AN ABSTRACT OF THE DISSERTATION OF

David J. Castro for the degree of Doctor of Philosophy in Toxicology presented on February 20, 2009.
Title: Critical Windows of Transplacental Carcinogenesis: Identifying Efficacious Approaches to Chemoprevention with Dietary Phytochemicals.

Abstract approved:

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David E. Williams

Polycyclic aromatic hydrocarbons (PAHs) are a re-emerging class of environmental pollutants. The persistent nature of these highly toxic compounds along with their ubiquity in the environment creates an unavoidable route of exposure. The developing fetus and neonate are a particularly susceptible population due to their incomplete complement of xenobiotic metabolizing enzymes and limited DNA repair capacity. We've previously found that administration of dibenzo[*a,l*]pyrene (15 mg/kg), a potent PAH, late in gestation results in the induction of lymphoma, lung and liver cancer in the subsequent offspring.

Considering the lipophilicity of DBP, the relative contribution of *in utero* and lactational exposure in DBP-dependent carcinogenesis warranted investigation. We adopted a cross-foster design; litters born to DBP-treated dams were exchanged at birth with litters from control dams. Exposure to DBP *in utero* (~2 days) incurred more risk, resulting in increased lymphoma mortality and lung tumor multiplicity compared to exposure only through breast milk. To elucidate the enzymes

responsible for DBP bioactivation, we incorporated a strain of mice with the targeted disruption of the *cyp1b1* gene. In support of our hypothesis concerning the role of fetal cyp1b1 in DBP transplacental carcinogenesis, we observed a clear pattern of decreasing survival time with increasing cyp1b1 gene dosage.

More importantly, we have utilized this model to provide evidence that addition of phytochemicals to the maternal diet can ameliorate these DBP-dependent cancers. Incorporation of green tea in drinking water provided evidence that caffeine has a major role in the protective effects of green tea. Epigallocatechin-3-gallate was also effective in limiting the lung tumor burden in the offspring. However, the most effective protocol of all was co-administration of chlorophyllin (CHL). Incorporating CHL in the maternal diet had no impact of DBP transplacental carcinogenesis, yet if it was administered concurrently with DBP there was a 50% increase in lymphoma survival and nearly a 50% reduction in lung tumor multiplicity, further supporting a mechanism involving complex-mediated reduction in carcinogen uptake. These results present a new paradigm of dietary cancer chemoprevention which focus on early stages of development and also provides a strategy to potentially lessen tumor burden later in adult life.

Critical Windows of Transplacental Carcinogenesis: Identifying Efficacious Approaches for Chemoprevention with Dietary Phytochemicals

by
David J. Castro

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

David J. Castro, Author

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CONTRIBUTION OF AUTHORS

Dr. David E. Williams provided guidance and critical review of experimental design, data analysis and manuscript preparation. Dr. Cliff Pereira assisted in the experimental design of each tumor study and performed statistical modeling and analysis with the aid of Jack N. Giovanini for the studies in chapters 2,3,4. Drs. Katrina M. Waters and Bobbie-Jo Webb-Robertson provided the statistical modeling and analysis in chapter 5. Dr. Christiane V. Löhr and Kay A. Fischer were responsible for the histological analysis in each tumor study. Dr George S. Bailey was involved in the data interpretation and manuscript preparation of the tumor study in chapter 5. Drs. Roderick H. Dashwood and Gayle A. Orner assisted in the experimental design and manuscript preparation of chapter 4. Dr. Frank J. Gonzalez provided the mice utilized in chapter 3. Drs. William M. Baird and Sharon K. Krueger assisted in the manuscript preparation of chapter 3.

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Chapter 1. Introduction

Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are a class of multiphasic organic compounds composed of fused aromatic rings (Registry, 1995; Finlayson-Pitts and Pitts, 1997) . They are generated by the incomplete combustion of organic material and are a major product of fuels used for energy production (coal, diesel, gasoline, etc.). In fact, PAHs were the first environmental/occupational chemical linked to human cancers (chimney sweeps in 18th century London and scrotal cancer)(Cherniack, 1992). Due to the reliance on fossil fuels in the developing world, PAHs have persisted as ubiquitous environmental contaminants. For example, China's vast industrial network and power-plant system rely on coal for ~70% to 75% of their energy needs compared with 14%, 22%, and 53% for Japan, the United States, and India, respectively (Millman *et al.*, 2008). As a consequence, numerous environmental, biomedical and molecular/epidemiologic studies have been undertaken with these environmental pollutants. Some of the most intensively studied PAHs include benzo[*a*]pyrene (BaP), 7,12-dimethylbenz[*a*]anthracene (DMBA), and dibenzo[*a,l*]pyrene (DBP). Indeed, each of these compounds have been identified as chemical carcinogens in numerous animal models. PAHs are thought to

exert mutagenic and carcinogenic effects in biological systems upon metabolic activation to chemically reactive species (Shimada and Fujii-Kuriyama, 2004). The aryl hydrocarbon receptor (AHR) has a high affinity for planar aromatic compounds and has been shown to mediate the activation of numerous PAHs. In what can be defined as an adaptive pathway, the AHR binds its ligands and up-regulates a battery of xenobiotic metabolizing enzymes including cytochrome P450s (CYP) and phase 2 enzymes such as glutathione-S-transferases (GST).

Dibenzo[*a,l*]pyrene[1] is of particular interest because it carries such high carcinogenic potential. The presence of a "fjord region" in the chemical structure lends to the formation of reactive epoxy-dihydrodiols that are very potent mutagens and carcinogens (Luch *et al.*, 1999a; Melendez-Colon *et al.*, 2000) (figure 1-1).

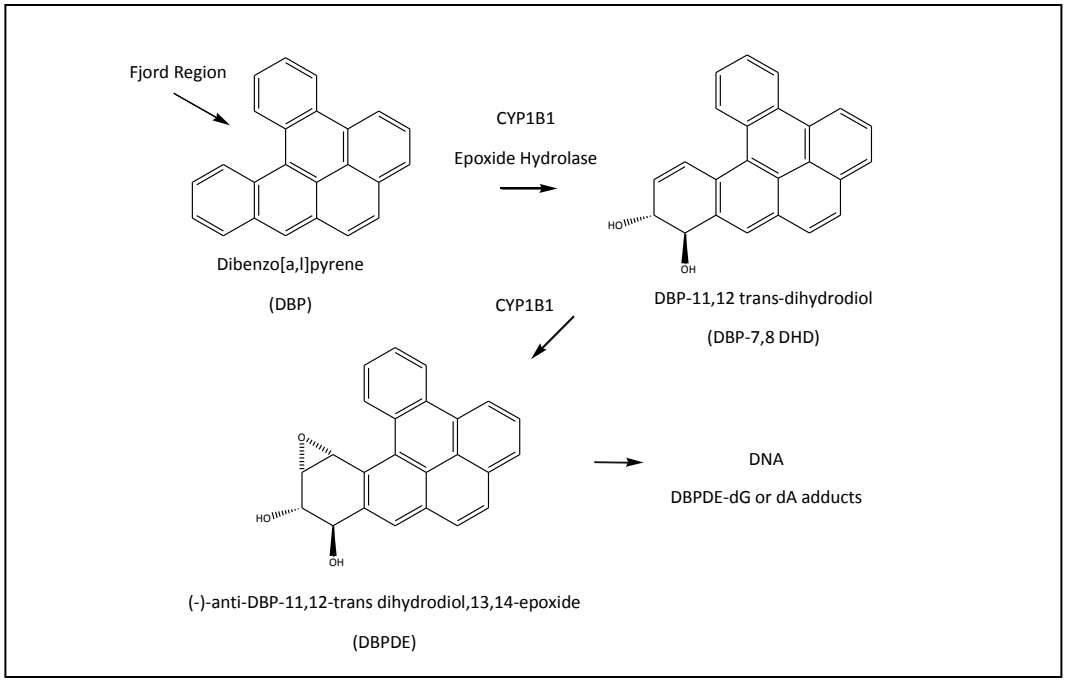


Figure 1-1. Cyp1b1-dependent Metabolic Activation of DBP

[1]CAS # 191-30-0: Dibenzo[def,p]chrysene

In the case of DBP, the most carcinogenic metabolite is primarily formed via cyp1b1 mediated metabolism. The relative contribution of cyp1b1 has been supported by *in vitro* studies with expressed enzymes and more convincingly with cyp1b1 null mice. Cyp1b1 is expressed constitutively (and following ahr-mediated induction) in various extrahepatic target tissues including bone marrow, ovaries, lung, skin, and thymus (Spivack *et al.*, 2001; Choudhary *et al.*, 2003). Accordingly, in adolescent mice, the absence of the *cyp1b1* gene provided protection against lymphoma, ovarian, and skin cancer following oral administration of DBP (1.07 mg/Kg given once daily 5 times a week for 3 weeks by gavage; total dose of 16 mg/Kg) (Buters *et al.*, 2002; Luch *et al.*, 2002).

Transplacental Carcinogenesis

Although anthropogenic contaminants such as PAHs could have detrimental effects on all members of a population, the very young are at particular risk. Experimental and human evidence indicates that the fetus and neonate are more susceptible to carcinogens in part due to underdeveloped DNA repair and detoxication capacity as well as the delicate balance of rapid proliferation and apoptosis required at such an early stage of development (Goldman, 1995; Anderson *et al.*, 2000; Charnley and Putzrath, 2001; Whyatt *et al.*, 2001). Moreover, fetal and childhood exposure to carcinogens can increase risk of disease in adulthood. The cellular or tissue damage incurred or "initiated" will have the opportunity to develop

to malignancy or acquire additional damage over the course of life (Ha *et al.*, 2003; Anderson, 2004b; Gauderman *et al.*, 2007).

It is important to note that the etiology of 80-90% of childhood cancers are unknown, but considerable evidence exists for maternal exposure to environmental chemicals as contributors to development of childhood leukemias and lymphomas (Wild and Kleinjans, 2003; Anderson, 2004a; Bunin, 2004). In fact, these two types of cancers are the second leading cause of deaths after accidents, in children in the US (Lightfoot and Roman, 2004) . As such, rodent transplacental cancer models have been developed to investigate the impact of chemicals for which there are significant human exposures. These chemicals belong to many different classes, including PAHs, nitrosamines, amino acid pyrrolysis products and heavy metals. In the majority of studies, *in utero* exposures to chemical carcinogens produce cancers in middle age adult offspring. For example, *in utero* exposure to the food mutagen, 2-amino-1-methyl-6-phenylimidazol[4,5-*b*]pyridine (PhIP), increased the incidence and multiplicity of mammary adenocarcinomas in 47 week old rats (Hasegawa *et al.*, 1995). Similarly, the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), yielded increases in lung tumorigenesis in offspring when exposed *in utero* (Anderson *et al.*, 1989a).

The investigation of PAHs as transplacental carcinogens originated with work back in the mid 1980's with the synthetic PAH, 3-methylcholanthrene (3-MC) (Anderson *et al.*, 1985). When given 3-MC transplacentally, 3-MC was an effective lung and liver carcinogen in the adult offspring. More importantly, they were able to

show that the potency and efficacy of 3-MC was a function of the ahr. Because the mouse has four *ahr* alleles, 3 of which code for a dominant "responsive" receptor, they were able to design a breeding strategy to examine the contribution of both the maternal and fetal ahr phenotype in the tumor response. At the conclusion of the study, genotyping established that the cancer risk was greater for the offspring if the mother was non-responsive and if the fetus was responsive (independent of maternal genotype)(Anderson *et al.*, 1989b; Miller *et al.*, 1989; Miller *et al.*, 1990). The interpretation of these findings was that a non-responsive mother was not as capable of inducing enzymes necessary for the metabolism and excretion of 3-MC, thereby leading to greater bioavailability to the fetus. In turn, a responsive fetus was more susceptible because the 3-MC activation of the fetal ahr was more effective at induction of enzymes required for bioactivation (cyps).

Our laboratory has developed a similar mouse model of transplacental carcinogenesis. However, we have extended this model by utilizing DBP, an environmentally relevant PAH carcinogen. Interestingly, rather than the sole development of lung adenomas and hepatocellular carcinomas at ~1 year of age, our mice develop an aggressive lymphosarcoma of T-cell origin producing mortalities as early as 12 weeks of age (Yu *et al.*, 2006b). With respect to the ahr, as with the 3-MC study, both maternal and fetal ahr phenotype affected the carcinogenic response of DBP. Offspring of non-responsive dams had greater mortalities compared to those born from responsive dams. Among the fetal populations, independent of maternal environment, ahr responsiveness enhanced risk of developing the thymic lymphoma.

Because cyp1b1 is a downstream target of the ahr, these results are in agreement with documented findings that cyp1b1 has the highest activity of all the cyps in bioactivation of DBP to its most mutagenic and carcinogenic metabolite (Crespi *et al.*, 1997; Ralston *et al.*, 1997; Luch *et al.*, 1998; Luch *et al.*, 1999a; Luch *et al.*, 1999b; Melendez-Colon *et al.*, 1999; Kleiner *et al.*, 2004; Mahadevan *et al.*, 2005). The expression of cyp1b1 in the fetal target tissues; thymus, lung, and liver, especially late in gestation, further points to the importance cyp1b1 in DBP-initiated transplacental carcinogenesis (Choudhary *et al.*, 2003; Choudhary *et al.*, 2005).

Cancer Chemoprevention

In addition to environmental factors, increasing evidence has shown that dietary factors can have a significant influence on cancer risk. In the seminal review by Doll and Peto (1981) it was first estimated that diet contributed to about 30% of cancers in the U.S. In subsequent years, a number of epidemiological studies have demonstrated that consumption of cruciferous vegetables, such as cabbage, broccoli, brussels sprouts, and cauliflower, are inversely associated with frequency of several cancers (Verhoeven *et al.*, 1996; van Poppel *et al.*, 1999). As a result, a great deal of research has focused on plant-derived compounds, known as phytochemicals, to establish mechanisms of protection against cancer.

Isothiocynates and indoles are degradation products of glucosinolates, which are present naturally in cruciferous vegetables and have been widely studied for their chemopreventative properties (Murillo and Mehta, 2001). A ground-breaking study

by Wattenburg and Loub provided the first evidence of dietary indole-3-carbinol (I3C) chemoprotection against PAH-induced cancer (Wattenberg, 1977). Since then, I3C has been shown to prevent or reduce the risk of cancer in a number of animal models of carcinogenesis (reviewed in Dashwood, 1998). Several mechanisms may account for the anti-cancer properties of I3C including changes in cell cycle progression, apoptosis, carcinogen bioactivation and DNA repair (reviewed in Kim and Milner, 2005).

In our mouse model of DBP-dependent transplacental carcinogenesis, we have recently demonstrated that maternal dietary treatment with I3C can be effective in reducing DBP-dependent lymphoma mortality as well as the severity of lung adenomas in middle age mice (Yu *et al.*, 2006c). Interestingly, the level of protection was not associated with ahr status, especially considering I3C is an established ligand of the ahr. Regardless, these findings introduce an effective strategy in reducing the incidence of cancers in children and possibly lessen tumor burden later in adult life. These findings also warrant the examination of other known cancer chemoprotective agents towards this potential application of transplacental chemoprevention against PAH-dependent cancers.

Green Tea Polyphenols

Tea is second only to water in terms of worldwide popularity as a beverage. Green tea is made from fresh tea leaves through a specific blanching, twisting, and drying process that precludes the oxidation of green leaf polyphenols. The dried tea

leaves contain polyphenols such as flavanols (catechins), flavandiols, and phenolic acids. The major green tea catechins include epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG), epicatechin (EC), and caffeine. These polyphenols may constitute up to 30% of the dry leaf weight (Graham, 1992).

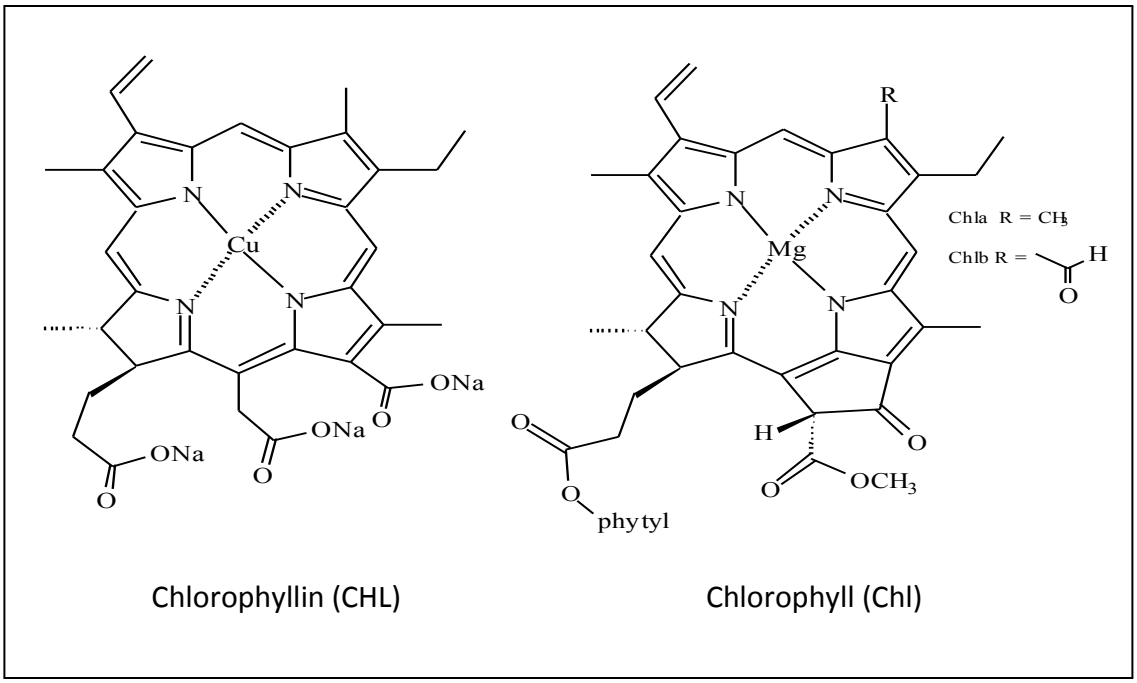
The ability of green tea to antagonize the carcinogenicity of chemicals has been shown repeatedly in numerous animal models (reviewed by Chung *et al.*, 2003). This includes a wide variety of structurally diverse chemical carcinogens; PAHs (Wang *et al.*, 1994), nitrosocompounds (Wang *et al.*, 1992) , heterocyclic amines (Xu *et al.*, 1996; Orner *et al.*, 2002) and hydrazines (Inagake *et al.*, 1995). A number of mechanisms have been postulated for the chemoprotective efficacy of teas, including protection against oxidative damage, scavenging of electrophiles, inhibition of cell proliferation, stimulation of apoptosis, inhibition of enzymes responsible for procarcinogen bioactivation and induction of enzymes catalyzing the detoxication of carcinogens (reviewed in Clark and You, 2006) . As the most abundant catechin component, EGCG has been identified as the key constituent in green tea's preventive activity against cancer (reviewed in Khan *et al.*, 2006). However, the mechanisms of action for EGCG have been investigated mostly in cell culture systems. Utilizing human cancer cell lines, EGCG has been shown to inhibit mitogen-activated protein kinase cascades, DNA methyltransferase, epidermal growth factor receptor signaling and others (Fang *et al.*, 2003; Vittal *et al.*, 2004; Hou *et al.*, 2005; Hussain *et al.*, 2005).

In spite of the numerous studies investigating the biological activities of EGCG and other tea constituents, the exact mechanisms of cancer prevention by tea and tea constituents still remain elusive. As we move forward, it is important to demonstrate the efficacy of tea and tea constituents in relevant animal models of tumorigenesis. This could shed light on why epidemiological studies have generated inconsistent results; some of which associated tea with reduced cancer risk, whereas others found that tea lacks efficacy against certain human cancers (Mukhtar and Ahmad, 1999; Chung *et al.*, 2003). The employment of a transplacental cancer model could potentially address issues associated with bioavailability (intestine and placenta) while allowing for the contribution of multiple molecular targets in the inhibition of carcinogenesis.

Chlorophyllin and Chlorophylls

Chlorophyllin (CHL) is the water soluble derivative of the natural plant pigment chlorophyll (Chl). CHL and Chl belong to the family of porphyrins, consisting of four pyrroline subunits interconnected via their α carbon atoms by way of methane bridges. The removal of phytol chains and replacement of magnesium with copper distinguishes CHL from Chl (Fig. 1-2).

The utility of CHL extends to various arenas. It is the active ingredient in a number of preparations intended to reduce odors associated with incontinence, commonly used in geriatric patients (Young and Beregi, 1980; Nahata *et al.*, 1983). It is also available as a topical preparation, useful for both treatment and odor control



Chlorophyllin (CHL) Chlorophyll (Chl)

Figure 1-2. Structures of CHL and Chl

of wounds, injuries and other skin conditions. Importantly, neither CHL nor Chl are associated with significant human toxicity. Most of the current research with CHL has focused on understanding its potential as an anti-carcinogen/anti-mutagen. The antimutagenic activity of CHL has been identified against a broad range of direct- and indirect-acting mutagens, including aflatoxins (Dashwood *et al.*, 1991; Breinholt *et al.*, 1995a) , polycyclic aromatic hydrocarbons (Arimoto *et al.*, 1993; Harttig and Bailey, 1998), heterocyclic amines (Dashwood, 1992; Dashwood *et al.*, 1996), alkylating agents (Guo and Dashwood, 1994) and several miscellaneous compounds.

Common amongst porphyrins is their ability to bind metals and form complexes. However, several mechanisms have been postulated for the anti-mutagenicity associated with CHL; including scavenging of free radicals and active

oxygen species, inhibition of CYPs involved in procarcinogen bioactivation and tight complex formation with the mutagen/carcinogen preventing uptake across cell membranes (reviewed in Sarkar *et al.*, 1994). Because of its high toxic threshold and effectiveness against aflatoxin B_1 (AFB_1) hepatocarcinogenesis in animal models, CHL was utilized in a human clinical intervention trial in Qidong, China, where dietary AFB_1 exposure is a serious concern (Yu, 1995). A relatively small dose of CHL (100 mg), given with meals, for a relatively short period of time (3 months) was effective at reducing the levels of aflatoxin-N^7-guanine adducts in urine samples by more than half (Egner *et al.*, 2001). Subsequent studies done in rainbow trout and rats have indicated that the predominant mechanism of protection involves the reduction of carcinogen uptake from the GI thereby reducing the bioavailable dose (Pratt *et al.*, 2007; Simonich *et al.*, 2007; Simonich *et al.*, 2008). Due to the observed chemopreventive efficacy in numerous animal models/species, evaluating the efficacy of CHL in a transplacental carcinogenesis model would be invaluable for such a critical/susceptible stage of life.

Hypothesis and Objectives

Based on the information presented above, we hypothesize that the transplacental carcinogenicity of the environmental PAH, DBP, is mediated by cyp1b1 bioactivation in the thymus resulting in dysregulation of genes important in normal T-cell development. The incorporation of our DBP-transplacental model should shed light on the issue of fetal versus maternal metabolism in PAH-transplacental

carcinogenesis. Further, although DBP could be expected to partition into breast milk due to its lipophilicity, we hypothesize that the majority of exposure occurs transplacentally and the fetus is more sensitive than the infant. The development of our transplacental mouse model of carcinogenesis also allows us to investigate the effect of dietary phytochemicals in the modulation of DBP-dependent carcinogenesis. We hypothesize that maternal consumption of green tea will provide chemoprotection against DBP-dependent transplacental carcinogenesis. The mechanisms investigated upon green tea consumption will establish the role of the green tea catechins, caffeine and EGCG, in the chemoprotective potential of green tea. Similarly, we hypothesize that CHL will ameliorate the carcinogenicity of DBP in our transplacental mouse model. Additionally, we hypothesize that CHL and Chl, will be more effective as pure compounds rather than delivery via a natural component of freeze-dried spinach. The study will also be able to identify the contribution of systemic effects of CHL and investigate the importance of complexation as a mechanism of CHL chemoprevention. Overall, these studies will provide information about the mechanisms for DBP-dependent transplacental carcinogenesis and provide insight into the potential for chemoprevention in reducing PAH-dependent cancer mortality.

Chapter 2

Lymphoma and Lung Cancer in Offspring Born to Pregnant Mice dosed with Dibenzo[*a,l*]pyrene: The Importance of *In utero* vs. Lactational Exposure

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ABSTRACT

The fetus and neonate cannot be viewed as "little adults"; they are highly sensitive to toxicity from environmental chemicals. This phenomenon contributes to the fetal basis of adult disease. One example is transplacental carcinogenesis. Animal models demonstrate that environmental chemicals, to which pregnant women are daily exposed, can increase susceptibility of the offspring to cancer. It is uncertain to what degree *in utero* vs. lactational exposure contributes to cancer, especially for hydrophobic chemicals such as polyhalogenated biphenyls, ethers, dioxins, furans, etc., which can partition into breast milk. We developed a pregnant mouse model in which exposure to the polycyclic aromatic hydrocarbon (PAH), dibenzo[*a,l*]pyrene (DBP), during late gestation, produces an aggressive T-cell lymphoma in offspring between 3 and 6 months of age. Survivors exhibit multiple lung and liver (males) tumors. Here, we adopt a cross-foster design with litters born to dams treated with DBP exchanged with those born to dams treated with vehicle. Exposure to DBP *in utero* (about 2 days) produced significantly greater mortality than residual DBP exposure only through breast milk (3 weeks of lactation). As previously observed, pups in all groups with an $ahr^{b\text{-}1/d}$ ("responsive") genotype were more susceptible to lymphoma mortality than $ahr^{d/d}$ ("non-responsive") siblings. At termination of the study at 10 months, mice exposed *in utero* also had greater lung tumor multiplicity than mice exposed only during lactation. Our results demonstrate

that short exposure to DBP during late gestation presents a greater risk to offspring than residual exposure to this very hydrophobic PAH following 3 weeks of nursing.

INTRODUCTION

The fetus and neonate are exposed to a number of toxic chemicals in the environment through a variety of exposure routes, often dietary (Autrup, 1993; McLachlan, 1993; Birnbaum, 1994; Sasco and Vainio, 1999; Landrigan *et al.*, 2002; Schonfelder *et al.*, 2002; Perera *et al.*, 2003; Massart *et al.*, 2005; Wang *et al.*, 2005; Fenster *et al.*, 2006; Perera *et al.*, 2006; Schnaas *et al.*, 2006; Smith *et al.*, 2006). It has been recognized for a number of years that the fetus and neonate exhibit increased sensitivity to a number of these environmental chemicals (Landrigan *et al.*, 2004). Regulatory agencies have adopted the policy that a child is not a "little adult" and most acceptable exposure levels incorporate a safety factor to specifically recognize that fact (Landrigan *et al.*, 2004). The study of epigenetics and imprinting has shown that exposure to agents during early stages of development can program gene expression such that susceptibility to disease in later life is impacted (Ho and Tang, 2007; Prins *et al.*, 2008).

A number of animal models of transplacental cancer have been developed (Brouwer *et al.*, 1995; Miller *et al.*, 2000; Anderson, 2004a; Anderson, 2004b; Miller, 2004; Liu *et al.*, 2007). Epidemiology studies in human populations find high

exposures to environmental chemicals associated with impacts on offspring including birth weight, behavioral endpoints and diseases including cancer, although not all studies show a positive correlation (Autrup, 1993; McLachlan, 1993; Birnbaum, 1994; Sasco and Vainio, 1999; Whyatt *et al.*, 2001; Jacobson *et al.*, 2002; Schonfelder *et al.*, 2002; Whyatt *et al.*, 2002; Eskenazi *et al.*, 2003; Perera *et al.*, 2003; Wild and Kleinjans, 2003; Miller *et al.*, 2004; Soechitram *et al.*, 2004; Whyatt *et al.*, 2004; Massart *et al.*, 2005; Wang *et al.*, 2005; Dallaire *et al.*, 2006; Fenster *et al.*, 2006; Leem *et al.*, 2006; Perera *et al.*, 2006; Schnaas *et al.*, 2006; Smith *et al.*, 2006).

Exposure to environmental carcinogens will occur *in utero* if the chemical can effectively cross the placenta and during nursing if capable of partitioning into breast milk. The beneficial effects of breast feeding for infants have been repeatedly demonstrated (Turck, 2005). Still, many women worry about chemical exposure to their nursing infants given the number of chemicals, including polyhalogenated biphenyls, dioxins, ethers, dibenzofurans, etc., measured in breast milk; the levels of some being positively correlated to adverse impacts in children (McLachlan, 1993; Landrigan *et al.*, 2002; Massart *et al.*, 2005).

Our laboratory has developed a mouse model of transplacental cancer with the potent polycyclic aromatic hydrocarbon (PAH), dibenzo[*a,l*]pyrene (DBP). Administration of DBP during late gestation produces a high rate of mortality in the offspring from an aggressive T-cell lymphoma (Yu *et al.*, 2006b). If the offspring do not succumb to the lymphoma, 100% have multiple lung tumors and most males liver lesions. We developed this model as a potential novel approach to dietary

chemoprevention. Inclusion of known cancer chemopreventive agents in the maternal diet (e.g., indole-3-carbinol (I3C) or green tea) or co-administration with chlorophyllin markedly reduced lymphoma mortality and/or lung cancer, even though the offspring were not exposed to these chemopreventive agents in their diet (Yu *et al.*, 2006c; Castro *et al.*, 2008b; Castro *et al.*, 2009).

In further characterization of this model, we felt it important to address the question of how much of the cancer in the offspring was due to DBP bioavailability and bioactivation in fetal target organs during the 2 days of *in utero* exposure compared to neonatal exposure and bioactivation during 3 weeks of nursing. This information is important in estimating risk of maternal diets and supplements and drug or xenobiotic exposure during nursing and in design of optimal chemoprevention strategies.

We report here that the brief *in utero* exposure contributed to a greater degree than residual DBP partitioning into breast milk. This observation was accomplished by switching the litters at birth so that they were exposed only *in utero* or only during breast feeding. We also included a group where the litters were not cross-fostered to compare the results to offspring that could accumulate the PAH through both routes of exposure. These results applied to both DBP-dependent lymphoma mortality and to lung tumor multiplicity and highlight the importance of reducing exposures as much as possible to toxic chemicals in the environment during pregnancy and to the potential for supplementation of the maternal diet with cancer chemoprotective agents.

METHODS

Chemicals and diets

DBP was obtained from the NCI carcinogen repository at the Midwest Research Institute (Kansas City, MO) and was at least 98% pure as determined by HPLC. The semipurified diets, AIN93G and AIN93M, were purchased from Research Diets (New Brunswick, NJ). Ethidium bromide was purchased from Sigma Chemical Co. (St. Louis, MO). Direct PCR lysis reagent and proteinase K was purchased from Viagen Biotech Inc. (Los Angeles, CA). Taq PCR master mix was purchased from Promega Co. (Madison, WI) and Novex TBE Gels were purchased from Invitrogen Technologies (Carlsbad, CA).

Animals and treatment protocols

Eight-week old B6129SF1 female and 129S1/SvImJ male mice (Jackson Laboratories, Bar Harbor, ME) were housed at the Laboratory Animal Resource Center at Oregon State University. The mice were maintained in a pathogen free environment at 20 ± 1˚ C and 50 ± 10% humidity, with a light dark cycle of 12 h in micro-isolator cages (Life Products, Inc., Seaford, DE) with CareFRESH bedding. During breeding, gestation, and lactation mice were allowed free access to AIN93G diet. After acclimation for 1 week, mice were bred and appearance of the vaginal plug determined to be day 0. Pregnant mothers were treated on the 17^{th} day of gestation by oral gavage with either vehicle (corn oil, 5 mL/kg body weight) or 15

mg/kg DBP in the same volume of corn oil. On the first day following parturition, a litter born to a dam receiving DBP was cross-fostered with a litter born to a mother receiving corn oil vehicle. Another subset of litters was not cross-fostered and represented either total DBP exposure (transplacental and lactational) or vehicle control. Figure 2-1 details the group assignments, experimental timeline, as well as the number of offspring in each experimental group. Upon weaning, mice remained on AIN93G diet until switching to AIN93M at 3 months of age. If any signs of distress or pain (or any sign of morbidity) were observed, mice were immediately euthanized with an overdose of CO_2 and a necropsy performed. No lymphomas were observed in offspring that were born to mothers receiving vehicle alone (data not shown). At 10 months of age, the study was terminated and any surviving mice euthanized and necropsied. All procedures used in the handling, treatment and husbandry of mice were approved by the Oregon State University Institutional Animal Care and Use Committee.

Histopathology

Upon necropsy, heart, thymus, lung, spleen, liver, kidney, abnormal lymph nodes, testes or ovaries, colon and skin were fixed in 10% formalin, stained with hematoxylin and eosin and analyzed as described previously (Yu *et al.*, 2006b; Yu *et al.*, 2006c; Castro *et al.*, 2008a). The incidence of lymphoma leading to morbidity as well as lung lesions were scored by gross necropsy and a sub-set from each group confirmed by histopathology.

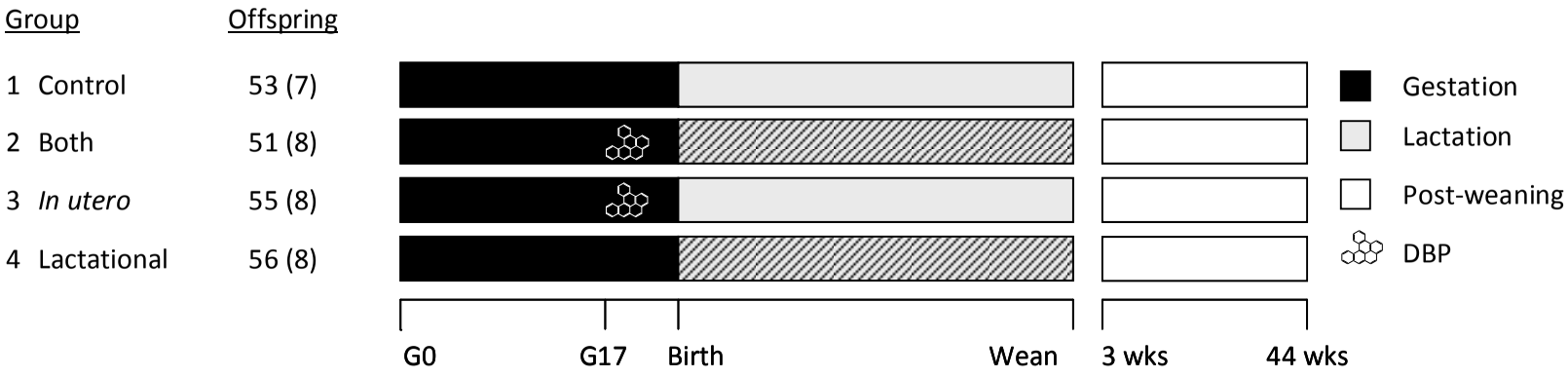

Figure 2-1. **Experimental design of DBP exposure in offspring**. Pregnant mice were administered DBP or corn oil vehicle at GD 17. Offspring were cross-fostered within 12 h from birth and consequently exposed to DBP (diagonal bars) via placenta (group 3) and/or via nursing (groups 2 and 4).

Genotyping for aryl hydrocarbon receptor (ahr) b-1 and d alleles

We previously demonstrated that the mouse *ahr* genotype influenced offspring susceptibility to lymphoma mortality (Yu *et al.*, 2006b). Our breeding scheme was designed such that, on average, litters should be comprised of half $ahr^{b-1/d}$ and half $ahr^{d/d}$ siblings. At necropsy, an 8 mm ear-punch was collected and lyzed overnight at 55˚C in 100 µl of DirectPCR Lysis Reagent containing proteinase K, followed by 45 min at 85˚C. The resulting lysate was briefly centrifuged prior to undergoing a PCR reaction with allele-specific primers to permit one-tube genotyping of the *ahr* alleles as previously described (Yu *et al.*, 2006b). PCR products were separated and visualized on Novex 8% Tris-borate EDTA gels. Ahr responsiveness ($ahr^{b-1/d}$) was confirmed by the presence of two PCR products of 159 bp (ahr^{b-1}) and 148 bp (ahr^{d}). Non-responsive mice had a single product of 148 bp. A molecular weight ladder of *Msp*I cut pBR322 DNA and ethidium bromide was used to stain the DNA, followed by UV visualization.

Statistical analysis

The experimental unit for dietary treatments is the dam and offspring responses are, therefore, clustered by litter. Offspring survival curves were compared between treatments using Cox proportional hazards regression and a robust sandwich adjustment to the score test to account for any within-litter dependence. The proportional hazards assumption appeared reasonable based on assessment of Martingale residuals in the SAS phreg procedure (version 9.2).

Offspring tumor multiplicity data were modeled with methods appropriate for counts clustered into litters. Results shown are from a generalized linear mixed model with random litters, log link and negative binomial conditional distribution which was fit with the SAS glimmix procedure (version 9.2). P-values are from an overall model for all survivors. Estimates of multiplicity ratios comparing *ahr* genotypes within each treatment are from separate model fits for each treatment. Very similar results and the same conclusions were reached using two other modeling approaches for counts: (1) Transform response to the square root of the tumor count, then apply linear mixed models with random litters using the SAS mixed procedure and (2) generalized linear mixed model with random litters, log link, Poisson conditional distribution and empirical residual variation (quasilikelihood). All approaches revealed strong evidence of within-litter dependence and the need, therefore, to retain random litters in the models.

The effect of *in utero* DBP on birth weights and litter size was investigated by comparing the lactational litters (no DBP *in utero*) to the litters from the other two treatments (both of which received DBP *in utero*). For per offspring birth weights linear mixed models with random litters was used (SAS mixed procedure). For per dam litter sizes one-way ANOVA was used.

RESULTS AND DISCUSSION

The experimental design of the cross-foster study resulted in 4 groups of offspring and a total of 215 mice. The timeline of exposure as well as the number of dams and offspring used for each experimental group is given in Figure 2-1. As seen previously (Yu *et al.*, 2006b; Yu *et al.*, 2006c; Castro *et al.*, 2008a), treatment of pregnant mice with DBP did not elicit acute maternal or fetal toxicities; there was also no evidence of reduction in litter size or birth weight ($p>0.4$ for all comparisons, data not shown)**.** Offspring born to dams treated with DBP exhibited the first mortalities at about 6 weeks of age but, as previously observed (Yu *et al.*, 2006b), the rate of mortality increased markedly between 12 and 24 weeks of age (Figure 2-2). In litters where offspring exposed to DBP *in utero* were switched at birth and nursed by dams treated only with vehicle, the survival curves demonstrated significantly less ($p=0.013$) mortality than pups in litters nursed by their own mother. There were surprisingly few DBP-dependent mortalities in litters born to control mothers that we cross-fostered to mothers that had been treated with DBP. These results suggest that only a small portion of the lymphoma risk was from DBP exposure in breast milk (pups exposed in utero only had significantly lower survival ($p=0.0087$) than pups exposed only during nursing). It is possible that a relatively small amount of the DBP partitions into breast milk. If the DBP is eliminated fairly rapidly from maternal compartments (e.g., t½ on the order of hours), there may be little left to be available

to the pups through nursing. We are conducting further studies employing ^{14}C-DBP to address these pharmacokinetic issues.

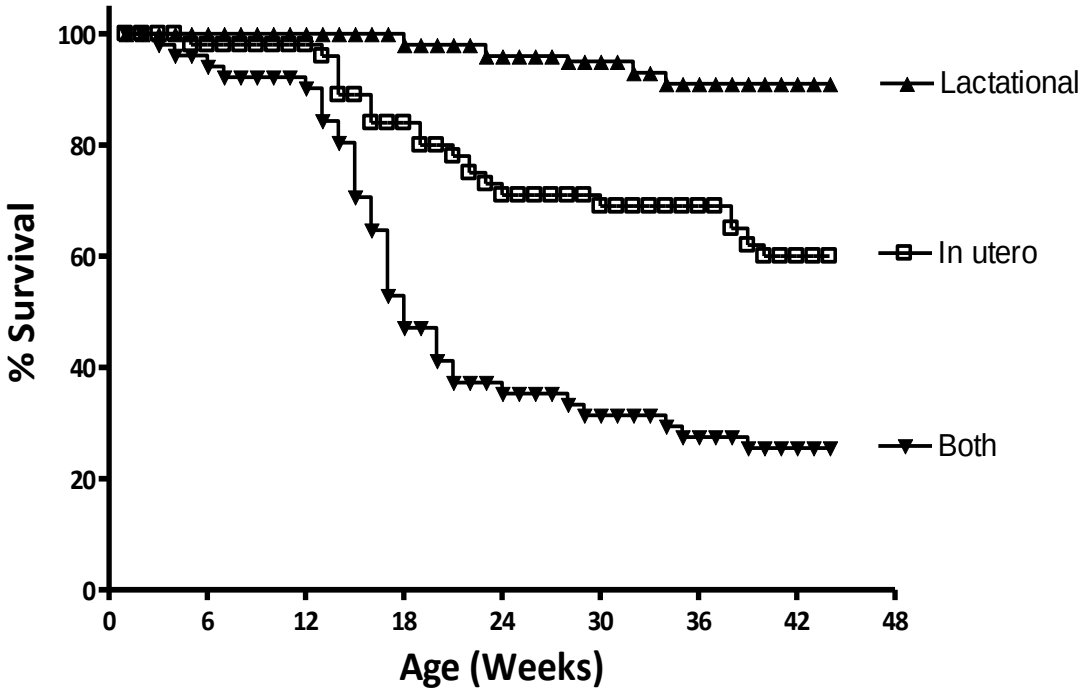

Figure 2-2. Survival curves for DBP-dependent lymphoma morbidity in offspring born to dams treated with 15 mg/kg at gestation day 17. The litters either remained with their mother during nursing (▼) or were cross-fostered. Some litters born to mothers dosed with DBP were immediately transferred to mothers treated with vehicle only (□, *in utero* exposure only); conversely, some litters were taken immediately after birth from mothers treated with vehicle and transferred to DBP-treated mothers (▲ , lactational only) for the entire 3 weeks of nursing. There is strong statistical evidence of treatment differences (p=0.009 for lactational only vs. *in utero* and p=0.013 for *in utero* only vs. both using Cox regression with a robust score test)

In this model of transplacental PAH-dependent cancer, we utilized a C57B6129F1 female back-crossed to a 129 male. This experimental design allowed us to examine the role of the fetal *ahr* genotype in tumor response as litters should have equal numbers of Ahr responsive and non-responsive pups. This allowed us to test hypotheses concerning chemopreventive agents (e.g., I3C) functioning through the Ahr. We previously found a maternal $ahr^{b\text{-}1/d}$ genotype reduced risk to the fetus (probably through enhanced maternal metabolism and reduced bioavailability to the fetus), but a fetal $ahr^{b\text{-}1/d}$ genotype increased the risk of to the offspring irrespective of the mother's genotype (Yu *et al.*, 2006b; Yu *et al.*, 2006c). In this study, we again found a responsive fetal *ahr* genotype reduced the lymphoma survival in all groups (offspring exposed *in utero* and lactationally, 19.2 vs. 32%; *in utero* only, 50 vs. 70.4% and lactationally only, 86.7 vs. 96.2%, respectively, Table 2-1). If the lymphoma survival curves shown in Figure 2-2 were plotted for each genotype, it would clearly demonstrate that ahr-responsive pups in all three groups have reduced lymphoma survival ($p=0.0217$ using a Cox proportional hazards regression with treatment as strata and with a robust test score to adjust for litters; data not shown). The thymus (mouse and human) expresses high levels of Cytochrome P450 (Cyp) 1b1 in late gestation and shortly following parturition (Choudhary *et al.*, 2003; Choudhary *et al.*, 2005). Cyp1b1 has the greatest activity of any CYP in bioactivation of DBP to carcinogenic metabolites, particularly DBP-(-)-*anti*-(11*R*,12*S*,13*S*,14*R*)-dihydrodiol epoxide (Luch *et al.*, 1998; Shimada *et al.*, 2001a; Shimada and Fujii-Kuriyama, 2004). In an earlier publication, employing *cyp1b1* knockout mice, we demonstrated a

Table 2-1

Influence of Genotype on Offspring Survival and Lung Tumor Multiplicity in 10 Month Survivors

	AhR Responsive (b-1/d)		AhR Non-Responsive (d/d)		Multiplicity Ratio
Group	Survival (%)	Lung tumor Multiplicity	Survival (%)	Lung tumor Multiplicity	b-1/d over d/d (± 95% CI)
Both	19.2 (5/26)	15.2 ± 4.7	32.0 (8/25)	14.1 ± 3.0	1.12 (0.66, 1.92)
In utero	50.0 (14/28)	15.7 ± 1.7	70.4 (19/27)	8.4 ± 0.9	1.54 (1.20, 1.97)
Lactational	87.1 (27/31)	3.1 ± 0.5	96.0 (24/25)	2.5 ± 0.3	1.06 (0.69, 1.64)

strong gene dosage effect. Cyp1b1 null offspring were completely resistant, wild-type siblings sensitive and the hets intermediate with respect to lymphoma-dependent mortality (Castro *et al.*, 2008a). In this study, we do not see complete resistance in offspring with the *ahr*$^{d/d}$ genotype. The *ahr*^{d} allele is still capable of induction by potent ahr ligands or by higher doses of ligands with more modest binding affinities. DBP may function in either of these modes and induce Cyp1b1 to some degree in the *ahr*$^{d/d}$ offspring. Alternatively, these "non-responsive" mice may exhibit some lymphoma-dependent mortality due to bioactivation by other CYPs, perhaps with contributions from peroxidases (Cavalieri and Rogan, 1990) and/or aldo-keto reductases (Palackal *et al.*, 2001). Fetal Cyp1b1 expression in thymus may also be controlled by an ahr-independent mechanism, perhaps involving methylation of the *cyp1b1* promoter region (Nakajima et al., 2003; Tokizane et al. 2005). These potential mechanisms require further study to assess their contribution to DBP carcinogenesis in this model.

Surviving offspring born to dams treated with DBP at 15 mg/Kg exhibit a 100% incidence of lung tumors with a multiplicity per tumor bearing animal of approximately 15. As with the lymphoma mortality, offspring exposed to DBP only *in utero* exhibited greater lung tumor multiplicity than offspring in litters that were exposed to DBP only through nursing (Figure 2-3). When exposure was *in utero*, the surviving offspring that were ahr "responsive" had significantly more lung tumors than their "non-responsive" siblings ($p<0.002$)(Table 2-1). However, the genotype difference was not as apparent with litters exposed to DBP only through nursing, 3.3

and 2.6 for responsive and non-responsive offspring (p>0.5). One possible explanation is that DBP is bioactivated in the lung more efficiently by Cyp1a1 which is induced to a greater degree through the ahr than Cyp1b1. If the reduced lung tumor multiplicity in the lactational only exposure is caused by a lower dose to the infant due to high maternal clearance, this would also explain the lack of impact from ahr signaling. Another possibility would be increased expression of phase II detoxication enzymes following parturition in the lung.

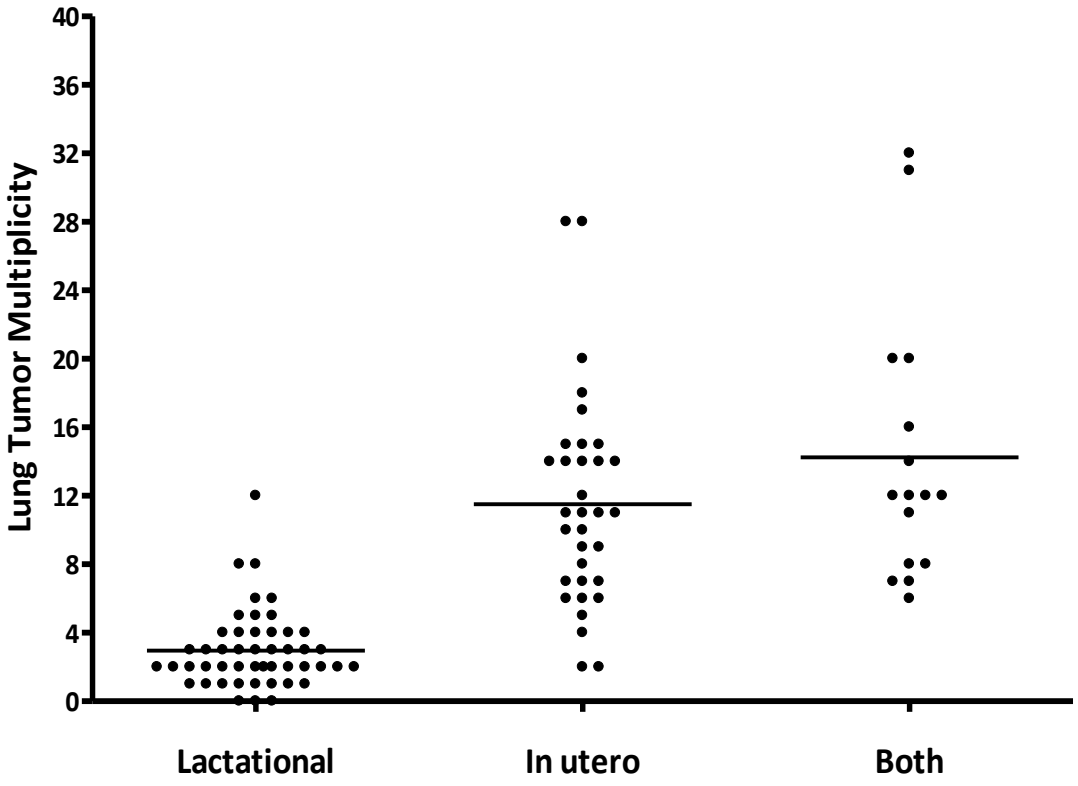

Figure 2-3. DBP-dependent lung tumor multiplicity in offspring. All mice surviving to 10 months of age were euthanized and necropsied. Lung lesions (predominantly adenomas) were quantified and confirmed by histopathology for each of the respective exposure groups (in utero, lactational, and both). Bars indicate group mean, dots represent individual animals.

PAHs represent a "re-emerging" environmental concern. Due to high energy demands, the excess burning of coal and petroleum is increasing the emission of PAHs. For example, 70% of the electricity generated in China and 50% in the U.S., comes from coal burning (Xu *et al.*, 2006). These two countries are also the two largest automobile markets. Particulate matter (PMs), containing PAHs, reach the U.S. west coast from China in less than a week. As women of child-bearing age will be increasingly exposed, we chose PAHs to study in our model of transplacental cancer.

Cancer is the number 1 cause of disease-related death in children (2nd overall, after accidents) and lymphomas/leukemias are the most common of these cancers (Anderson *et al.*, 2000). According to the Leukemia and Lymphoma Society, over 800,000 Americans have leukemia, lymphoma or myeloma with an estimated 135,500 new cases and over 50,000 deaths annually (http://www.leukemia-lymphoma.org). Lung cancer is still the number one cause of cancer mortality in the U.S. with an estimated 213,380 new cases in 2007 and 160,000 deaths (Jemal *et al.*, 2007). The poor survival rate (15%) in lung cancer patients reinforces the need to develop effective prevention strategies.

Our transplacental model of a PAH-dependent T-cell lymphoma and lung cancer, demonstrating that a brief exposure *in utero* is primarily responsible for a robust response, is remarkable given that the cancers occur in what would be the equivalent in humans of young adults (lymphoma) or middle-age (lung cancer). Furthermore, we have developed effective chemopreventive strategies in this model

by supplementation of maternal diet (Yu *et al.*, 2006c; Castro *et al.*, 2008b). In order to devise the most efficacious approaches to chemoprevention, we need to know when the greatest period of sensitivity is. We now show that, in this model, *in utero* exposure carries the greatest risk. This may not be true for carcinogens with different pharmacokinetic properties or pathways of bioactivation. Our long-term goal is to translate this work into potential strategies for providing maximum protection to the fetus from transplacental carcinogens by supplementation or optimizing the maternal diet.

ACKNOWLEDGEMENTS

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Chapter 3

Fetal Mouse *Cyp1b1* and Transplacental Carcinogenesis from Maternal Exposure to Dibenzo[*a,l*]pyrene

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ABSTRACT

Dibenzo(*a,l*)pyrene (DBP) is among the most potent carcinogenic polycyclic aromatic hydrocarbons. Previously, we showed that DBP administration to pregnant mice resulted in high mortality of offspring from an aggressive T-cell lymphoma. All mice that survive to 10 months of age exhibit lung tumors with high multiplicity. Recombinant cytochrome P450 (cyp) 1b1 from mice and the homologue 1B1 in humans exhibit high activity toward the metabolic activation of DBP. Targeted disruption of the *cyp1b1* gene protects against most DBP-dependent cancers. Mice heterozygous for the disrupted *cyp1b1* allele were used to examine the effect of *cyp1b1* gene dosage on DBP transplacental carcinogenesis. Dams were treated with 1 or 15 mg/kg of DBP or 50 mg/kg of benzo(*a*)pyrene. *Cyp1b1*-null offspring did not develop lymphoma, whereas wild-type and heterozygous siblings, born to dams given the high dose of DBP, exhibited significant mortalities between 10 and 30 weeks of age. At 10 months, all groups had lung adenomas or carcinomas [9.5%, 40.3%, 25.6%, and 100% incidences for controls, benzo(*a*)pyrene, 1 and 15 mg/kg DBP, respectively]. *Cyp1b1* status did not alter benzo(*a*)pyrene-dependent carcinogenesis. At 1 mg/kg DBP, *cyp1b1* status altered the incidence of lung tumors (19.0, 27.8, and 28.6% for nulls, heterozygous, and wild-type, respectively). At 15 mg/kg, tumor multiplicities in *cyp1b1* wild-type (9.3) and heterozygous (9.5) offspring were nearly twice that of *cyp1b1*-null siblings (5.0). These data confirm that cyp1b1 bioactivation

of DBP occurs in fetal target tissues, following transplacental exposure, with the thymus and lung as primary and secondary targets, respectively.

INTRODUCTION

The fetus and infant are at increased risk, relative to adults, upon exposure to many environmental chemicals. Yet, only exposures to ionizing radiation and diethylstilbestrol to pregnant women have been sufficiently well documented as causing cancer in their children (Anderson, 2004a; Anderson, 2004b). However, an increasing number of carcinogens have been shown to be effective transplacentally in animal models, including arsenic (Shen *et al.*, 2007), 4-(methynitrosamino)-1-(3-pyridyl)-1-butanone (Anderson *et al.*, 1989a) , 3'-azido-3'-deoxythymidine (Walker *et al.*, 2007), and cooked food mutagens such as 2-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine (Hasegawa *et al.*, 1995) and polycyclic aromatic hydrocarbons (PAH) (Miller, 2004). These data, in addition to the fact that human epidemiologic studies correlate maternal chemical exposure with cancer in children (Anderson, 2004a; Anderson, 2004b), highlight the importance of understanding the mechanism(s) of transplacental carcinogenesis.

We recently developed a mouse model of transplacental carcinogenesis in which maternal exposure to the potent PAH, DBP, resulted in high mortality in offspring at a relatively young age from a T-cell lymphoma. At 10 months, the

offspring exhibited a 100% incidence of lung adenomas and carcinomas and most of the males had liver lesions as well (Yu *et al.*, 2006b; Yu *et al.*, 2006c).

Recombinant mouse cyp1b1 and human CYP1B1 have the highest activity toward conversion of DBP to the (-)-anti-DBP-11,12-trans dihydrodiol,13,14-epoxide metabolite, a "fjord" region diol-epoxide thought to be responsible for the high mutagenic and carcinogenic potency of DBP (Luch *et al.*, 1998; Shimada and Fujii-Kuriyama, 2004). *In vivo* evidence also shows that disruption of the *cyp1b1* gene protected adult mice from DBP cancer at most sites (Buters *et al.*, 2002; Luch *et al.*, 2002).

We tested the hypothesis that DBP is transplacentally available to the fetus and is bioactivated by cyp1b1 in thymus and lung. Cyp1b1 is expressed at relatively high levels in both fetal thymus and lung (Choudhary *et al.*, 2003; Choudhary *et al.*, 2005). The finding that fetal thymus in humans exhibits the highest CYP1B1 expression of any tissue (Choudhary *et al.*, 2005) highlights the relevance of this model for human exposures. We bred *cyp1b1* heterozygote (*cyp1b1*$^{+/-}$) so that all litters should have a 1:2:1 ratio of wild-type/heterozygote/nulls to assess fetal *cyp1b1* gene dosage on DBP transplacental carcinogenesis. *Cyp1b1* knockouts were completely resistant to DBP transplacental lymphoma mortality. In lung, *cyp1b1* genotype influenced tumor multiplicity only at the high dose of DBP, whereas tumor burden was unaffected by genotype in all other groups.

These results confirm that DBP transplacental lymphoma in mouse is due to cyp1b1 bioactivation in fetal thymus. This finding gains further significance in human

fetal risk assessment considering that, in humans, CYP1B1 expression is greater in thymus than in mouse during late stages of ontogeny.

METHODS

Mouse husbandry, carcinogen treatment, necropsy and pathology

Cyp1b1 null mice on both a B6129F1 and a 129 genetic background were obtained from the National Cancer Institute and bred to produce female heterozygote on a B6129F1 background and male heterozygote on a completely 129 genetic background. All colonies were housed in the Laboratory Animal Resource Center at Oregon State University at 20 ± 1°C and 50 ± 10% humidity and a light/dark cycle of 12 h in microisolator cages with CareFRESH bedding (Absorption Corp.). During breeding, gestation, and lactation, mice were fed powdered AIN93G diet (Research Diets) *ad libitum*. When these mice were 8 weeks old, we bred the B6129F1 *cyp1b1*$^{+/-}$ females to 129 *cyp1b1*$^{+/-}$ males. Gestation day 0 was established by appearance of the vaginal plug. Offspring were fed pelleted AIN93G diet for the first 3 months and then AIN93M diet (Research Diets) *ad libitum* until euthanized. On the 17th day of gestation, pregnant mice were treated with vehicle (corn oil, 5 mL/kg body weight), 1 or 15 mg/kg DBP, or 50 mg/kg benzo(*a*)pyrene (Midwest Research Institute, Kansas City, MO) in corn oil by gavage. To monitor the health status of the mice, sentinels were housed in the colony and tested for viral or bacterial pathogens

and parasites. Upon signs of morbidity, pain, or distress, the mice were euthanized with an overdose of CO_2, exsanguinated, and necropsied. At 10 months of age, any surviving mice were euthanized and necropsied. All procedures for treatment, housing, and euthanasia of the mice used in this study have been approved by the Oregon State University Institutional Animal Care and Use Committee and successfully carried out by the investigators (Yu *et al.*, 2006b; Yu *et al.*, 2006c). Tissues were fixed in 10% formalin, stained with H&E, and analyzed by light microscopy. We previously identified the lymphoma to be a T-cell lymphoblastic lymphoma and the lung tumors as hyperplasia, adenoma, adenoma with progression, and carcinoma; the liver tumors were identified as foci and adenoma (Yu *et al.*, 2006b).

Genotyping of mice for the Cyp1b1 allele

At necropsy, an 8-mm ear punch was collected and lysed overnight at 55°C in 100 µL of DirectPCR Lysis Reagent containing proteinase K (Viagen Biotech, Inc.), followed by 45 min at 85°C. The lysis reaction was centrifuged for 10 s and used directly in a PCR reaction with allele-specific primers (Table 3-1) to permit one-tube genotyping for the wild-type *cyp1b1* allele. A 25 µL PCR reaction contained 1x GeneAmp buffer and 0.25 units AmpliTaq Gold polymerase (Promega Co.), 0.2 mmol/L of each deoxynucleotide triphosphate, 0.2 µmol/L of each primer, and 2 µL DNA. PCR cycling conditions were an initial 5 min at 95°C enzyme activation step, followed by 35 cycles of 30 s at 95°C to denature the DNA, 30 s at 55°C for primer

annealing, and 45 s at 72°C for extension. A final cycle with a further 10 min extension at 72°C concluded the reaction. PCR products were separated on Novex 8% Tris-borate EDTA gels (Invitrogen Technologies). *Cyp1b1* heterozygotes yielded two PCR products of 365 and 460 bp (NEO), respectively. Wild-type *cyp1b1* mice had a single product of 365 bp. A molecular weight ladder of *Msp*I cut pBR322 DNA (New England Biolabs) and ethidium bromide (Sigma Chemical Co.) was used to stain the DNA, followed by UV visualization.

Table 3-1. Primer Sequences for Cyp1b1 and Neomycin Selection Marker

Marker	Primer		Product Size
Cyp1b1[a]	1b1-3	5'-ttt gcc tgt cac cat tcc ac-3'	
	1b1-3R	5'-acg act tgg gct taa tgg tc-3'	365 bp
Neomycin	NEO-1	5'-tga atg aac tgc agg acg ag-3'	
	NEO-2	5'-cca cag tcg atg aat cca ga-3'	460 bp

[a]Wild-type mice (*Cyp1b1*$^{+/+}$) have a single band at 365 bp, knockout mice (*Cyp1b1*$^{-/-}$) a single band at 460 bp and the hets (*Cyp1b1*$^{+/-}$) both bands.

Statistical Analysis

Litter sizes per dam were compared between treatments using the exact Kruskal-Wallis test (SAS 9.1.3 Npar1way procedure). Because the experimental unit is the pregnant female, litters were accounted for in modeling of individual offspring

data. Birth weights were compared between treatments using linear mixed models with litters as a random factor (SAS mixed procedure). Survival curves were compared between *cyp1b1* groups using a robust score test in Cox proportional hazard regression with litters as clusters in the model using S-plus 7.0. (Therneau and Grambsch, 2000; Yu *et al.*, 2006b).

Lung tumor incidences in the three treatment groups [benzo(*a*)pyrene, DBP1, and DBP15] were each compared with the incidence in the control group using quasi-likelihood logistic regression where the observed variation between litters is used to account for overdispersion (SAS genmod procedure). For comparing tumor incidences between the *cyp1b1* gene dosages within the DBP1 treatment group, both logistic regression ignoring litters and quasi-likelihood logistic regression for grouped binomial data (accounting for litters) were used and both gave very similar results (SAS genmod procedure). Offspring tumor multiplicity was compared between *cyp1b1* gene dosages within the DBP15 treatment group with a linear mixed model fit to the log transformed tumor count for each offspring. Because there was large variability in the *cyp1b1* group differences from litter to litter ($P = 0.0011$), the mixed model included a random litter-by-*cyp1b1* interaction (SAS Mixed procedure). Residuals (either simple or deviance residuals) were examined and found reasonable for each linear model and generalized linear model fit.

RESULTS

Treatments of dams with DBP did not result in any significant maternal or fetal toxicities. The PAHs had no significant effect on litter size ($P = 0.43$, Kruskal-Wallis test) or birth weight ($P > 0.5$; data not shown). These results are consistent with previous studies in which no toxicity was observed at the 15 mg/kg DBP maternal dose (Yu *et al.*, 2006b; Yu *et al.*, 2006c; Castro *et al.*, 2008b).

As previously observed, offspring born to mothers treated with 15 mg/kg DBP exhibited lymphoma-dependent mortality between 10 and 30 weeks of age (Fig. 3-1A). In support of our hypothesis concerning the role of fetal cyp1b1 in DBP transplacental carcinogenesis, we observed a clear pattern of decreasing survival times with increasing *cyb1b1* gene dosage (Fig. 3-1B). None of the *cyp1b1*-null mice succumbed to the DBP transplacental lymphoma mortality. Siblings with both wild-type alleles exhibited sensitivity comparable with what was previously observed, whereas mice heterozygous for wild-type *cyp1b1* allele exhibited a survival curve almost exactly intermediate between the nulls and the wild-type siblings. There is strong evidence of a trend consisting of decreasing probability of survival with increasing *cyp1b1* gene dosage ($P = 0.0002$ score test ignoring litters and $P = 0.018$ robust score test with litters as clusters). The number of litters and offspring, as well as their genotype ratio, is shown in Table 3-2.

Table 3-2. Cyp1b1 Genotype Composition

Group	Dams	Offspring	Genotype Ratio ($1b1^{-/-}$: $1b1^{+/-}$: $1b1^{+/+}$)
Control	7	42	1.24 : 2.20 : 0.56[a]
BP	11	77	0.88 : 1.92 : 1.20
DBP (1 mg/kg)	11	78	1.08 : 1.84 : 1.08
DBP (15 mg/kg)	11	79	0.88 : 2.04 : 1.16

[a]This is not significantly different from expectations (1.00) based on a Chi-square test with a two-tailed P value.

We included a lower dose of DBP in case, at the high dose, cyp1b1 is saturated and cyp1a1 and cyp1a2 could contribute. Again, this is doubtful as previous studies have shown cyp1a enzyme to be expressed at very low levels in fetal tissue during the final trimester (Choudhary *et al.*, 2005; Xu *et al.*, 2005; Walker *et al.*, 2007), although it is still capable of responding to induction. The benzo(*a*)pyrene group was included as a negative control. Benzo(*a*)pyrene, like DBP, is a potent carcinogenic PAH, but is bioactivated primarily by cyp1a1 and cyp1a2 rather than by cyp1b1 (Shimada *et al.*, 2001a; Kleiner *et al.*, 2004; Shimada and Fujii-Kuriyama, 2004). For this reason, we expected to see few, if any, lymphomas and no significant difference between siblings of different *cyp1b1* genotypes with respect to benzo(*a*)pyrene-dependent transplacental carcinogenesis.

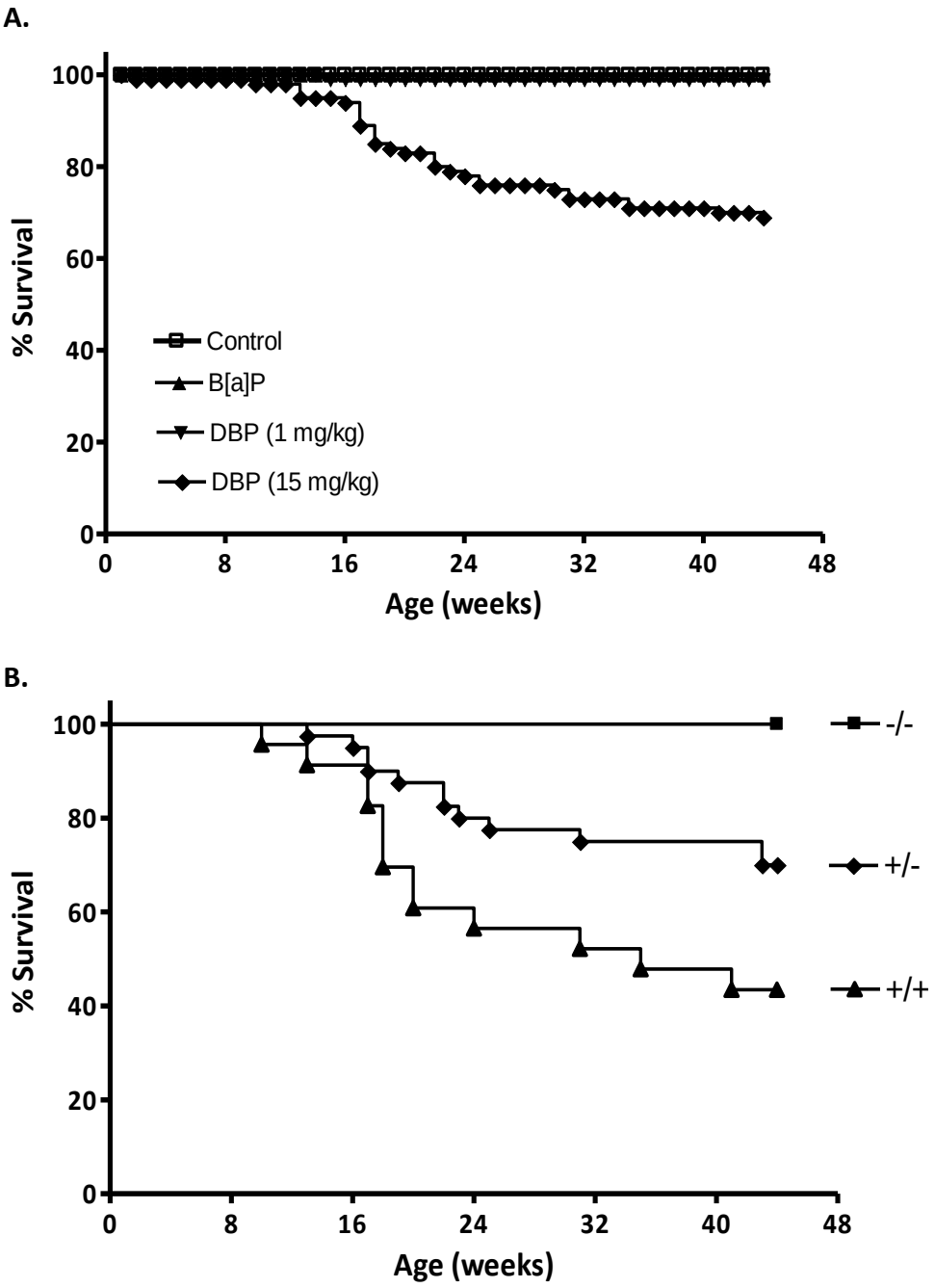

Figure 3-1. DBP-Dependent Lymphoma Mortality. A) Offspring, born to dams treated on the 17th day of gestation with corn oil (5 ml/Kg; □), BP (50 mg/Kg; ▲), DBP (1 mg/kg; ▼), or DBP (15 mg/Kg; ♦). B) Offspring of null (■), heterozygous (♦), and wild-type (▲) cyp1b1 genotype, born to dams given 15 mg/kg DBP. All mice were euthanized upon signs of morbidity as described in Methods.

Neither benzo(*a*)pyrene nor the low dose of DBP produced any lymphoma-dependent mortality over the time course examined (3-1A). All treatments produced higher incidence of lung adenomas and carcinomas than the spontaneous rate in controls, and the rates in the benzo(*a*)pyrene ($P = 0.01$, quasi-likelihood F test) and DBP15 ($P < 0.0001$) groups were significantly higher than the incidence in the controls at 10 months of age (Table 3-3). Due to overdispersion between litters, DBP1 is not significant ($P = 0.11$) compared with controls. The observed incidences are consistent with there being an effect of genotype, but the differences are too small to be statistically significant. Interestingly, all mice born to mothers receiving 15 mg/kg DBP had 100% incidence of lung tumors at the end of the study, irrespective of genotype. However, only at the high dose of DBP was there a *cyp1b1* genotype-dependent effect lung tumor multiplicity (Fig. 3-2; Table 3-3). Mice of the wild-type and heterozygous genotype had nearly double the numbers of tumors per mouse compared with the null offspring (9.3, 9.5, and 5.0; Table 3-3). After adjusting for litter variation (random litter-by-*cyp1b1* effect), there was some evidence of a *cyp1b1* effect ($P = 0.08$).

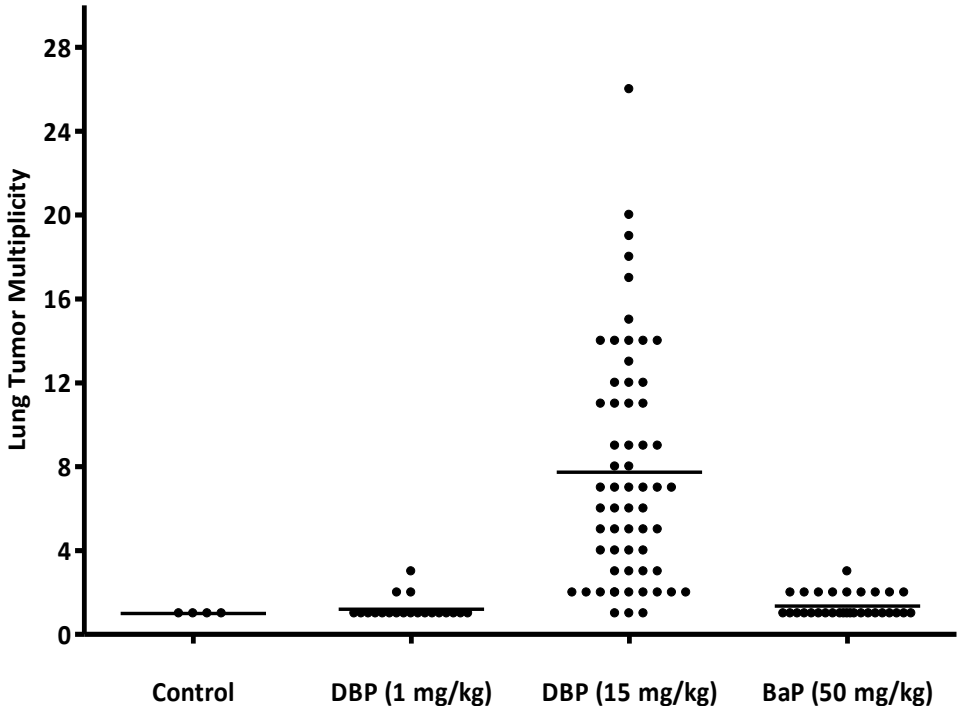

Figure 3-2. Lung Tumor Multiplicity at 10 Months. Offspring were euthanized at the conclusion of the study (10 months) and lung lesions (predominantly adenomas) quantified by histopathology. Offspring born to mothers administered corn oil (control), 1 mg/kg DBP, 15 mg/kg DBP, and 50 mg/kg B[a]P. Bars indicate group mean, dots represent individual animals.

Table 3-3. 10 Month Lung Tumor Multiplicity Sorted by *cyp1b1* Genotype

	Incidence		Multiplicity
Controls			
Wild-type	0.0 %	(0/6)	N/A
Hets	13.0 %	(3/23)	1.00 ± 0.00
Nulls	7.7 %	(1/13)	1.00 ± 0.00
BP (50 mg/Kg)			
Wild-type	39.1 %	(9/23)	1.33 ± 0.15
Hets	40.5 %	(15/37)	1.33 ± 0.15
Nulls	41.2 %	(7/17)	1.43 ± 0.17
DBP (1 mg/Kg)			
Wild-type	28.6 %	(6/21)	1.00 ± 0.00
Hets	27.8 %	(10/36)	1.40 ± 0.20
Nulls	19.0 %	(4/21)	1.00 ± 0.00
DBP (15 mg/Kg)			
Wild-type	100.0 %	(10/10)	9.30 ± 1.46
Hets	100.0 %	(28/28)	9.54 ± 1.03
Nulls	100.0 %	(16/16)	5.00 ± 0.96

DISCUSSION

Childhood cancer accounts for <1% of all cancers but these 12,400 cases annually in the United States translate to the second leading cause of death (2,300) after accidents, in children in the United States (Anderson, 2004a; Anderson, 2004b). Leukemias and lymphomas are the most common type of childhood cancer, followed by tumors of the nervous system. The etiology of 80% to 90% of childhood cancers is unknown (Wild and Kleinjans, 2003; Anderson, 2004a; Bunin, 2004; Lightfoot and Roman, 2004), but considerable evidence exists for maternal exposure to environmental chemicals as contributors to development of childhood leukemias and lymphomas (Alexander *et al.*, 2001; Ma *et al.*, 2002; Perera *et al.*, 2002; Reynolds *et al.*, 2002; Flower *et al.*, 2004; Kato *et al.*, 2004; Quintana *et al.*, 2004; Shu *et al.*, 2004; De Roos *et al.*, 2005; Infante-Rivard *et al.*, 2005). The two environmental *in utero* exposures that have definitively been linked with increased cancer in children and young adults are diethylstilbestrol and ionizing radiation (Anderson, 2004a). In addition, it may well be that exposure *in utero* or during infancy may not result in cancer during childhood, but may predispose the individual to cancers developing later in life. Indeed, studies with diethylstilbestrol in rodent models have shown that transplacental exposure significantly enhanced the risk of development of tumors in older animals upon exposure to chemical carcinogens (Anderson, 2004b). In many of the rodent transplacental cancer models, *in utero* exposure produces cancers in

middle-aged adult offspring (reviewed in Miller, 2004); that is, the significance of this research is not limited to childhood cancers.

With respect to the suitability as a model for human transplacental carcinogenesis, mice have turned out to be an excellent model in the case of DES (Anderson, 2004b). Many environmental chemicals for which there are significant human exposures are transplacental carcinogens in rodents, including the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (Hasegawa *et al.*, 1995) , benzo(*a*)pyrene (Anderson *et al.*, 1995), and the tobacco-specific carcinogen 4-(methynitrosamino)-1-(3-pyridyl)-1-butanone (Anderson *et al.*, 1989a). The infant mouse has also been used as a cancer model with enhanced sensitivity compared with the adult for a number of chemical carcinogens such as diethylnitrosamine, PAHs, and aflatoxin B_1 (Vesselinovitch *et al.*, 1972; Vesselinovitch *et al.*, 1984; Rodriguez *et al.*, 1997). The sensitivity of the infant mouse to chemical carcinogens is relevant for the present discussion as we administered a very lipophilic carcinogen during late pregnancy in our model. We have recently done a cross-fostering experiment to estimate how much of the DBP-dependent lymphoma mortality was due to *in utero* exposure and how much to exposure through nursing (21 days). The results indicate a slightly greater contribution from the much shorter *in utero* exposure (data not shown).

Currently, over 320,000 Americans have leukemia, lymphoma, or myeloma with an estimated 135,500 new cases annually and 85,580 deaths (Stewart *et al.*, 2006). Although the etiology of the majority of such cancers is unknown, chemical

exposures have been identified as one definitive risk factor (reviewed in Anderson, 2004a). The mouse can serve as a useful model for human lymphoma (Teitell and Pandolfi, 2004) with the recognition that certain lymphoma pathologies are species specific and molecular markers may be distinct. A classification scheme devised under the direction of the Mouse Models of Human Cancers Consortium has been published and can be used for comparison with the WHO classification of lymphomas (Morse *et al.*, 2002). We have, for the first time in any animal model, documented that exposure of pregnant mice to a single dose of a potent PAH, DBP, results in a very aggressive T-cell lymphoma in the offspring beginning at 10 weeks of age (Yu *et al.*, 2006b; Yu *et al.*, 2006c; Castro *et al.*, 2008b). Our hypothesis is that maternal transfer to the fetus results in cyp1b1-dependent bioactivation to DBP-(–)-*anti*-(11*R*,12*S*,13*S*,14*R*)-dihydrodiol epoxide and other reactive metabolites of DBP in the thymus producing a number of DNA adducts. This model should recapitulate human fetal exposure to PAHs from maternal diet and airborne particles, including direct and second-hand tobacco smoke as a causative factor in leukemias/lymphomas in children and young adults.

We hypothesize that cyp1b1 bioactivation of DBP occurs in fetal target tissues, primarily thymus with the secondary target of lung and that this pathway plays a greater role than other potential means of bioactivation of PAHs, such as peroxidation (Cavalieri and Rogan, 1990) and the aldo-keto reductase pathways (Palackal *et al.*, 2001), both of which produce oxidative damage to DNA. In previous studies, using DBP or 3-methylcholanthrene in models, in which either the dam or the

fetus were ahr "nonresponsive" or "responsive," it was shown that the responsive dam reduced the risk to the fetus apparently by decreasing the bioavailability through enhanced maternal metabolism and clearance (Miller, 2004; Yu *et al.*, 2006b). However, the issue of fetal versus maternal metabolism in PAH-transplacental carcinogenesis is not settled. By using crosses of *cyp1b1* heterozygote on the same genetic background as our DBP-transplacental model, we were able to show that DBP-dependent transplacental lymphoma mortality was dependent on the presence of at least one wild-type *cyp1b1* allele and there was a very tight correlation between *cyp1b1* gene dosage and mortality. In the lung, the effect of the cyp1b1 expression was apparent, but not as marked, possibly due to some contribution from CYPs in the 1a subfamily. It should be kept in mind, however, that lung tumor incidence and multiplicity determined at 10 months of age in the high-dose DBP group is complicated from a statistical standpoint by the earlier lymphoma deaths. In offspring born to mothers treated with benzo(*a*)pyrene or low-dose DBP, the statistical analysis is cleaner as we are not dealing with only a population of lymphoma survivors.

The results clearly show that the cyp1b1 genotype does not influence benzo(*a*)pyrene-dependent lung cancer in this model. Lung cancer is the major leading cause of cancer-related deaths for both sexes in the United States with 173,700 new cases in 2004 and 160,400 deaths. The 5-year survival rate is poor for lung cancer (15%) and any prevention approach would be beneficial. CYP1B1 has been found to be, at least in part, under epigenetic control (Nakajima *et al.*, 2003;

Tokizane *et al.*, 2005) and it has been suggested that CYP1B1 would make an excellent target for prevention strategies (Guengerich *et al.*, 2003) . In our previous studies, we showed that the addition of indole-3-carbinol, green tea, or caffeine to the maternal diet during pregnancy and nursing significantly reduced lung cancer multiplicity in 10-month-old offspring from mothers treated with 15 mg/kg DBP (Yu *et al.*, 2006c; Castro *et al.*, 2008b). The results presented in this study provide further evidence that fetal cyp1b1 plays an important role in DBP transplacental carcinogenesis, primarily with lymphoma and to a lesser degree with lung tumors. We conclude that fetal cyp1b1 could be a target for cancer prevention and additional work with specific inhibitors, such as 2,3,4,5-tetramethoxystilbene may prove fruitful in that respect. In addition, as with most other CYPs in humans, 1B1 exists in the population as a number of nonsynonymous allelic variants (Shimada *et al.*, 1999; Shimada *et al.*, 2001a; Shimada *et al.*, 2001b; Roos and Bolt, 2005; Wenzlaff *et al.*, 2005). The Swiss Protein entry for human CYP1B1 (http://ca.expasy.org/uniprot/Q16678) lists 21 nonsynonymous gene variants, found in at least 25 allelic combinations. The Cancer Genome Anatomy Project data for human CYP1B1 (http://snp500cancer.nci.nih.gove) identified four nonsynonymous SNPs with >5% frequency in a least one population. The same nonsynonymous SNPs were also most common in CYP1B1 data from the National Institute of Environmental Health Sciences Environmental Genome Project (http://egp.gs.washington.edu) (Livingston *et al.*, 2004). If humans recapitulate the responses observed here, transplacental exposure to carcinogenic PAHs, such as DBP or benzo(*a*)pyrene, will

lead to lymphoma or lung cancer later in life. The CYP1B1 genetic polymorphism may play a role in the target and severity of the transplacental carcinogenesis.

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Chapter 4

Chemoprevention of dibenzo[*a,l*]pyrene transplacental carcinogenesis in mice born to mothers administered green tea: primary role of caffeine

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ABSTRACT

Our laboratory recently developed a mouse model of transplacental-induction of lymphoma, lung and liver cancer by the polycyclic aromatic hydrocarbon, dibenzo[*a,l*]pyrene (DBP). Pregnant B6129SF1 females, bred to 129S1/SvIm males, were treated on day 17 of gestation with an oral dose of 15 mg/Kg DBP. Beginning on day 0 of gestation, dams were given (*ad lib*) buffered water, 0.5% green tea, 0.5% decaffeinated green tea, caffeine or EGCG (both at equivalent concentrations found in tea). The concentration of the teas (and corresponding caffeine and EGCG) was increased to 1.0% upon entering the second trimester, 1.5% at onset of the third trimester and continued at 1.5% until pups were weaned at 21 days of age. Offspring were raised with normal drinking water and AIN93G diet. Beginning at 2 months of age, offspring experienced significant mortalities due to an aggressive T-cell lymphoma as seen in our previous studies. Ingestion of caffeinated, but not decaffeinated, green tea provided modest but significant protection ($p=0.03$) against mortality. Caffeine provided a more robust ($p=0.006$) protection, but EGCG was without effect. Offspring also developed DBP-dependent lung adenomas. All treatments significantly reduced lung tumor multiplicity relative to controls ($p<0.02$). EGCG was most effective at decreasing tumor burden ($p=0.005$) by over 40% compared to controls. Induction of Cyp1b1 in maternal liver may reduce bioavailability of DBP to the fetus as a mechanism of chemoprevention. This is the

first demonstration that maternal ingestion of green tea, during pregnancy and nursing provides protection against transplacental carcinogenesis.

INTRODUCTION

In animal models, a number of carcinogens are capable of inducing cancer in offspring of mothers exposed during pregnancy (reviewed in Anderson, 2004a). In humans, ionizing radiation and diethylstilbestrol are the two agents for which the evidence for such transplacental carcinogenesis is convincing (Anderson, 2004a), although a number of studies exist providing indirect evidence that environmental exposures of pregnant women to chemical carcinogens are associated with increased risks of cancer in children (Anderson *et al.*, 2000; Alexander *et al.*, 2001; Ma *et al.*, 2002; Flower *et al.*, 2004; Jensen *et al.*, 2004).

Previous studies by Drs. Anderson and Miller and co-workers demonstrated that exposure of the dam during the third trimester to the synthetic polycyclic aromatic hydrocarbon (PAH), 3-methylcholanthrene (3-MC), in crosses of B6D2F1 and D2 mice, produced lung and liver cancers in offspring at one year of age (Miller, 1994). We employed a different strain of mice and a different PAH. Treatment of B6129F1 mice, bred to 129 mice, resulted in high mortalities beginning at 2-3 months of age from an aggressive T-cell lymphoma (Yu *et al.*, 2006b). If the mice survived to 10 months of age without developing lymphoma, 100% exhibited lung neoplasia and

most males had liver tumors (Yu *et al.*, 2006b). The significance of this model is enhanced by the fact that leukemias and lymphomas are the major childhood cancers and cancer is the major cause of death in children other than accidents (Anderson, 2004a).

In our first chemoprevention study employing this model, offspring born to mothers fed indole-3-carbinol (I3C), a major phytochemical found in cruciferous vegetables (and available as a dietary supplement), exhibited significant protection against the DBP-dependent lymphoma mortality (Yu *et al.*, 2006c). Furthermore, there was a significant reduction in lung tumor multiplicity at 10 months of age (Yu *et al.*, 2006c). Therefore, the addition of a chemoprotective phytochemical to the maternal diet could provide significant reduction in cancer, not just in the age of mice corresponding to young adults (3-6 months in mice), but in middle-age (10 months), as well.

In the present study, we utilized this model to examine the potential for green tea to act as a chemoprotective agent against transplacental DBP carcinogenesis. Tea is the second most consumed beverage world-wide (Clark and You, 2006). Studies with a number of animal models have demonstrated the efficacy of teas (black, green, white) in chemoprevention of cancers (Cao *et al.*, 1996; Dreosti *et al.*, 1997; Chung, 1999; Suganuma *et al.*, 1999; Yang *et al.*, 2002; Liao *et al.*, 2004; Yang *et al.*, 2005; Lu *et al.*, 2006b). Epidemiology studies in humans have yielded equivocal results with respect to the cancer chemoprotective properties of tea (Yang *et al.*, 2002).

Epigallocatechin-3-gallate (EGCG) is a major polyphenol constituent, especially abundant in green and white (the least processed) teas. EGCG is thought to be one of the most effective constituents in modulation of disease, including cancers, by tea (Ahmad *et al.*, 1997; Yang *et al.*, 2000; Okabe *et al.*, 2001; Lambert and Yang, 2003; Nakazato *et al.*, 2005; Navarro-Peran *et al.*, 2005). Another major constituent of green tea is caffeine and some cancer chemoprevention studies suggest that caffeine plays a major role in the beneficial (Ito *et al.*, 2003; Hayashi *et al.*, 2005; Yang *et al.*, 2005) and, perhaps, harmful (Baker *et al.*, 2005; Al-Wadei *et al.*, 2006) effects observed. Caffeine is of special interest in our model, as concerns about its potential harmful effects on the fetus have led agencies such as the FDA and the March of Dimes to recommend pregnant women refrain from its consumption during pregnancy. Caffeine is a teratogen in rodents, but only at high doses (Nehlig and Debry, 1994). The risk to the fetus from maternal ingestion of caffeine remains a concern, but the overall evidence would suggest that caffeine has not been responsible for a significant number of malformations in babies born to mothers consuming moderate amounts (Christian and Brent, 2001; Nawrot *et al.*, 2003; Browne, 2006).

We now report that caffeinated, but not decaffeinated, green tea given to the pregnant and nursing mouse significantly ($p=0.03$) reduced the mortality in her offspring from transplacental DBP-induction of lymphoma. EGCG, administered at the same concentration had no effect. Caffeine (again, administered at the concentration found in green tea), exhibited the greatest chemoprotection ($p=0.006$)

against lymphoma mortality. All treatment groups reduced lung tumor multiplicity and size and was most marked with EGCG.

These results confirm that this model can be very useful for the study of transplacental (and, perhaps, lactational) chemoprotection against cancer by modulation of the maternal diet. Offspring born to mothers consuming chemoprotective phytochemicals exhibit significant protection against PAH-dependent cancers in young adults and out to at least middle age, even though they never consume the phytochemical following weaning.

MATERIALS AND METHODS

Chemicals and diets

DBP was provided by the Carcinogen Repository, supported by the National Cancer Institute, at Midwest Research Institute (Kansas City, MO) and was at least 98% pure as determined by HPLC. The semipurified diets, AIN93G and AIN93M, were purchased from Dyets, Inc. (Bethlehem, PA). Green tea and decaffeinated green tea were obtained from Stash Teas (Portland, OR). EGCG was a gift from DSM Nutritional Products (Basel, Switzerland). Caffeine was purchased from Sigma Chemical Co. (St.Louis, MO). 2,3,4,5 – Tetramethoxystilbene (TMS) was purchased from Cayman Chemical Co. (Ann Arbor, MI).

Preparation of teas and treatment of mice

The teas used in this study were analyzed for the composition of various polyphenols and caffeine by HPLC as previously described (Santana-Rios *et al.*, 2001). The teas were prepared by adding 0.5 g tea leaves per 100 ml buffered water (0.5% w/v) with a brew time of 2 min. Citric acid (0.5% w/v) was used to buffer the solution to pH 7.6 in order to stabilize polyphenols. Solutions of EGCG and caffeine were prepared by addition of each to the citric acid buffered solution (pH 7.6) at the concentration present in the whole tea (0.13 and 0.09 mg/ml for EGCG and caffeine, respectively, for a 0.5% tea, etc.). The administration of the teas, EGCG and caffeine began at day 0 (detected by appearance of the vaginal plug) of pregnancy and were given at 0.5% initially. We have observed that mice begun immediately at 1.5%, tend to exhibit aversion. We administered the test solutions at 0.5% for the first trimester of pregnancy and then increased the concentration to 1.0% for the second trimester and to 1.5% for the third trimester. The 1.5% solutions were continued throughout nursing (21 days post-partum). We observed no overt adverse effects of this treatment regiment on either the dams or the pups (e.g., no difference in birth weight, litter size, etc.).

Eight-week old B6129SF1 female and 129S1/SvImJ male mice (Jackson Laboratories (Bar Harbor, ME) were housed at the Laboratory Animal Resource Center at Oregon State University at 20 ± 1˚ C and 50 ± 10% humidity, with a light dark cycle of 12 hours each in micro-isolator cages (Life Products, Inc., Seaford, DE) with CareFRESH bedding. After acclimation for one week, mice were bred and

appearance of the vaginal plug was determined to be day 0. The mice were given 0.5 % citric acid buffered water, teas, EGCG or caffeine to drink and AIN93G diet *ad libitum*. After birth, offspring were nursed for 21 days and dams continued on 1.5% tea and EGCG or caffeine equivalents. After weaning, offspring of each sex from the same litter were housed together (up to 5 per cage) and administered water and AIN93G diet *ad libitum*. At 3 months of age, the diet was changed to AIN93M. The number of dams and offspring in each experimental group is shown in Table 4-1. We also analyzed 5 additional groups that were identical with the exception that dams were administered vehicle alone on day 17 of gestation. No lymphomas were observed in offspring born to mothers receiving vehicle alone (data not shown).

The health of the colony was monitored by sentinels tested for viral or bacterial pathogens and parasites by the University of Missouri Research Animal Diagnostic Laboratory (Columbia, MO). All tests were negative throughout the course of the study. If any signs of distress or pain (or any sign of morbidity) were observed, mice were immediately euthanized with an overdose of CO_2 and a necropsy performed. Of those that did not survive to the end of the study, 13.9 % died between monitorings and were not useful for necropsy. The proportion not useful was similar across all treatments ($p>0.7$ Chi-square test). At 10 months of age, the study was terminated and any surviving mice euthanized and necropsied. All procedures used in the handling, treatment and husbandry of mice were approved by the Oregon State University Institutional Animal Care and Use Committee.

Table 4-1. Numbers of Dams and Offspring in Each Experimental Group

Group	Dams	Offspring	Survived Study
Controls	16	108	8
Green Tea	14	100	15
Decaffeinated Tea	16	116	15
Caffeine Alone	14	105	24
EGCG Alone	17	110	8

Day 0 of gestation was set as the first day a vaginal plug was observed. Dams were immediately started on 0.5% brewed (2 min) tea (in 0.5% citric acid, pH 7.6) as their sole drinking source. The concentrations were increased to 1% for the second trimester and to 1.5% at the start of the third trimester and this concentration was continued until weaning. All dams received DBP (15 mg/Kg) as a single dose by gavage (corn oil 5 ml/Kg) on day 17 of gestation. There was no difference between groups with respect to litter size or birth weight.

Histopathology

At necropsy, the heart, thymus, lung, spleen, liver, kidney, abnormal lymph nodes, testes or ovaries, colon and skin were collected, fixed in 10% formalin, stained with H&E and analyzed by light microscopy. In our previous studies with this model (Yu *et al.*, 2006b; Yu *et al.*, 2006c) we established that the lymphoma producing the mortalities in the mice at 3-6 months of age was of T-cell origin (CD3+,B220-) with various phenotypes (CD4-,CD8+; CD4+,CD8+). These were classified as T-cell lymphoblastic lymphomas. These lymphomas were very aggressive, resulting in invasion of numerous organs by the transformed lymphocytes (Yu *et al.*, 2006b; Yu *et al.*, 2006c).

In our previous study, all offspring born to mothers treated with DBP developed lung tumors by 10 months and most males had liver neoplasia as well (Yu *et al.*, 2006b; Yu *et al.*, 2006c). In this study, we carefully examined the lung for each

mouse necropsied and determined the lung multiplicity as a function of age, out to the 10 month termination point.

Statistical analysis

Overall strategy for analysis of offspring responses included: 1) accounting for cluster (litter) effects when there is any evidence of them being present, 2) assessment of treatment effects both overall and then with pairwise comparisons to the control, 3) assessment of gender and aryl hydrocarbon receptor (ahr) effects, both main effects and interactions with other factors. The statistical software was SAS for Windows 9.1.3 and S-plus 7.0 Windows.

For analysis of the offspring survival curves, treatment groups were compared using Cox regression with robust score tests to account for the fact that pups were clustered into litters (S-Plus coxph function with cluster term, (Therneau and Grambsch, 2000)). There was evidence of litter effects in 3 of the 5 treatments. Pairs of survival curves reasonably satisfied the proportional hazards assumption based on graphical and analytical checks (Cantor, 1996) with the Lifetest and Phreg procedures in SAS. There was no evidence of gender or ahr effects on survival.

For analysis of number of tumors per offspring, the data set modeled included two subsets: (1) survivors and (2) animals surviving long enough to have the opportunity to develop multiple tumors. For the second subset, a criterion of requiring every treatment group to have at least 50% of the animals with multiple tumors resulted in an inclusion cutoff for survival of at least 150 days. Overdispersed

(quasilikelihood) Poisson regression with a log link and age at death as a simple linear covariate was chosen as the final model due to a reasonable pattern for the deviance residuals, homogeneity of variance between subsets and easily interpretable (multiplicative) effects. Over the range of ages being modeled, there was no evidence of curvilinearity or differences in slope between treatments (no age-by-treatment interaction). Models were fit with the SAS Genmod and Glimmix procedures and p-values reported are for approximate F-tests and t-tests. There was no evidence of litter effects for this response (zero estimate for litter variance component).

For analysis of maternal Cyp1b1 activity, data were log transformed prior to analysis due to increasing standard deviation with mean of response and right skew for repeated measurements. After averaging across 3 measurements within each of 3 trials to achieve one mean response per dam, treatments were compared by one-way ANOVA followed by pairwise contrasts (t-tests) with the control treatment using the SAS Mixed procedure.

RESULTS

Maternal consumption of green tea and green tea components protects against mortality in offspring from transplacental DBP-induced lymphoma

As previously reported by our laboratory (Yu *et al.*, 2006b; Yu *et al.*, 2006c), offspring of these crosses, born to mothers administered DBP, exhibited a high rate of mortality between 3-6 months of age from an aggressive T-cell lymphoma. Using robust tests to account for any litter effects, there was clear evidence of treatment effects on offspring survival (p=0.018 overall 4 d.f. test). The survival curves for offspring born to mothers given drinking water, caffeinated tea and decaffeinated tea are shown in Figure 4-1 (top panel). A modest, but significant (p=0.03) protective effect was observed for offspring born to mothers drinking regular green tea throughout pregnancy and nursing. If the mothers were given decaffeinated green tea, the protection was not significant (p=0.25).

Figure 4-1 (bottom panel) depicts the survival of offspring born to mothers drinking EGCG or caffeine solutions (at the equivalent tea concentration). There was no protective effect for the offspring born to mothers consuming EGCG, but the protective effect with caffeine was striking and highly significant (p=0.006).

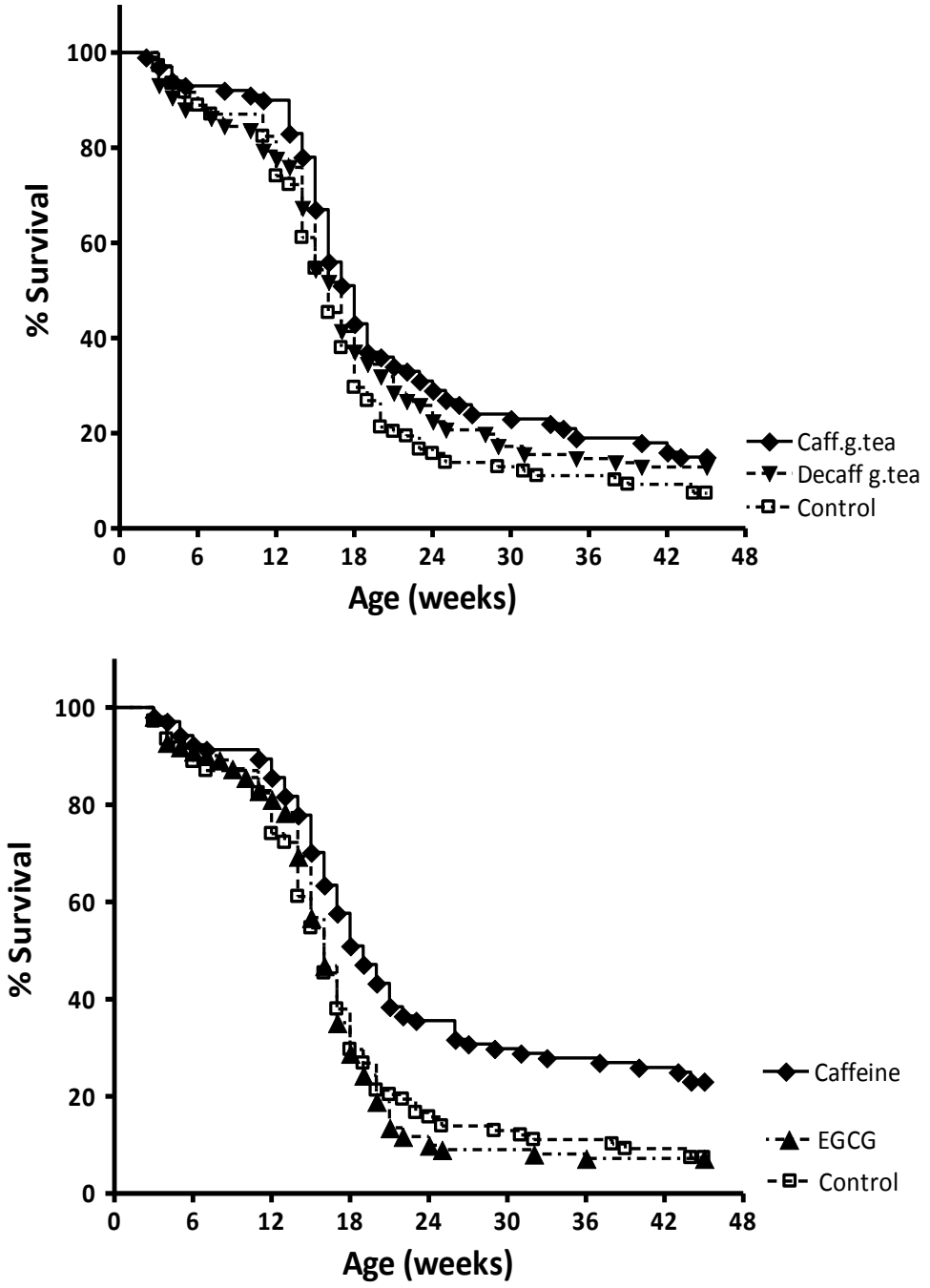

Figure 4-1. Survival curve of offspring born to mothers given DBP. DBP was administered at a dose of 15 mg/Kg by gavage (corn oil) on day 17 of gestation. The pregnant and nursing dams were administered *ad libitum* citric acid buffered water (□), green tea (♦, top panel), decaffeinated green tea (▼, top panel), caffeine (♦, bottom panel) and EGCG (▲, bottom panel). The concentrations and periods of administration are given in Materials and Methods.

Maternal Consumption of green tea and green tea components protects against DBP-dependent transplacental lung cancer throughout lifetime

As we previously reported, DBP in this model is also a transplacental lung carcinogen. In our study of the chemoprotective effect of I3C in this model, we reported a 35% reduction in lung tumor multiplicity (Yu *et al.*, 2006c) at 10 months of age in mice not succumbing to lymphoma. In this study, we monitored lung tumor multiplicity from 4-10 months of age (Figure 4-2). Both survivors and those surviving more than 150 days (~21 weeks, see Methods) exhibited reduced tumors per animal in all treatments compared to the control. After adjusting for age and gender as significant covariate ($p<0.0001$ quasilikelihood F-test), there was clear evidence of treatment effects on tumors per animal ($p=0.01$, overall F-test). In contrast to the impact on lymphoma survival, EGCG was the most effective at reducing the tumor multiplicity by an average of over 40% compared to controls. Tumor burden was also reduced by 27%, 32%, and 36% in the caffeine, caffeinated green tea, and decaffeinated green tea drinking regimens ($p<0.02$). These results imply that the mechanism(s) of cancer chemoprotection by tea and components of tea for lymphoma and lung in this model are distinct. In order to rigorously test the transplacental chemoprevention potential of tea and tea components with respect to lung cancer, it would be necessary to employ a model, such as the 3-MC model in B6D2F1 X D2 crosses (Miller, 1994). This experimental design produces lung tumors in both sexes and liver tumors in males without the confounding lymphoma mortality.

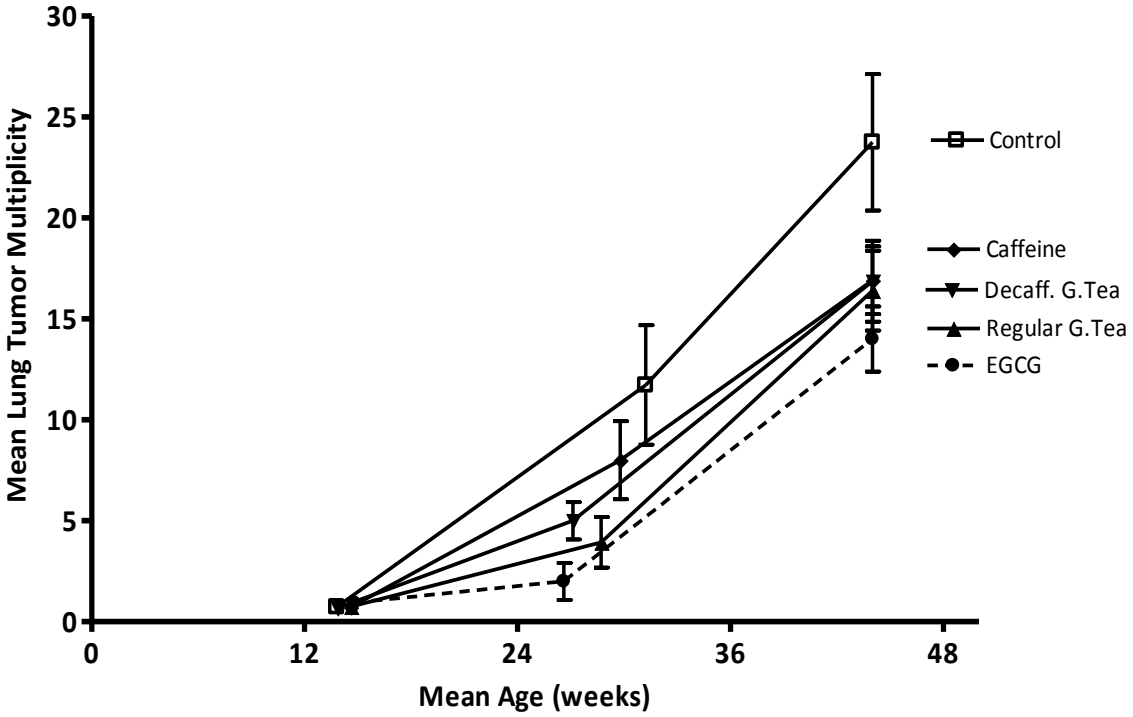

Figure 4-2. Lung Tumor Multiplicity. Offspring were euthanized due to lymphoma-dependent morbidity (4-9 months) or at the conclusion of the study (10 months) and lung lesions (predominantly adenomas) quantified by histopathology. Offspring born to mothers given citric acid buffered drinking water (□), green tea (▲), decaffeinated green tea (▼), caffeine (♦) or EGCG (●). Numbers euthanized in the first group (<21 wks) were 72, 55, 70, 53, and 80. For the second group (>21 wks and <44 wks) 11, 14, 14, 12, and 4 were euthanized. Numbers surviving to the end are given in table 1. The concentrations and timing of administration to the dams are given in Materials and Methods.

Influence of drinking regimens on maternal Cyp1b1 activity

Measurement of Cyp1b1 activity in maternal liver microsomes (Figure 4-3, top panel) via a P450-Glo assay revealed strong evidence of differences between treatments ($p<0.0001$, overall ANOVA). Dams administered caffeinated green tea and caffeine alone showed significant (over 2-fold) increases in Cyp1b1 activity compared to controls ($p=0.01$ and $p=0.024$ respectively). Hepatic microsomes from dams administered EGCG exhibited some reduction in Cyp1b1 activity ($p=0.066$). However, analysis of mRNA for Cyp1A1 and Cyp1b1 revealed no significant changes in transcript levels among the treatments (data not shown). In order to confirm the contribution of Cyp1b1, 2,3',4,5' – Tetramethoxystilbene (TMS) was co-incubated under the same assay conditions. The presence of TMS effectively diminished Cyp1b1 activity to near background levels in a clear dose-response manner (Figure 4-3, bottom panel). These results suggest that at least one mechanism for the transplacental chemoprevention observed in this study is a reduction of DBP bioavailability to the fetus due to induction of DBP metabolism in maternal liver. Cyp1b1 exhibits the greatest activity of all the Cyps examined with respect to DBP metabolism (Shimada *et al.*, 1996; Crespi *et al.*, 1997; Shimada *et al.*, 1997; Luch *et al.*, 1998; Luch *et al.*, 1999a; Melendez-Colon *et al.*, 1999; Shimada *et al.*, 2001a; Buters *et al.*, 2002; Shimada and Fujii-Kuriyama, 2004). In addition, we employed Cyp1b1 (breeding heterozygous mice) knockout mice to demonstrate a clear gene dose effect; null mice were resistant to DBP compared to wild-type siblings, and hets exhibited intermediate sensitivity (results not shown).

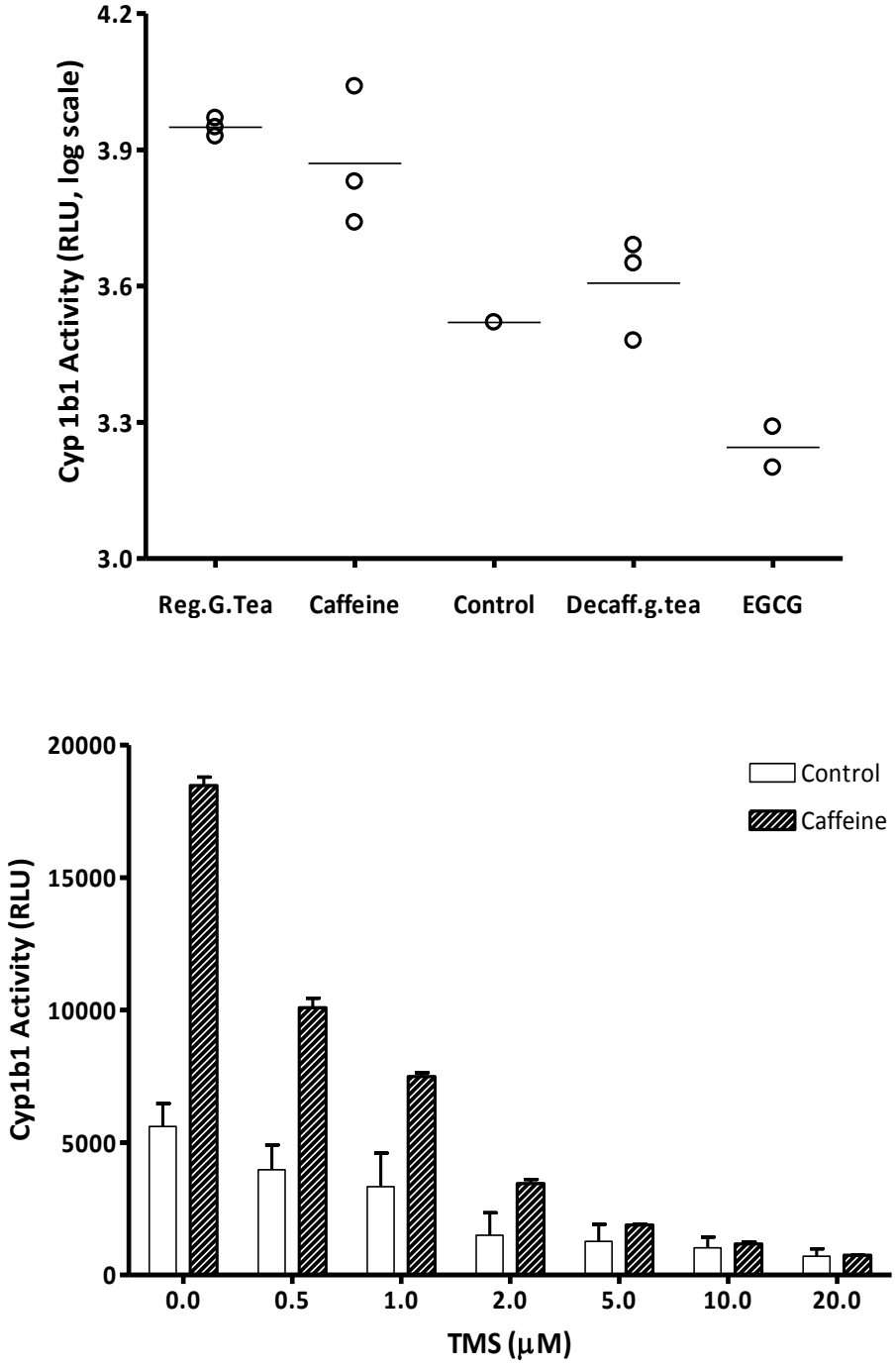

Figure 4-3. Effects of drink regimens on maternal hepatic Cyp1b1 activity. At GD 17 a subset of dams were sacrificed and livers were extracted for preparation of microsomal fractions. Activity of Cyp1b1 was assayed fluorometrically via reaction with a luminogenic Cyp1b1 substrate (luciferin-CEE, P450-Glo assay). Values are expressed in total luminescence (RLU, log scale) for each drink regimen. Top Panel; Bars indicate the mean of log transformed Cyp1b1 activity for the respective dams. Circles represent individual dams. Bottom Panel; Reactions were performed in the presence or absence of the Cyp1b1 inhibitor, 2,3,4,5-tetramethoxystilbene (TMS).

DISCUSSION

Tea is the second most widely consumed beverage in the world and green tea (Camellia sinesis), the more popular tea in Asia, has been demonstrated in a number of animal models to have chemoprotective effects (Cao *et al.*, 1996; Dreosti *et al.*, 1997; Chung, 1999; Suganuma *et al.*, 1999; Yang *et al.*, 2002; Liao *et al.*, 2004; Yang *et al.*, 2005; Clark and You, 2006; Lu *et al.*, 2006a; Lu *et al.*, 2006b). In these models, green tea has been shown to be effective against cancers of the breast, colon, skin, oral cavity, esophagus, forestomach, small intestine, pancreas and lung (reviewed in Yang *et al.*, 2002). The lung has received much of the attention in mouse models of green tea chemoprotection. The A/J mouse is commonly used for these studies as they are prone to lung cancer development and studies can be conducted in 4-6 months, rather than a year or more required in resistant strains. In the A/J mouse model of lung cancer, green tea is chemoprotective against numerous carcinogens requiring bioactivation including the tobacco-specific nitrosamine, 4-(methynitrosamino)-1-(3-pyridyl)-1-butanone (NNK), diethynitrosamine (DEN), the PAH (benzo[a]pyrene, BaP), as well as direct-acting carcinogens such as nitrosomethylurea and cisplatin (Yang *et al.*, 2002; Yang *et al.*, 2005).

Initial studies on the mechanism of cancer chemoprevention by green tea highlighted the antioxidant properties of polyphenol constituents such as EGCG. Indeed, green tea and EGCG are effective *in vitro* and *in vivo* in the reduction of biomarkers of carcinogen-induced oxidative damage, such as the level of 8-oxo-

deoxyguanosine (Chung, 1999). Other potential mechanisms of action (reviewed in Yang *et al.*, 2002) are induction of apoptosis (Ahmad *et al.*, 1997; Yang *et al.*, 2000; Nakazato *et al.*, 2005), inhibition of angiogenesis (Liao *et al.*, 2004), inhibition of cell proliferation or cell cycle arrest (Ahmad *et al.*, 1997; Yang *et al.*, 2000; Clark and You, 2006), induction of phase II enzymes through the Nrf-2 pathway (Clark and You, 2006) and alteration in cell signaling through NF-κB (Okabe *et al.*, 2001). Fewer studies with green tea have focused on leukemias and lymphomas. *In vitro*, green tea (EGCG) induces apoptotic cell death in transformed human B-cells (Nakazato *et al.*, 2005) and in a mouse lymphoma cell line (Ahmad *et al.*, 1997).

In our mouse model of DBP transplacental carcinogenesis, offspring born to mothers treated with this PAH during late gestation develop an aggressive T-cell lymphoma producing a high rate of mortality between 3-6 months of age (Yu *et al.*, 2006b; Yu *et al.*, 2006c). In an earlier study, we demonstrated a marked chemoprotective effect if offspring were born to mothers consuming indole-3-carbinol in diet during pregnancy and nursing (Yu *et al.*, 2006c). We now report that green tea, given to the mother throughout pregnancy and nursing, also provides significant protection to offspring with respect to lymphoma-dependent mortality. Removal of caffeine from this green tea resulted in no significant protection. Consistent with the whole tea results, offspring born to mothers consuming caffeine alone exhibited a striking protection against development of lymphoma, whereas, the major polyphenol (present in both caffeinated and decaffeinated green tea),

EGCG, was without effect. It would appear that the major chemoprotective effect of green tea in this transplacental model of DBP-lymphoma is due to caffeine.

These results are consistent with other animal models comparing whole tea with caffeine, *i.e*., caffeine appears to be the major chemoprotective component (Yang *et al.*, 2005). Although the exact mechanism of the inhibition by caffeine is not entirely known, we postulate that the induction of Cyp1b1 results in the decreased bioavailability of DBP in the target organs. This mechanism has also been shown to underlie the inhibition of lung tumorigenesis initiated by the nicotine-derived carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), in rats treated with various levels of caffeine (Chung *et al.*, 1998). It is possible, however, that other mechanisms may also be involved and can be attributed to caffeine's broad range of biochemical and physiological activities (IARC, 1991). In addition, caffeine has been demonstrated to be a teratogen in animal models, including the mouse (Nehlig and Debry, 1994). The FDA and March of Dimes recommend that pregnant women limit intake of caffeine. In order to develop candidates for human transplacental chemoprotection, we need to have a thorough understanding of the dose-response relationship for beneficial and adverse effects in order to do proper risk assessment.

As we reported previously, administration of DBP in this model, not only resulted in T-cell lymphoma, but all mice not succumbing to the lymphoma developed lung cancer. In this study, we also saw a 100% incidence of lung cancer in offspring born to DBP-treated mothers. The time course of lung tumor multiplicity demonstrated that offspring born to mothers in any of the four treatment groups

exhibited a markedly and significant delay in the appearance of lung tumors. The greatest effect and the longest delay were observed in offspring born to mothers given EGCG alone. Mortality from lung cancer is greater than any other cancer in the U.S. for both sexes. A significant delay in the appearance of lung tumors could save billions in health care dollars.

In addition to PAHs, arsenic has been demonstrated to be a transplacental lung carcinogen in mice (Shen *et al.*, 2007). Evidence suggests that transplacental exposure to air pollutants (including PAHs) are transplacental carcinogens in humans as well (Perera *et al.*, 2002). In humans, the chemopreventive or therapeutic properties, with respect to lung cancer, of green tea are equivocal (Shim *et al.*, 1995; Yang *et al.*, 2002; Witschi *et al.*, 2004; Bonner *et al.*, 2005; Laurie *et al.*, 2005). Very few studies on transplacental cancer chemoprevention have been done. In addition to our model (Yu *et al.*, 2006c), the addition of oriental food-seasoning spices (Garam masala) or mustard seed oil to the maternal diet of mice provides chemoprotection against 7,12-dimethylbenz[*a*]anthracene-dependent transplacental carcinogenesis (Rao and Hashim, 1995; Hashim *et al.*, 1998).

In summary, administration of green tea, decaffeinated green tea, or the major bioactive components of green tea, EGCG and caffeine, to pregnant and nursing mice exposed to the potent PAH carcinogen, DBP, provided significant cancer chemoprotection for her offspring. In the case of lymphoma, caffeinated green tea or caffeine alone were protective, whereas decaffeinated green tea of EGCG alone were without effect. In the case of lung, all the treatments decreased lung tumor

multiplicity and size and EGCG was the most effective. It is remarkable that offspring can be protected out to at least middle age following *in utero* or breast milk exposure to a phytochemical chemoprotective agent. These results suggest a possible "imprinting" or epigenetic mechanism.

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Chapter 5

Identifying Efficacious Approaches to Chemoprevention with Chlorophyllin, Purified Chlorophylls and Freeze-dried Spinach in a Mouse Model of Transplacental Carcinogenesis

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ABSTRACT

The carcinogenic potential of dibenzo[a,l]pyrene (DBP) has been well characterized in numerous animal models. We have previously documented that a single dose of 15 mg/Kg DBP to pregnant mice late in gestation (GD 17) produces an aggressive T-cell lymphoma as well as lung and liver cancer in offspring. The current study examines the chemopreventative properties of chlorophyllin (CHL) and chlorophyll (Chl) in this transplacental carcinogenesis model. Pregnant B6129SF1 females, bred to 129S1/SvIm males, received purified diets incorporated with either 2000 ppm CHL, 2000 ppm Chl, or 10% freeze-dried spinach beginning at gestation day 9. Lymphoma-dependent mortality was not significantly altered by maternal consumption of any of the diet and little effect on lung tumor burden in mice surviving to 10 months of age was observed. However, co-administration of CHL at 380 mg/Kg with DBP by gavage (molar ratio of 10:1, CHL:DBP) provided significant protection against DBP initiated carcinogenesis. Offspring born to dams receiving CHL co-gavaged with DBP exhibited markedly less lymphoma-dependent mortality ($p< 0.001$). The degree of protection by CHL, compared to controls dosed with DBP in tricaprylin (TCP) as the vehicle, was less marked, but still significant. Co-administration of CHL (TCP as vehicle) also reduced lung tumor multiplicity in mice by approximately 50% and this was observed throughout the study ($p< 0.005$). This is the first demonstration that CHL can provide potent chemoprotection in a

transplacental carcinogenesis model and supports a mechanism involving complex-mediated reduction of carcinogen uptake.

INTRODUCTION

Chlorophyllin (CHL) is a water-soluble derivative of chlorophyll (Chl) in which magnesium has been replaced with copper and the phytol chains lost. CHL has been safely used in human medicine (e.g., Derifil, primarily to control body odor in geriatric patients) for many years (Young and Beregi, 1980) and is available as a dietary supplement. Chl is present in our diet in green, leafy vegetables, reaching levels of 5.7% in spinach (Dashwood, 1997). Although the potential for CHL and Chl to act as anti-mutagens *in vitro* had been previously published (Negishi *et al.*, 1997) the cancer chemopreventive properties of CHL and Chl *in vivo* were first demonstrated in the aflatoxin B_1 (AFB_1) hepatocellular carcinoma (HCC) rainbow trout model (Breinholt *et al.*, 1995a; Dashwood *et al.*, 1998; Breinholt *et al.*, 1999; Reddy *et al.*, 1999; Pratt *et al.*, 2007; Simonich *et al.*, 2008) and later in a rodent model (Simonich *et al.*, 2007). Physical complexation with the carcinogen reduces bioavailability to target organs (Arimoto *et al.*, 1993; Breinholt *et al.*, 1995b), whereas extended preloading with dietary CHL prior to a single carcinogenic treatment with AFB_1 is ineffective (Breinholt *et al.*, 1999). This mechanism should be essentially species-independent and, therefore, effective in humans. Indeed, in a human clinical intervention trial in

Qidong, China, where dietary AFB_1 exposure is a serous concern (Yu, 1995), a dose of 100-300 mg of CHL, given with meals, for only 3 months was effective at reducing the urinary biomarker of AFB_1-dependent DNA adduction by more than half (Egner *et al.*, 2001). CHL costs pennies a day with no significant side effects being reported, making it extremely attractive for intervention due to the high rate of compliance.

It has been difficult to conduct cancer chemoprevention studies *in vivo* with Chl, mainly due to prohibitive costs and chemical instability. A counter-current chromatography method was recently reported (Jubert and Bailey, 2007), enabling the production of 23 g of highly pure Chl a/b from 90 Kg of spinach leaves in a single run. In addition to demonstrating chemoprevention against AFB_1-dependent HCC, in both trout and rat (Simonich *et al.*, 2007; Simonich *et al.*, 2008), the Chl purified via this method markedly reduced AFB_1 exposure in humans following oral co-administration (Bailey, et al., unpublished data), using ^{14}C-microdosing and accelerator mass spectrometry (AMS) as the means of detection (Brown *et al.*, 2005).

Our laboratory has developed a mouse transplacental cancer model (Yu *et al.*, 2006b; Yu *et al.*, 2006c; Castro *et al.*, 2008a; Castro *et al.*, In press) that has proven useful for chemoprevention studies. Incorporation of indole-3-carbinol (I3C, a major chemopreventive phytochemical from cruciferous vegetables) (Yu *et al.*, 2006c) or green tea (Castro *et al.*, 2008b) into the maternal diet or drinking water, respectively, provided marked protection for offspring with respect to development of DBP-dependent lymphoma and lung cancer. Although DBP-dependent lymphoma mortality is dependent upon fetal Cyp1b1 (Castro *et al.*, 2008a), chemoprevention by

I3C was independent of the aryl hydrocarbon receptor (ahr) genotype (Yu *et al.*, 2006c). In the case of tea, it appeared the major effect was from caffeine, as neither decaffeinated green tea nor EGCG provided protection, whereas, caffeine alone provided the greatest protection.

We now report that co-administration to pregnant mice of CHL by gavage with the potent polycyclic aromatic hydrocarbon (PAH), DBP, results in marked protection from mortality in offspring beginning at 3-6 months of age from an aggressive T-cell lymphoma and significantly reduced transplacental DBP-dependent lung tumor multiplicity as well. However, if CHL or Chl, either as pure compounds or as a component of freeze-dried spinach, were incorporated into the maternal diet before, during, and after gavage with DBP alone, no chemoprotection toward the offspring was observed.

MATERIALS AND METHODS

Chemicals and diets

Chlorophyllin (CHL), tricaprylin (TCP), and dichloromethane were purchased from Sigma Chemical Co. (St. Louis, MO). The chlorin content of CHL was based on the manufacturer's assay of 4.5% copper and assertion that all copper was present as copper-chlorins. DBP was provided by the National Cancer Institute sponsored Carcinogen Repository, at Midwest Research Institute (Kansas City, MO) and was at

least 98% pure as determined by HPLC. The semipurified diets, AIN93G and AIN93M, were purchased from Research Diets, Inc. (New Brunswick, NJ). Chlorophyll (Chl) was prepared as described below.

Preparation of chlorophyll

The chlorophyll used in this study was extracted from baby spinach purchased from local organic growers. A detailed description of the extraction process can be found elsewhere (Jubert and Bailey, 2007). Briefly, after removal of stems, the leaves were washed with cold water, freeze dried, washed twice with petroleum ether (boiling point 30-60°C) and solids extracted twice using methanol/petroleum ether (3:1, v/v). Combined extracts were transferred to a separatory funnel and washed with saturated NaCl. A repeat wash of the aqueous layer with petroleum ether was re-combined to give the final extract and again washed with saturated NaCl, filtered, and evaporated *in vacuo* (< 30°C). On average, 30 g of freeze-dried spinach yielded 300 mg of Chl. This Chl extract (90% pure by HPLC) contained trace amounts of other pigments (carotenoids), as well as some oils, fats, and waxes derived from the spinach leaves. Separate testing of those non-chlorophyll fractions have revealed no protection against DBP carcinogenesis (Bailey et al., unpublished observation).

Preparation of test solutions

Concentrated stocks (> 5mg/ml) of DBP were first prepared in dichloromethane and reconstituted to working concentrations in corn oil or TCP

gavage vehicles. CHL is virtually insoluble in corn oil; thus, CHL solutions were prepared and diluted to the administered concentration in TCP gavage vehicle.

Animals and Treatment Protocols

Eight-week old B6129SF1 female and 129S1/SvImJ male mice (Jackson Laboratories (Bar Harbor, ME) were housed at the Laboratory Animal Resource Center at Oregon State University. Mice were allowed to acclimate for 1 week at 20 ± 1˚ C and 50 ± 10% humidity, with a light/dark cycle of 12 hours in micro-isolator cages (Life Products, Inc., Seaford, DE) with CareFRESH bedding. During breeding, gestation, and lactation, mice were fed powdered AIN93G diet *ad libitum*. Upon breeding, gestation day 0 was established by the appearance of the vaginal plug. Beginning on the 9th day of gestation, pregnant mice were randomly assigned to one of the following feeding regimens; 2000 ppm CHL, 2000 ppm Chl, 10% dietary spinach, or control diet (powdered AIN93G). On the 17th day of gestation, pregnant mice were treated with vehicle (corn oil, 5 mL/kg body weight) or 15 mg/kg DBP. Another subset of mice on control diet were administered CHL (380 mg/kg) as a co-gavage with DBP (15 mg/kg) to give a molar ratio of CHL:DBP of 10. Pregnant mice were continued on the corresponding feeding regimens through the completion of nursing (21 days post parturition). After weaning, offspring of each sex from the same litter were housed together (up to 4 per cage) and fed pelleted AIN93G for the first 3 months and then pelleted AIN93M diet *ad libitum* until euthanized. The number of dams and offspring in each experimental group is shown in Table 5-1.

Table 5-1: Effect of Treatment and Genotype on Survival of Offspring

		Genotype ratio	% Survival		
Maternal Diet	# Offspring	b-1/d : d/d	Overall	b-1/d	d/d
control-A	51	1.04 : 0.96	25.5	19.2	32.0
control-B	60	0.93 : 1.07	50.0	41.4	58.1
Spinach	108	0.92 : 1.08	22.2	19.2	25.0
CHL	101	0.98 : 1.02	16.8	16.0	17.6
Chl	96	1.08 : 0.92	26.0	26.0	26.1
co-CHL	106	1.01 : 0.99	76.6	75.9	77.4

Day 0 of gestation was set as the first day a vaginal plug was observed. Dams were placed on the listed dietary regimens beginning at gestation day 9 as their sole diet source. Dams designated in the following groups received DBP (15 mg/Kg) as a single dose by gavage (corn oil 5 ml/Kg) on day 17 of gestation (Control-A, Spinach, CHL, and Chl). The remaining dams (Control-B and co-CHL) received DBP at identical levels in the TCP gavage vehicle. The group (co-CHL) in which the CHL (in TCP) was administered with DBP had a significantly higher survival rate than the control groups employing either corn oil or TCP as the vehicle.

We observed no overt adverse effects of any treatment regimen on dams or offspring (e.g., no difference in birth weight, litter size, gender ratio, etc.).

To monitor the health status of mice, sentinels were housed within the colony and periodically tested for viral or bacterial pathogens and parasites. All tests, conducted independently under contract to the University of Missouri Research Animal Diagnostic Laboratory (Columbia, MO), were negative throughout the course of the study. Any indication of morbidity, distress or pain resulted in immediate euthanization with an overdose of CO_2, exsanguination and necropsy. Remaining survivors were euthanized and necropsied at 10 months of age, as in our prior work (Yu *et al.*, 2006b; Castro *et al.*, 2008a; Castro *et al.*, In press). All procedures used in the handling, treatment and husbandry of mice were approved in advance by the Oregon State University Institutional Animal Care and Use Committee.

Histopathology

At necropsy the heart, thymus, lung, liver, spleen and kidney were removed, as well as other tissues if they appeared abnormal by gross pathology. The tissues were fixed in 10% formalin, stained with H&E and analyzed by light microscopy. The previously identified T-cell lymphoblastic lymphoma produces high rates of mortality in this transplacental model (Yu *et al.*, 2006b; Castro *et al.*, 2008a; Castro *et al.*, In press). The lymphomas were very aggressive, resulting in invasion of numerous organs by transformed lymphocytes. In addition to lymphoma, mice surviving to 10 months of age developed lung tumors and most males had liver lesions, including foci, hepatocellular adenomas, and, rarely, hepatocellular carcinomas. The lung lesions were initially scored by gross necropsy and a subset of each group submitted for histopathology. As identified previously, the lung lesions were diagnosed as hyperplasia, adenomas, adenoma with progression, and carcinomas (Yu *et al.*, 2006b; Castro *et al.*, 2008a; Castro *et al.*, In press).

Genotyping for ahr^{b-1} and ahr^{d} alleles

At necropsy, an ear punch was collected and lysed overnight at 55°C in a solution of DirectPCR Lysis Reagent containing proteinase K (Viagen Biotech, Inc, Los Angeles, CA). The resulting lysate was briefly centrifuged prior to undergoing a PCR reaction with allele-specific primers to permit one-tube genotyping of the *ahr* alleles as previously described (Yu *et al.*, 2006b). PCR products were separated and visualized on Novex 8% Tris-borate EDTA gels (Invitrogen Technologies, Carlsbad, CA).

Statistical analysis

In statistical analysis of the offspring responses of various litters we corrected for cluster (litter) effects if there was evidence of such an effect. This statistical approach is more completely described in (Castro *et al.*, 2008b).

Survival curves

To evaluate the survival curves we used a log-rank test, also known as the Mantel-Haenszel or Mantel-Cox test (Gibbons, 1985). The survival curves of each group were evaluated (Figure1); p-values that are significantly different at $\alpha=0.05$ from control A (diets, corn oil vehicle) and control B (co-gavage, TCP vehicle) are highlighted in Table 5-1. In addition, in some cases it is relevant to adjust these p-values since multiple hypothesis tests are being performed.

Multiplicity data

To evaluate lung tumor data, we used a Wilcoxon Rank Sum Test (alternatively known as the Mann-Whitney U test), which is a nonparametric version of a t-test for equal means. We used this because an initial evaluation of the multiplicity data for each group showed that, in many cases, the data could not be considered normally distributed. We compared the lung multiplicity information for all groups (Table 5-2). Since we were performing multiple comparisons, which increases the likelihood of observing a significant comparison simply by chance, the p-value cut-offs for significance were modified by a Bonferroni correction.

RESULTS

CHL and Chl effects on DBP-dependent lymphoma mortalities

As previously documented by our laboratory, administration of a single dose of DBP on day 17 of gestation did not elicit acute maternal or fetal toxicities (Yu *et al.*, 2006b; Yu *et al.*, 2006c; Castro *et al.*, 2008a; Castro *et al.*, 2008b; Castro *et al.*, In press). Offspring born to mothers treated with DBP exhibited lymphoma-dependent mortality beginning at 10-12 weeks of age (Figure 5-1). When B6129F1 dams are crossed with 129 sires, half the offspring should have the ahr "responsive" phenotype (genotype, $ahr^{b\text{-}1/d}$) and half the "non-responsive" (genotype, $ahr^{d/d}$). Consistent with our previous findings, offspring identified as ahr responsive typically had lower rates of survival compared to their non-responsive littermates (Table 5-1). Interestingly, administration of Chl or CHL, in the diet or by co-administration, eliminated that genotypic difference in offspring survival irrespective of any overall survival effect.

Maternal dietary exposure to freeze-dried spinach, CHL, or Chl (in AIN 93G) did not significantly alter the overall survival rates of offspring born to mothers treated with DBP (Figure 5-1). It should be noted that, as in previous studies with this model (Yu *et al.*, 2006b; Yu *et al.*, 2006c; Castro *et al.*, 2008a; Castro *et al.*, 2008b; Castro *et al.*, In press), there were no mortalities in offspring born to dams administered the corn vehicle (rather than DBP) among all the treatments (data not shown).

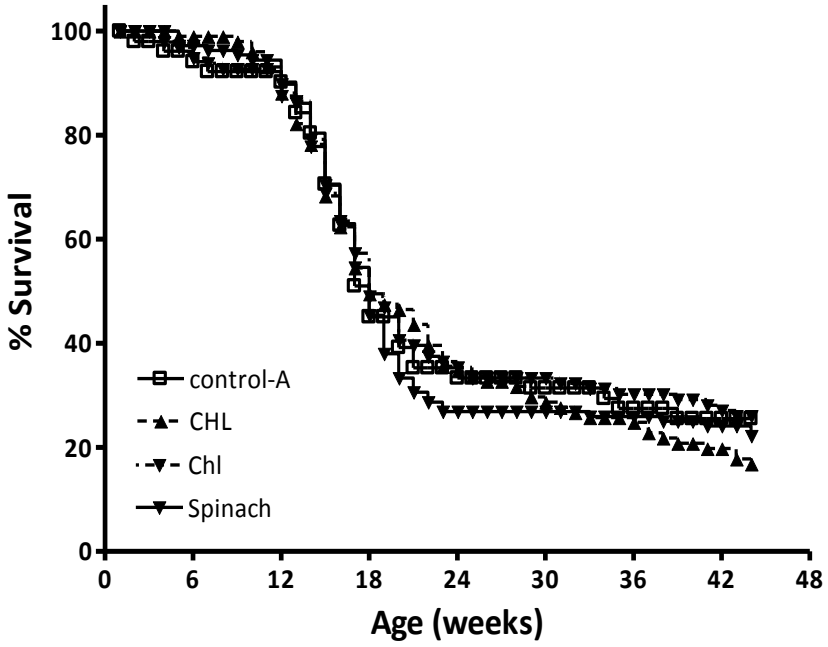
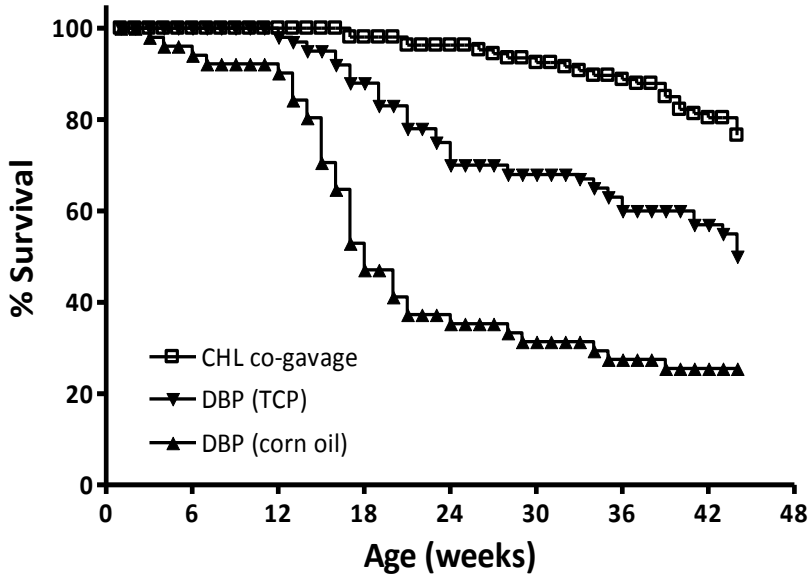


Figure 5-1. Effect of Maternal Dietary Treatment on DBP-dependent mortality. DBP was administered at a dose of 15 mg/Kg by gavage (corn oil) on day 17 of gestation. The pregnant and nursing dams were administered powdered AIN93G diets supplemented with nothing (control (□)), 2000 ppm CHL (▲), 2000 ppm Chl (▼), or 10% freeze-dried spinach (♦).

When dams were co-administered CHL with DBP, the protection against lymphoma mortality in the offspring was highly significant ($p < 0.001$, Figure 5-2A). The impact of co-administered DBP with CHL was apparent irrespective of offspring genotype (Figure 5-2B and Table 5-1). To our surprise, mortality was lower in offspring born to mothers dosed with DBP alone using TCP instead of corn oil as the vehicle (Figure 5-2A). The difference was significant ($p<0.01$), although less than in the mothers that were dosed with DBP in corn oil ($p<0.001$). This result highlights the importance of including appropriate vehicle controls in transplacental chemoprevention studies, as performed here, since the TCP vehicle alone apparently can alter DBP bioavailability to the fetus.

A)



B)

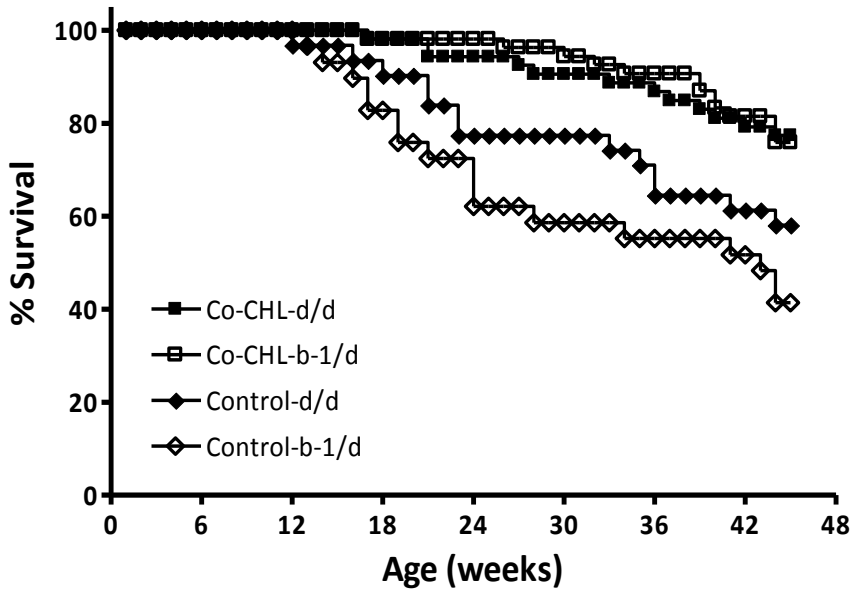


Figure 5-2. A) Impact of co-administration of CHL on DBP-dependent mortality. Pregnant and nursing dams were administered control diets and gavaged at gestation day 17 with DBP in corn oil (▲), TCP (▼), or DBP concurrently with 380 mg/kg CHL (□). CHL was co-administered at a dose of 380 mg/kg by co-gavage with DBP (15 mg/kg) to give a molar ratio of 10 (CHL/DBP). **B) Influence of Genotype and CHL co-gavage on DBP-dependent mortality.** Offspring born to dams receiving DBP by gavage (TCP as the vehicle) and genotyped as $ahr^{b\text{-}1/d}$ (◊) or $ahr^{d/d}$ (♦); offspring born to dams receiving DBP concurrently with 380 mg/kg CHL (in TCP) and genotyped as $ahr^{b\text{-}1/d}$ (□) or $ahr^{d/d}$ (■).

CHL and Chl effects on DBP-dependent transplacental lung cancer

As we have previously reported with this model, all mice exposed *in utero* to DBP and surviving to 10 months of age exhibited multiple lung lesions. The chemoprotective properties of I3C and green tea have been examined in this model and effectively reduced lung tumor burden by 35% and 32% in mice reaching 10 months of age (Yu *et al.*, 2006c; Castro *et al.*, 2008b). In the current study, maternal consumption of spinach, CHL, or Chl in the diet did not provide any significant protective effects against DBP-dependent lung cancer. However, as with DBP-dependent lymphoma mortality, the ability of CHL to protect against lung tumor burden did prove significant if it was co-administered with DBP. Mice born to these mothers receiving the co-gavage of CHL and surviving to 10 months of age had approximately 51% fewer lung tumors (Table 5-2, $p<0.01$). Interestingly, although the vehicle used for delivery of DBP (corn oil vs. TCP) had an impact on lymphoma mortality, mice surviving to 10 months of age had very similar levels of lung tumor multiplicity, 14.5 vs. 15.2 for corn oil and TCP, respectively.

Table 5-2: Effect of Treatment and Genotype on Lung Tumor Multiplicity

Maternal Diet	Overall	Responsive (b-1/d)	Non-responsive (d/d)
Control-A	14.5 ± 2.6	15.2 ± 4.7	14.1± 3.0
Control-B	16.0 ± 1.3	13.8 ± 1.6	17.5 ± 1.8
CHL	14.8 ± 1.5	13.0 ± 1.5	16.3 ± 2.5
Chl	16.1 ± 1.2	16.5 ± 1.7	15.8 ± 1.8
Spinach	19.4 ± 1.6	16.5 ± 1.9	21.4 ± 2.2
Co-CHL	8.6 ± 0.7	8.9 ± 0.8	8.3 ± 1.0

Data is presented as mean ± SE for multiplicity (number of lung tumors per mouse) all groups are significantly different from co-CHL. Control-A, Control-B, CHL, Chl, and Spinach are not significantly different from one another. Co-CHL b-1/d and co-CHL d/d are not significantly different than one another.

Additionally, we also documented the time-course of lung tumor multiplicity from 3 to 10 months of age. Co-treatment of CHL with DBP provided protection throughout the entire duration of the tumor study (Figure 5-3). If reduction in DBP bioavailability accounted for the extent of transplacental chemoprotection observed with corn oil vs. TCP, one would have to account for the difference between fetal target tissues. The genotype of the offspring was not a significant factor in the degree of chemoprevention observed by co-administration of CHL (Table 5-2). A potential explanation could lie in differences in DBP dose-response among fetal target organs and endpoints in this model. As previously reported in a 10,000 animal dose-dose matrix experiment (Pratt *et al.*, 2007), DBP dose-responses for liver tumor incidence, and tumor multiplicity in the rainbow trout model were not linear, but instead reached a plateau or optimum at higher DBP doses. As a consequence, CHL chemoprevention in this organ was observed only at CHL doses sufficiently high to bring the "effective", or bioavailable DBP dose below the plateau or optimum region. By contrast, tumor incidence in trout stomach was linear over the entire DBP dose range studied, and CHL chemoprevention was observable at every DBP dose.

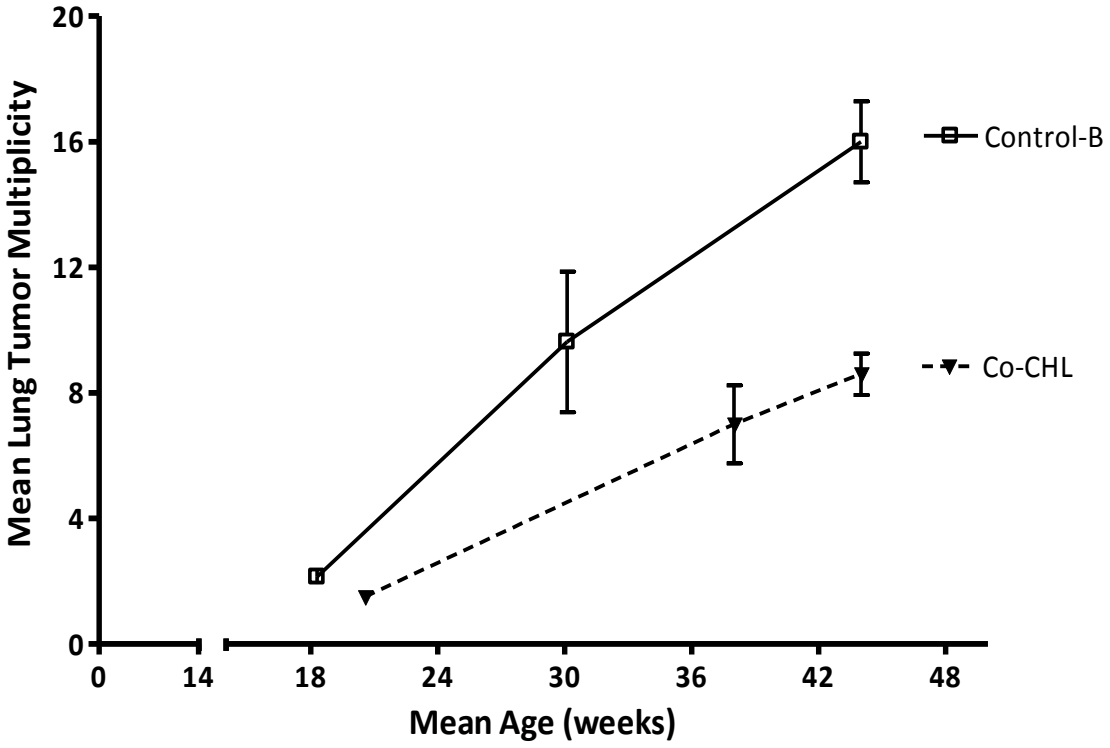


Figure 5-3. Effects of Co-administered CHL on Lung Tumor Burden throughout the Study. Offspring were euthanized due to lymphoma-dependent morbidity (3-9 months) or at the conclusion of the study (10 months) and lung lesions (predominantly adenomas) quantified by histopathology. Data indicate offspring born to mothers given 15 mg/kg DBP alone by gavage (in TCP) (□) or concurrent with 380 mg/kg CHL (in TCP) (▼).

DISCUSSION

Exposure during pregnancy and lactation to chemicals in the environment, including tobacco smoke, has been associated with increases in incidences of disease, malformations or behavior in offspring (Jedrychowski and Flak, 1997; Keeler *et al.*, 2002; Perera *et al.*, 2003; Flower *et al.*, 2004; Shu *et al.*, 2004; Perera *et al.*, 2005; Chang *et al.*, 2006; Choi *et al.*, 2006; Perera *et al.*, 2006). A number of chemicals have been shown to be transplacental carcinogens in rodent models reviewed in (reviewed in Anderson *et al.*, 2000; Anderson, 2004a) and epidemiology studies suggest this phenomenon occurs in exposed human populations as well (Jedrychowski and Flak, 1997; Anderson *et al.*, 2000; Alexander *et al.*, 2001; Keeler *et al.*, 2002; Ma *et al.*, 2002; Perera *et al.*, 2002; Reynolds *et al.*, 2002; Perera *et al.*, 2003; Anderson, 2004a; Flower *et al.*, 2004; Lightfoot and Roman, 2004; Shu *et al.*, 2004; Perera *et al.*, 2005; Chang *et al.*, 2006; Choi *et al.*, 2006; Perera *et al.*, 2006).

PAHs are formed from the incomplete combustion of organic materials including the burning of coal, petroleum products or tobacco (reviewed in Luch, 2005) and have been listed as human carcinogens by International Agency for Cancer Research (IARC, 1983). Increasing energy requirements, especially in countries such as China, are resulting in greater use of coal for energy production; indeed, China derives 70% of its energy from burning coal and the consumption is greater than the U.S., European Union and Japan combined (Tao *et al.*, 2006; Xu *et al.*, 2006; Primbs *et al.*, 2007; Zhang and Smith, 2007). In recent years, Chinese physicians have noted an

increased incidence of diseases which they associate with increased fuel emission (Mumford *et al.*, 1995; Watts, 2006; Yu *et al.*, 2006a; Zhao *et al.*, 2006; Zhang and Smith, 2007). The U.S. derives about 50% of its energy from coal. China and the US also lead the world in automobile use, another important source of environmental PAHs. With respect to environmental exposure to PAHs in the U.S., it should also be noted that emissions from China reach the U.S. west coast in about 6 days from the point of origin, during which they undergo a process of "aging", reacting with sulfur, oxygen and nitrogen to yield more genotoxic PAH derivatives (Feilberg *et al.*, 2002; Reisen and Arey, 2005).

The fetus and the neonate are at increased risk from the toxicological effects of PAHs and exposure of women who are of child-bearing age and women known to be pregnant or nursing should be of concern. We developed a mouse model in which a single treatment with DBP, a few days prior to parturition, produced a severe T-cell lymphoma between 3-6 months of age in offspring (Yu *et al.*, 2006b; Castro *et al.*, 2008a; Castro *et al.*, In press). If the mice do not succumb to the lymphoma, 100% develop multiple lung tumors and the majority of males also exhibit liver tumors (Yu *et al.*, 2006b; Castro *et al.*, 2008a). Utilizing Cyp1b1 null mice, we demonstrated that DBP-induced transplacental lymphoma mortality is dependent upon Cyp 1b1 expression (Castro *et al.*, 2008a). In the human fetus, as in mice, the thymus exhibits the highest expression of CYP1B1 of any organ during late gestation, and among all CYP isoforms CYP1B1 has the highest activity towards the conversion of DBP to carcinogenic metabolites (Luch *et al.*, 1998).

We further developed this model for the study of transplacental chemoprevention by dietary agents. Feeding pregnant and lactating mice I3C (Yu *et al.*, 2006c), or providing green tea or caffeine in the drinking water (Castro *et al.*, 2008b), resulted in significant protection for offspring against DBP-dependent T-cell lymphoma mortality and lung tumor multiplicity. One caveat with this model with respect to statistical analysis of the effect of chemopreventive agents on lung tumors, is the fact that, as many mice die at an early age from lymphoma, we are not statistically assessing a true representative population. In order to assess transplacental chemoprevention efficacy in lung without the confounding lymphoma mortality we would have to conduct studies in a different strain such as the A/J mouse. However, we find this remarkable given that, once weaned, offspring were never exposed to the chemopreventive agent. Therefore, all of the chemopreventive benefits had to be from *in utero* exposure and/or through breast milk. The lymphoma is fatal to mice corresponding in human age to a young adult, with lung tumors developing approximately at the equivalent of human middle age. Thus, modification of the mother's diet during pregnancy (and perhaps lactation) may provide long-term protection from chemical carcinogenesis following *in utero* exposure, to middle age and beyond.

Previously, we demonstrated the chemopreventive potential of both CHL and Chl in the trout model and in the rat with AFB_1 and DBP as the carcinogen (Breinholt *et al.*, 1995a; Breinholt *et al.*, 1995b; Dashwood *et al.*, 1998; Breinholt *et al.*, 1999; Reddy *et al.*, 1999; Pratt *et al.*, 2007; Simonich *et al.*, 2007; Simonich *et al.*, 2008). A

preliminary clinical intervention trial in China, where dietary AFB_1 exposure is high and HCC represents the major cause of cancer mortality, showed significant protective effects of CHL tablets taken orally at meal time (Egner *et al.*, 2001). Results to date point to the importance of simultaneous co-administration of CHL with the carcinogen, supporting the complexation theory for chemoprevention (Arimoto *et al.*, 1993; Breinholt *et al.*, 1995b). Unpublished data in human volunteers given ultra-low doses of AFB_1 in studies employing AMS demonstrated a marked reduction in carcinogen bioavailability when co-administered with CHL or Chl (Bailey et al., unpublished). These results do not exclude additional potential mechanisms of CHL chemoprevention, including modifications of carcinogen metabolizing enzymes (Yun *et al.*, 1995). The study in China documented that some CHL was systemically bioavailable in humans (Egner *et al.*, 2000), and oral CHL was capable of inhibiting PAH-induced skin cancer in mice when the carcinogen was applied topically (Park and Surh, 1996).

In our mouse model of transplacental cancer, co-administration of CHL provided marked protection against DBP-dependent T-cell lymphoma mortality and lung tumor burden. By design, CHL, Chl and freeze-dried spinach were incorporated into the synthetic AIN93G diet to test for possible systemic effects on tumor development. As mice are nocturnal, most diet would be consumed at night, and there would be little if any agent left in the stomach to interfere with DBP uptake when it was administered hours later. The results point to the importance of complexation as a mechanism of CHL chemoprevention, and presumably also for Chl.

The latter possibility remains to be confirmed, since we did not include a group with Chl co-administration. However, the results also do not exclude a transient effect, such as inhibition of phase I or phase II xenobiotic metabolizing enzymes, or epigenetic mechanisms of chemoprevention, such as alterations in DNA methyl transferase or histone deacetylase, that might return to baseline when DBP dosing took place. In a similar fashion, the administration of CHL by gavage could impact enzymes important for bioactivation/detoxication and potentially exert competitive inhibitory effects at such high concentrations. However, as the major route of PAH exposure (in non-smokers) is dietary (Yu *et al.*, 2006a), our results would indicate that CHL should be administered with each meal for maximum efficacy, as in the China intervention study (Egner *et al.*, 2001; Egner *et al.*, 2003). With respect to the impact of the ahr genotype, co-administration of CHL appeared to eliminate the genotype sensitivity rather than making it more complex. However, there is no statistical difference between CHL co-treatment with respect to response by the b-1/d and d/d ahr genotypes.

In summary, CHL, which is inexpensive and appears to lack toxicity in humans, was demonstrated to be effective in the reduction of transplacental cancer risk if given with the PAH carcinogen DBP. This protection was evident even with tumors that appeared well into adult life, and is a further example of the "fetal basis of disease". Cancer is the number two cause of death in children/young adults (accidents being number one) and lymphoma/leukemias are the most common of these cancers. Lung cancer is the major cause of cancer mortality in both sexes in the

U.S. and has a relatively poor prognosis (five-year survival rate of 15%). For these reasons, chemopreventive strategies that begin early in development have the potential to reduce the suffering (as well as the health care dollars) associated with cancer, and perhaps other chronic diseases.

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Chapter 6. Conclusion

In these studies we utilized a transplacental mouse model to characterize the carcinogenic potential of an environmentally relevant carcinogen, dibenzo[*a,l*]pyrene (DBP). Administration of DBP to a pregnant mouse late in gestation was previously found to produce a severe T-cell lymphoma in the subsequent offspring between 10-30 weeks of age. In addition to the lymphoma as a target, all surviving mice develop lung cancer and most of the males liver tumors at 10 months of age. This was the first demonstration in any preclinical model that exposure of pregnant mice to a single dose of a PAH can result in an aggressive lymphoma in offspring, a disease also observed in humans.

DBP has a structure that lends itself to be very lipophilic, as such, it can be expected to partition into the breast milk at reasonably high concentrations. We preformed a cross-fostering study to determine how much risk was due to exposure *in utero* during the 2 days between DBP administration (17^{th} day of gestation) and birth, compared to 3 weeks of nursing. The results observed strongly point to *in utero* exposure as presenting the greatest risk. However, there also appears to be a synergistic relationship between *in utero* and lactational DBP exposure with regard to lymphoma dependent mortality. Mice exposed only briefly late in gestation had higher lymphoma dependent mortality rates than mice exposed only via breast milk (40% vs. 9.9%). If mice were exposed to DBP for both periods of exposure, mortality rates increased to nearly 75%, clearly not the sum of the two individual exposure

periods. A likely explanation could be attributed to the limited detoxication capacity of the offspring following the varying exposure periods which in turn alter the burden DBP on the limited DNA repair capabilities. As with lymphoma mortality, offspring exposed to DBP only *in utero* exhibited greater lung tumor multiplicity than offspring in litters that were exposed to DBP only through nursing (11.5 vs. 3.0). Interestingly, the lung tumor multiplicity associated with the 2 days of *in utero* only exposure was not significantly different than that observed if the offspring received both *in utero* and lactational exposure to DBP (11.5 vs. 14.0).

When comparing the role of the ahr amongst the groups, we again found that a responsive fetal *ahr* genotype reduced the lymphoma survival regardless of the period of exposure. With respect to lung tumor multiplicity, only groups that were exposed to DBP *in utero* were found to have significant genotypic differences. If the offspring were only exposed to DBP through nursing, the *ahr* genotype did not have an impact on lung tumor multiplicity, possibly due to the minimal bioavailable dose or the increased expression of phase II enzymes responsible for detoxification. Collectively, the results suggest that a relatively small amount of DBP partitions into breast milk, presumably due to the rapid elimination from maternal compartments. However, the study also suggests that the response observed depends not only upon the differential target organ dosimetry, but also on the developmental sensitivity of the mouse.

Our mouse model of transplacental carcinogenesis has also been informative with respect to the role of the ahr receptor and its downstream gene targets that are

known to contribute to PAH metabolism (e.g., cyp1a1, cyp1a2, cyp1b1 for bioactivation). As alluded to earlier, we have employed breeding strategies that generate a balanced population of offspring that carries either a "responsive" *ahr* encoding allele (Ahr^b) or only a "non-responsive" encoding allele (Ahr^d). With our model, ahr responsiveness has consistently enhanced the risk of developing lymphoma upon maternal DBP administration. This is in agreement with the understanding that planar PAHs have high affinity for the ahr and are bioactivated primarily by cyps in the 1 family. We believe that these downstream enzymes become induced upon DBP administration and readily bioactivate DBP in the fetal target organs, primarily thymus with secondary targets of lung and liver.

Both *in vivo* and *in vitro* studies have shown that cyp1b1 has the highest activity toward the conversion of DBP to its ultimate carcinogen, the diol-epoxide. In addition, cyp1b1 is expressed at high levels in extrahepatic tissues of untreated animals, including fetal thymus and lung (mouse and human), especially late in gestation (Choudhary *et al.*, 2003; Choudhary *et al.*, 2005). For these reasons, we specifically chose to look at the contribution of cyp1b1 in our transplacental model of carcinogenesis. By utilizing crosses of cyp1b1 heterozygotes on the same genetic background as our previous studies, we were able to show that DBP-transplacental lymphoma mortality was dependent on the presence of at least one wild-type allele and there was a tight correlation between cyp1b1 gene dosage and mortality. However, this correlation was not as apparent in the lung, possibly due to some contribution from cyps in the 1a family. In this study we also included a

toxicologically equivalent dose of benzo[*a*]pyrene (BP) as a negative control. As expected, BP-dependent lung cancer was not influenced by *cyp1b1* status as it is primarily bioactivated by cyp1a1 and cyp1a2.

These results provide convincing evidence that cyp1b1 plays a major role in determination of DBP carcinogenesis, primarily with respect to lymphoma dependent mortalities. The characterization of this model strengthens the notion that gestational exposure to PAHs from maternal diet and airborne particles is a causative factor in the prevalence of leukemias/lymphomas in children and young adults. The discovery that cyp1b1 plays a significant role in the transplacental carcinogenicity of this representative PAH in turn identifies a potential target for prevention strategies. As a corollary, this study also suggests that a CYP1B1 genetic polymorphism could play a role in the target and severity of DBP initiated transplacental carcinogenesis.

Cancer chemoprevention studies have been implemented in various models of cancer with numerous agents of prevention. In the present studies, we utilized this model to examine the potential for green tea and chlorophyllin to act as chemoprotective agents against transplacental DBP carcinogenesis. A number of laboratories have documented potential mechanisms for chemoprevention for both of these phytochemicals with great success. However, neither of these phytochemicals have been employed in a transplacental model of carcinogenesis.

In order to test the efficacy of green tea, we conducted a study in which the pregnant mice were administered green tea, decaffeinated green tea, caffeine, or epigallocatechin-3-gallate (EGCG) throughout gestation and lactation. Caffeine and

EGCG equivalents were included to examine the contribution of these key tea constituents that are suggested to play major roles in the beneficial effects observed by teas. We observed significant protection to offspring with respect to lymphoma-dependent mortality via maternal consumption of green tea during pregnancy and nursing. Removal of caffeine from the green tea resulted in no significant protection. Similarly, there was no effect of administering the major green tea polyphenol, EGCG. However, the greatest protection against lymphoma-dependently mortality was observed in offspring born to mothers receiving caffeine alone, further substantiating the chemopreventive role of caffeine. Although the exact mechanism of the inhibition of caffeine is not entirely known, we postulate that the increased activity of maternal cyp1b1 upon caffeine consumption results in the decreased bioavailability of DBP to fetal target organs. However, we do not advocate any changes to the recommendations bestowed by Food and Drug Administration and the March of Dimes regarding caffeine consumption in pregnant women. Our results highlight the need for a thorough understanding of mechanism of action and dose-response for beneficial and adverse effects in order to do a proper risk assessment.

In this study we also saw a 100% incidence in lung cancer in offspring born to DBP-treated mothers. The time course of lung tumor multiplicity demonstrated that offspring born to mothers in any of the four drinking regimens exhibited a marked and significant delay in the appearance of lung tumors. In contrast to the lymphoma mortality, the greatest effect and the longest delay were observed in offspring given EGCG alone. These results imply that the mechanisms of cancer chemoprevention by

tea and components of tea for lymphoma and lung in this model are distinct. Regardless, these findings are remarkable given the limited duration of exposure and the significant protection observed throughout lifetime of these offspring.

In our latest study, we tested the efficacy of purified chlorophyllin (CHL), purified chlorophylls (Chl) or freeze-dried spinach as chemopreventive agents. Chl is of great interest due to its conserved blocking mechanism of chemoprotection and ubiquity in green plants. In order to assess the physical complexation mechanism of protection we included a group which received a co-gavage of CHL and DBP at a 10:1 molar ratio. To test for possible systemic effects on tumor development, each of the individual forms of Chl were separately incorporated in the maternal diet. Consistent with the proposed mechanism, only co-administration of CHL provided marked protection against DBP-dependent T-cell lymphoma mortality and lung tumor burden. However, since the CHL, chl, or freeze-dried spinach was consumed by these nocturnal animals at night, a lag period between consumption and DBP administration exists and potentially affords little chance for complexation to occur. Because of this lag, we also cannot rule out a transient effect that might return to baseline when DBP dosing took place. This includes alterations of bioactivation/detoxification enzymes or possibly modulation of epigenetic machinery.

Collectively, we have demonstrated in this DBP transplacental model of lymphoma that cancer chemoprevention agents are a viable approach to reduce the impact of environmental carcinogens on the sensitive fetus or infant, providing

protection well into middle and old age. As expected, gestational exposure to environmental carcinogens appears to be the critical window of sensitivity. Considering our findings regarding cyp1b1 dependent bioactivation of DBP, the capacity to bioactivate and detoxify the carcinogen appears to be a key determinant in the subsequent tumor burden upon fetal/infant exposure in the mouse. We have yet to test this model with other chemical carcinogens, but we believe there is great promise in this approach and high potential for positively impacting human health. More research is required to further evaluate the mechanism and significance of our transplacental chemoprevention strategy. It is likely that multiple mechanisms contribute to the efficacy of our chemopreventive agents, which may explain the varying levels of protection in the different target organs. However, we find it remarkable that the addition of cancer chemopreventive agents to the maternal diet can provide protection to the offspring for a good portion of their lifespan, even though they themselves never consume the phytochemical.

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