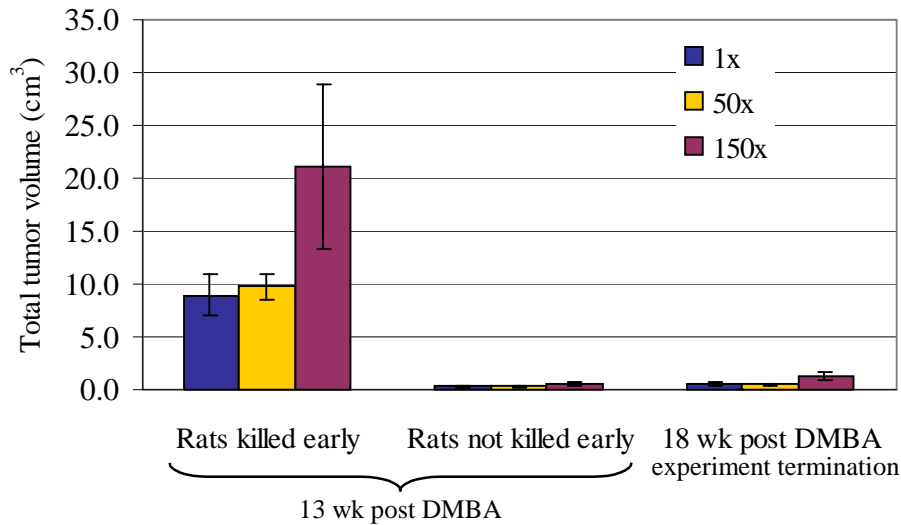


Figure 3.2. Average total tumor volume following treatment with 7, 12-dimethylbenz(a)anthracene (DMBA) in female Sprague Dawley rats fed diets containing either 7 (1x), 350 (50x), or 1050 (150x) mg PN-HCl/kg diet. Several rats (1x, n = 7; 50x, n = 9; 150x, n = 10) were killed prior to the termination of the study due to overwhelming tumor burden. Several rats (1x, n = 19; 50x, n = 22; 150x, n = 18) remained in the study until termination of the experiment at 18 wk post DMBA. No significant differences were observed between dietary treatment groups at any time point. Tumor volume at 13 wk post DMBA was significantly greater in each dietary treatment group in the rats killed early compared to those not killed early ($P < 0.002$).

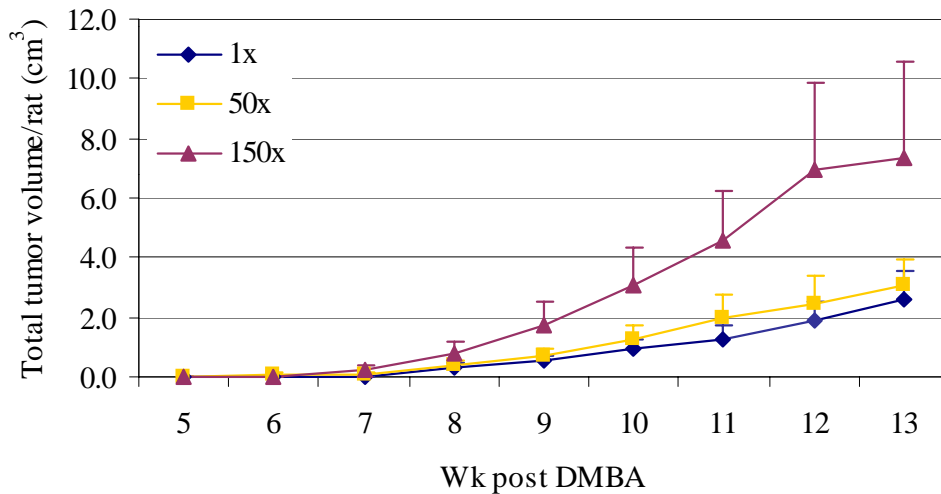


Figure 3.3. Average total tumor volume following treatment with 7, 12-dimethylbenz(a)anthracene (DMBA) in female Sprague Dawley rats fed diets containing either 7 (1x), 350 (50x), or 1050 (150x) mg PN-HCl/kg diet. No significant differences in tumor volume were observed at any time point. Tumor growth rate is significantly different between groups ($P < 0.05$).

PLP and PL concentrations. Liver PLP and PL concentrations did not differ significantly between groups (**Table 3.3**). Plasma PLP and PL concentrations were significantly affected by dietary supplementation with 150x the NRC recommendation of B₆ (**Table 3.3**). Rats fed the 150x diet had significantly greater plasma PLP levels than rats fed the 1x diet ($P < 0.05$). Rats fed the 150x diet also had significantly higher plasma PL concentrations than the rats fed the 1x and 50x diets ($P < 0.0001$ and $P < 0.001$, respectively). Although mammary tissue PLP concentration did not significantly differ between dietary treatment groups, PL concentrations were significantly higher in the 150x group compared to the 1x group ($P < 0.05$, **Table 3.3**). No significant differences were found in tumor PLP between the diet groups (**Table 3.4**). However, tumor PL was significantly increased in both the 50x and 150x groups compared to the 1x group ($P < 0.005$ and $P < 0.0005$, respectively). Measures of tumor PLP and PL concentration were determined from the largest tumor in each rat. Tumor PL was also positively correlated with the volume of the largest tumor in each rat ($r = 0.35$, $P < 0.05$). Other significant correlations are shown in **Table 3.5**.

Table 3.3

PLP and PL concentrations in liver, plasma, and mammary tissue of female rats orally gavaged with 7, 12-dimethylbenz(a)anthracene and fed different concentrations of PN^{1,2}

Diet ³	Liver		Plasma		Mammary Tissue	
	PLP (nmol/g wet tissue)	PL (nmol/g wet tissue)	PLP (nmol/L)	PL (nmol/L)	PLP (nmol/g wet tissue)	PL (nmol/g wet tissue)
1x	20.6 ± 0.7 ^a (n = 25)	4.3 ± 0.2 ^a (n = 17)	262.9 ± 8.7 ^a (n = 14)	311.8 ± 15.8 ^a (n = 15)	1.4 ± 0.1 ^a (n = 15)	1.3 ± 0.2 ^a (n = 11)
50x	21.6 ± 0.5 ^a (n = 28)	4.2 ± 0.2 ^a (n = 23)	279.5 ± 13.7 ^{a,b} (n = 21)	420.5 ± 21.4 ^a (n = 20)	1.8 ± 0.2 ^a (n = 19)	1.4 ± 0.1 ^{a,b} (n = 13)
150x	20.9 ± 0.8 ^a (n = 25)	4.7 ± 0.3 ^a (n = 16)	308.0 ± 11.3 ^b (n = 19)	581.9 ± 40.2 ^b (n = 21)	1.7 ± 0.2 ^a (n = 16)	1.7 ± 0.1 ^b (n = 10)

¹PLP, pyridoxal phosphate; PL, pyridoxal; PN, pyridoxine. ²Values are means ± SEM.

³1x, 7 mg PN-HCl/kg diet; 50x, 350 mg PN-HCl/kg diet; 150x, 1050 mg PN-HCl/kg diet. Values in the same column with unlike superscripts are significantly different, plasma PLP ($P < 0.05$), plasma PL ($P < 0.001$), mammary tissue ($P < 0.05$).

Table 3.4

PLP and PL concentration of 7, 12-dimethylbenz(a)anthracene-induced rat mammary tumors in rats fed different concentrations of PN^{1,2}

Diet ³	Tumor ⁴	
	PLP	PL
	(nmol/g wet tissue)	
1x	5.0 ± 0.2 ^a (n = 18)	0.9 ± 0.1 ^a (n = 17)
50x	5.1 ± 0.2 ^a (n = 16)	1.5 ± 0.1 ^b (n = 16)
150x	5.6 ± 0.2 ^a (n = 18)	1.6 ± 0.1 ^b (n = 17)

¹PLP, pyridoxal phosphate; PL, pyridoxal; PN, pyridoxine. ²Values are means ± SEM.

³1x, 7 mg PN-HCl/kg diet; 50x, 350 mg PN-HCl/kg diet; 150x, 1050 mg PN-HCl/kg diet

⁴The largest tumor from each rat was analyzed for its PLP and PL concentrations. Values in the same column with unlike superscripts are significantly different (P < 0.002).

Table 3.5

Correlations between PLP, PL, and tumor assessment data^{1,2}

Parameter	Correlated with:	r	Trend ³
Liver PLP	Plasma PLP	0.37	+
	Plasma PL	0.46	+
	Number of tumors	0.49	-
	Total tumor volume	0.42	-
Liver PL	Mammary tissue PLP	0.33	-
	Number of tumors	0.40	-
Plasma PL	Mammary tissue PL	0.52	+
Mammary tissue PLP	Number of tumors	0.39	+
	Total tumor volume	0.38	+
Tumor PL	Tumor volume ⁴	0.35	+

¹All shown correlations are significant at P < 0.05. ²PLP, pyridoxal phosphate; PL, pyridoxal. ³+ denotes a positive correlation, - denotes negative correlation. ⁴Volume of the largest tumor in each rat.

Discussion

The growth inhibitory effects of supplemental B₆ on fibrosarcoma and melanoma cells in culture (Disorbo et al., 1985; Disorbo & Nathanson, 1983; Shultz et al., 1982) have also been observed in animal models with either subcutaneous injection or diet as the mode of supplemental B₆ delivery (Disorbo et al., 1985; Gridley et al., 1987). However, the growth inhibition of mammary carcinoma produced by supplemental B₆ in cultured cells was not reproduced in the present study. Moreover, we have shown that dietary B₆ supplementation at the 150x level promoted the growth of mammary tumors. It is evident that the concentration of PL reaching the mammary carcinoma cells via the plasma was low (0.3 to 0.6 μM) compared to the concentrations of PL (100 to 500 μM) previously shown to produce growth inhibition of mammary carcinoma cells in culture (Shultz et al., 1982; Davis & Cowing, 2000). It is also interesting to note that in many of the *in vitro* studies examining B₆ supplementation on cancer cells, the control media contained 5 μM PL, approximately 10 times higher than that found in the plasma of rats fed the 150x diet in this study. That a concentration 10-fold higher than circulating PL levels is required for maintenance of breast cancer cells in culture suggests poor stability of this compound *in vitro*. Therefore, it was reasonable to hypothesize that a growth inhibitory concentration of B₆ was achievable in a physiological system. This is further supported by previous *in vivo* studies showing B₆ inhibition of tumor growth. To our knowledge this is the first study examining the effects of dietary B₆ supplementation on mammary cancer in an animal model.

In a previous study, mice were fed diets containing 0.2, 1.2 (control), 7.7, or 74.3 times their PN requirement for 4 wk prior to and 4 to 7 wk after being inoculated with H238 fibrosarcoma cells (Gridley et al., 1987). Tumor growth was inhibited in mice consuming the deficient or supplemented diets compared to those being fed the control diet. In contrast, we found that dietary B₆ supplementation at 150x the NRC requirement enhanced the growth of mammary tumors. Gridley and coworkers also reported a significant effect of dietary supplementation on tumor PLP concentrations. Mice consuming the deficient diet had lower tumor PLP concentrations than the controls ($P < 0.001$), and tumor PLP concentrations of the supplemented mice were higher than

controls ($P < 0.005$). Additionally, these researchers observed an inverse relationship between tumor PLP concentrations and tumor volume in mice fed supplemental PN, suggesting that increased intracellular PLP suppressed the growth of the tumors. A similar relationship was not observed in our study. Interestingly however, in the present study, tumor volume was significantly and positively correlated with tumor PL concentration, suggesting that elevated intracellular PL enhanced tumor growth. The contrasting results obtained from *in vivo* experimentation with B₆ supplementation and cancers of the skin and of the breast could stem from differences in experimental design (timing of B₆ supplementation in relation to tumor induction), animal model utilized (species, tumor induction protocol) and characteristics of the target tissue investigated (antigenicity, metabolism).

Dietary B₆ supplementation did not significantly affect liver PLP or PL concentrations. Liver PLP concentrations obtained in this study are in agreement with a report by Schaeffer and coworkers (1995) in which rats were fed diets supplemented with up to 175X their B₆ requirement, however these researchers did not measure liver PL. This suggests that administration of DMBA, a compound that is metabolized by the liver, did not significantly effect B₆ metabolism in this organ. Also in agreement with Schaeffer et al. (1995) plasma PL increased with supplementation, albeit only significantly in the 150x group. We also observed a significant increase in plasma PLP, which was not reported by these investigators. However, Gridley and coworkers (1987) reported significant increases in liver PLP in response to dietary B₆ supplementation in mice with or without cancer. This contrast in results could be due to species differences, or the chemically-induced cancer protocol used in our study.

Due to the strict control of B₆ vitamer concentrations in normal cells, no significant differences in PLP or PL levels were expected in mammary tissue of virgin rats. However, mammary tissue PL levels did show a significant increase in response to the 150x diet ($P < 0.05$). The ability of mammary tissue to accumulate B₆ may relate to pregnancy and lactation. A similar trend in lactating mammary tissue B₆ concentration resulted when Kirksey and Susten (1978) fed female Sprague Dawley rats diets either deficient in (1.2, 2.4, and 4.8 mg PN-HCl/kg) or supplemented with (9.6 and 19.2 mg PN-HCl/kg) B₆. Stepwise significant increases in mammary tissue, and milk total B₆

concentration occurred in rats fed the low B₆ diets. B₆ concentrations of both mammary tissue and milk from the supplemented rats continued to increase, although not significantly. Also during pregnancy, plasma PLP levels are lower than that of non-pregnant females (Cleary et al., 1975). In one study, this reduction in PLP has been reported to be accompanied by an increase in plasma PL (Barnard et al., 1987). Coburn et al. (1992) found that human mammary tissue PLP and PL levels also appear to follow this pattern in pregnancy. Thus, it is evident that mammary tissue can accumulate B₆ during pregnancy and lactation. Although virgin rats were utilized in this study a significant increase in mammary tissue PL concentration was observed in response to supplementation. This could relate to the ability for changes in nutrient uptake that occur in this tissue in response to pregnancy and lactation.

Based on previous *in vitro* and *in vivo* studies we hypothesized that mammary carcinoma cells in our study would accumulate PLP and PL in response to dietary B₆ supplementation. While both PLP and PL concentrations in the tumors increased with B₆ supplementation, significant differences were observed only in PL concentrations between the 1x and 50x, and 1x and 150x groups. Additionally, we hypothesized that this accumulation of B₆ in the tumors would inhibit their growth. In fact, dietary B₆ supplementation at the 150x level promoted the growth of tumors.

Previously it has been reported that women with advanced breast cancer have lower plasma PLP concentrations than women with either no or early breast cancer (Potera et al., 1977). While we did not find this relationship to be significant in the present study it was observed that as overall tumor burden increased (number and total volume of tumors) liver PLP decreased. Liver PL also decreased as the number of tumors per rat increased. In addition, positive correlations were found between liver PLP and plasma PLP, and liver PLP and plasma PL. Therefore, as liver PLP increased, plasma PLP and PL increased and indicators of tumor burden decreased. However, it is noteworthy that liver PLP and PL did not change significantly with dietary treatment, and plasma PLP and PL were highest in the 150x group which also had the greatest mean total tumor volume. Although these correlations are statistically significant, the r values presented in Table 3.5 show that these relationships do not fully account for the variation observed between parameters.

In conclusion, the growth inhibitory effects of B₆ on mammary carcinoma previously observed *in vitro* were not reproduced in the *in vivo* study described here. This suggests that dietary B₆ supplementation at 50 or 150 times the NRC estimated requirement was not able to raise intracellular mammary carcinoma PLP concentrations to growth inhibitory concentrations observed *in vitro*. High doses of supplemental B₆ are frequently used to treat a variety of conditions. For instance, doses of B₆ from 100 to 200 mg/d (75 to 150 times the RDA for females) have been recommended for the treatment of carpal tunnel syndrome and premenstrual syndrome (Ellis 1987; Wyatt et al., 1999). If physiological processes similar to those that occurred in our study are present, the use of pharmacological doses of B₆ could potentially promote the growth of tumors in humans. Therefore, the effects of high-dose B₆ supplementation by humans should be further investigated.

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Chapter 4: Implications and Future Research

We have demonstrated that, in the chemically-induced rat model of mammary carcinogenesis, dietary supplementation of up to 150 times the requirement for B₆ does not inhibit the growth of tumors. Furthermore, this supplementation may actually promote tumor growth. These results suggest that large supplemental doses of B₆ may also promote the growth of mammary tumors in humans. Since it is common practice for many women to take pharmacological doses of B₆ for the treatment of various conditions, it is important to determine the relevance of the results obtained from this study in relation to humans. In the future, an epidemiological study examining the development and growth of mammary tumors in women who consume pharmacological doses of B₆ for the treatment of other conditions compared to those who do not, would give some indication of whether this should be a concern in the human population.

A potential limitation of this study is the use of the DMBA model of mammary carcinogenesis. DMBA and B₆ are both metabolized in the liver, therefore it is possible that DMBA had an effect on the normal metabolism of B₆. In view of the fact that all rats in this study were treated with DMBA, we cannot determine the effects of DMBA or dietary B₆ supplementation alone on normal B₆ metabolism. However, tissue B₆ vitamer concentrations observed in the present study are in general agreement with Schaeffer et al. (1995) who fed healthy rats diets supplemented with similar amounts of B₆, thereby suggesting that DMBA had little effect on B₆ metabolism.

Due to overwhelming tumor burden, a number of rats were euthanized prior to the termination of the experiment. This may have influenced our results. It is possible that tumors in these animals may have regressed with time, but that pattern of growth has not been seen in previous studies involving B₆ supplementation. Other limitations include dose of supplemental B₆ used, in the present study only two levels of supplementation were employed, and the highest level did not appear to produce any toxic effects. A more stratified scheme of supplementation, with levels reaching beyond those used in our study may be of some help in determining if dietary supplementation can produce growth inhibition without other toxic effects. As described above, vitamin A and D analogues have been successfully utilized to circumvent toxicity related to treatment with

pharmacological doses of these vitamins. Similarly, B₆ analogues may be useful in producing concentrations that are effective in inhibiting the growth of mammary carcinoma without harmful side effects.

Tucaresol, a Schiff base forming molecule that is structurally similar to B₆ and well tolerated by humans, has previously been shown effective in the treatment of both melanoma and colon cancer (Kirkwood et al., 1997, Rhodes et al., 1995). Oral doses of 200 mg of tucaresol are well tolerated by humans and can elevate plasma tucaresol concentrations from 44 to 184 μM (Rolan et al., 1993) suggesting that tumor growth inhibiting concentrations of this analogue may be achievable. An *in vitro* study by Davis (2001) examined the effects of tucaresol on mammary carcinoma cell growth and showed that this analogue was effective in producing greater growth inhibition than equal concentrations of PL. In order to examine mechanistic differences between tucaresol and PL growth inhibition of breast cancer, Davis also investigated the effects of these compounds on the expression of cathepsin D and HER2, markers of poor prognosis and tumor aggressiveness in breast cancer. While PL supplementation significantly reduced the expression of cathepsin D compared to vehicle-treated controls, tucaresol was effective in reducing the expression of both cathepsin D and HER2 to levels significantly lower than that of PL supplemented or control cells. Currently in our lab, tucaresol is being tested for its effectiveness against breast cancer in an *in vivo* model.

The therapeutic efficacy of dietary vitamin B₆ for breast cancer is questionable given the potential growth-promoting effects of physiologically achievable concentrations. In light of the results obtained here, investigation of the effects of supplemental B₆ on cancer growth in humans is critical. In addition, identification of the mechanisms through which B₆ and similarly structured molecules modulate tumor growth may uncover new targets for the development of novel therapeutic compounds to treat human cancer.

Appendix A: Methods

Experimental Design

Female Sprague-Dawley rats (35 – 37 d old, Harlan, Indianapolis, IN) were housed 3/cage and kept under quarantine for 1 wk, during which time they were fed Teklad 2018 diet (Harlan Teklad, Madison, WI). Rats were then weighed, ear tagged, randomly divided into three groups (A, B, and C), and fed a purified rodent diet containing 7 mg PN-HCl/Kg diet for 7 d (AIN-93M, Dyets, Inc., Bethlehem, PA). Throughout the experiment rats were housed in stainless steel mesh bottom cages in a light (12h/d) and temperature-controlled (24°C) barrier facility. Animal care and use was approved by the Virginia Tech Animal Care Committee.

At 50 d of age, each rat (n = 95) received a single 15 mg dose of 7, 12 - dimethylbenz(a)anthracene (DMBA, ICN Biomedicals, Inc., Costa Mesa, CA) suspended in 1 mL of sesame oil (Sigma, St. Louis, MO) by oral gavage. DMBA (500 mg) was dissolved in sesame oil (33.3 mL) in a brown bottle to avoid exposure to light, which would cause inactivation. The bottle was placed in simmering water on a hot plate inside a fumehood for approximately 30 min, and was allowed to cool before being administered to the rats. A 16-gauge, 3 inch stainless steel animal feeding needle was used to administer the DMBA intragastrically (Popper & Sons, Inc., New Hyde Park, NY). Beginning immediately after DMBA administration, rats were housed individually to allow for measurement of food intake. Experimental diets were fed beginning 7 d after DMBA administration.

Group B (n=30) received a diet containing 7 mg PN-HCl/kg diet (1X), or the estimated rat requirement for growth and reproduction (NRC, 1995). Groups C (n=34) and A (n=33) received diets containing 350 and 1050 mg PN-HCl/kg diet, or 50 and 150 times the NRC recommendation (50X, 150X), respectively. All rats had free access to food and water.

The diets were continued for 17 wk; food intake and body weight was recorded weekly throughout the experiment. General health was monitored for signs of vitamin B₆ toxicity. Beginning 4 wk after DMBA administration, rats were palpated twice per wk to identify mammary tumors; the appearance and location of each tumor was recorded. On

a weekly basis each tumor was measured with vernier calipers; the largest diameter (length) and its perpendicular counterpart (width) were recorded. Relative tumor volume was then calculated using the formula $[(\text{width})^2 \text{ length}]/2$. The investigators involved in feeding, palpating, and tumor measurement were blinded to the diet groups. Moribund rats were killed prior to the end of the experiment.

Upon termination of the experiment, rats were fasted overnight, anesthetized using isoflurane (Abbott Laboratories, North Chicago, IL), and killed by exsanguination. Using a 21-gauge needle and 5 mL syringe, blood was collected via closed cardiac puncture into sodium-heparin vacutainer tubes, kept on ice, and protected from light until the time of processing. Plasma was obtained via centrifugation of whole blood at 1500 relative centrifugal force (RCF), 4°C, for 15 min and was then stored protected from light at -80°C in 1 mL aliquots in 2 mL microcentrifuge tubes. Necropsy included a brief examination of the internal cavity, removal of all mammary tumors, at least one unaffected mammary gland, liver, spleen, one kidney, and one lung. All tissues were weighed. A section from all tumors, and several livers of animals from each dietary treatment group were placed in 10% buffered formalin. Samples were then embedded in paraffin blocks and processed for histological examination using H&E staining. One unaffected mammary gland, all tumors, and the liver from each rat were wrapped in foil, flash frozen in liquid nitrogen, and stored at -80°C until prepared for high performance liquid chromatography (HPLC) analysis of vitamin B₆ concentration.

Sample Preparation

PL and PLP concentrations in liver, mammary tissue, tumor, and plasma were determined using the HPLC method of Ubbink et al. (1985) and Mahuren & Coburn (1990). Provided that enough tissue was obtained, a sample of each type of tissue was assayed from each rat.

Due to the photosensitivity of the B₆ vitamers all of the following procedures were carried out under yellow lights. Approximately 100 mg of liver tissue was placed into a 14 mL polypropylene tube and homogenized (10sec, speed 4, PRO 250, PRO Scientific Inc., Monroe, CT) in 3 mL of 5% trichloroacetic acid (TCA) to facilitate the precipitation of proteins. The resulting homogenate was then centrifuged for 10 min at

1000 RCF, 4°C. The supernatant was removed to a glass 8 mL tube, 150 µL of 0.5 M semicarbazide was added and the sample was vortexed briefly and incubated in a 40°C water bath for 15 min. The addition of semicarbazide facilitates the formation of PL and PLP semicarbazones which fluoresce more intensely and are more stable than the free forms of PL and PLP, thereby facilitating measurement. Next, to remove any fat-soluble components, 3 mL of ether was added, the sample was vortexed and centrifuged at 1500 RCF, 4°C for 10 min. The upper layer was then discarded and the previous step repeated. After removing the upper ether layer for the second time, 3 mL of methylene chloride was added, the sample vortexed and centrifuged at 1500 RCF, 4°C for 10 min. Finally, the upper aqueous layer was removed to a 5 mL glass volumetric flask and brought up to volume with double-distilled, deionized water. The diluted samples were then mixed thoroughly and an aliquot was removed to two 1.5 mL microcentrifuge tubes. The samples were then centrifuged in a microcentrifuge for 5 min at approximately 12000 RCF. Next, to ensure the removal of any particles that may interfere with the chromatography, the majority of each sample was placed into another set of microcentrifuge tubes. Samples were then wrapped in foil and stored at -80°C until the time of analysis.

Mammary gland tissue was processed in the same manner as liver except that due to intra-gland variability, the tissue was first minced before a representative sample was taken. In an attempt to further reduce sampling error, two samples were taken from each mammary gland. Additionally, due to the lower concentrations of PL and PLP compared with liver samples, approximately 120 mg of mammary tissue was used and no dilution was made prior to HPLC analysis.

Tumor samples were processed similarly to liver, except that the aqueous layer obtained after the addition of methylene chloride was not diluted due to the lower concentration of B₆ in tumors compared with the liver. The largest tumor, by volume, from each rat was analyzed provided that it was large enough to retrieve at least 850 mg of tissue.

Plasma was processed in a slightly different manner than the liver or mammary gland tissue. To 1 mL of plasma, 500 µL of 10 % TCA was added, the sample was vortexed and centrifuged as above. Semicarbazide (50 µL) was then added and

processed as described above for tissues. The aqueous layer obtained after the addition of methylene chloride was not diluted, but rather weighed in a microcentrifuge tube to determine the approximate volume obtained. Due to the small volume of aqueous solution obtained, the extraction was completed in duplicate for each rat in order to assay for both PL and PLP.

Standards

A stock solution of 10 mg PL-HCl or PLP/L double-distilled, deionized water was made. From this stock solution each standard was made. For example, to make a standard of 1.0 ng PLP/100 μ l, 100 μ L of the stock was diluted to 100 mL with double-distilled, deionized water. An aliquot of this standard solution was placed in a glass 8 mL tube and 50 μ L of 0.5 M semicarbazide was added for every 1 mL of standard solution. The tube was then vortexed and the solution was incubated in a water bath at 40°C for 15 min. Aliquots of 200 μ L of the standard solutions were placed in 500 μ L microcentrifuge tubes and stored at -20°C protected from light for up to 30 d. A standard curve consisting of 4 to 5 points was generally used.

High Performance Liquid Chromatography

Previously prepared samples and standards were thawed protected from light, mixed thoroughly, and an aliquot was injected via an autoinjector (temp. set-point 4°C, Waters 717 plus Autosampler, Waters Corporation, Milford, MA) onto a Zorbax® ODS 4.5 mm x 25 cm analytical column (MAC-MOD Analytical, Chadds Ford, PA). Injections of 50 μ L in triplicate and 100 μ L in duplicate were used for the measurement of PL and PLP, respectively. The mobile phase used was 0.05 M potassium dihydrogen phosphate (KH₂PO₄) with 8 % acetonitrile (v/v), pH adjusted to 2.9 using orthophosphoric acid. The flow rate for the mobile phase was 1.1 mL/min at a pressure no greater than 2200 PSI (Waters 600 Controller, Waters Corporation). A solution of 4 % sodium hydroxide (w/v) was used for post column alkalisation to enhance fluorescence; the flow rate for this was 0.1 mL/min (Perkin Elmer Series 10 Liquid Chromatograph). The fluorescence detector (Waters 470, Scanning Fluorescence Detector, Waters Corporation) was set at excitation and emission wavelengths of 367 nm

and 478 nm, respectively. Chromatographic data was processed using Millennium³² version 3.20 (Waters Corporation).

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