AN ABSTRACT OF THE DISSERTATION OF

Mary Margaret Pratt for the degree of Doctor of Philosophy in Toxicology presented on January 22, 2003. Title: Chlorophyllin Chemoprevention Against Dibenzo[*a,l*]pyrene-Initiated Multi-organ Carcinogenesis in the Rainbow Trout Model

Abstract approved by: Redacted for Privacy

George S. Bailey

Chlorophyllin (CHL), a water-soluble derivative of the green plant pigment, chlorophyll, is an effective antimutagen and anticarcinogen in various model systems when used as a modulator against a class of carcinogens that, in general, have a structure consisting of at least three fused rings. Dibenzo[*a,l*]pyrene (DBP), an extremely potent environmental carcinogen, has been isolated from urban air samples, tobacco smoke, and coal smoke condensate. A study was conducted to evaluate the complex interrelationships among dietary DBP doses with co-exposure to a range of CHL doses. In order to achieve adequate statistical power in the generation of multiple dose-response curves, this dose-dose matrix experiment utilized over 12,000 rainbow trout. The resulting DNA adducts were assessed and evaluated as biomarkers of exposure to discern their relationship with the final tumor outcome.

CHL was highly effective in reducing DBP-initiated DNA adduct formation in the liver and stomach and strongly inhibited tumor formation in the liver (56 -

79% inhibition), stomach (30 - 68%), and swim bladder (over 80% at the highest DBP dose). Molecular dosimetry revealed adduct formation to be predictive of final tumor response in both organs regardless of CHL dose. Other parameters evaluated were consistent with CHL-mediated protection.

A clinical CHL preparation, evaluated in a human population subsequent to the seminal demonstration of CHL chemopreventive properties against AFB_1 in trout (1), revealed CHL to be just as effective in reducing biomarkers of alfatoxin exposure to humans (2). Dietary administration of this clinical preparation along with DBP in the rainbow trout demonstrated CHL protective capacity against DBP-initiated multi-organ DNA adduct formation and final tumor incidence.

Sucrose was evaluated, deemed unlikely to be sequestered in a complex with CHL, and was used as a control in a pharmacokinetic study evaluating the biodistribution of DBP with and without CHL. The results provide evidence against a non-specific masking mechanism for CHL-mediated blocking of DBP (or aflatoxin)-initiated tumorigenesis.

CHL at multiple doses provided significant protection against multi-dose DBP-initiated DNA adduction and tumor formation in multiple organs. CHL-mediated protection, primarily by reduced carcinogen biouptake and consistent with a complexation mechanism, is supported by these results.

Chlorophyllin Chemoprevention Against Dibenzo[*a,l*]pyrene-Initiated Multi-organ Carcinogenesis in the Rainbow Trout Model

by

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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.

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Mary Margaret Pratt, Author

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CONTRIBUTION OF AUTHORS

George S. Bailey	Guidance and critical review of experimental design, data analysis, and manuscript. Incredible insight and patience.
Jerry D. Hendricks	Instruction in and assistance with gross pathology and histologic analysis of tumor tissue.
Ashok P. Reddy	Guidance in designing a 12,000 animal tumor study and instruction in postlabeling techniques.
Cliff Pereira	Statistical modeling and analysis.

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Chapter 1

Introduction

Objectives

The information presented in this first chapter is intended to provide a general summary of the basis for the work being presented followed by an overview of carcinogenesis. Included in this overview will be a brief description of the multistage model of carcinogenesis, a brief history of the evolution of the understanding of cancer molecular biology, and some background into how the research presented in the body of this thesis developed and is intended to contribute to the understanding of cancer chemoprevention mechanisms. A better understanding of how cancer occurs is essential to gaining a better understanding of how cancer risk can be reduced. This is the primary objective of the following thesis.

A General Background for this Thesis

The work presented herein evolved from research investigating the chemopreventive properties of naturally-occurring dietary compounds and an

interest in finding ways of using these compounds to reduce the cancer risk of individuals unavoidably exposed to dietary carcinogens.

The incidence of liver cancer in the United States is quite low relative to other areas of the world, in particular, parts of Southeast Asia and Africa. This high incidence of liver cancer has been attributed primarily to a relatively high incidence of infection by hepatitis B and C virus and is compounded by the unavoidable dietary exposure to aflatoxin (AFB_1) (3). The connection to AFB_1 exposure was further strengthened by the demonstration that biomarkers of AFB_1 exposure, specifically AFB_1-N^7-guanyl DNA repair products, were found in the urine of individuals living in those areas where there are high levels of AFB_1 contamination in the foodstuffs (4, 5). Following the demonstrated success of the chlorophyll derivative, CHL, in reducing AFB_1-initiated liver tumorigenesis in the rainbow trout (1), a clinical trial was undertaken to evaluate the effectiveness of CHL in reducing the biomarkers of AFB_1 exposure in humans (6). The results of this study, published in November 2001, indicate that CHL was highly effective in reducing the urinary biomarkers of AFB_1 exposure (2).

With the clinical study in China underway, another report was published regarding the effectiveness of CHL in reducing liver tumorigenesis in the rainbow trout model following initiation with another potent carcinogen, dibenzo[*a,l*]pyrene (DBP) (7). DBP is mutagenic (8) and an extremely potent carcinogen in mouse mammary gland and rat skin models (9). DBP has been identified in tobacco smoke

(10), urban air samples collected in the United States (11) and in coal smoke (12). Following the DBP study in trout, it was suggested that the same mechanism of protection may be responsible for the effectiveness of CHL against both AFB_1 (13) and DBP (7). By the time the trout DBP report was published, work had already begun on another study, which is reported in Chapter 2 of this thesis. This new study sought to evaluate multi-organ tumorigenesis following dietary administration of multiple doses of CHL, including the clinical formulation used in China, in combination with a range of DBP doses. In an effort to further understand the protective mechanism of CHL, Chapter 3 of this thesis describes an experiment that attempted to evaluate the pharmacokinetic behavior of DBP, alone and in simultaneous gavage administration with a concentration of CHL in a molar ratio that was shown to strongly inhibit tumorigenesis in the liver. Also reported in Chapter 3 is the result of a control experiment using sucrose, which was deemed unlikely to form a complex with CHL, to assess the possibility that the mechanism of CHL protection may involve a nonspecific masking of carcinogen uptake.

A Multistage Model for Carcinogenesis

What is cancer?

Cancer is not a term that describes a singular disease, and likely it has no singular cure. It may be more appropriately thought of as a disease process,

consisting of a series of steps, some reversible, some not. If this step-wise progression is not reversed or otherwise terminated, a mass of self-replicating material results that is not under the control of the body which supplies it with a place to grow, along with the nutrients necessary for continued growth. Over time, the tumor grows until it breaks through its confines, releasing transformed cells into the surrounding tissue, thus becoming a malignancy. When cells from the malignant tumor are able to migrate into the bloodstream or lymphatic system, metastasis occurs, resulting in the transport of dysregulated cells to remote tissues in the body where they begin to grow anew as a secondary cancer. The growth of these tumors can eventually overwhelm the body in which they exist, resulting in the failure of the organ systems in which the tumors reside, followed by illness and death. This process is summarized in a model consisting of three distinct stages: initiation, promotion, and progression. The reader is referred to reviews of this subject by Alphonse Sirica (14), and Pitot and Dragan (15), from which the following was summarized.

Initiation

The first event in the multistage process of carcinogenesis involves damage to DNA and is referred to as initiation. DNA damage can occur from exposure to exogenous factors, such as ultraviolet light, certain viruses, or chemical compounds (16), but can also result from exposure to reactive oxygen species and other factors

generated endogenously (17). Distortions in the shape of the DNA molecule can be caused by any of these processes, resulting in a disruption in the normal course of the cell cycle and transcription. The damaged DNA is frequently recognized by components of the cellular DNA repair systems and, in most cases, the damage is repaired. An important part of the initiation process is the failure to accurately repair DNA damage. If the cell survives the DNA damage but the repair process was inaccurate, a mutation results.

A point mutation occurs when a single base in the DNA strand is damaged and/or improperly replaced with a normal, but incorrect nucleotide. If this occurs in a non-transcribed region, the consequences are minimal and the cell is likely to continue with its existence as if no mutation had occurred. If, however, the mutation occurs in a region that codes for a gene product or is in a promoter region where the transcription factors attach and assemble, the results can lead to an incorrect DNA sequence being replicated during the subsequent round of cell division, with potential outcomes in the daughter cell including a lack of transcription for that gene, transcription of a full-length but nonfunctional or malfunctioning product, or a truncated product which may or may not possess any functionality.

A frameshift mutation occurs when one or more bases are deleted or inserted improperly, resulting in the shifting of the genetic reading frame, rendering the gene product completely non-functional. In the event of a three-base deletion or

insertion, the frameshift results in the production of a protein product that has the correct sequence of amino acids, except for one omission or addition, assuming the omitted bases are contained within the same codon. Such a product may or may not have some functionality.

Initiation is considered an irreversible process; once the mutation is fixed by the passing of the altered DNA sequence to the progeny cells, there is no known process whereby it is deliberately repaired. Initiation, however, is only the first step of the process. If the subsequent steps do not occur, an initiated cell with a mutation in a coding region may be of no consequence to an organism.

Promotion

The process of encouraging a cell with mutated DNA to continue living and proliferating is defined as promotion, which is the second stage in the multistage carcinogenesis model. By definition, promoters are not mutagenic compounds nor do they need to interact directly with DNA. In this sense, promoters are epigenetic in their mode of action. Mechanisms of promotion include receptor-mediated changes in gene expression, the alteration of signal transduction pathways or the disruption of the mitotic spindle apparatus. Initiated cells, in the absence of a promoter, will likely not proliferate and become a tumor. Initiated cells exposed to an appropriate promoter will begin the process of clonal expansion, even if the promoter is applied long after initiation occurs. The progeny of the original,

mutated cell are referred to as clones because each progeny cell, unless subject to another mutation, is an exact clone (copy) of the original.

Besides damage to DNA, there are two other major differences between initiation and promotion. First, promotion is reversible. The withdrawal of the promoting agent from the proliferating mass of cells can result in a stoppage and reversal of cell proliferation. Secondly, during later stages of promotion, the growing mass of cells, also known as a neoplasm (new growth), becomes large enough to be visually discerned.

Despite the fact that initiation and promotion are discreet steps in the development of cancer, it is actually quite rare that a compound doesn't function in both capacities. Most carcinogens are referred to as "complete carcinogens" and are capable of both initiation and promotion.

Progression

The third defined step of multistage carcinogenesis is progression, which is characterized by increasing growth of the cell mass and increasing genetic and karotype instability. During the early stage of progression, cells are still sensitive to environmental agents. Additionally, progressor agents, including asbestos fibers and benzene, can be applied to advance promoted cells to this stage. Cells in the early stages of progression can still be benign, but a characteristic of late-stage

progression is invasion of transformed cells into adjacent tissues, which is referred to as malignancy.

For a neoplasia to reach this point, a failure of the immune system is involved as well. The immune system of an organism operates by being able to distinguish "self" from "non-self." This simple concept, on the surface, is actually a very complex system of biochemical checks and balances which, if functioning normally, should result in the destruction of exogenous invaders while doing no harm to "self". When an initiated cell undergoes development through progression, it has become a mass that is, technically, not native to the host in that it is of a different genetic composition. A normally functioning immune system should recognize this non-normal cell as "non-self" and destroy the cell as it would an invading bacteria.

Progression of Cancer Molecular Biology

Three theories of the origins of cancer

The following historical discussion summarizes a detailed review by Robert Weinberg in The Molecular Basis of Cancer (18). The reader is directed to Weinberg's review for more complete information.

In the early1970s there were three prevailing theories of carcinogenesis. The first portrayed cancer as a disease of abnormal differentiation, which is the

process whereby an undifferentiated cell, which has the genetic potential to become any cell in the body, is directed by endogenous signaling to become a specific type of cell, such as a skin or muscle cell. This theory of abnormal differentiation held that, for cancer to occur, the normal development of the cell was somehow disrupted, implying that cancer was essentially an epigenetic process in that no concomitant change in the genetic structure was assumed.

A second theory held that cancer resulted from exposure to a retrovirus, which would insert its viral genetic information into a cell that would redirect cellular processes to benefit the existence of the virus. With the virus-directed changes in genetic content, the cell would be transformed from a normal to a cancerous state.

A third theory evolved due to increasing observations of the relationship between carcinogens (those compounds found to transform normal tissue into malignant neoplasms) and mutagens (which are compounds that produce changes in the DNA base sequence). In particular, the observation in the Bruce Ames laboratory of the relationship between mutagenic potency of compounds and the ability of these compounds to induce tumors in laboratory animals directed the attention of cancer researchers toward non-viral processes that result in damage to genetic material. This latter theory came to dominate the thinking of most cancer researchers, however, research involving the viral theory of cancer subsequently contributed to the understanding and description of oncogenes (genes capable of

producing cancer) and proto-oncogenes (normal genes that have the potential to produce cancer).

The src oncogene

In the 1970s, investigations designed to better understand the transformation-inducing replication strategy of the Rous sarcoma virus (RSV) led to the identification, by Varmus and Bishop, of *src* as the gene containing the cancer-causing information. Further studies of this virus, and the *src* gene in particular, led them to believe that *src* likely had its origins in the genome of the experimental host animal, the chicken. In fact, the *src* gene, in its original, unaltered state, was found not only in the genome of chickens, but also in the genome of all vertebrates. To distinguish between the viral and cellular versions, the name v-*src* was bestowed upon the viral, oncogenic, version of the gene and c-*src* was the name given to the host, or cellular, proto-oncogene.

The discovery of v-*src*, and its origins as c-*src*, led to the realization that the genetic potential for the carcinogenic process in humans actually lay within the human genome. The illustration of c-*src* showed that when a proto-oncogene is inserted into an abnormal chromosomal environment, incurring small genetic sequence changes in the process, the potential exists for this gene to induce cancer. It follows that, in an otherwise normal cell, alteration of the cellular control

pathways and/or alteration the chromosomal environment could cause abnormal expression of the gene, resulting in the induction of cancer *in situ*.

The ras oncogene

The subsequent use of gene transfer, or transfection, showed that oncogenes could be transferred from a tumor cell to a healthy cell without the involvement of a retrovirus. In this manner, the *ras* family of oncogenes, so named because of their association with rodent sarcoma viruses, was identified. Subsequent investigations led to the discovery that the difference between the *ras* gene associated with a normal, healthy cell, and the tumor-associated *ras* was a single base pair.

The mutated *ras* gene and its function in carcinogenesis, we now know, is typical of those genes in which mutations or dysregulation have been associated with carcinogenesis. Genes such as *src*, *ras*, *myc*, and *p53* are components of signaling pathways that are important in controlling the cell cycle and proliferation. The *ras* gene product, in particular, is one of the earlier components of a signaling cascade that, when mutated, results in an oncogenic transformation and loss of cell cycle control. The *ras* gene, is significant not only in that it was one of the first oncogenes identified, but also in that mutated *ras* has been identified in over 30% of all human neoplasms (19). Additionally, the Ki-*ras* gene has been identified in the rainbow trout (20), and *ras* mutational data in response to several carcinogens has been published (21).

Cancer Chemoprevention

There are two primary defined mechanisms of cancer chemoprevention. The primary difference between the two is the point of action of each within the multistage model of carcinogenesis. A thorough review by Stoner, *et al*, of both mechanisms has been summarized below (22).

Blocking

Interception and removal or destruction of a carcinogen prior to initiation, before DNA damage has a chance to occur and become fixed, is referred to as blocking. Chemoprevention by blocking includes changes in the expression of both phase I and II metabolizing enzymes, alteration in the rate of DNA repair, and the scavenging of a reactive oxygen species and other free radicals (23). All of these blocking mechanisms can serve to reduce the likelihood of DNA damage by preventing a chemical carcinogen from reaching the target tissue. This is accomplished by altering the activity of detoxifying or bioactivating enzymes in the target tissue, upregulating the rate of DNA repair, or reducing the quantity of reactive oxygen species. Dietary protective factors, including ascorbic acid (Vitamin C), alpha tocopherol (Vitamin E), and tea polyphenols, are antioxidants that have been shown to improve general health and decrease damage to DNA resulting from exposure to free radicals (24-26). Chlorophyll and its derivatives

have also shown promise as blocking agents (27, 28). More will be said later in this text regarding CHL.

It is difficult, in humans, to assess the immediate effectiveness of blocking agents, though biomarkers of exposure are being developed that can, in some situations, give a fairly prompt indication of reduced exposures. These will be discussed shortly.

Suppression

Suppressing agents act by reducing the consequences once an initiation event has occurred (23). Cancer prevention clinical trials generally assess the effectiveness of suppressing agents, though some blocking agents have endpoints that can be evaluated (29). It is more common to see discussions of the effectiveness of suppressing agents because of their visible effect on tumor size and number, though surrogate endpoints are actively being sought (29). Agents such as non-steroidal anti-inflammatory drugs (NSAIDS) can slow or even reverse abnormal cell growth. Other compounds can act by causing cell growth arrest or the induction of apoptosis. Clinical chemotherapeutic agents historically have been generalized suppressing agents that act by killing rapidly proliferating cells. This has the unfortunate side-effect of toxicity toward proliferating cells that are not cancerous, such as cells in hair follicles. Research continues in the quest to identify

suppressing agents that are more specific to cancerous tissue and less damaging to normal tissue.

Indicators of Carcinogen Exposure

Biomarkers

For the purposes of biomonitoring, it is useful to identify an intermediate, or surrogate endpoint, or biomarker, for assessing the extent of exposure to a toxin or carcinogen. (30, 31). By definition, biomarkers are not the final endpoint of interest, rather, they are "measurable biochemical, physiological, cytological, immunological or molecular changes in a biological system related directly or indirectly to exposure as well as measurable levels of metabolites in body fluids or compartments" (32).

With respect to carcinogens, biomarkers frequently are a means of assessing DNA damage using techniques designed to detect nucleotides damaged in a particular fashion (such as oxidative damage), by a particular carcinogen, or class of carcinogens. When a carcinogen is able to bind, chemically, to DNA, an adduct is formed. Detection of DNA adducts are commonly used as biomarkers of carcinogen exposure.

For an experiment utilizing a radiolabeled carcinogen, detection of DNA damage involves the isolation of DNA and quantification of the amount of

radiolabeled DNA relative to total DNA. In other experimental situations, or when it is desirable to assess environmental exposures, the DNA can be subjected to chemical labeling techniques that target damaged nucleotides. The DNA is enzymatically digested and the adducted nucleotides can be separated from the normal nucleotides, either chromatographically or by solvent extraction. The label, which frequently consists of radioactive phosphorus (^{32}P or ^{33}P) (32-35), can then be detected either to assess total adducts or they can be subjected to a chromatographic procedure to separate the different adducted nucleotides or different conformations of the carcinogen.

DNA adduction can also be evaluated by analyzing the urine for DNA repair products. Individuals chronically exposed to DNA-binding carcinogens have been shown to excrete DNA repair products containing carcinogen metabolites adducted to DNA (36). This technique has been used to assess the effectiveness of oltipraz and CHL in their ability to reduce DNA damage caused by dietary AFB_1 exposure (2, 6). A primary benefit of using urinary biomarkers is the lack of invasiveness of the sample collection.

Other techniques for the detection of DNA adducts have been reviewed and discussed (37).

Molecular Dosimetry

If a biomarker can, experimentally, be shown to be a quantitative indicator of the final endpoint of interest, the biomarker can then be used not only to assess the imminence of the final endpoint, but also as a quantitative predictor of magnitude of that endpoint (38, 39). This is extremely useful in that quantitative detection of carcinogen biomarkers can be used to assess the degree of success of an intervention (2, 6) long before the final endpoint, in this case, metastatic cancer, can fully develop. A quantitative biomarker is also a much better indicator of the toxic potency of a compound than is the administered dosage of that compound. The actual, *in vivo*, tissue dosage, as evidenced by a quantitative biomarker, is a more direct link to a certain endpoint.

The Rainbow Trout Model

Origin

The use of rainbow trout for the purpose of cancer research came about as a result of an epizootic of liver tumors in hatchery fish in the Pacific Northwest in the late 1950s and early 1960s (40, 41). The normally low background tumor incidence was substantially elevated to the point that an investigation was undertaken to determine the cause for this increase in liver tumor incidence. The culprit was

finally identified as the mycotoxin, AFB_1, which was a contaminant in the cottonseed oil used in the formulation of the fish diet (40).

AFB_1 is a metabolite produced by the fungus, *Aspergillus flavus*, which is commonly found on various seed crops, and grows particularly well in warm, moist conditions. The identification of AFB_1 as the carcinogen in the diets of fish and livestock contributed to the discovery of AFB_1 in the human food supply and its identification as a very potent human hepatocarcinogen (3).

Utility of the Rainbow Trout Model

There is substantial utility in using rainbow trout or other fish models for the purposes of medical research. A detailed review is available (41). The following summary draws heavily from this review.

Results gleaned from the use of fish models represent yet another vertebrate species in which affects, or lack thereof, can be demonstrated. Because of the lower costs associated with animal husbandry, considerably greater numbers of individuals can be used experimentally, thus achieving greater statistical power in the experimental results. Another benefit of using rainbow trout in particular is their demonstrated sensitivity to carcinogens such as AFB_1, *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG), and 7,12-dimethylbenz[*a*]anthracene (DMBA).

The sensitivity of rainbow trout to AFB_1 has been attributed to a negligible constitutive or inducible glutathione transferase activity with specificity for AFB_1-

8,9-epoxide, and the fact that they are excision-repair deficient. These features of rainbow trout metabolism are beneficial in that smaller amounts of carcinogen or toxins are required in order to elicit a treatment response. For a time it was thought rainbow trout lacked the metabolic activity that could make them a desirable model for use with compounds requiring bioactivation. This was later disproved and, in fact, rainbow trout possess a great number of metabolizing enzymes. The process of isolating, identifying, and characterizing these enzymes continues. An excellent review of this ongoing process has been published (42)

As is the case with mammals, rainbow trout can also be used as a multi-organ model for carcinogenesis. Tumors of the liver, stomach, kidney, and swim bladder have all been identified in rainbow trout following exposure to chemical carcinogens. The body of literature is increasing regarding histologic classification of these lesions. Multiple exposure routes, including dietary, bath exposure, embryo injection, IP injection, and oral gavage can all be utilized in the rainbow trout model.

Chlorophyllin

Background

The relevance of studying the relationship of dietary compounds to carcinogenesis can be found from a reading of "The Causes of Cancer" (16).

Included in this report is a discussion relating the proportion of cancer deaths attributed to various environmental factors. Tobacco is, of course, well represented, but the most noteworthy figure is the estimation that 35% of all cancer deaths could be attributed to dietary imbalance. This imbalance includes unavoidable exposure to constituents that are present naturally in food, such as the caloric and fat content, and exposure to those compounds that are formed during the processing or cooking of food (43-45).

There are also a fair number of dietary protective agents that could reduce the risk of cancer (43-45). A diet rich and fruits and vegetables contains dietary fiber and antioxidants, both of which have been associated with reduced risk of cancer. The British habit of having a cup of tea bodes well for the drinker because antioxidants found in tea, and green tea in particular, have been shown to reduce colon cancer risk. Further, studies have shown that chlorophyll derivatives also have protective properties. Inadequate consumption of these protective compounds could increase an individual's risk of acquiring cancer at some point in their life.

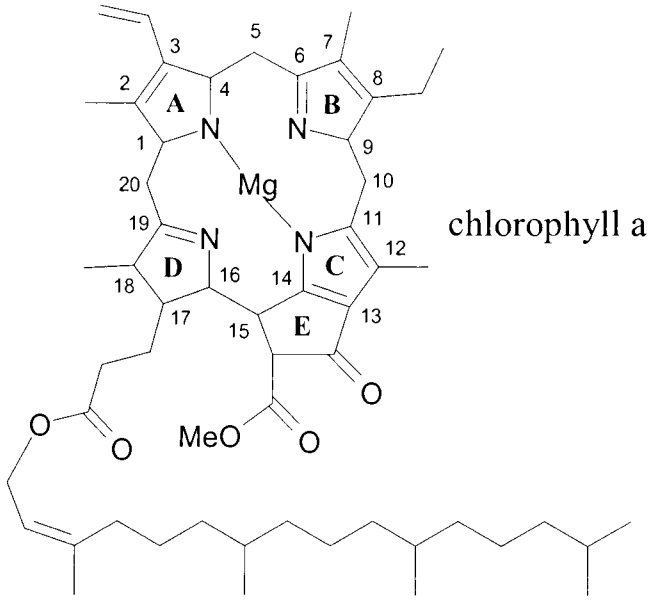
A Derivative of Chlorophyll

All green plant material is colored by the pigment, chlorophyll, to which humans are exposed on a daily basis, and have been for thousands of years. Of particular interest here is the derivative known as chlorophyllin (CHL). A lengthy review of the literature relating early attempts to characterize chlorophyll and the

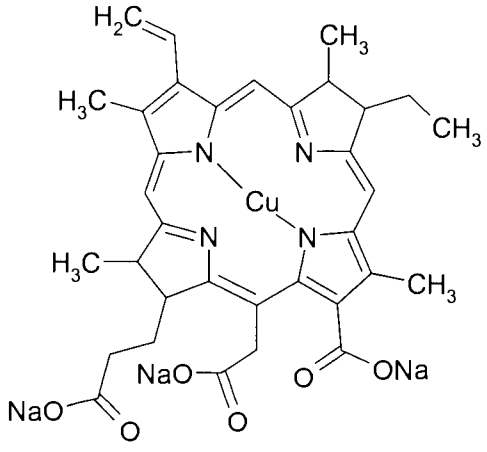
eventual derivation of the product referred to as CHL was published in 1955 (46). Included in this review is a description of the preparation of CHL from chlorophyll by a saponification process. The actual composition of CHL has since been characterized as a mixture composed of two primary components, copper chlorin-e_6 and copper isochlorin e_4 (e_6 and e_4, respectively), and several other pigmented compounds in smaller quantities (47, 48) (Figure 1-1). Whether e_6 or e_4 is present in greatest quantity apparently varies between manufacturers. This has implications for the observed effectiveness of the preparation (49, 50). Unless specified otherwise, this discussion will refer to any preparation simply as "CHL".

CHL has been prepared and used for medicinal purposes since the early part of the 20th century. A report in the American Journal of Surgery from 1940 recommends the use of CHL as an antiseptic "to be applied as often as necessary without fear of skin irritation (51)." The preparation of chlorophyll derivatives and their many uses is discussed extensively (46). Later reported uses of CHL include controlling body and fecal odors (52-54). Early concerns about the safety of chlorophyll derivatives were addressed and it was concluded that there was no evidence for toxicity, either in general or due to the release of the complexed metal ion (55). Additionally, there have been no documented reports of adverse effects with CHL usage in the 50+ year history of use by humans.

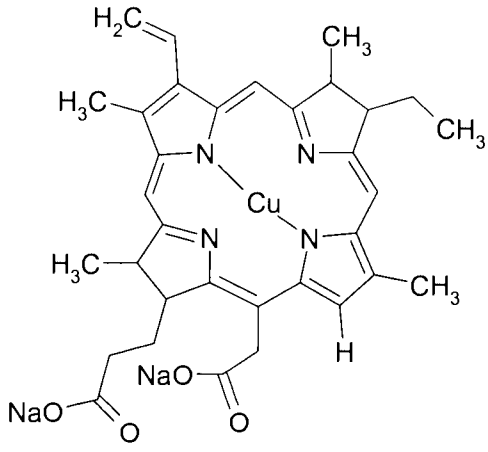
As research was increasingly being devoted to the potential of naturally occurring dietary compounds to function as anticarcinogenic agents, CHL was



chlorophyll a



trisodium copper chlorin e_6



disodium copper isochlorin e_4

Figure 1-1. Structures of chlorophyll a and the major components of chlorophyllin

investigated because of its close relationship with chlorophyll. In 1988 a review was published citing the various biological activities of chlorophyll and its derivatives (56). Besides stating the effectiveness, particularly of the water-soluble "chlorophyll copper complex" in its various uses, Chernomorsky and Segelman also noted the lack of toxicity observed in experimental animal models.

Chlorophyllin antimutagenicity

In 1986, Ong, *et al*, reported the antimutagenic properties of CHL against a variety of environmental and dietary complex mixtures, and suggested a mechanism related to CHL's antioxidant properties (57). Further investigation into the antimutagenic potential of CHL showed its effectiveness against 3-hydroxy-amino-1-methyl 5*H*-pyrido[4,3-b] indole, a metabolically active form of the heterocyclic amine, 3-amino-1-methyl 5*H*-pyrido[4,3-b] indole (Trp-P-2), in the Salmonella assay (58). This provided evidence of the effectiveness of CHL in the absence of metabolizing enzymes.

CHL has been reported to inhibit the activities of metabolizing enzymes (59, 60), however other studies have shown that CHL is effective even against direct-acting mutagens and carcinogen metabolites (61, 62).

A hypothetical blocking mechanism by complex formation between CHL and the mutagen was suggested by several studies involving heterocyclic amines, AFB_1, and other mutagens (58, 63-66). The complexation mechanism was again

implicated with the demonstration of Sepaharose- and chitosan-supported CHL *in vitro* binding of numerous compounds, most strongly to those compounds having three or more fused rings in their structure (65, 67). In a related study, a dietary combination of CHL and Trp-P-2 prevented the formation of wing spots in the *Drosophila* mutagenicity assay (66).

Chlorophyllin anticarcinogenicity

Carcinogenesis assays with CHL also demonstrated the protective capacity of CHL in multiple model systems. Studies in the rainbow trout model were the first to demonstrate the anticarcinogenic activity of CHL. Following administration in the diet with AFB_1, a significant reduction in liver adduct formation and subsequent tumor response was observed (1). Subsequent studies in the same model reported significant reduction in AFB_2 (a less toxic surrogate of AFB_1) uptake and biodistribution following co-gavage with CHL (68). It was also reported that CHL had no effect on metabolizing enzymes but was effective at inhibiting AFB_1 liver adduct formation only when both were contained in the same solution, either in a bath or in an oral co-administration (69).

Dietary administration of CHL with 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) to rats showed that CHL was most effective in reducing biomarkers of exposure when administered concurrently with IQ. This was evidenced by an 80% reduction in DNA binding in the liver and 63% reduction in

colon DNA binding observed at the highest dose of CHL evaluated (70). A subsequent study with the same two compounds demonstrated the effectiveness of CHL administered in the drinking water in delaying Zymbals gland tumor formation, inhibiting tumor formation in the small intestine, colon, and liver and the reduction of tumor multiplicity in the liver against dietary administration of IQ in the rat model (71). However, a slight increase in skin tumor incidence with administration of CHL was reported.

This adverse effect was not without precedent. CHL has been shown to have promotional effects against dimethylhydrazine (DMH) in the rat colon (72). A subsequent study in the same model supported these results with DMH rendering an increased multiplicity of colon tumors with low doses of CHL, though it was suggested that this effect was dependent on the initiator, test species, and protocol (73). Additional work in the rat colon carcinogenesis model with IQ and DMH has led to the suggestion that lower doses of CHL could affect the balance between apoptosis and proliferation in a post-initiation protocol (74), possibly in association with β-catenin mutations (75).

CHL in drinking water resulted in protocol-dependent inhibition of DNA adduct formation in the colon following gavage or dietary treatment with 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (76). The results of this study led the authors to conclude that CHL had shown some evidence of suppression in the post-initiation phase, but that the protocol was not optimized to permit complex

formation between PhIP and CHL. Thus, the protection against initiation was less than anticipated. Anti-promotional activity by CHL was also reported against 12-O-tetradecanoyl phorbol-13-acetate (TPA)-promoted, DMBA-initiated mouse skin carcinogenesis model (77).

Moving from animal models to actual human usage, a double-blinded, placebo-controlled clinical study with CHL was recently completed in China where urinary biomarkers of AFB_1 have been validated and used (6, 36). The clinical study showed 300 mg/day doses of CHL to be effective by producing a 55% reduction in AFB_1-N^7-guanine DNA repair products detectible in the urine (2). Again, no toxicity or side effects, other than green excrement, were reported.

Dibenzo[*a,l*]pyrene

Background

Subsequent to the flurry of work in this laboratory with AFB_1, another carcinogen containing at least three fused rings was identified for investigation. Dibenzo[*a,l*]pyrene (DBP, Figure 1-2) is a polycyclic aromatic hydrocarbon (PAH)combustion product which has been detected in cigarette smoke (10) and coal smoke (12). Several chromatographic peaks identified as "dibenzopyrenes" have also been isolated from urban air particulates (11).

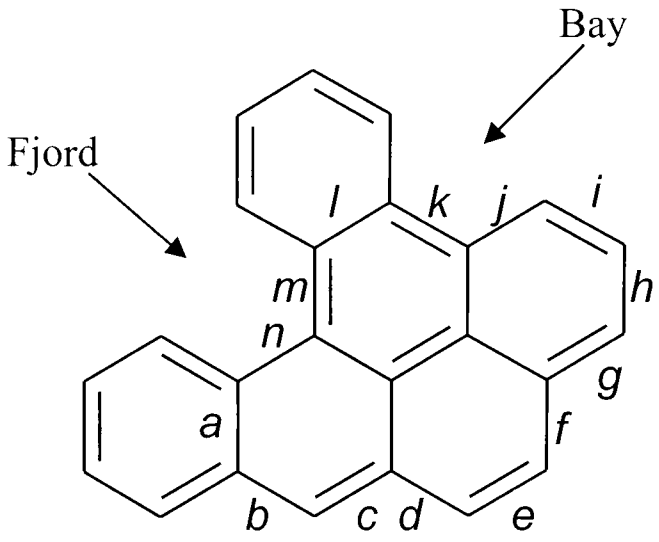


Figure 1-2. Structure of dibenzo[*a,l*]pyrene. Alphabetic characters surround the pyrene portion of the structure and indicate the chemical bond designations. Fjord and bay regions are indicated.

A potent carcinogen

Research into the carcinogenicity of DBP was very limited until the late 1980s, likely due to a misidentification of another, less potent carcinogen as DBP. In 1966, a study was published correcting the error (9). In 1989 an article was published relating the substantial toxicity and mouse skin and mammary carcinogenicity following DBP-initiation and TPA promotion (78). A follow-up article two years later using the same model systems reported that DBP was the strongest PAH carcinogen ever tested (9). Interest in studying the health effects of DBP increased thereafter.

A thorough review of PAHs and cancer risk assessment was published very recently, which discusses the nature of PAHs, probable sources, and possible methods of evaluating exposure risk from these compounds (79). The term "polycyclic aromatic hydrocarbon" (PAH) is descriptive of the family of compounds it represents. In essence, these compounds are all variations on a central theme of between 3 and 6 six-member rings. Until recently, benzo[*a*]pyrene (BaP) was considered to be the most potent PAH carcinogen. A system of evaluating the carcinogenic potential of these compounds was based on that of BaP, which was assigned a Toxic Equivalency Factor (TEF) of 1. As reviewed by Bostrom, different evaluators assigned TEF's to DBP ranging from 1 to 100 (79), indicating not only the variation in the TEF determination, but also indicating the degree of perceived DBP potency as a carcinogen.

Figure 1-2 shows the structure of DBP, which consists of a core, four-ringed pyrene group with two additional benzyl rings attached at the "*a*" and "*l*" positions. DBP is considered a procarcinogen, which means, without bioactivation, this compound does not exert a carcinogenic effect. DBP is bioactivated by cytochrome P450 enzymes (CYP), with the 1A1 isoform (CYP1A1) being the primary bioactivating enzyme (80). The metabolites of this bioactivation (Figure 1-3) vary in carcinogenic potency, with the most potent being (-)-*anti(R,S,S,R)* and (+)-*syn(S,R,S,R)*-DBP, which were found to bind exclusively to deoxyadenosine residues of DNA in MCF-7 cells (35).

Given its potency as a carcinogen, it is interesting to note that DBP was a less potent mutagen in the Salmonella assay than numerous other compounds, including BaP (8). However, a later assessment of mutagenicity using a human cell line engineering to constitutively express CYP1A1 found DBP to be approximately 24 times more mutagenic than BaP (81) demonstrating the substantially increased potency of DBP upon bioactivation by CYP1A1.

A structure-activity relationship has been demonstrated with PAHs showing that those compounds having a "bay" or "fjord" region in their structure exhibit greater mutagenic and carcinogenic potency (79). As shown in Figure 1-2, DBP contains both of these features. The presence of the fjord region diol-epoxide metabolite has been associated, for DBP in particular, with very potent

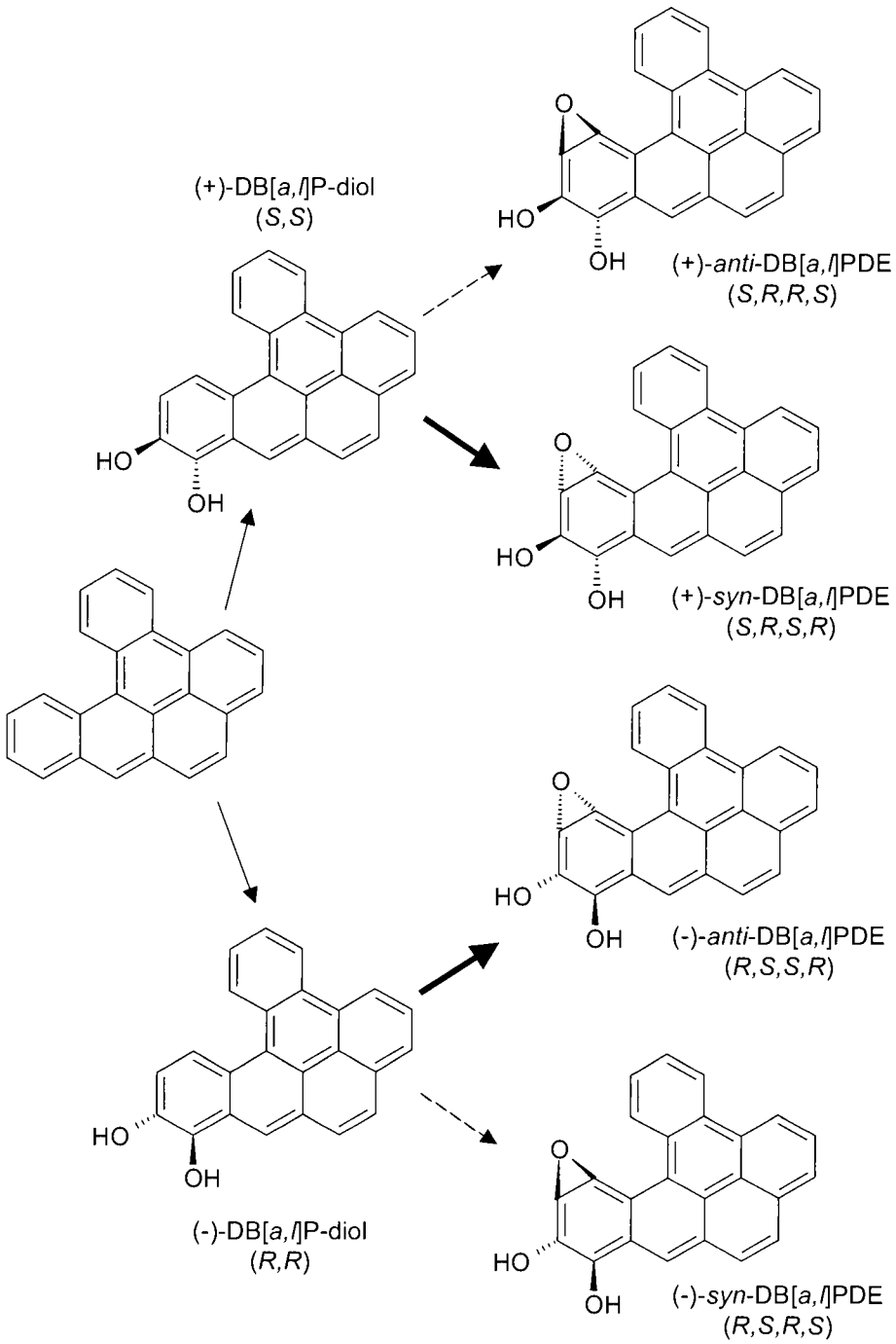


Figure 1-3. Bioactivation pathway of DBP to ultimate carcinogens in MCF-7 cells. Bold arrows indicate observed metabolic pathways. Adapted from Ralston, *et al.* (35).

tumorigenicity in newborn mice (82). For PAH in general, compounds containing fjord regions are considered more active as mutagens and carcinogens (79).

Human Exposure to DBP

The extent of human exposure to DBP is uncertain. Likely because it has been detected in such small quantities, it hasn't received the attention of those PAHs existing in greater quantity. As reported in the Bostrom review, a recent Swedish study did not consider DBP in assessing environmental PAHs, however, a recent study in California did include DBP and concluded that DBP was the main contributor of BaP-based toxic equivalents (79).

DBP has been identified in the indoor air of homes in Xuan Wei county, China, where unvented coal and wood burning stoves are used for cooking and heating. Urinary biomarkers of PAH exposure have been identified from those individuals who live in these homes (12). This area of China has a high mortality from lung cancer. In Henan Province, China, and Linxian County, in particular, where unvented, coal-burning stoves are also a common source of household heating, there is a long history of "YeGe", which is essentially translated as "esophageal cancer" (83, 84). DBP was not specifically identified in an analysis of soot extracts from the coal-burning stoves in this region, however, the authors freely admit the possibility that their analysis may have missed detecting some co-eluting compounds (83).

Obviously, PAHs pose a lung cancer risk due to their nature as combustion products. It is pointed out by Bostrom, however, that PAH intake by diet is larger than via inhalation, probably due to the deposition of particulate matter on food (79). Despite the relatively low levels of DBP identified in the environment, and the extreme potency of DBP suggests that it should be considered a significant environmental hazard, particularly in areas where air pollution due to combustion products is elevated.

CHL Inhibition of DBP-initiation carcinogenesis

Previous experiments

A study conducted in this laboratory with medaka revealed that this fish model is sensitive to DBP-initiated carcinogenesis and that DBP was an effective hepatocarcinogen (85). Concurrent work in this laboratory also demonstrated the sensitivity of rainbow trout to DBP-initiated multi-organ carcinogenesis (7). This same study also showed that CHL administered in the diet at a single concentration significantly inhibited DBP- initiated carcinogenesis in liver, stomach, and swim bladder, and inhibited hepatic DNA adduct formation in the trout. *In vitro* models have shown similar protective effects by CHL against DBP-initiated DNA adduction (86, 87). CHL inhibited DBP-initiated mutagenesis and was shown, *in*

vitro to be capable of non-covalent complex formation with DBP (7). This study was limited in that it examined only one dose of CHL at one dose of DBP.

Experimental Objectives

The primary objective of this research project was to evaluate the dose-dose interactions between CHL and DBP, determine to what extent CHL would be protective against DBP-initiated carcinogenesis, and to further define the mechanism by which this protection occurs. Given that CHL has been shown to inhibit DBP-initiate tumorigenesis in the trout model at a single dose combination (7), specific questions to be addressed in the following studies include:

- What effect does dietary DBP dose have on tumor response in each organ?
- What is the effect of dietary co-administration of CHL on DBP-initiated tumor response?
- What effect does dietary DBP dose have on DNA adduct response in each organ?
- Does dietary CHL dose change the nature of the DNA adduct response?
- How do the tumor and adduct responses relate to each other? Can DNA adduct response be used as a quantitative predictor of final tumor response?
- What is the effect of dietary DBP exposure on body weight, liver weight, and liver somatic index? What is the effect of CHL on these parameters?

- What is the spectrum of liver tumor phenotypes? Is there a change in spectrum associated with DBP or CHL dosage?
- Is there a DBP-dependent effect on tumor multiplicity and size? Is there a CHL-dependent effect on tumor multiplicity and size?
- Where does CHL exert its protective effect, and by what mechanism? Can some potential mechanisms be eliminated from consideration?
- Is there evidence suggesting toxicity at high CHL doses?

Obtaining answers to these questions will provide insight into potency and mechanism of CHL in its apparent ability to protect against DBP-initiated carcinogenesis. A demonstration of the effective protection by CHL across a broad spectrum of endpoints has implications outside the laboratory given the detection of DBP in the environment, its carcinogenic potency in various model systems, and the demonstrated safety and effectiveness of CHL in clinical trials.

Chapter 2

Modulation of dibenzo[*a,l*]pyrene-initiated liver, stomach, and swim bladder tumor response by dietary chlorophyllin in rainbow trout

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Abstract

Chlorophyllin (CHL), a water-soluble, sodium/copper derivative of chlorophyll, is a potent antimutagen against BaP, DMBA, and the heterocyclic amines, IQ and PhIP[1]. CHL anticarcinogenicity was first observed against dietary AFB_1 initiation in the rainbow trout model and subsequently seen following oral exposure in the rat model and again in the rainbow trout model against a single dietary dose of the potent carcinogen, dibenzo[*a,l*]pyrene (DBP). More recently, a double-blinded, placebo-controlled clinical study concluded that oral treatment with CHL significantly reduced urinary biomarkers of AFB_1 exposure in a human population. Here we report an expanded analysis of DBP in the trout model that evaluated the complex interrelationships among dietary carcinogen doses with co-exposure to a range of CHL doses. In order to achieve adequate statistical power in the generation of multiple dose-response curves, this dose-dose matrix experiment utilized 12,350 rainbow trout. CHL was prefed for one week, co-fed with DBP for four weeks, followed by one week of CHL post-feeding. CHL doses were 0, 1500, 3000, 4500, or 6000 ppm. At individual doses of CHL, DBP was fed at doses of 0, 10.1, 28.4, 80.0, 225 or 371.5 ppm. Each dose combination of CHL/DBP was evaluated with three or more tanks of 100 fish per tank. Following 28 days of carcinogen feeding, livers and stomachs were removed from a subset of fish from

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[1]Abbreviations used: AFB_1, aflatoxin B_1; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; BaP, benzo[*a*]pyrene; DMBA, 7,12-dimethylbenz[*a*]anthracene.

each tank to assess DNA adducts as biomarkers of exposure and to discern their relationship with the final tumor outcome. Eleven months after initiation, the livers, stomachs, and swim bladders were removed from the remaining 8711 trout and examined microscopically for the presence of tumors. DBP administered in the diet resulted in a dose-dependent increase in DNA adduction and tumor formation in all organs. A marked decrease in adduct formation and tumor incidence with increasing CHL dose in all organs evaluated was observed. The effect of CHL on tumor formation can be summarized using percent inhibition defined as $100(1\text{-}TD_{50}$ ratio), assuming a parallel-offset model for the majority of CHL doses evaluated. Increasing doses of CHL shifted the DBP dose-response curves toward higher TD_{50} values yielding tumor inhibition ranging from 55-79% in liver and 30-68% in the stomach. A molecular dosimetry plot of logit incidence vs log DBP-DNA adducts for liver and stomach indicated that these biomarkers are quantitative predictors of eventual tumor outcome over all DBP and CHL dose combinations in this model. Increasing CHL dose also reduced tumor multiplicity in the liver at all doses of DBP evaluated, and was associated with a small decrease in tumor size at the highest dose of DBP. There was no dose-effect of DBP or CHL on tumor spectrum or liver somatic index. Concurrently, we evaluated the clinical CHL preparation (Derifil) used in the double-blinded, placebo-controlled intervention study in individuals chronically exposed to AFB_1. The Derifil preparation was effective in reducing DNA adduct formation and final tumor incidence in all organs evaluated

with a potency that was very similar to the higher doses of the reagent grade CHL. Responses to the placebo preparation were essentially indistinguishable from the controls, except for a small, but statistically significant decrease in liver tumor formation and multiplicity. These results support the hypothesis that CHL, as either the reagent grade product or the clinical preparation, is highly effective in reducing DBP-initiated DNA adduct formation and subsequent tumor formation in a manner that is largely consistent with reduced bioavailability to all target organs.

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Introduction

Carcinogens to which people are exposed come from numerous sources, including pollution, ultraviolet light, tobacco products, and, most disturbingly, the diet. It has been estimated that 35% of cancer deaths can be attributed to dietary imbalance (16), including a lack of protective dietary factors. Carcinogenic compounds in the diet need not arise from pesticides or industrial contaminants. Specific examples include heterocyclic amine mutagens/carcinogens that are formed during the cooking process (88). The mycotoxin and potent human carcinogen, aflatoxin (AFB_1), is produced by the Aspergillus fungus, which is a common contaminant on improperly stored grains and seed crops, such as corn and peanuts, and has been associated with high rates of liver cancer in parts of Asia and Africa. Polycyclic aromatic hydrocarbons (PAHs) can be formed on foods during the cooking process or can be deposited on foods as smoke condensate (79). This is a problem in areas, particularly rural China, where unvented stoves are used for indoor cooking and heating (89). The inhabitants of these regions, where this type of heating is used, experience high rates of lung (12) and esophageal cancer (84). Dibenzopyrenes have been identified in airborne particulates collected from urban areas (11). Dibenzo[*a,l*]pyrene (DBP) has specifically been identified as a combustion product in coal smoke (12) and tobacco smoke (10). Though present in small quantities, it has been shown to be a potent mutagen (81, 90), among the most potent carcinogens in rodent models (9, 91) and a potent carcinogen in fish

models[2] (85). Work in the rainbow trout model has shown that CHL can substantially reduce single dose, DBP-initiated liver tumorigenesis (7).

CHL has been used in human populations for over 50 years for medicinal purposes with no reported adverse effects (46, 51-55). CHL was a very effective antimutagen against several heterocyclic amines (57, 58, 62, 64-66, 92-95), a potent agent for the reduction of carcinogen-DNA binding (70). The anticarcinogenic properties of CHL were first demonstrated in the rainbow trout model in a dietary co-exposure protocol with AFB_1 (1) with confirmation of CHL-mediated protection in a subsequent rodent study against 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) (71). Subsequent to the CHL-AFB_1 work in trout, a double blinded, placebo-controlled clinical study was undertaken in AFB_1-endemic areas of China where AFB_1-N^7-guanine DNA adduct repair products have been validated as biomarkers of AFB_1 exposure (4). Taken orally in the amount of 300 mg per day, CHL reduced the recovery these biomarkers by > 50% (2).

In this study we report the effectiveness of CHL against DBP-initiated tumorigenesis in a multi-organ rainbow trout model. Similar to mammals, rainbow trout express a wide range of cytochrome P450 metabolizing enzymes (42), some of which have been shown to bioactivate DBP (96). Rainbow trout have proven a useful and sensitive model for carcinogenesis studies (41), with a primary benefit being reduced husbandry costs which allows for the use of greater numbers of

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[2]Bailey, G. S., *et al.*, Unpublished data.

individuals for greater statistical power. This dose-dose matrix study was designed to evaluate the complex interrelationships among DBP doses delivered across multiple doses of CHL, assess DNA adduct formation and evaluate the usefulness of these adducts as a biomarkers of exposure in discerning their relationship to eventual tumor outcome. In order to conduct this study with sufficient statistical power, 12,350 animals were used in 23 DBP-CHL dose combinations. Included as two of these treatments were the CHL tablet (Derifil) and placebo formulations that were used in the recent clinical study against AFB_1 in China.

Materials and Methods

Materials

DBP was obtained from the National Cancer Institute (NCI) Chemical Carcinogen Reference Standard Repository at Chemsyn Laboratories (Lenexa, KS). CHL, proteinase K, nuclease P1, prostatic acid phosphatase, apyrase, and snake venom phosphodiesterase were purchased from Sigma Chemical Co. (St. Louis, MO). T4 nucleotide kinase was purchased from United States Biochemical (Cleveland, OH). RNAase A and T1 cocktail was purchased from Ambion (Austin, TX). Radiolabeled ATP ([γ-^{33}P]) was purchased from New England Nuclear (Boston, MA). Organic chemicals used were HPLC grade and were purchased from EM Science (Gibbstown, NJ). The dose of Sigma CHL used in this study was

based on the copper content stated on the label and corrected to the actual copper chlorin content of 42.05%. Derifil and placebo tablets were generous gifts of Rystan Inc. (Little Falls, NJ). The CHL content of the Derifil tablets was 100 mg per tablet, with a stated purity of 98% copper chlorins[3]. DBP was dissolved in the oil component of the trout semi-synthetic Oregon Test Diet (OTD) (40, 97) where DBP was required. CHL, Derifil, and placebo tablet powder were dissolved in the water portion of the diets. The concentrations of both DBP and CHL are expressed in parts per million (ppm) relative to the dry weight portion of the diet. For those diets containing DBP or CHL, or both, the oil and water containing specified amounts of DBP and CHL, respectively, were used in the same fashion as the oil and water portions of the diets containing neither DBP nor CHL. Both DBP and CHL are light-sensitive compounds and were handled, when possible, under subdued lighting or under lighting with a 400 nm cut-off. Diets were prepared every 2 weeks and stored at -20°C until 1 day prior to feeding, at which time they were removed to 4°C. A separate experiment examined DBP recovery from diets with and without CHL following storage at -20°C for a period of up to 3 years. (see below).

Animals

Shasta strain rainbow trout (*Oncorhynchus mykiss*) were spawned, raised,

[3]Thomas Kensler, personal communication.

and treated at the Food Toxicology and Nutrition Laboratory (FTNL), Oregon State University. The fish were fed OTD during the time between spawning and dietary initiation. The fish used were from four separate groups of brood stock that were spawned on the same date. In total, 12,350 trout were selected and distributed, 130 fish per tank, to 95 tanks in random order. Each tank contained equal representation of fish from the four groups of brood stock. The fish were acclimatized in their respective tanks for 1 week prior to the commencement of CHL prefeeding.

Dosage Determination

The concentrations of DBP used were based in part on a previous study, which showed that 500 ppm DBP exceeded a maximum tolerated dose, that ample tumor response was available at 200 ppm DBP, and that 4000 ppm CHL co-exposure greatly inhibited tumor response at this single DBP dosage (7). Based on this single data point, we made assumptions regarding the extent of inhibition of various doses of CHL in conjunction with assumptions regarding the extent of tumor formation with varying doses of DBP. The expected tumor incidence at each proposed combination of CHL and DBP was used to calculate the number of replicate tanks at each treatment needed to produce equal variances between all doses. The number of tanks was then rounded to multiples of three for the purpose of designing a sampling schedule that would minimize the effects of time on tumor formation. The actual concentrations of DBP used in the present study represent

log equal intervals, with the exception of the 371.5 ppm dose, which is only a half-log interval from 225 ppm. A DBP dose of 632.8 ppm, the concentration of DBP that is a full log interval above 225 ppm, would have been unwise, given the high mortality reported at 500 ppm, and prohibitively expensive DBP cost.

Study Design

Figure 2-1 shows the overall dose-dose matrix design of this study. CHL was prefed 1 week at 0, 1500, 3000, 4500, or 6000 ppm, then co-fed with varying doses of DBP for 4 weeks, followed by 1 week of post-initiation feeding with CHL. DBP doses were 0, 10.1, 28.4, 80.0, 225.0, or 371.5 ppm. After 4 weeks of CHL and/or DBP feeding, five fish were sampled from each tank with livers and stomachs removed for DNA adduct analysis. This exposure duration was selected based on studies indicating that hepatic AFB_1-DNA (98) and DBP-DNA adducts[4] accumulate linearly over a 4-week dietary exposure in trout, a species deficient in global excision repair. After sampling for adduct analysis, the remaining trout were fed OTD for the duration of the study, which was terminated 11 months after study initiation. Each CHL-DBP dose combination (23 total, including clinical formulations) was evaluated in triplicate or multiples thereof in order to equalize the variances of the anticipated tumor incidences.

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[4]Bailey, G. S., *et al*, Unpublished data.

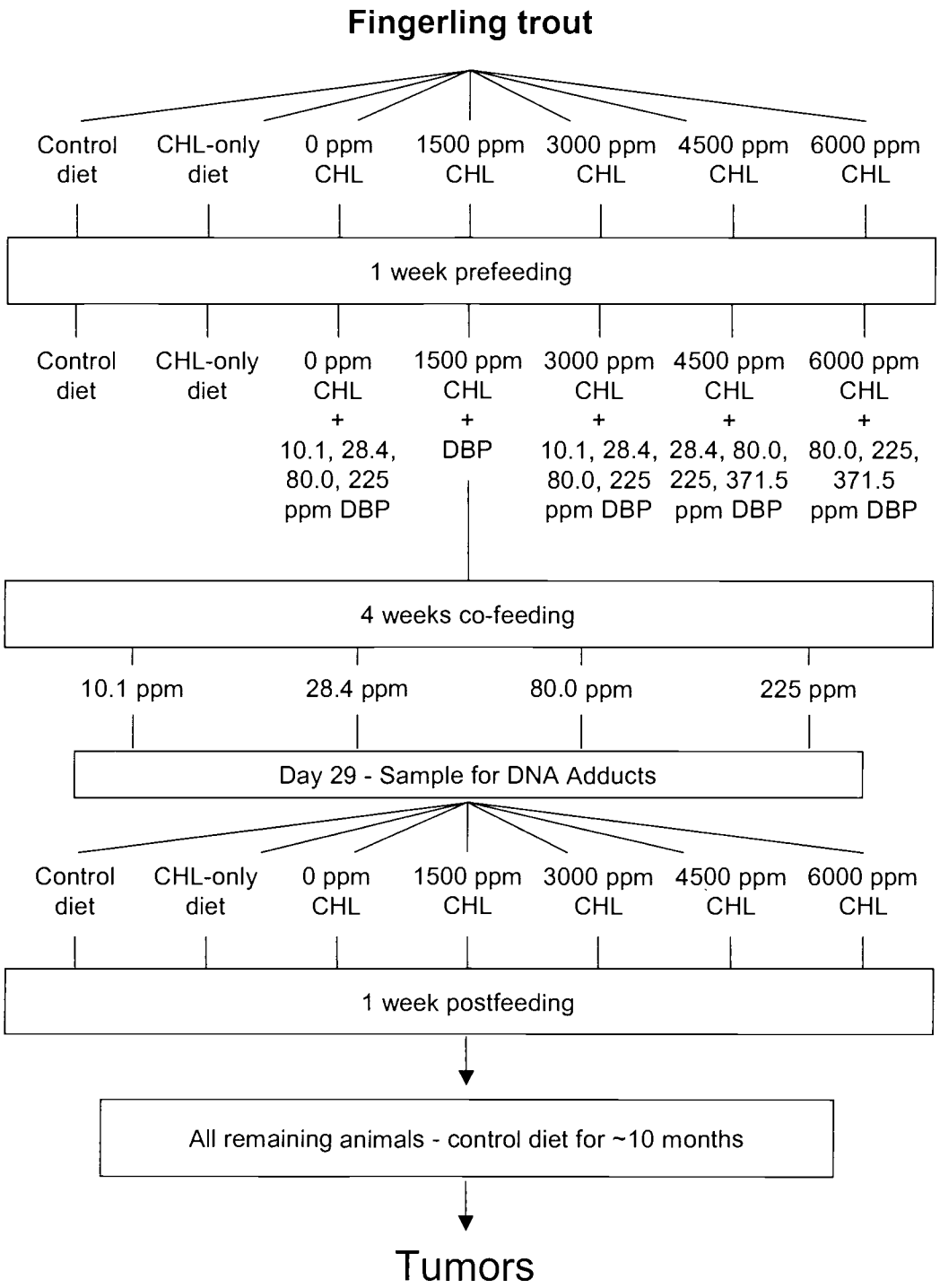

Figure 2-1. Study design.

Isolation of Hepatic and Stomach DNA and Determination of DBP-DNA Adduction

Twenty-nine days after commencement of carcinogen feeding, five fish were removed from each tank and euthanized. The livers and stomachs were removed and pooled by tank and organ. All collected samples were quick-frozen in liquid nitrogen then stored at -80°C. Pooled tissue samples were placed in a mortar and ground with a teflon pestle in a buffer consisting of 100 mM Tris, 100 mM KCl, 10 mM EDTA, and 10% glycerol at pH 8. The nuclear material was isolated by centrifugation at 1000 g and 4°C for 10 minutes, separated from the resulting supernatant, then resuspended in 4 ml 1% sodium dodecyl sulfate (SDS)/20 mM EDTA. For liver samples, the nuclear material was digested with RNAase (16 µl of a solution containing 6.2 units/µl each of RNAase A, RNAase T1, and alpha-amylase) and Proteinase K (100 µl at 20 µg/µl, pH 8, freshly prepared), followed by phenol-chloroform extraction and precipitation with ice-cold ethanol. Following the removal of ethanol, purified DNA was redissolved in TE buffer and stored at -80°C until ^{33}P-postlabeling. Stomachs were homogenized as above, digested with RNAase Cocktail and Proteinase K (100 µl at 20 µg/µl, pH 8, freshly prepared), extracted using NaCl and chloroform-isoamyl alcohol, then precipitated with ethanol. Purified stomach DNA was also dissolved in TE and stored at -20°C.

The purified DNAs were postlabeled by a modification of previously described procedures (33, 99). For each sample, 10 µg of DNA were dissolved in 5 µl deionized water and incubated with nuclease P1 (0.4 units) and prostastic acid

phosphatase (0.35 units) in 1.0 µl nuclease P1 buffer (a solution consisting of 125 mM sodium acetate, pH 5.2, and 30 mM zinc chloride) at 37°C for 45 minutes. The samples were then concentrated to 5 µl before the addition of T4 Polynucleotide kinase (0.6 µl of 30 units/µl stock) in 2.0 µl T4 kinase buffer (a solution consisting of 0.5 M Tris-NaOH, pH 9.6, 8 mM spermidine, 100 mM magnesium chloride, and 100 mM dithiothreitol), followed by the addition of [γ-^{33}P]ATP and another incubation of 1 hour at 37°C. The addition of 15 mU snake venom phosphodiesterase and 0.1 U apyrase to the mixture was followed by a final, 1-hour 37°C incubation. Samples were stored overnight at -20°C. The DBP-DNA adducts were separated from the non-adducted nucleotides using C-18 SepPak cartridges, as previously described, with minor modifications (99). Before use, the cartridges were conditioned with 10 ml methanol, 10 ml deionized water, and 10 ml loading buffer. Postlabeled samples were diluted to 7 ml in loading buffer and applied to their respective cartridges, followed by the reapplication of the eluent to ensure maximal retention of the DBP-DNA adducts. The cartridges were then rinsed with 3 x 10 ml deionized water, followed by the elution of adducts with 3 ml of 5% NH_4OH in methanol. To quantify the total adducts, 3 x 10 µL aliquots of the eluent were taken for LSC analysis. To determine the percentage of adducts contained in the sample, the remainder was concentrated to near dryness and reconstituted with approximately 50 µL, each, of deionized water and methanol to a nominal final volume of 100 µL. The samples were vortexed, transferred to autosampler vials,

and placed in the sample compartment (maintained at 10°C) of the Waters 2690 HPLC. Analysis of the samples was performed using a C-18 column (Waters Symmetry 2.1 x 150 mm) in a 35°C, temperature-controlled compartment. A gradient elution was performed using methanol:acetonitrile (9:1) (solvent A) and 0.1 M ammonium phosphate buffer (pH 5.5) (solvent B), beginning at 20% A, with a constant flow rate of 0.4 ml/minute. The mobile phase changed according to the following gradient: 0 min, 20% A; 12.5 min., 22.5% A; 17.5 min., 23.5% A; 45 min., 47.5% A; 62.5 min., 57.5% A; 72.5 min., 80% A; and 75 min., 20% A. Between samples, the column was equilibrated for 15 minutes. An inline Packard TR505 radiometric detector (0.5 ml cell, 1.6 ml/min scintillant) was used to detect the postlabeled adducts.

Determination of Tumor Response

Following sampling for DNA adduction, the remaining fish in each tank were fed OTD and allowed to grow for approximately 10 additional months, at which time the fish were evaluated for tumor production. The actual commencement of the final sampling was determined by a planned, 6 tank pre-sacrifice sampling 9 months after initiation to evaluate the extent of tumor formation at the highest doses. The time required for final sampling was expected to be 8 weeks, therefore, a sampling schedule was devised so that the mean time of sampling for all tanks in each treatment group was 48 weeks post-initiation, plus or

minus 1 day. In addition, the daily ration of OTD was reduced to maintenance rations in an attempt to further reduce any effect of time on fish growth and final tumor incidence. The fish were sacrificed by immersion in a lethal concentration of tricaine methanesulfonate (MS-222) in water. The fish were then weighed individually and bled by cutting several gill arches. Body and liver weights were recorded, and livers and stomachs were examined under a dissecting microscope for the presence of suspect tumors. Swim bladders and kidneys were screened and inspected under the dissecting microscope if abnormal features were noted. (It has been observed that over 95% of trout liver tumors and 100% of stomach, swim bladder, and kidney tumors are surface oriented and readily detectible by visual inspection .) Liver tumors were sized and counted. All livers and other organs with suspected tumors were fixed in a Bouin's solution and processed by routine histological techniques. One slide from each organ having one or more suspect tumors, as determined by gross pathology, was prepared for histology. By this procedure, tumor incidence was based on confirmed neoplasms, whereas multiplicity > 1.0 included unconfirmed gross lesions as well. Tumors were classified according to the criteria established by Hendricks et al. (41, 100, 101). Tumor incidence is expressed as the percentage of fish observed having one or more confirmed tumors at each dose. Tumor multiplicity, which was not exhaustively determined in the liver, is defined as mean number of tumors per organ for those animals having tumors. The tumor spectrum was reported

according to the percentage of each tumor phenotype found at that dose. The percentage reported is the number of that particular phenotype at the designated dose, divided by the total number of all tumors at that dose.

Analysis of stored diets

To evaluate the potential for DBP degradation when stored in OTD, both with and without CHL, a subsampling of diets stored 3 years at -20°C was analyzed following the protocol of Loveland *et al.* (102).

Statistical procedures

Comparisons of body weight, liver weight, liver somatic index, tumor multiplicity, tumor size, and adduct profile were evaluated using single factor ANOVA and two-sample t-tests assuming unequal variances. Two-tailed *P* values are stated. Tumor incidence data was modeled using generalized linear mixed models (GLMM) in SAS (SAS Institute, Inc., Cary, NC). The GLIMMIX macro was used to allow simple overdispersion at the between-tank level.

Results

Diet analysis

Portions of the diets used in this study were archived and stored at -20°C. The results of the analyses on four of these diets following 3 years of storage showed that the DBP was very stable, and not subject to degradation in the presence of CHL. The nominal doses of DBP were 27.9 ± 1.4 and 27.4 ± 1.2 ppm for the target dose of 28.4 ppm at 0 and 4500 ppm CHL, respectively. Nominal doses of 221 ± 19 and 197 ± 6 ppm DBP were found in the 225 ppm DBP diets at 0 and 4500 ppm CHL, respectively. Each diet was analyzed in triplicate.

CHL influence on DNA adduct profile

The profiles of stable DBP-DNA adducts in the liver were virtually identical to those reported in earlier studies from this laboratory where DBP-DNA adducts were investigated using ^{33}P-postlabeling (33). Increasing concentrations of DBP in the diets resulted in an increase in total adducts (Table 1). Increasing concentrations of CHL at each dose of DBP resulted in a relatively uniform decrease in the adduct profile. In general, 70-80 % of all liver adducts eluted prior to 35 minutes in a region characterized as containing polar adducts. The highest DBP dose, 225 ppm, (Figure 2-2A) is representative of the liver adduct profiles, which consist primarily of polar adduct peaks eluting within the 10-20 minute

Table 1. Liver and stomach DNA-adduct response in trout fed varying doses of DBP and CHL

[DBP] (ppm)	[CHL] (ppm)	nmole adducts/mole DNA [a]					
		Liver adducts [b]			Stomach adducts [b]		
		Total	% Polar	% Nonpolar	Total	% Polar	% Nonpolar
0.0	0	138.7 (16.4)	73.0 (2.8)	23.6 (3.2)	292.6 (94.2)	33.0 (14.2)	62.0 (14.5)
0.0	6000	135.3 (33.0)	51.9 (5.6)	45.1 (6.8)	158.7 (21.3)	29.4 (11.2)	67.5 (10.5)
10.1	0	452.4 (87.8)	75.4 (3.8)	23.2 (3.7)	249.7 (28.8)	23.3 (3.2)	74.6 (2.6)
28.4	0	1844.6 (196.1)	80.9 (1.3)	18.6 (1.4)	565.6 (66.8)	17.8 (2.3)	80.6 (2.7)
80.0	0	5498.1 (398.8)	82.2 (1.5)	17.4 (1.6)	1404.8 (160.2)	10.4 (1.3)	89.0 (1.5)
225.0	0	14200.3 (4390.6)	78.9 (1.6)	20.3 (1.4)	2954.1 (313.5)	10.9 (3.6)	88.9 (3.6)
10.1	1500	339.3 (57.2)	69.1 (1.5)	29.6 (1.9)	195.3 (59.8)	31.5 (1.1)	65.0 (0.8)
28.4	1500	776.5 (282.7)	74.1 (2.9)	23.5 (1.6)	373.7 (77.0)	28.8 (1.6)	68.9 (1.4)
80.0	1500	3016.2 (215.5)	79.5 (2.2)	19.7 (2.4)	742.3 (286.8)	18.1 (6.3)	79.7 (6.1)
225.0	1500	6709.8 (3471.3)	72.1 (4.8)	27.0 (4.7)	1942.9 (48.1)	26.2 (6.1)	73.4 (6.2)
10.1	3000	280.1 (45.3)	67.4 (3.6)	28.2 (3.1)	288.2 (156.8)	22.5 (8.4)	72.8 (7.1)
28.4	3000	739.9 (134.8)	70.5 (0.2)	27.9 (0.6)	422.7 (56.2)	22.3 (3.6)	74.7 (3.8)
80.0	3000	1590.3 (67.5)	72.6 (1.6)	26.3 (1.4)	862.1 (347.1)	25.3 (6.5)	71.5 (6.4)
225.0	3000	3163.9 (938.2)	68.8 (4.2)	30.3 (4.2)	1196.4 (218.1)	18.3 (1.9)	81.1 (2.1)
28.4	4500	335.5 (86.6)	74.8 (9.3)	23.4 (10.1)	249.0 (48.6)	30.2 (7.0)	67.8 (6.7)
80.0	4500	1643.0 (208.1)	76.8 (3.3)	22.0 (2.8)	536.7 (95.2)	27.4 (5.9)	71.0 (5.6)
225.0	4500	2369.8 (346.8)	69.6 (7.5)	29.5 (7.3)	1043.5 (115.6)	25.4 (6.0)	73.7 (6.0)
371.5	4500	2189.0 (1301.5)	38.0 (19.3)	60.4 (18.8)	1630.1 (353.5)	21.4 (5.3)	76.9 (4.2)
80.0	6000	1292.9 (227.4)	70.8 (7.6)	28.4 (7.6)	453.4 (92.0)	26.1 (4.7)	72.0 (5.2)
225.0	6000	2063.0 (579.7)	72.1 (4.7)	27.3 (4.9)	957.6 (80.7)	25.5 (4.6)	73.4 (4.6)
371.5	6000	3145.1 (407.1)	73.9 (3.6)	25.3 (3.5)	1487.7 (276.5)	21.9 (4.3)	76.5 (4.3)
80.0	Derifil	774.4 (105.1)	70.6 (1.5)	28.4 (1.3)	735.9 (220.5)	28.5 (3.8)	69.3 (3.8)
80.0	Placebo	1539.0 (648.9)	60.5 (9.4)	38.8 (9.2)	1382.2 (272.7)	10.1 (1.4)	89.1 (1.0)

[a] Mean (SE)

[b] Adduct levels not corrected for background.

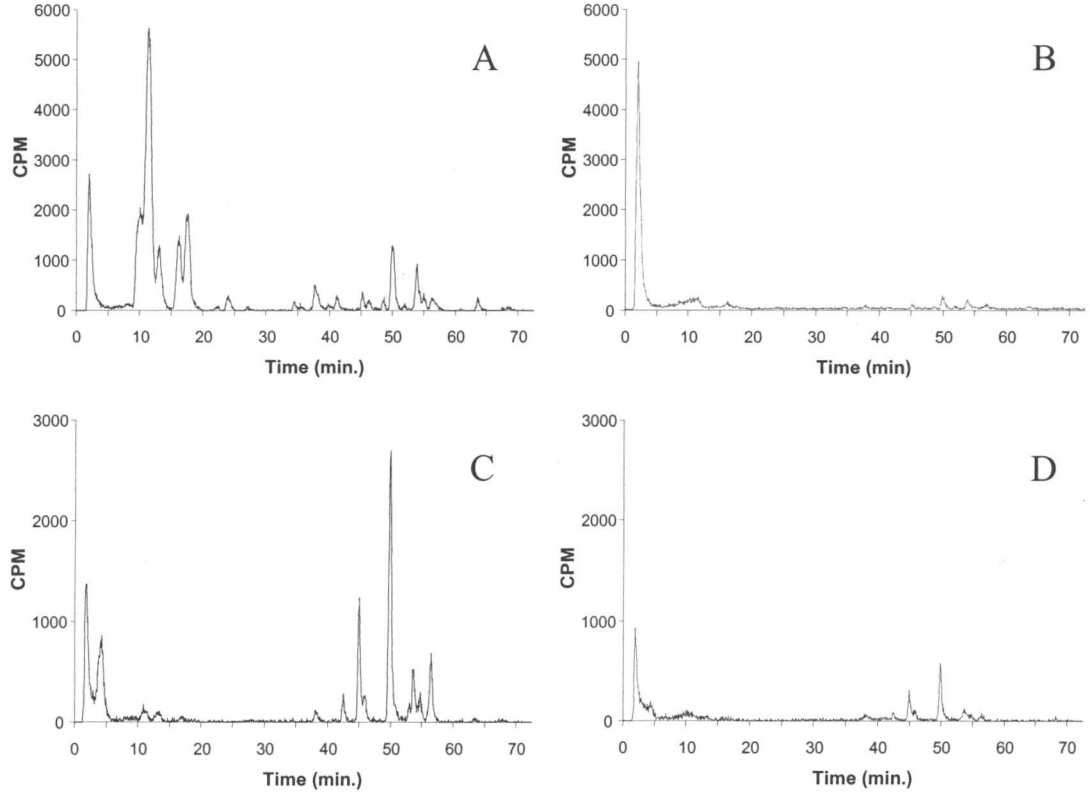

Figure 2-2. Chromatograms of *in vivo* DBP-DNA adducts from rainbow trout after ^{33}P postlabeling and HPLC analysis. Liver adduct profiles were generated by four-week dietary exposure to either 225 ppm DBP alone (A) or 225 ppm DBP and 6000 ppm CHL (B). Stomach adducts resulting from four-week dietary exposure to either 225 ppm DBP only (C), or 225 ppm DBP and 6000 ppm CHL (D) are shown.

range, and several, smaller non-polar peaks eluting in the 35-65 minute range. The addition of the 6000 ppm CHL results in the chromatogram shown in Figure 2-2B, which illustrates the magnitude and uniform nature of the decrease in adduct profile. There was no evidence of a dose effect by either DBP ($P > 0.15$) or CHL ($P > 0.2$) on the adduct profiles.

In the stomach, the majority (in excess of 70% in most cases) of DBP-DNA adducts eluted in the non-polar 35-65 minute range, with very few adducts in the polar 10-20 minute range (Figure 2-2C). Overall, the nmoles DBP adduct/mole DNA in the stomach for any DBP-CHL dose combination was approximately 30-50% of that seen in the liver. As in the liver, addition of CHL to the diets resulted in a largely dose-dependent, uniform decrease in peak height across the stomach adduct profile (Figure 2-2D). An exception is noted, however, in that the addition of the lowest dose of CHL resulted in a small, but statistically significant ($P = 0.003$) increase in polar DNA adducts in the stomach, despite a decrease in total adducts recovered (Table 1). This small elevation in percentage of polar adducts and a corresponding significant decrease in non-polar adducts ($P \leq 0.02$) in the presence of CHL was maintained with increasing CHL concentrations (For polar adducts, $P \leq 0.01$ at 4500 and 6000 ppm CHL).

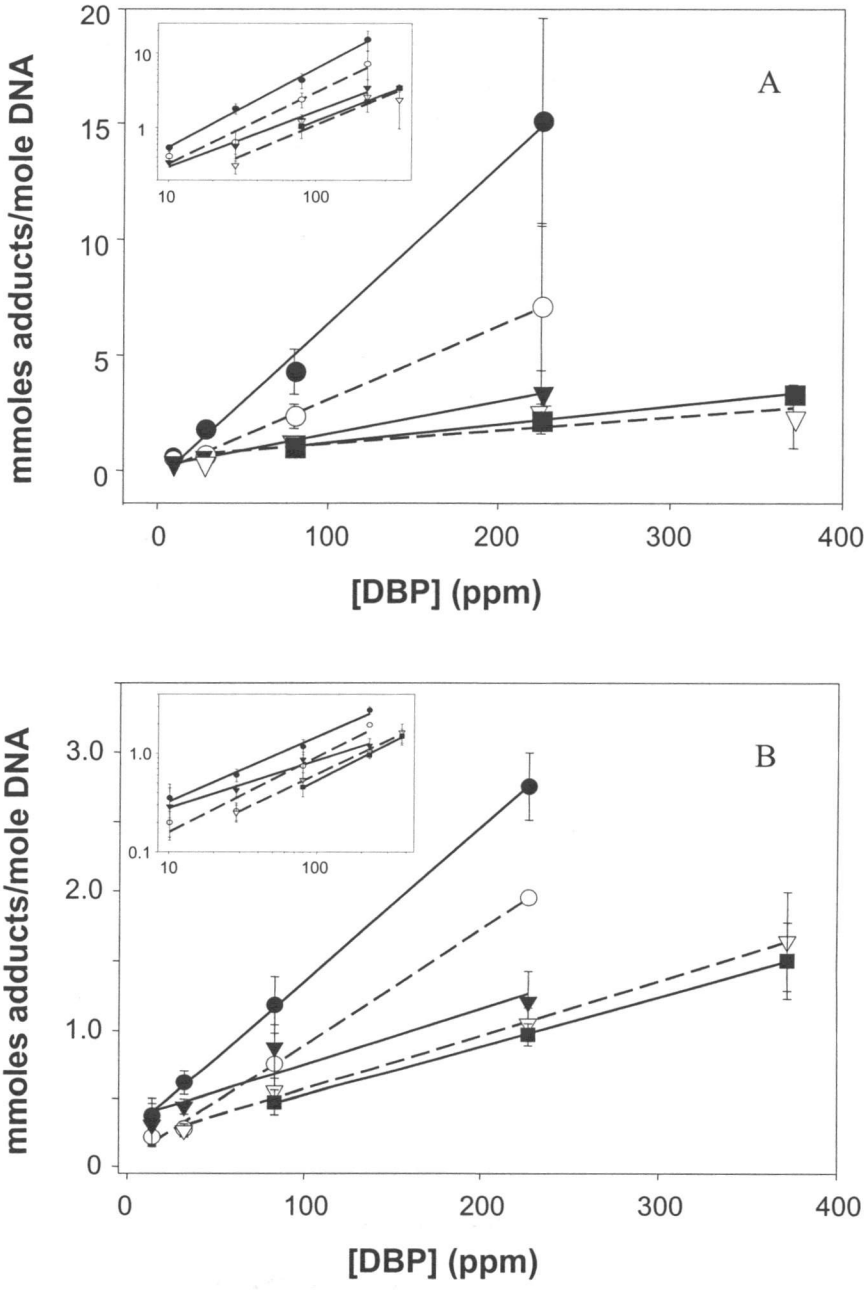

Figure 2-3. DNA Adduct Response. Graphs depict liver (panel A) and stomach (panel B) adducts following 4 weeks of dietary DBP and CHL adminstration. Symbols represent 1500 (○), 3000 (▼), 4500 (▽), and 6000 (■) ppm CHL. Incremental doses of CHL are represented by alternating solid and dashed lines. Inset shows log[DBP] vs. log adducts.

DBP-initiated adduct formation and inhibition by CHL

Four weeks of dietary DBP treatment resulted in a dose-dependent formation of total DBP-DNA adducts in the liver and stomach of rainbow trout as quantified by ^{33}P-postlabeling (Table 1). Figure 2-3 illustrates the dose-response and effect of CHL in reducing adduct formation for both organs. A general decrease in slope with increasing CHL concentration indicates the reduction in adduct formation is dose dependent in both organs. The CHL-mediated percentage inhibition of adduct formation can be determined by evaluating the change in slope using % Inhibition = $(1-(\text{slope}_{CHLX}/\text{slope}_{CHL0}))*100$, for CHLX = CHL dose and CHL0 = control. Using this formula, the reduction in liver adducts at CHL doses of 0, 1500, 3000, 4500, or 6000 ppm were 53, 79, 93, or 90%, respectively. In the stomach, the adduct response was inhibited by 25, 63, 65, or 69% at the same respective doses of CHL.

Body weight and somatic indices in response to treatments

Table 2 shows the carcinogen and modulator doses, the number of replicate tanks at each dose, and the number of fish actually sampled for tumors at the conclusion of the study, which totaled 8711 trout. In addition to the tumor data, the mean body weight of fish at tumor sampling is shown. No trend of significant change in the body weight of the fish occurred due to increasing doses of either DBP or CHL ($P > 0.1$, either compound). A trend of increasing body weight during

Table 2. Tumor incidence, liver somatic index, and tumor type among animals fed varying doses of DBP and CHL

[DBP] (ppm)	[CHL] (ppm)	# Tanks	N	Tumor Incidence [a,b]			Mean Body Weight (g) [a]	Liver Somatic Index [a,c]	# Liver Tumors Examined	% of Liver Tumors Examined[d]					
				Liver	Stomach	Swim Bladder				HCC	HCA	MC	MA	CCC	CCA
0.0	0	3	300	-	-	-	109.6 (1.8)	0.69 (0.06)	-	-	-	-	-	-	-
0.0	6000	3	295	-	-	-	112.8 (8.1)	0.58 (0.02)	-	-	-	-	-	-	-
10.1	0	3	300	2.67 (0.67)	-	-	105.6 (1.3)	0.69 (0.04)	9	33.3	66.7	-	-	-	-
28.4	0	3	300	25.33 (3.84)	1.33 (0.33)	1.33 (0.33)	105.1 (1.6)	0.60 (0.01)	135	40.0	48.9	9.6	0.7	0.7	-
80.0	0	3	300	57.00 (3.61)	7.67 (1.20)	3.00 (1.53)	107.1 (2.8)	0.61 (0.01)	587	17.4	74.8	4.1	3.1	-	0.7
225.0	0	3	298	60.40 (5.20)	41.61 (1.33)	23.83 (3.84)	108.6 (1.4)	0.67 (0.02)	338	29.3	55.3	12.7	2.4	0.3	-
10.1	1500	6	599	0.83 (0.50)	0.33 (0.33)	-	104.8 (1.4)	0.67 (0.02)	6	33.3	33.3	16.7	-	16.7	-
28.4	1500	3	300	8.33 (2.33)	0.33 (-)	-	103.0 (3.2)	0.62 (0.03)	30	50.0	30.0	13.3	3.3	-	3.3
80.0	1500	3	300	25.67 (4.48)	2.00 (0)	-	104.5 (1.0)	0.61 (0.02)	185	31.4	62.2	5.4	1.1	-	-
225.0	1500	3	300	56.67 (0.33)	28.33 (3.67)	3.67 (0.67)	103.9 (1.7)	0.63 (0.01)	442	26.0	67.2	5.4	0.9	0.5	-
10.1	3000	12	1197	1.34 (0.21)	0.08 (-)	-	105.5 (1.4)	0.63 (0.01)	16	68.8	-	31.3	-	-	-
28.4	3000	3	300	2.67 (0.88)	-	0.67 (0.67)	108.2 (2.3)	0.65 (0.03)	78	28.2	62.8	3.8	5.1	-	-
80.0	3000	3	282	21.28 (0.58)	1.06 (0.50)	-	114.0 (10.3)	0.66 (0.03)	106	30.2	59.4	2.8	2.8	4.7	-
225.0	3000	3	269	56.51 (4.67)	17.10 (4.70)	2.60 (0.67)	120.8 (8.4)	0.62 (0.02)	527	24.1	67.9	6.8	0.8	0.4	-
28.4	4500	9	863	5.10 (0.75)	0.58 (0)	0.12 (0.11)	113.4 (4.3)	0.63 (0.01)	78	29.5	57.7	10.3	-	2.6	-
80.0	4500	3	296	14.19 (2.65)	2.03 (0.58)	-	105.1 (3.1)	0.65 (0.02)	67	38.8	50.7	7.5	3.0	-	-
225.0	4500	3	276	40.94 (1.86)	9.42 (2.67)	0.36 (0.33)	113.8 (6.2)	0.68 (0.01)	341	34.0	62.8	2.6	0.3	0.3	-
371.5	4500	3	274	52.55 (4.16)	18.98 (2.96)	1.46 (0.88)	113.9 (4.6)	0.61 (0.01)	355	29.6	61.4	5.9	2.0	0.6	0.6
80.0	6000	6	584	12.67 (0.49)	1.20 (0.25)	-	107.3 (2.9)	0.62 (0.01)	141	31.9	58.2	6.4	2.1	-	1.4
225.0	6000	3	300	27.33 (2.67)	5.67 (2.60)	0.67 (0.67)	103.5 (2.3)	0.66 (0.04)	200	24.5	66.5	2.5	5.5	0.5	0.5
371.5	6000	3	298	48.99 (0.67)	16.44 (5.78)	0.34 (0.33)	107.1 (0.9)	0.62 (0.02)	417	25.2	67.1	6.5	1.0	-	0.2
80.0	Derifil	3	287	11.50 (1.16)	0.70 (0.33)	-	109.1 (5.9)	0.68 (0.02)	71	23.9	67.6	7.0	0.0	0.0	1.4
80.0	placebo	2	193	37.82 (0.50)	11.40 (3.00)	2.07 (1.00)	104.3 (0.3)	0.62 (0.02)	192	15.1	75.0	6.3	3.1	0.5	0.5

[a] Mean (SE)

[b] SE = "-" indicates only one tank contained fish bearing tumors

[c] Somatic index is defined as mean liver weight (g)/mean body weight (g).

[d] HCC, hepatocellular carcinoma; HCA, hepatocellular adenoma; MC, mixed hepatocellular/cholangiocellular carcinoma; MA, mixed hepatocellular/cholangiocellular adenoma; CCC, cholangiocellular carcinoma; CCA, cholangiocellular adenoma.

the 8 weeks of tumor sampling was observed, however, with the exception of four tanks, the mean body weight stayed within ± 2 standard deviations from the overall mean. There was no evidence for increased body weight influencing final tumor incidence ($P > 0.3$). The liver somatic indices displayed in Table 2 were subject to a small, but significant decrease with time ($P < 0.01$), but were not affected by earlier treatment with either CHL or DBP ($P > 0.2$).

CHL modulation of DBP-initiated liver tumor formation

Administration of DBP in the diet of rainbow trout for 4 weeks resulted in a dose-dependent formation of tumors of the liver, stomach, and swim bladder observed 11 months after initiation commenced (Table 2). The highest tumor response was found in the liver, followed by the stomach and the swim bladder, respectively. The addition of CHL to the diets resulted in a dose-dependent reduction in tumor formation across all doses of DBP. A visual inspection of the unmodeled liver data, plotted on a log[DBP] versus logit tumor incidence scale, shows a strong tendency of the DBP dose-tumor response curves to move to the right with increasing CHL concentration (Figure 2-4). These results provide unambiguous evidence that CHL is a highly effective blocking agent when co-fed with DBP, and that cancer chemoprevention was achieved over the entire range of DBP doses administered.

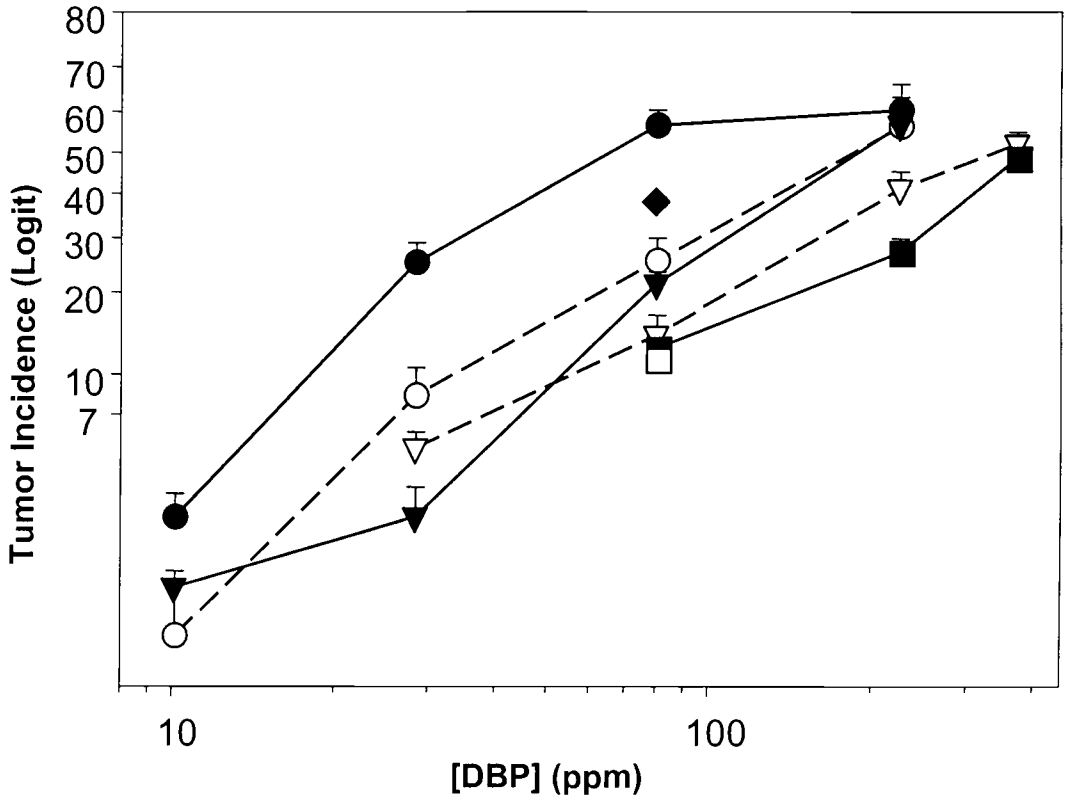

Figure 2-4. DBP-initiated liver tumor response as a function of CHL dose. For a period of four weeks, trout were fed experimental diets containing 10.1, 28.4, 80, 225, or 371.5 ppm DBP and 0 (●), 1500 ( ), 3000 (▼), 4500 ( ), or 6000 (■) ppm CHL. Additional treatments consisted of 80 ppm DBP plus either Derifil ( ) or placebo (◆) tablets. Trout were examined for tumor formation 11 months after initiation. Unmodeled dose-response curves of the incremental CHL concentrations are represented by alternating solid and dashed lines.

Modeling of liver tumor response

One aim of the study design was to determine if the magnitude of CHL protection remained constant over all DBP dose ranges. That is, we wanted to determine if one can legitimately extrapolate the degree of CHL protection at high DBP doses and incidences down to lower DBP doses and tumor incidences perhaps more representative of human cancer risks. We have previously stressed that the magnitude of cancer chemoprevention protection cannot be legitimately determined by calculating a simple tumor incidence ratio, and "percent inhibition" at any carcinogen dose, but requires, instead, a modeling of the effect of any CHL dose on the shape and position of the entire DBP dose-response curve (1, 98). This is because tumor incidences themselves are not linearly related to carcinogen dose, and any calculated "percent inhibition" ratio is consequently highly dependent on incidence of the carcinogen-only control group. Instead, the condition of equal magnitude of CHL chemoprevention over all DBP doses will be met for any CHL dose at which the Logit incidence vs. log DBP dose curves with and without CHL are parallel. However, our attempt to model the CHL dose-response curves in this study as linear and parallel, the same manner as in similar, previous studies (1, 98), was impeded due largely to a statistically significant departure of the DBP-only dose response curve from linearity in this study ($P < 0.01$). Logistic regression of the DBP-only data indicates that non-parallelism of the DBP+CHL curves cannot be rejected. Therefore, while the basis of this departure in this particular study is

not evident, it does preclude our normal data modeling analysis of the DBP dose-dependency for CHL chemoprevention, even with a data base from over 8700 animals.

That said, we note that the apparently nonlinear control curve from this study stands in contrast to other dose-response curves over this DBP dose range generated in unpublished studies from our laboratory. One of these, involving many more animals, shall be referred to as the ED01 study. The ED01 study[5] overlapped in time with the present study, and used DBP in the same doses as the present study as well as four lower doses of DBP in log-even intervals. The number of replicate tanks used for each dose in the ED01 study exceeded the number of replicate tanks used for each dose in the present study, specifically, 17, 13, 10, and 4 tanks of trout for DBP doses of 10.1, 28.4, 80, and 225 ppm, respectively. The present study evaluated only three tanks of fish at each of these doses. Once again, inclusion of the ED01 225 ppm DBP dose contributes to the curvilinearity of the dose response ($P < 0.01$). This reproducible lack of linearity suggests saturation in the DBP hepatocarcinogenicity at or above dietary DBP concentrations of 225 ppm in the trout model.

Given the greater linearity of the ED01 study's DBP-only "control" curve, and its overlap with the present study, we were interested in examining the effects on modeling when using that "control" curve instead of the one generated in this

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[5]Bailey, G. S. *et al*, unpublished

study, which used fewer replicate tanks per dose. Figure 2-5 shows the overall result when this substitution was made and the inset shows the difference between the ED01 data and the control data from this study. Excluding the 3000 ppm CHL curve, the resulting dose-response curves were all successfully modeled as parallel (lack of fit $P > 0.1$) showing that, within the range of incidences evaluated, the reduction in tumorigenesis in response to incremental doses of CHL at 1500, 4500, and 6000 ppm is not dependent on the concentration of DBP. For reasons unknown, the dose response at 3000 ppm CHL in this experiment could not be modeled as linear or parallel.

A related aim of the study design was to generate estimates for CHL dose-response potency to inhibit tumor formation, at any or all DBP doses. For this analysis, the dose of carcinogen required to produce ρ% tumor incidence is defined as the TDρ value. For ρ = 50%, the TD50 values generated for each dose of CHL can be used to calculate the magnitude of tumor inhibition for the respective doses of CHL using the following: % Inhibition = 100 x (1- ($TD50_{CHL0}$/$TD50_{CHLX}$)) where CHL0 refers to the treatment with no CHL and CHLX = [CHL]. (We note that, when the dose-response curves are linear and parallel, the percent inhibition calculated from any tumor incidence within the linear range will be equivalent.) In the liver, using TD50 values obtained from the modeling in Figure 2-5, tumor inhibitions of 56, 73, and 79% at CHL doses of 1500, 4500, and 6000 ppm,

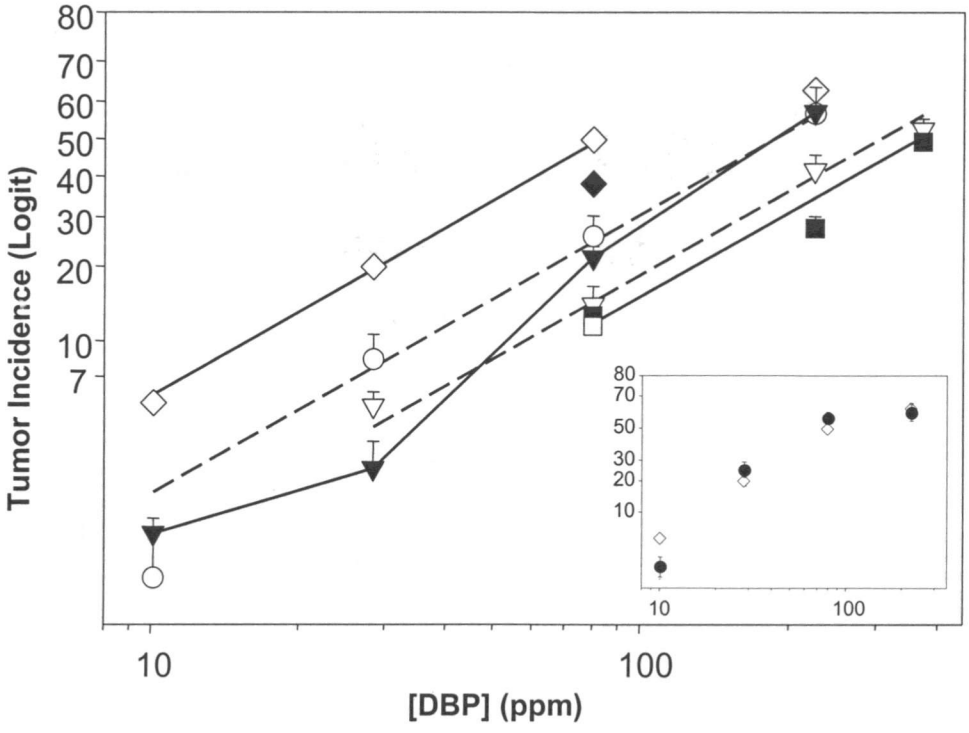

Figure 2-5. Dose-response curves using the ED01 data as control. Symbols represent 0 (◇ - ED01 data), 1500 (○), 3000 (▼), 4500 (▽), or 6000 (■) ppm CHL and Derifil (□) or placebo (◆) tablets. Trout were examined for tumor formation 11 months after initiation. Dose-response curves of the incremental CHL concentrations are represented by alternating solid and dashed lines. The 3000 ppm CHL response could not be modeled as linear and is, therefore, displayed unmodeled. The control 225 ppm DBP data is omitted from the modeling due to a reproducible lack of linearity, or apparent saturation in DBP hepatocarcinogenicity at or above 225 ppm in the trout model. Inset shows the data point from both this study's 0 ppm CHL response (●) and the ED01 study.

respectively, were obtained. The 3000 ppm CHL response could not be modeled as linear, however, protection consistent with that dose of CHL is apparent.

Stomach and swim bladder tumor response

The stomach tumor response was considerably lower than that of the liver (Table 2). Despite this fact, a visual inspection of the data shown in Figure 2-6 reveals the same trend of dose-response curves moving to the right with increasing dosage of CHL. Unlike the liver data, the stomach data was successfully modeled as linear and parallel (lack of fit $P > 0.5$) without reference to the ED01 data, thus allowing a calculation of inhibition of tumor formation using TDρ values within the linear range of the dose-response curves. In the stomach, the inhibition was 30, 49, 62, and 68% for the same respective CHL doses evaluated in the liver. Figure 2-7 depicts a side-by-side comparison of inhibition values for liver and stomach. The low tumorresponse in the swim bladder made it impossible to model dose-response curves in the same manner. An inhibition calculation, based solely on the decrease in tumor incidence at the DBP concentration of 225 ppm, reveals an inhibition of 85, 89, 98, and 97% for each respective incremental dose of CHL in the swim bladder.

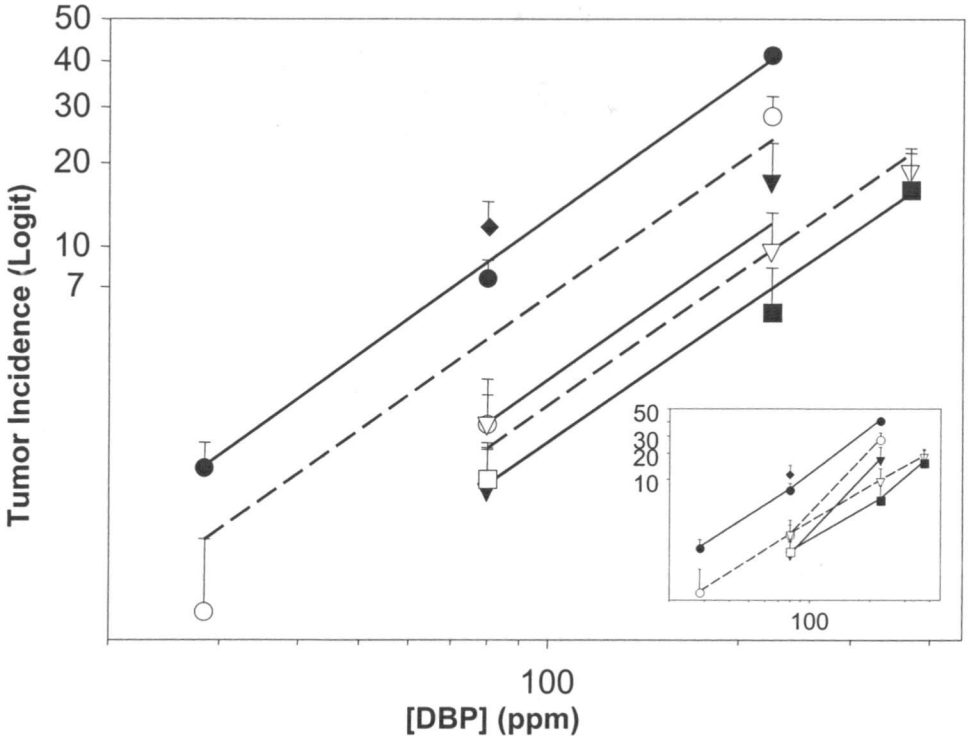

Figure 2-6. DBP-initiated stomach tumor response modeled as a function of CHL dose. Rainbow trout were treated in the diet for 4 weeks. Diets contained 10.1, 28.4, 80, 225, or 371.5 ppm DBP and 0 (●), 1500 (○), 3000 (▼), 4500 (▽), or 6000 (■) ppm CHL. Additional treatments consisted of 80 ppm DBP plus either Derifil (□) or placebo tablets (◆). Incremental CHL dose-response curves are represented by alternating solid and dashed lines. Inset shows data without linear, parallel modeling.

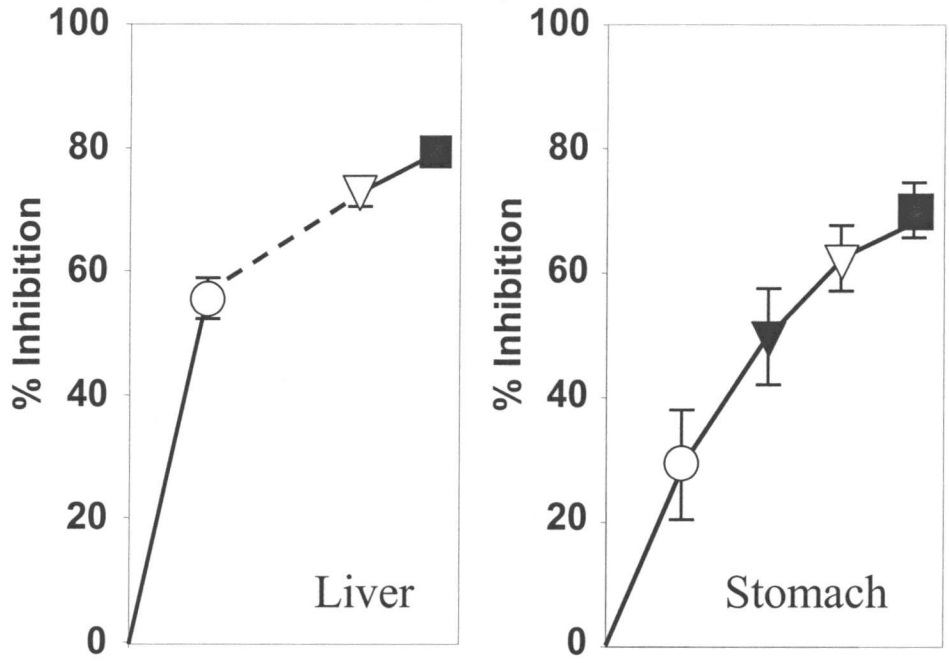

Figure 2-7. Percent inhibition of final tumor incidence in liver and stomach at dietary CHL concentrations. Diets contained 10.1, 28.4, 80, 225, or 371.5 ppm DBP and 1500 (○), 3000 (▼), 4500 (▽), or 6000 (■) ppm CHL. The inability to model the liver response at 3000 ppm CHL as linear precludes the use of that data in this figure, however, protection consistent with the CHL dose is visually evident in Figures 2-4 and 2-5. Error bars represent standard error.

CHL effect on liver tumor spectrum

Approximately two-thirds of the tumors found in the liver were either hepatocellular adenomas or carcinomas (HCA or HCC, Table 2). Of the remaining tumors, most were mixed hepatocellular-cholangiocellular neoplasms (MC/MA) and a very small quantity were cholangiocellular neoplasms. There was no significant DBP dose-dependent effect on the percentage of HCA or MC ($P \geq 0.1$, both phenotypes) and the response to the inclusion of CHL in the diets was identical to that seen in response to DBP alone ($P \geq 0.1$, both phenotypes), regardless of the CHL concentration ($P \geq 0.1$, both phenotypes). All tumors of the stomach and swim bladder were papillary adenomas, irrespective of CHL.

CHL effects on apparent tumor multiplicity and size

Although liver pathology was not exhaustive, apparent liver tumor multiplicity was evaluated at four doses, 10.1, 28.4, 80.0, and 225 ppm DBP. There was strong evidence for increased multiplicity as the DBP dose increased from 10.1 to 225 ppm ($P < 0.003$). At 10.1 ppm DBP, CHL doses of 0, 1500, and 3000 ppm yielded multiplicities of 1.07, 0.33, and 1.24, respectively, with only the 1500 ppm dose being significantly different from the others ($P < 0.01$). At 28.4 ppm DBP, the averaged tumor multiplicities at 0, 1500, 3000, and 4500 ppm CHL were 2.3, 1.5, 1.4, and 1.6, respectively, with some evidence of a significant decrease between 0

and 3000 ppm ($P = 0.08$). It should be noted that the number of fish with 1 or more tumors was quite limited at these DBP doses ($n < 50$ for 4 out of 7 treatments, $n < 100$ for 6 of the 7 treatments). Figure 2-8 illustrates the changes noted at the 80 and 225 ppm DBP doses. In general, there was a decrease in tumor multiplicity at 80 ppm DBP (5.03 ± 0.16) with increasing CHL dose, though, only the interval between 0 and 1500 ppm CHL (2.74 ± 0.02) exhibited a significant decrease ($P = 0.01$). Evaluation of mean multiplicity across all CHL doses (2.30 ± 0.21) at 80 ppm DBP suggests a significant reduction in multiplicity with inclusion of CHL in the diet ($P < 0.002$). A similar evaluation at 225 ppm DBP (3.17 ± 0.51) across all CHL doses (4.17 ± 0.68) yields insufficient evidence of a change in multiplicity ($P = 0.12$). Comparison between CHL = 0 (3.16 ± 0.51) and 3000 ppm (5.07 ± 0.14) suggests increased multiplicity ($P < 0.03$), however, between 3000 ppm and 6000 ppm (3.20 ± 0.19) there is evidence of decreased multiplicity ($P < 0.005$)

An increase in tumor size with DBP dose was apparent, with a significant increase in mean size evident between 80 (0.96 mm ± 0.04) and 225 (1.46 mm ± 0.01) ppm DBP ($P = 0.05$). At 80 ppm DBP and below there was little evidence that CHL had any effect on tumor size ($P > 0.3$). The addition of each dose of CHL at 225 ppm DBP resulted in mean tumor sizes of 1.1 mm ± variances ≤ 0.07. Grouping the CHL responses at 225 ppm DBP and comparing the result with the control strongly suggests that inclusion of CHL reduces tumor size (1.10 mm ± 0.02, $P < 0.001$) in a treatment that otherwise produces a significant increase.

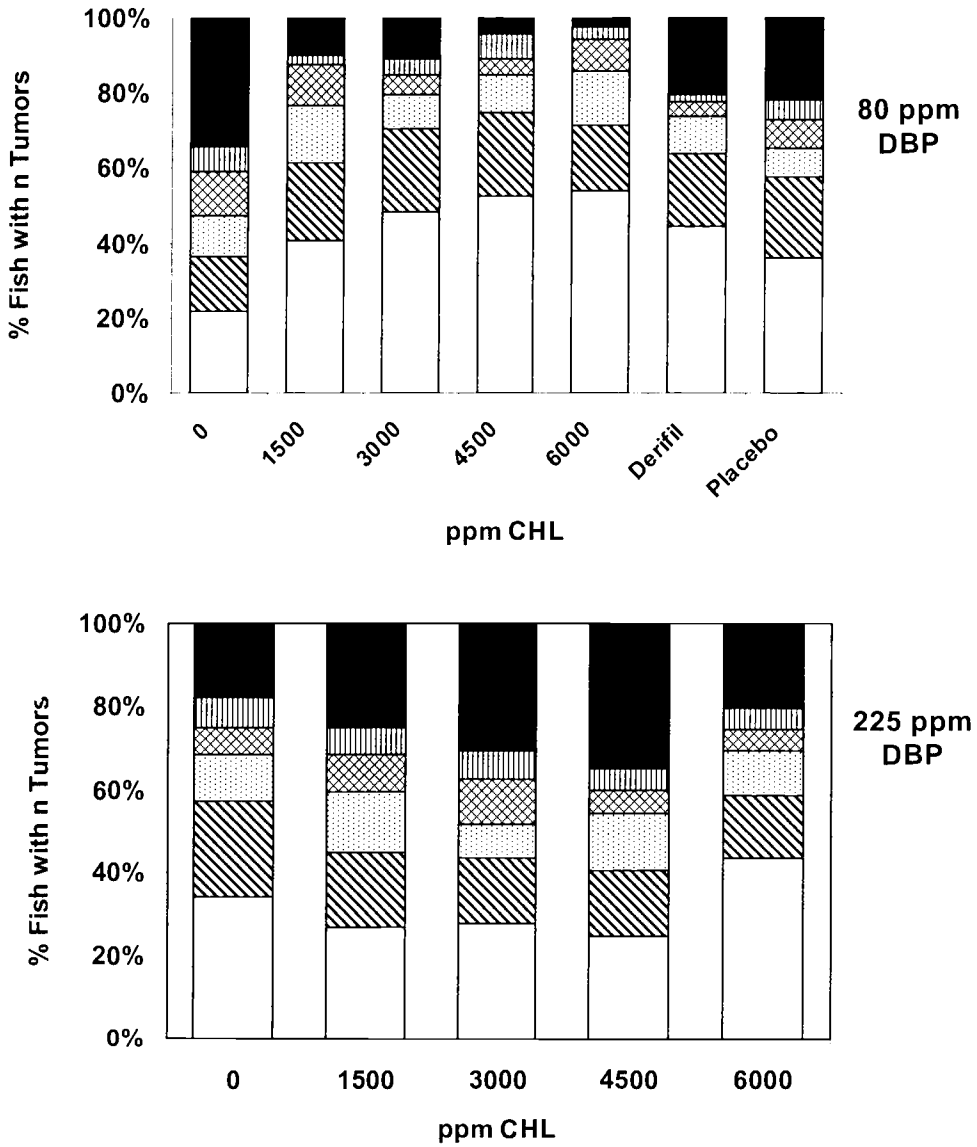

Figure 2-8. Change in tumor multiplicity with CHL dose at 80 ppm (top) and 225 ppm (bottom) DBP. The columns show number of tumor-bearing fish with 1 ( ), 2 (▒), 3 ( ), 4 (▒), 5 ( ), and 6+ (■) tumors per fish. The top panel displays the changes in multiplicity observed with the 80 ppm DBP dose, and represents 206, 120, 99, 72, and 120 fish at CHL doses of 0, 1500, 3000, 4500, and 6000 ppm, respectively. The lower panel shows the multiplicity response at 225 ppm DBP for the same CHL concentration increments, representing 228, 199, 181, 146, and 112 animals per dose, respectively.

Molecular dosimetry

We next asked if the CHL-mediated reduction in DBP-DNA adduct formation may be an appropriate early biomarker to predict CHL effects on final tumor outcome. There are several approaches that can be used for this assessment (69, 98). Figure 2-9 depicts the relationship between log DNA adduction and Logit final tumor incidence in the liver and stomach, for each combination of CHL and DBP dose. This analysis has the advantage of making no assumptions with regard to the linear nature of the tumor or adduct dose-response curves. As seen in Figure 2-9, a linear relationship is discernable in these plots with r^2 values of 0.83 and 0.86 for the liver (panel A) and stomach (panel B), respectively. These data provide evidence that total target organ DBP-DNA adducts are in direct proportion to final tumor incidence, and that CHL-mediated reduction of adducts during the carcinogen exposure period is an accurate biomarker for CHL-mediated reduction in tumor initiation and final tumor outcome. The closer the data points to a common line, the stronger the evidence for adduct reduction being a quantitative predictor of final tumor incidence.

Response to the CHL tablet and placebo

Along with the Sigma CHL preparation, we had the opportunity to include in our evaluation the CHL tablets (Derifil) and placebos from the same lot used in

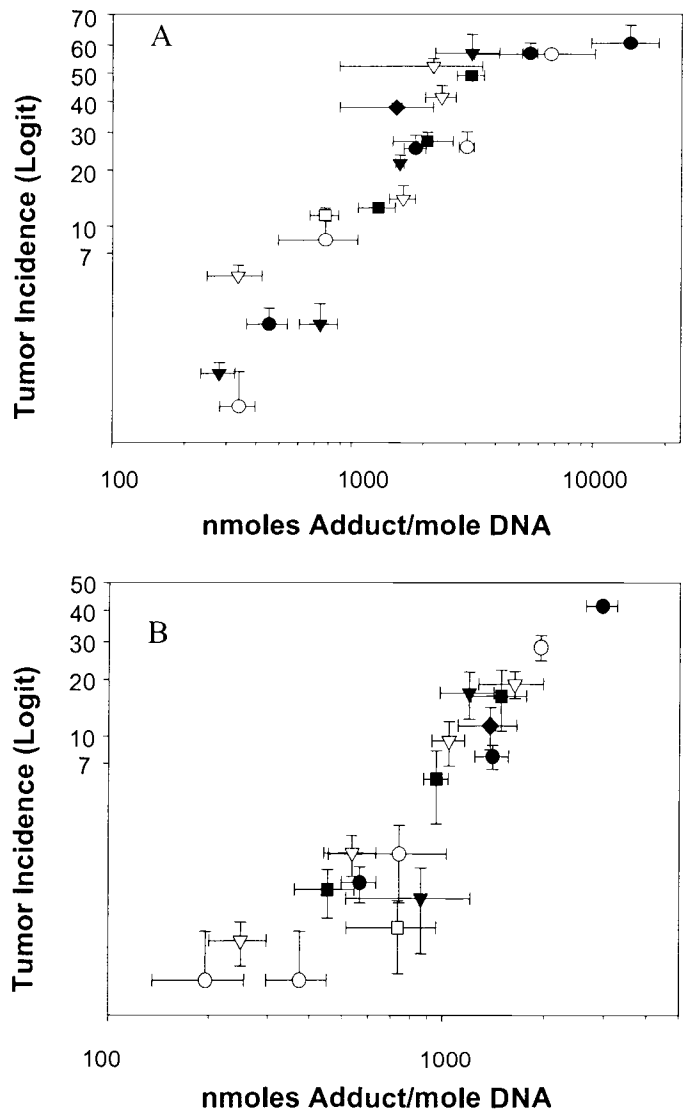

Figure 2-9. Molecular dosimetry of log DNA adducts verses Logit final tumor incidence in liver (panel A) and stomach (panel B). Symbols represent 1500 ( ), 3000 (▼), 4500 ( ), or 6000 (■) ppm CHL, Derifil ( ) or placebo tablets (◆). Data points and error bars represent means and standard errors, respectively, from replicate tanks at each dose. The linear trends in both the liver (r^2 = 0.83) and stomach (r^2 = 0.85) are indications that adduct formation is a quantitative indicator of final tumor incidence in both organs.

the clinical trial in an AFB_1-endemic area of China (2). Both Derifil and placebo tablets were powdered, mixed into the diets, and evaluated for effectiveness at the 80 ppm DBP exposure dose. The level of Derifil was chosen to approximate the total chlorins in the 4500 ppm Sigma CHL diets.

Table 1 shows that the quantity of DNA adducts in the liver and stomach following the Derifil and placebo treatments. The response of the Derifil preparation on DNA adduction in the liver appears to be greater than that seen in the 6000 ppm CHL treatment, however, a large variance renders this difference insignificant ($P > 0.1$). The apparent placebo effect ($P < 0.04$) on the reduction of liver adducts should be tempered by the fact that there was a large variation in the quantity of adducts detected between the 2 tanks in which the placebo was evaluated. The evident reduction ($P = 0.07$) in mean stomach adduct formation following Derifil treatment was most similar to that seen with the 3000 ppm CHL treatment ($P = 0.8$). The quantity of adducts recovered following placebo treatment was not different from the control ($P = 0.9$).

As shown in Table 2 and Figures 2-5 and 2-6, inclusion of the powdered Derifil tablets substantially reduced liver and stomach tumor incidences to essentially the same extent as did the 6000 ppm dose of Sigma CHL ($P > 0.4$ for comparison between Derifil and 6000 ppm CHL in both organs). At the same dose of carcinogen, the placebo showed a significantly lower liver tumor response when directly compared with the 80 ppm DBP-only control ($P < 0.02$), however, the

tumor response was less than that seen at 1500 ppm CHL. In the stomach, the tumor response to the placebo was most similar to the control ($P > 0.4$). In the swim bladder, no tumors were produced in the Derifil treated animals and the placebo response was consistent ($P > 0.6$) with the control. As with the Sigma CHL, there was no significant effect on mean body weight or tumor spectrum with either the Derifil or placebo preparations ($P > 0.1$ for all comparisons), however there was some evidence for a Derifil-associated increase in liver somatic index ($P = 0.07$).

As with DBP and CHL, there was no evidence for an effect of either Derifil or the placebo on the size of tumors formed ($P > 0.4$). There was, however, weak evidence of a Derifil-associated reduction in tumor multiplicity ($P = 0.09$, Figure 2-8). The placebo treatment was also associated with a significant reduction in tumor multiplicity relative to the control ($P = 0.02$).

The Derifil and placebo responses are also included in the molecular dosimetry figures for both the liver and stomach (Figure 2-9). Both the Derifil and placebo data appear to mingle with the other data from this study.

Discussion

In this study, we utilized the unique, sensitive rainbow trout model to perform the largest cancer chemoprevention study ever performed at Oregon State University, in which over 12,000 animals were used to evaluate the protective

effects of CHL against DBP DNA damage and tumorigenesis. The results of this study show that dietary administration of CHL, concurrent with dietary DBP, results in significant reduction of DBP-DNA adduct formation in the two organs examined, liver and the stomach. The effectiveness of CHL inhibition of DBP-DNA adduct formation is CHL dose-dependent at all doses of carcinogen in both the liver and the stomach. Evaluating tumor formation 11 months following initiation, it is also evident that CHL similarly causes a dose-dependent decrease in final tumor formation at all doses of carcinogen.

Mechanisms of CHL chemoprevention

Several studies investigating the protective effects of CHL have reported the formation of a complex between the compound of interest and CHL (13, 95, 103). Given the fact that DBP has been shown to form *in vitro* complexes with CHL with a K_d = 1.59 μM, similar to the 1.4 μM K_d of AFB_1 (13), it is not unreasonable to infer that the same complexation mechanism may be at work affording protection against both AFB_1 and DBP. In this study, we report significant DNA adduct and tumor inhibition in the liver and the stomach in a CHL dose-dependent manner. Though beyond the scope of this study to discern precise mechanisms, this response is, at least in the liver, similar to that seen with AFB_1's response to CHL, again suggesting a similar mechanism of protection.

In the AFB_1 studies (1, 98), molecular dosimetry showed that the extent of

tumor protection at higher CHL doses was significantly greater than would have been predicted from DNA adducts formation, indicative of additional CHL chemoprotection mechanism at higher doses. In comparison, the molecular dosimetry of CHL versus DBP in the present study gave no consistent indication of a departure between adduct reduction and tumor response at higher concentrations of CHL. In these AFB_1 studies, the adduct response to doses of CHL >3000 ppm apparently underestimated CHL effect on final tumor response, as evidenced by the resulting data point lying consistently and significantly to the right of the other data points. In the present study, none of the CHL doses consistently lay to the right (or the left) of the general, linear trend, suggesting that adduct formation is a quantitative predictor of final tumor incidence regardless of CHL dose and at all but the control 225 ppm dose of DBP in this model system.

Although reduced DBP uptake may be the major mechanism for CHL protection in this model, examination of the chromatographic data from the adduct analysis suggests that CHL treatment might also produce some type of *in situ* metabolic effect in the stomach. Though small in magnitude relative to the overall reduction in adducts, a small, but significant increase in the percentage of polar adducts was noted with the addition of CHL in the stomach, particularly at the higher doses of DBP. Otherwise, the contrasting adduct profiles in the liver and the stomach indicate that different DBP bioactivation pathways are occurring in the two organs. In fact, the greater non-polar character of adducts in the stomach

resembles that seen in MCF-7 cells upon treatment with DBP (104).

There was no evidence that the dosage of DBP or the presence or dosage of CHL caused any change in the DBP-initiated tumor spectrum. It is interesting to note that the primarily hepatocellular tumors in DBP-treated trout of this study, and in a previous DBP study in trout (7), are an exception, though not without precedence (105). The tumor spectrum reported here differs from reports that trout liver tumors were primarily MC following exposure to carcinogens such as AFB_1, DMBA, *N*-Nitrosodiethylamine, and a benzo[a]pyrene metabolite (1, 106-109). The significance of this observation is unknown as the mechanisms driving the formation of particular tumor phenotypes are not fully understood.

Clinical application for CHL in cancer prevention

Another chapter in the history of human usage of CHL was opened in the last few years with the completion of a double-blinded, placebo-controlled biomarker intervention clinical trial to assess the ability of CHL to function as a protective agent against dietary AFB_1 as a risk factor for liver cancer (2). Once again, there were no reports of adverse side effects with the consumption of 300 mg per day of CHL administered as three Derifil tablets at mealtimes for four months. The placebo tablets were covered with a green coating and were visually identical to the Derifil tablets. The results from the clinical study showed that CHL, in the form of the Derifil tablets, was highly effective in reducing the amount of urinary

AFB_1-N7-guanyl DNA repair product that forms in response to AFB_1 exposure (6, 36), compared with the group receiving the placebo. The study did not examine whether this reflected a reduction in AFB_1 bioavailability, or interference with metabolic activation *in situ* in the liver.

The present study confirms that the CHL, in both the Sigma preparation and the Derifil tablets, was fully effective in reducing tumor response as well as DNA binding in the liver and stomach following dietary initiation with the potent carcinogen, DBP, as we had previously shown for Sigma CHL against AFB_1 in the trout (1). In this model we were also able to show that the reduction in DNA binding is a quantitative predictor of final tumor incidence in both the liver and the stomach. We also report that CHL affords protection against tumor formation in a third, remote organ, the swim bladder, which is exposed to the carcinogen via the circulatory system. The response to the placebo preparation was largely consistent with that of the controls, indicating, that the green coloring used in the coating of the tablets is not biologically active against tumor formation in the same manner as is the CHL. The balance of the placebo formulation was simply the inactive ingredients found in the Derifil tablets.

Acknowledgments

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Chapter 3

Mechanisms of chlorophyllin inhibition of tumor initiation by the potent carcinogen, dibenzo[*a,l*]pyrene; a pharmacokinetic study in the rainbow trout model

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Abstract

The antimutagenic and anticarcinogenic effects of chlorophyllin (CHL) are well documented in a variety of model systems, though the mechanisms of these protective effects continue to be investigated. Numerous *in vitro* studies have shown that non-covalent complex formation occurs between between CHL and Trp-P-2, IQ, AFB_1, AFB_2[6], and the potent carcinogen, dibenzo[*a,l*]pyrene (DBP). It has been suggested that complex formation between the mutagen/carcinogen and CHL is a primary mechanism of CHL-mediated protection. CHL was shown, *in vivo*, to reduce the uptake and biodistribution of AFB_2 in a manner consistent with complexation, but also not inconsistent with non-specific masking of the carcinogen. To further investigate the nature of CHL-mediated protection, an investigation was undertaken of the pharmacokinetic behavior of DBP alone and in combination with CHL in a liquid gavage treatment. To address the possibility that CHL may block uptake by a non-specific masking mechanism, we investigated sucrose as a surrogate approximating the size of DBP and AFB_1, but not anticipated to form a strong molecular complex with CHL. The nominal concentration of DBP used, 200 μM, was comparable to doses of DBP used in a recent dietary carcinogenesis study in which co-administration of approximately 2.9 mM CHL reduced tumor formation in the liver and stomach between 55-79% and 30-68%,

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[6]Abbreviations used: Trp-P-2, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2.

respectively. Assuming the primary CHL-mediated protective mechanism involves blocking of carcinogen uptake, the concentration of CHL used for the present study, 6.2 mM, would be expected to provide even greater protection. Groups of 35 fish were gavaged with an aqueous suspension containing a trace amount of toluene, 25% DMSO and 200 μM radiolabeled DBP alone or in combination with 6.2 mM CHL (as copper chlorins). Equilibrium dialysis further indicated sucrose would not complex with CHL, therefore, additional groups of fish were gavaged with an aqueous solution of 200 μM radiolabeled sucrose alone or in combination with 6.2 mM CHL. From each DBP and sucrose treatment, five fish were sampled at each of the following time points: 1, 3, 6, 12, 24, 48 or 72 hours. At each time point the stomach, pyloric cecae, intestine, blood, liver, and bile were removed and evaluated for the presence of radioactivity. In this model system, DBP was taken up and distributed systemically. The presence of CHL appeared to expedite the passage of DBP equivalents through the pharmacokinetic compartments. In the presence of CHL, DBP equivalents were passed more quickly out of the stomach and blood, with evidence of a significant reduction in AUC for both organs (P = 0.06 and 0.08, respectively). Sucrose, in the presence of CHL, also passed more rapidly through the pharmacokinetic compartments, though the magnitude of AUC change and alteration of the response curves was less pronounced than with DBP. The lack of a significant alteration in sucrose kinetics suggests a CHL blocking mechanism other than non-specific masking. Because of the nature of the liquid

gavage, we were unable to evaluate the pharmacokinetic response of DBP specifically with regard to complex formation with CHL. (Supported by USPHS grants ES03850, ES00210 and CA34732)

Introduction

Studies in recent years have yielded numerous reports of the usefulness and effectiveness of CHL, a water-soluble, sodium-copper derivative of the green plant pigment, chlorophyll, for a variety of health-related purposes. CHL has been used to treat human subjects for at least 50 years with no reported adverse effects. Medical uses have included as an aid in wound healing, as an oral treatment for body, fecal and urinary odors, oral hygiene (46, 51-53, 55), and, outside the field of medicine, CHL is employed as a commercial food dye. CHL was effective as an antimutagen against several types of compounds in multiple model systems (57, 58, 61, 62, 64-66). *In vivo* studies have shown the effectiveness of CHL in inhibiting target organ DNA binding and tumorigenesis in rainbow trout exposed to the very potent liver carcinogen, AFB_1 (1, 63, 69), DNA adduction and tumorigenesis or precancerous lesion formation in rats exposed to IQ (70, 71), and PhIP[7] (76).

In the rainbow trout model, CHL was also effective as a dietary inhibitor of liver tumorigenesis when co-fed with two doses of the very potent carcinogen, dibenzo[*a,l*]pyrene (DBP) (7). A subsequent study in the trout model showed CHL to be very effective in producing a dose-dependent reduction in both DNA adduct formation and multi-organ tumorigenesis by dietary treatment in combination with

[7]PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine.

multiple doses of DBP[8]. CHL reduced the absorption and biodistribution of aflatoxin B_2 (AFB_2), a non-carcinogenic surrogate for AFB_1, in trout following co-exposure via liquid gavage (68).Other studies have demonstrated that protection occurred only if CHL was given simultaneously with AFB_1 within the same solution, and that CHL did not detectably alter the activities of important hepatic metabolizing enzymes in the trout model (69). Additionally, heterocyclic amine mutagens were reported to strongly bind to CHL attached to a solid support (65, 67)

These data are consistent with reduced uptake as a general blocking mechanism for CHL, however, the specific mechanism through which this may be achieved is not known. Studies conducted *in vitro* have demonstrated the formation of strong, non-covalent complexes between CHL and Trp-P-2 (58), IQ (64), AFB_1 (13), AFB_2 (68), and DBP (7), and it is possible that such complex formation accounts for CHL-mediated reduction in carcinogen uptake. Presently, no evidence exists that *in vivo* DBP biouptake is impeded by CHL. Additionally, no *in vivo* study has been conducted with appropriate controls that can conclusively eliminate non-specific masking as an alternative mechanism for CHL-mediated protection. The purpose of the present study was to investigate the effects of CHL co-exposure on DBP uptake and biodistribution, and to determine if non-specific masking might

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[8]Pratt, M. M., Reddy, A. P., Hendricks, J. D., Pereira, C., and Bailey, G. S. Modulation of dibenzo[*a,l*]pyrene-initiated liver, stomach, and swim bladder tumor response by dietary chlorophyllin in rainbow trout. In preparation for submission, 2003.

be a contributing CHL protective mechanism against DBP and other carcinogens. To address the latter issue, we have investigated sucrose as a surrogate that would approximate DBP and AFB_1 in molecular size, but would not be expected to exhibit strong complex formation with CHL.

Materials and Methods

Chemicals

Radiolabeled DBP ([1, 2, 3, 4, 4a, 4b-^{14}C]-DBP, Lot No. CSL-01-019-64-28, SA = 53.5 mCi/mmole, 0.652 mCi/ml in toluene) was obtained from Chemsyn Laboratories (Lenexa, KS). (Note: DBP is a potent carcinogen. Handling, storage, and disposal procedures were in compliance with the National Institutes of Health and Oregon State University guidelines for class C carcinogens.) Radiolabeled sucrose ([^{14}C]-, Lot No. 245-107-495, SA = 495 mCi/mmole, 0.1 mCi/ml in 9:1 ethanol:water) was purchased from Moravek Biochemical Inc. (Brea, CA). Manufacture's HPLC analysis reported radiochemical purity of at least 98% for both compounds. CHL (Lot No. 77H0594) and unlabeled sucrose (Lot No. 93H2514) were purchased from Sigma Chemical Co. (St. Louis, MO). HPLC grade dimethylsulfoxide (DMSO, Lot No. KS01359CS) was obtained from Sigma-Aldrich (St. Louis, MO). All water used for reagent preparation was Millipore-filtered. Because commercially available CHL is a mixture of sodium-copper CHL

and several inorganic salts, all CHL concentrations used in this study were corrected to the lot-specific copper chlorin content (42%), based on the manufacturer's stated copper content (3.69%) and claim that no free copper existed in the mixture (7, 68, 69). The remaining constituents, according to Sigma, were inorganic salts, primarily NaCl. Because CHL in solution is prone to self-association, all CHL solutions were prepared immediately before addition to other solutions. Float-a-lyzer dialysis cartridges (500 μl) were a gift from Spectrum Laboratories (Rancho Dominguez, CA). Solvable, Soluene-350, and Hionic-Fluor scintillation cocktail were purchased from PE/Packard Biosciences. BCS-450 and Ready Organic scintillation cocktail were obtained from Beckman Coulter, Inc. (Fullerton, CA).

Treatment Dosage Determination

The concentration of DBP used in the study was a convenient dose to use given the specific activity of the [^{14}C]DBP and the amount of radioactivity that was desirable for delivery in the gavage treatments. Additionally, 200 μM DBP was comparable to the doses of DBP in an unpublished dietary tumor study where the presence of CHL resulted in a substantial decrease in both DNA adduction and subsequent tumor formation in multiple organs. Assuming 1 gram of diet occupies 1 ml of volume, the DBP concentration of 200 μM translates to a minimal dietary concentration of 60.4 ppm on a wet diet basis, or 173 ppm on a dry diet basis (for

OTD, wet diet weight = dry diet weight x 2.857). The tumor study doses of 80 and 225 ppm DBP (dry diet basis, unpublished data) convert to 94 and 265 μM DBP in the wet diets, respectively. This provided a reference range that included the 200 μM DBP dosage used in the present study. When incorporated into diets containing 80 and 225 ppm DBP, the tumor study CHL concentration of 6000 ppm (2.9 mM) resulted in a reduction in liver tumor incidence of 78 and 55%, respectively. A dissociation constant (K_d) value of 1.59 μM for the complex formed between CHL and DBP in a single-phase solution of 20% tetrahydrofuran in 0.1 M Tris buffer at pH 7.4 has been reported (7). Using the published K_d value, a calculation was performed which determined that a Cu CHL concentration of 2 mM was adequate to ensure < 99% complexation with 200 μM DBP in this solvent. The 6.2 mM CHL used here, when administered together with 200 μM DBP in a dietary formulation, would be expected to provide substantial protection against DBP-initiated DNA damage and tumorigenesis. We anticipated that use of these concentrations in a biologically compatible solvent for gavage would yield insight regarding the hypothesis that CHL-mediated blocking of DBP absorption and biodistribution is the primary mechanism of protection. By replacing DBP with the same molar concentration of sucrose, an agent presumed not to form a complex with CHL, the hypothesis of non-specific masking can be addressed as a possible mechanism of CHL-mediated protection.

Preparation of test solutions

DBP liquid gavage mixtures were prepared by using a Hamilton syringe to deliver 82 µl [^{14}C]DBP from the as-purchased toluene stock into a glass vial where the solution was concentrated to near dryness under a gentle stream of nitrogen. The concentrate was diluted with 1250 µl DMSO and mixed well. Two 500 µl aliquots of the resulting DMSO solution were removed to separate glass vials. To one vial was added 1500 µl water, resulting in a fine and apparently homogeneous suspension containing 204 µM DBP (1.1 µCi/100 µl, as determined from triplicate 25 µl aliquots evaluated by liquid scintillation counting (LSC)). The other vial received 1500 µl of a CHL solution (aliquotted from a freshly prepared stock solution containing 28.5 mg crude CHL in 2.0 ml water), resulting in a mixture containing 223 µM DBP (1.2 µCi/100 µl, verified by LSC) and 6.2 mM copper chlorins. These are nominal concentrations because the solvent did not appear to provide a true, one-phase solution for the DBP-only vial (the opaque nature of the CHL-containing mixture precluded visual evaluation of DBP solubility). Sucrose gavaging solutions were prepared by removing two 200 µl aliquots of [^{14}C]sucrose stock and placing each aliquot in separate glass vials. To each aliquot was added 900 µl of 400 µM unlabeled sucrose and 900 µl of either water or a freshly prepared CHL solution (23.8 mg CHL dissolved in 1 ml water). Both resulting

sucrose solutions contained 200 μM sucrose (1.0 μCi/100 μl, verified by LSC), with the latter solution containing 6.2 mM copper chlorins.

Animals

Shasta strain rainbow trout (*Oncorhynchus mykiss*) were reared, housed, and treated at the Food Toxicology and Nutrition Laboratory, Oregon State University, Corvallis, Oregon, as previously described (40). Prior to fasting, trout were fed the standard Oregon Test Diet (OTD) (40), which fulfills the nutritional requirements of the trout, and is the same formulation given to all animals used for tumor studies. All animals used were from the same spawn.

Biodistribution kinetics

For each treatment, 35 fish were fasted 7 or more days prior to treatment to minimize reflex regurgitation of gavage samples. (Note that at 12°C, several days were required for the trout to completely digest food, thus they were not "starved" as a rodent would be during such a period of fasting.) Five fish were netted at a time, anesthetized in weak solution of MS-222, then weighed to determine how much gavage mixture should be administered. A 100 μl Hamilton syringe with a 2", blunt-tipped (#3) needle was used to administer the gastric gavage of 1.0 μl per g of body weight. The anesthetized trout were held head up during gavage and for 10

seconds after withdrawal of the needle before being gently placed back in water. Five fish from each treatment were sacrificed by an overdose of MS-222, and six different tissues collected from each fish at each time point of 1, 3, 6, 12, 24, 48, and 72 hours after gavage. Blood was collected in pre-weighed, heparin-containing blood tubes and stored at 4°C until the tubes could be weighed to quantify blood recovery. Thereafter, 100 µl aliquots from each sample were distributed to triplicate 20 ml glass scintillation vials for solubilization by Solvable, according to the manufacturer's protocol, followed by LSC analysis. The remaining blood was transferred to polyethylene tubes and stored at -20°C. The stomach, pyloric cecae, intestine, liver, and bile/gall bladders were collected and stored in individual polyethylene sample tubes at -20°C. The weight of each empty sample tube was subtracted from the weight of the respective sample-containing tube to determine the actual sample mass recovered from the trout. The stomachs and pyloric cecae were removed from the sample tubes while still frozen and quickly sliced into three nominally equal sections. Each section was placed in a separate scintillation vial for solubilization. Whole livers and intestines were solubilized without slicing. The bile/gall bladder samples were thawed briefly, then centrifuged to draw all material to the bottom of the polyethylene tube. The samples were then refrozen. The frozen mass at the bottom of each sample tube was quickly transferred to a single 20 ml scintillation vial. Livers and bile/gall bladders were solubilized using 2 ml Solvable per scintillation vial and decolorized with hydrogen peroxide as directed by the

manufacturer's instructions. Stomach, pyloric cecae, and intestine were solubilized using 2 ml Soluene-350 or BCS-450 (both are toluene-based quarternary ammonium hydroxide solutions of the same molarity) per scintillation vial and prepared for LSC analysis following the manufacturer's instructions. Except for the blood, the entire amount of each collected tissue was solubilized and analyzed. All radioactivity was measured using a Beckman 6500 Liquid Scintillation Counter.

Effect of CHL on DBP biodistribution

After fasting at least 7 days, the fish were treated by liquid gavage mixtures containing either DBP only (204 μM, 1.1 μCi/100 μl) or DBP + 6.2 mM CHL (223 μM DBP, 1.2 μCi/100 μl). Five trout were collected at each sample point for both DBP only and DBP plus CHL treatments. Six different tissues were collected from each fish at 1, 3, 6, 12, 24, 48, and 72 hours after gavage. All tissues were processed as above for detection of radioactivity.

Effect of CHL on sucrose biodistribution

For both the control (sucrose only) and test (sucrose + 6.2 mM CHL) treatments, 35 fish were treated by liquid gavage with solutions containing 200 μM sucrose (1.0 μCi/100 μl). Five fish per treatment were sacrificed by overdose with

MS-222 at 1, 3, 6, 12, 24, 48, and 72 hours after gavage and six different tissues from each fish were removed and processed as above for detection of radioactivity

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Statistical Procedures

The data were modeled using a heterogeneous-variance linear model to allow for different variances at different time points. Contrasts of Area Under Curve (AUC) differences with and without CHL were evaluated to obtain the reported *P* values, which address the null hypothesis of no difference between the reported AUCs.

Results

Evaluation of sucrose interaction with CHL

To distinguish between specific (complex-mediated) and non-specific (masking) CHL protective mechanisms, a non-complexing compound of similar mass to AFB_1 (MW = 312.3) or DBP (MW = 302.4) needed to be identified. Two brief experiments were conducted to investigate the potential for sucrose (MW = 342.3) to form complexes with CHL. A single replicate spectroscopic study was performed that gave indirect evidence for a possible complexation interaction between sucrose and CHL (data not shown). To further investigate the possible interaction between sucrose (MW = 342.3) and CHL (MW = 722), two dialysis

cartridges, with a molecular weight cut off of 500 Da, were each filled with a solution containing 200 μM sucrose (1.25 μCi), with or without 4000 μM CHL. The dialysis cartridges were placed into separate containers filled with 600 ml of 200 μM unlabeled sucrose solution. The containers were stirred gently for a period of nearly 6 weeks. At various intervals during this time, triplicate aliquots were removed from each container for evaluation by LSC to determine the amount of radioactivity that had passed through the dialysis tubing into the outer solution. Additional low molecular weight species, in the form of 3000 mM NaCl, were introduced into the outer, unlabeled sucrose solution in 1000 mM increments in order to equalize osmotic pressure across the membrane. No visible leakage of CHL from the dialysis tubing was evident throughout the course of the experiment. The results, shown in Figure 3-1, indicate that no substantial complexation occurs between sucrose and CHL, as evidenced by inner and outer solutions achieving essentially the same equilibrium concentration of radiolabeled sucrose during the 30-day evaluation. It was, therefore, concluded that sucrose would be an appropriate control to evaluate the possibility of nonspecific masking by CHL.

Kinetics of sucrose biodistribution

Having identified sucrose as a suitable, non-complexing control agent, we then proceeded to address the question of CHL acting by a mechanism of

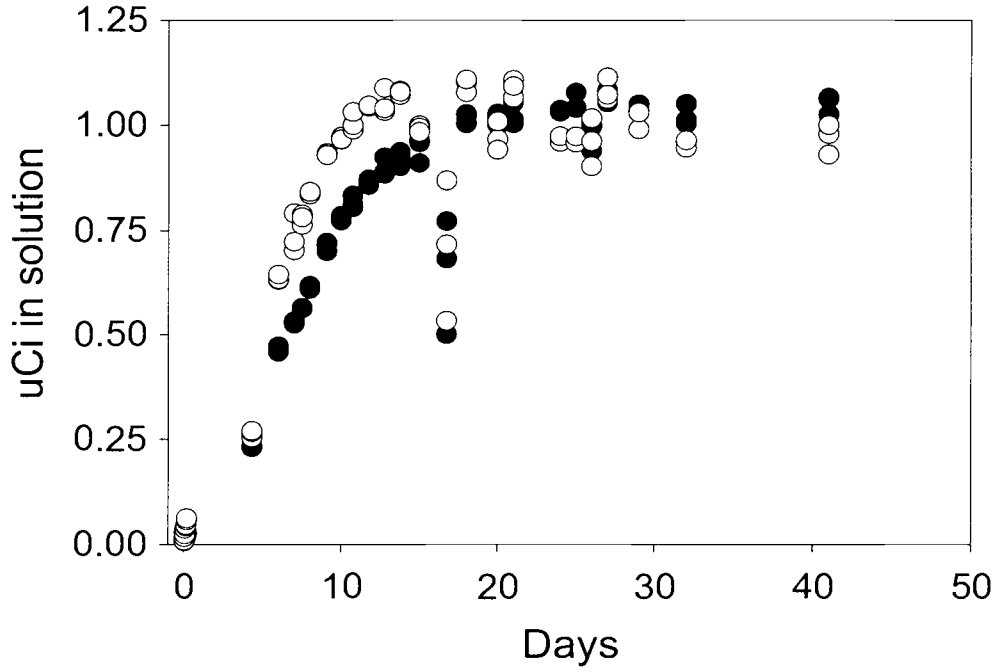

Figure 3-1. Sucrose dialysis. Dialysis tubing was filled with 200 μM [^{14}C]sucrose (1.25 μCi) with ( ) or without (●) 4000 μM CHL in a stirred outer solution containing 200 μM sucrose at room temperature. Triplicate 1 ml aliquots were removed and evaluated by LSC for radioactivity. All results are shown.

nonspecific masking. Two groups of 35 fish were each treated with 200 μM sucrose (1 μCi/100 μl) by gavage in the amount of 1.0 μl per g body weight. The control group received only the sucrose solution, whereas the test group was treated with a solution containing both sucrose and 6.2 mM CHL. For both groups, five fish were sacrificed 1, 3, 6, 12, 24, 48, and 72 hours after treatment. From each fish, the stomach, liver, bile/gallbladder, pyloric cecae, and intestine were sampled to establish the biodistribution of sucrose over time after gavage treatment, both with and without CHL. Figure 3-2 shows that sucrose, in the absence of CHL, passed out of the stomach and into the other organs very rapidly. Based on overall recovery from the organs examined, approximately 90% of the sucrose had passed through the monitored compartments by 72 hours after treatment with both control and test solutions.

The addition of 6.2 mM CHL to the gavage solution resulted in relatively minor changes in biodistribution in all the organs monitored, though the presence of CHL appears to be associated with a very slight increase in the rate of passage of sucrose out of the stomach and into the other organs. In the stomach, the AUC changed from 3.85 to 2.80, representing a decrease of 27% ($P = 0.02$) when CHL was added. The AUC was reduced from 6.07 to 5.20 ($P = 0.01$) and from 3.52 to 3.02 ($P = 0.14$), respectively, in the pyloric cecae and intestine, representing a decrease of 14% in both organs when CHL was included. In the blood, liver, and bile samples, the addition of CHL to the sucrose gavage solution resulted in

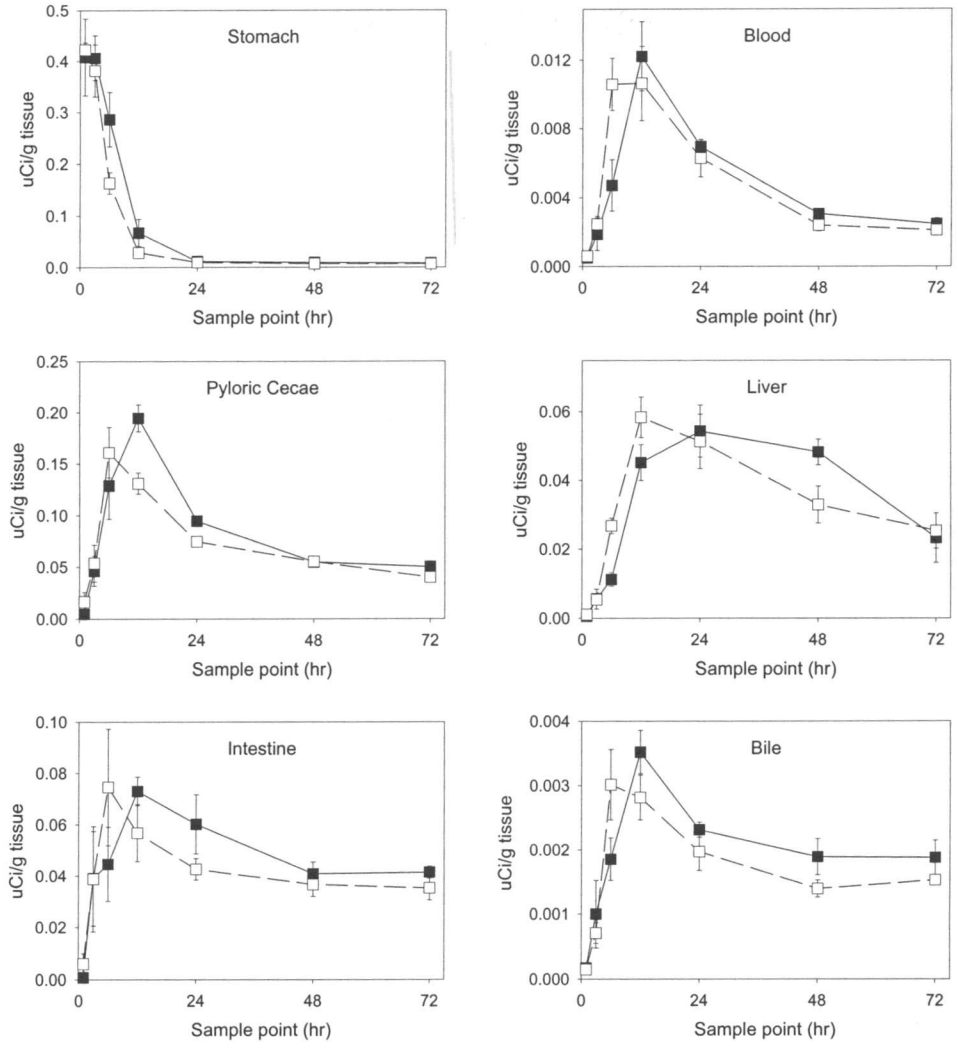

Figure 3-2. Pharmacokinetics of 200 μM [^{14}C]sucrose with (□) and without (■) 6.2 mM CHL in six tissues following oral co-gavage treatment of 1 μCi/g body weight. Five fish were sacrificed at each time point of 1, 3, 6, 12, 24, 48, or 72 hours after gavage. Samples of each tissue were individually collected, processed, and evaluated by LSC for radioactivity.

respective AUC decreases of 7, 7 , and 16% ($P = 0.62$, 0.47, and 0.04, resp.). Consistent with the small AUC changes, the shape of the biodistribution curves were minimally changed by the presence of CHL. We note that all changes were in the direction of CHL-mediated increases in sucrose uptake and biodistribution, not impairments that would be suggestive of a non-specific masking mechanism for CHL.

Kinetics of DBP biodistribution

The initial solvent used for the investigation of CHL-DBP complexation (7) was not biologically compatible. We chose here a water-DMSO solvent that we knew to be tolerated by trout, and would be of at least roughly comparable polarity. Unfortunately, however, water added to the carcinogen-DMSO solution during the preparation resulted in the DBP gavage solution taking on a milky appearance. This led to a brief investigation into the nature of an identical mixture. Left undisturbed for 1 hour, a small degree of separation was noted in this duplicate mixture. Upon subsequent centrifugation for 20 minutes, a clarification of the mixture plus the appearance of a thin film on the surface of the solution confirmed that a suspension had actually resulted upon addition of water to the DMSO solution. Triplicate aliquots carefully removed from the clear, bottom layer revealed that approximately 3.5% of the DBP used to prepare the solution was still present in the clear bottom layer of fluid. The remaining DBP was retained in the material that collected on top

of the clear DMSO-water solution. (The opaque nature of the CHL-containing mixture made it impossible to make similar observations, however, we assumed that the same two-phase suspension resulted upon addition of the CHL-containing water solution to the DBP-containing DMSO solution.) This dissimilar distribution of DBP and CHL into a two-phase gavage mixture precluded a direct examination of CHL effects in the presence of a known amount of DBP-CHL complexation in this initial study. We have yet to identify a biologically compatible solvent that would allow DBP and CHL to exist in a single-phase solution.

However, a study with this solvent may still provide useful information regarding non-specific CHL masking, irrespective of DBP dissolution, provided it can be shown that DBP uptake and biodistribution is robust. Figure 3-3 depicts the kinetics of [^{14}C]DBP uptake and biodistribution into major tissues following single gavage administration in the trout. Maximal DBP- derived ^{14}C concentrations were reached in the pyloric cecae, lower intestine, blood, and liver at the 24, 48, 24, and 24-hour time points, respectively. Radioactivity in the bile was still ascending at 72 hours, the last time point examined. The mean recovery of the nominally administered [^{14}C]DBP dose for all the samples collected through 72 hours was 71.6%. The remaining dose represents DBP lost into lesser pharmacokinetic compartments not assessed in this study (residual carcass; DBP and metabolites lost into the water through gill, fecal, and urinary excretion), as well as some material loss through initial reflex regurgitation by some animals shortly after gavage. This

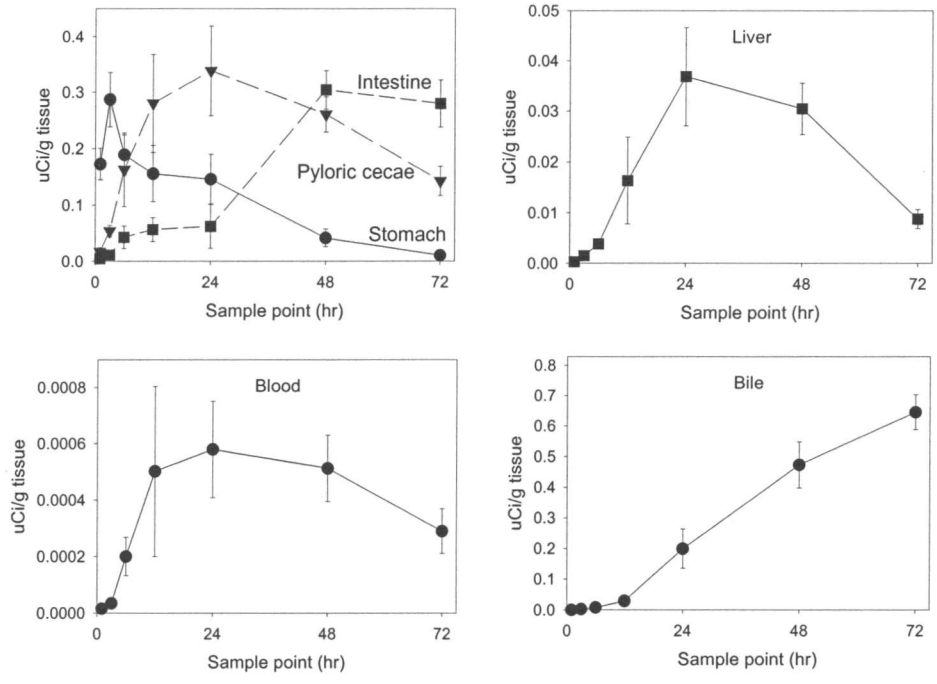

Figure 3-3. Pharmacokinetics of 200 μM [^{14}C]DBP following oral gavage treatment of 1 μCi/g body weight. Five fish were sacrificed at each time point of 1, 3, 6, 12, 24, 48, or 72 hours after gavage. Samples of each tissue were individually collected, processed, and evaluated by LSC for radioactivity.

latter phenomenon likely explains the recovery of just under 50% of nominal dose from the stomach among the individuals selected to sample at the 1-hour time point. In all, these data indicated that DBP was taken up well from the 2-phase gavage medium, that an observation period of 72 hours was sufficient to assess the kinetics of DBP uptake and biodistribution in this model, as it was for AFB_2 in true solution (68), and that the tissues/compartments examined represent an acceptable majority (72%) of the nominal dose administered.

DBP equivalents were taken up slowly from the stomach such that, at 24 hours, nearly 50% of the administered dose still remained in the stomach. In comparison, the study with AFB_2 and CHL revealed that, at 12 hours, the stomach contained only 5% of the AFB_2 that was present at 1 hour after gavage (68). The relatively slow departure of DBP from the stomach was echoed by the relatively slow passage of DBP into the pyloric cecae and the intestine. In light of the quantity of DBP recovered in other compartments, little was actually absorbed into the bloodstream and subsequently into the liver and bile. The appearance of radioactivity in the blood and liver was followed by a relatively slow decline in amount of DBP detected in these organs, whereas a steady increase in DBP was observed in the bile throughout the course of the 72-hour study.

Effect of CHL on the biodistribution of DBP

The addition of 6.2mM CHL into the gavage mixture in this study did not retard or reduce DBP uptake and biodistribution. Instead, CHL addition appeared to produce a very slight but noticeable increase in the rate of passage of DBP through the digestive tract and into systemic circulation (Figure 3-4). The presence of CHL caused more rapid emptying of the DBP from the stomach into the pyloric cecae and resulted in a reduction in stomach AUC from 6.898 units to 4.319 units (P = 0.06. If omitting 1 and 3 hour time points from comparison due to poor recovery, P = 0.02), a decrease of 37% compared to the 27% decrease noted with the sucrose treatment. In the pyloric cecae and intestine, the presence of CHL was associated with an increase in AUC from 17.47 to 27.74 ($P < 0.002$), and from 12.52 to 19.95 ($P = 0.01$) respectively, which translate to increases in AUC of 59% in both organs, a considerably larger change in the opposite direction from the 14% decrease noted in the same organs with the addition of CHL to sucrose. Not only did the presence of CHL in the blood result in a slightly more rapid absorption of DBP into the bloodstream, but also a more rapid decrease in DBP levels and a decrease in the AUC from 0.0317 to 0.0203, a change of 36% ($P = 0.08$). There was also a noteworthy change in the shape of the DBP pharmacokinetic curve in the blood when CHL is added. This was in sharp contrast to the relatively indistinguishable change in the shape of the sucrose curve when CHL was included, resulting in a 7% decrease in AUC. The observable changes in the curve shape in the liver and bile

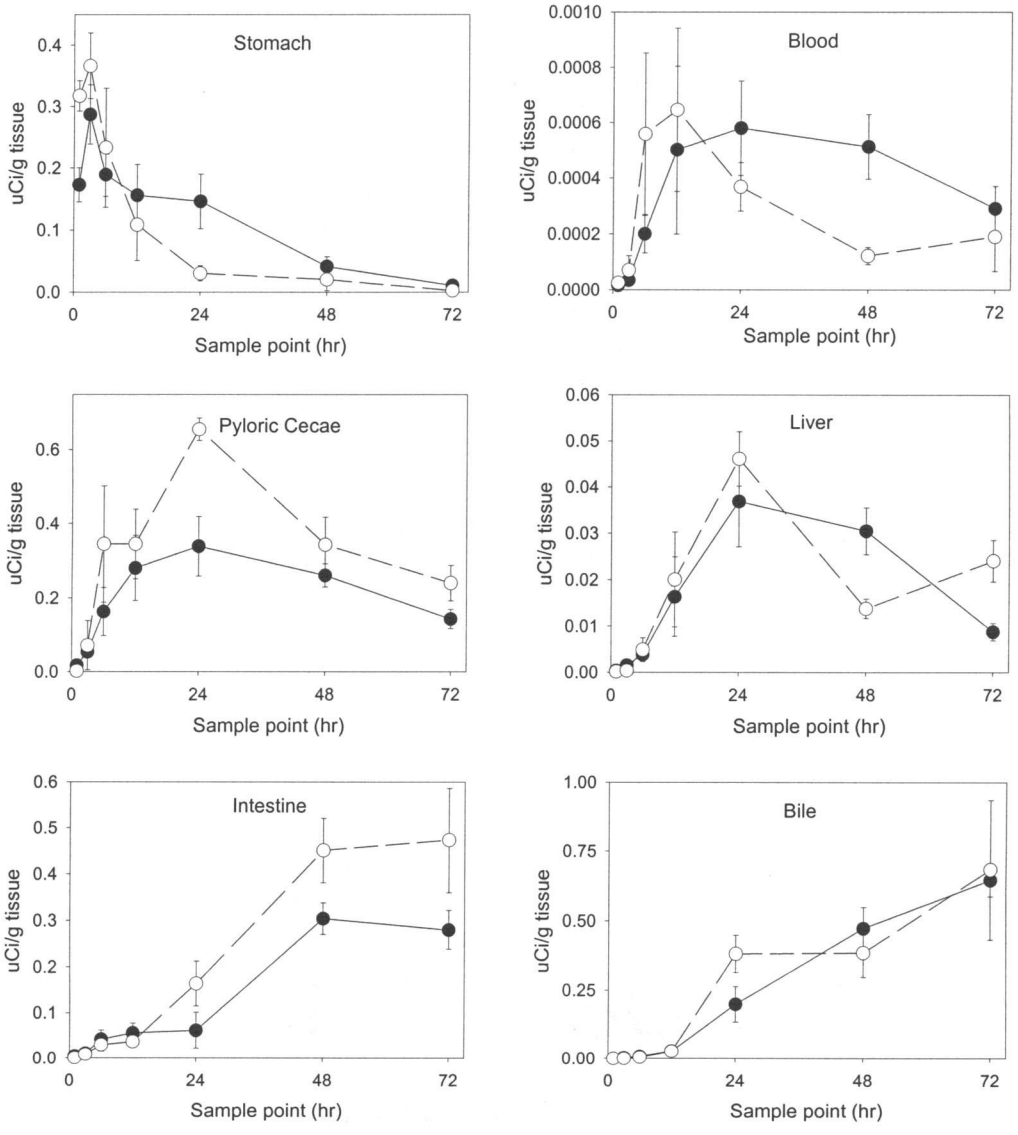

Figure 3-4. Pharmacokinetics of 200 μM [^{14}C]DBP with (○) and without (●) 6.2 mM CHL following oral gavage treatment of 1 μCi/g body weight. Five fish were sacrificed at each time point of 1, 3, 6, 12, 24, 48, or 72 hours after gavage. Samples of each tissue were individually collected, processed, and evaluated by LSC for radioactivity.

were associated with AUC changes of -1% and +7%, respectively, when CHL was added ($P > 0.7$, both organs). The mean recovery of [^{14}C]DBP from the evaluated organs of all animals over all time points was 71.6% in the controls and 85.8% in the CHL treated animals. There was no decrease in the recovery of radiocompound over time as was noted with the sucrose treatments during the 72-hour experiment.

Discussion

This experiment was designed to address the following questions: 1) can the protective effects of CHL against DBP, at dietary equivalent doses known to dramatically decrease DNA adduct formation and subsequent tumor response, be explained by a mechanism of nonspecific masking, and 2) what is the effect of CHL on the biodistribution of DBP when both are administered by gavage in a single solution and in the same concentrations used to address the first question?

To answer the first question, it was necessary to identify an appropriate control compound. Because our specific interest involved the CHL interaction with DBP, and by extension, AFB_1, it was desirable to identify a compound that is similar in size to DBP, but does not form a complex with CHL when combined in solution. An equilibrium dialysis study verified absence of a strong CHL-sucrose complex. We then determined that CHL added to a sucrose gavage solution had little effect on the biodistribution of sucrose, except for a slight increase in the rate of passage of the radiolabeled sucrose through the digestive tract. The presence of

CHL resulted in biodistribution curves of essentially the same shape as the control curves, but offset by a period of 3 to 6 hours. The small magnitude of AUC value change in all six organs evaluated provides evidence supporting the hypothesis that CHL anticarcinogenesis protection *in vivo* occurs through some mechanism other than nonspecific masking.

The second question addressed was how does the presence of CHL affect the biodistribution of DBP when both are given together in a liquid gavage treatment. In a similar study, it was concluded that CHL does alter the pharmacokinetics of AFB_2 by reducing the AUC as well as slowing the passage of AFB_2 through the digestive tract and into systemic circulation (68). The putative mechanism was presumed to be a complex formation between CHL and AFB_2, which was shown to have an *in vitro* K_d = 1.92 μM, similar to the dissociation constant of 1.4 μM reported for the complex between CHL and AFB_1 (13). An *in vitro* dissociation constant of 1.59 μM was reported for DBP and CHL in a solution consisting of 20% tetrahydrofuran in a Tris buffered solution (7). For the present study, attempts were made to produce a single-phase solution containing both CHL and DBP that would be palatable and tolerable to the fish. An initial experimental solution consisting of 75% DMSO and 25% water was not tolerated by the fish, and approximately 80% of the DBP-only gavage solution was lost, apparently by reflex regurgitation (data not shown). A second attempt using a 25% DMSO suspension was tolerated to a much greater extent, as evidenced by the average recovery of

nearly 72% of the radiolabeled material from tissues collected from the fish treated with DBP alone. However, this tolerated test material turned out to be a suspension and not a single-phase solution, rendering impossible an evaluation of the hypothesis involving complex formation. The material administered to the fish might be considered more similar to the diets used in the tumor study in that it was of multiphasic composition. However, the diets are colloidal in nature and have been shown to increase residence time of treatments in the digestive tract (68), which has implications of increased time of exposure. A dietary treatment consisting of a similar DBP molar concentration and approximately 50% of the CHL concentration used in this pharmacokinetic study resulted in a reduction in liver DNA adduction and tumor incidence of ranging from 50- 70%[9].

It should be noted that the nominal molar quantities of DBP and sucrose (200 μM) were very different from that used for the AFB_2 pharmacokinetic study (0.9 μM), as were the molar ratios between the test compounds and CHL (1:30 in this study versus 1:13,900 and 1:139:000 for the AFB_2 study). This difference may account, in part, for the contrasting observations of a delay in AFB_2 passage and uptake versus expedited DBP and sucrose passage and uptake in the presence of CHL. The precise explanation for this apparent disparity has yet to be determined.

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[9]Pratt, M. M., Reddy, A. P., Hendricks, J. D., Pereira, C., and Bailey, G. S. Modulation of dibenzo[*a,l*]pyrene-initiated liver, stomach, and swim bladder tumor response by dietary chlorophyllin in rainbow trout. In preparation for submission, 2003.

The fact that DBP and sucrose respond differently to the presence of the same dose of CHL is intriguing, despite the multiphasic nature of the DBP treatments. Admittedly, the observed differences may be partly due to the fact that sucrose was in the same solution with CHL, whereas DBP was not. An evaluation of DBP in a single-phase solution with CHL is required to properly address this issue. Even so, a few observations from this study are worth noting. The relatively rapid passage of the DBP "in suspension" through the stomach in the presence of CHL is suggestive of protection in that organ. The rapid reduction of peak DBP concentration in the blood when CHL is included in the gavage, suggests a reduction in systemic DBP bioavailability, however, given the reduction in blood AUC when CHL is included, it is interesting to note that the AUC in liver and bile were relatively unchanged. Consistent with the results of the AFB_2 study, the presence of CHL in the gavage with DBP resulted in a substantial increase in radiolabeled material found in the pyloric cecae and the intestine, as would be expected if CHL were acting to sequester the DBP and prevent absorption.

CHL is an effective blocking agent and, as such, is able to reduce DNA adduction and tumor incidence against large, near-planar polycyclic compounds, such as AFB_1 and DBP. The response of DBP to the presence of CHL was more pronounced than that of sucrose as evidenced by altered biodistribution response curves and more substantial AUC changes. This suggests that identical concentrations of DBP and sucrose respond by different mechanisms to the same

concentration of CHL. It cannot be concluded, based on this study, that the DBP response is due to complexation with CHL. The pharmacokinetic changes observed in the DBP response to CHL followed liquid gavage treatment in the form of a suspension in which complex formation with the published K_d value (7) could not be evaluated. Identifying a biologically compatible solvent formulation capable of holding both CHL and DBP in solution is necessary before the hypothesis of CHL protection by complexation with DBP can be properly addressed. We believe this study has provided adequate evidence, however, that CHL-mediated protection against DBP-initiated DNA adduction and tumorigenesis occurs by a mechanism other than the non-specific masking of carcinogen uptake and biodistribution.

Acknowledgments

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Chapter 4

Conclusions

The experiments reported in this thesis have demonstrated, once again the utility of the rainbow trout model for conducting studies of carcinogenesis. In a study initially utilizing over 12,000 animals, the effectiveness of CHL has been demonstrated by a dose-dependent reduction in tumor formation in multiple target organs following dietary initiation with the potent environmental carcinogen, dibenzo[*a,l*]pyrene. In both the liver and the stomach, the dose-response behavior with increasing CHL doses was successfully modeled as linear within the range of doses evaluated, with one exception. Despite the apparently non-linear tumor response in the liver at a single dose of CHL, protection against DBP-initiated tumorigenesis is still evident. Those CHL doses which were successfully modeled as linear, including those in the stomach, suggest that the protective effects of CHL were independent of the carcinogen dose.

Additional information gleaned from this study reasserted the potential usefulness of DNA adduct formation as a biomarker of exposure to DBP. The study reported in Chapter 2 indicates that DBP-DNA adduct formation was a quantitative predictor of final tumor response within the dose range and target organs evaluated. The utility of having reliable and predictive biomarkers would be extremely useful in terms of early detection of carcinogen (or toxicant) exposure and the potential

implementation of intervention strategies very early in the disease process. In particular, DNA adduct biomarkers are sensitive, specific to DNA damaging agents, and can also be associated with future consequences, unlike other biomarkers which merely detect exposure (30). A urinary biomarker consisting of AFB_1-N7-guanyl DNA adduct repair products (6) was successfully and quantitatively used to confirm the reduction in AFB_1 exposure in human populations (2, 31). Work in the trout model has demonstrated that hepatic AFB_1-DNA adducts can be used as quantitative predictors of eventual tumor outcome (1, 98). Validation of such biomarkers with subsequent tumor outcomes in humans suggests the possibility of early, accurate detection and, presumably, more successful intervention.

Consistent with the extensive history of CHL usage, no adverse effects were apparent in the fish receiving CHL in the diet. Interestingly, the fish receiving CHL were found to have green livers at the time of adduct sampling. This is an obvious indication that some components of the CHL preparation were being absorbed into the bloodstream. The metabolic implications of this CHL appearance in the liver beyond the trout model are uncertain. CHL was shown to affect the activity of certain metabolizing enzymes in human and rat liver microsomes (60), however, studies in the rainbow trout model indicated no effect on major hepatic metabolizing enzymes in the presence of a large dose of CHL (69). A recent study using CaCo-2 cells on porous cell culture inserts reports significantly higher apical

over basolateral efflux in this model system, along with a substantial degradation of copper chlorin e_6 and relatively little degradation of copper chlorin e_4 in the simulated digesta (110). Assuming similar behavior in trout, this suggests the green coloration observed in the trout liver consists primarily of copper chlorin e_4. Another recent study reported the isolation of a third component of clinical CHL mixture, copper chlorin e_4-ethyl ester, from the serum of individuals consuming CHL tablets as part of a clinical intervention trial (48). The demonstrated ability of CHL to block the formation of DNA adducts of direct-acting mutagens and carcinogen metabolites (95, 111), its fractional absorption into the blood stream, and its apparent deposition in the liver suggests CHL-mediated protection, even into the target organ. Further investigations characterizing and quantifying systemic CHL uptake in trout and other models may lead to further mechanistic insights.

During the tumor evaluation portion of these studies, samples of tumor tissue were collected for future evaluation of mutational spectra. The *ras* gene in rainbow trout has been shown to be mutated in multiple organs in response to carcinogen exposure (21), including DBP[10]. An evaluation of the *ras* gene, other potential genetic targets of this carcinogen, and any effects caused by the presence of CHL will, perhaps, be the subject of future studies.

The presence of CHL had no effect on the spectrum of tumor phenotypes observed in the DBP-treated trout. The DBP-initiated liver tumor spectrum,

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[10]Bailey, *et al.*, unpublished data.

however, differs from that observed in response to other carcinogens evaluated in the trout model, suggesting different cellular pathways for DBP-initiation. The relevance of this observation will, perhaps, be clarified in future studies, possibly in relation to mutational information in target genes.

The pharmacokinetic behavior of a dose of DBP known to produce ≥50% tumor incidence in the liver showed that this potent carcinogen is bioavailable following oral exposure. The pharmacokinetic response of DBP to CHL contained in the same gavage mixture is suggestive of a protective effect, particularly by the AUC reductions in the stomach and in the blood. However, an additional study will be needed in order to more fully evaluate the DBP pharmacokinetic response to the presence of CHL, particularly with regard to the formation of a complex between the two compounds.

There was relatively little evidence of an alteration in the pharmacokinetics of sucrose when administered in the same dosage as DBP. The dose of CHL used was in excess of those producing ≥ 50% inhibition of liver and stomach tumor formation against comparable doses of DBP in a dietary co-treatment[11]. The hypothesis of non-specific masking suggests a protective mechanism of reduced biouptake due to the presence of CHL. At the molar ratios evaluated, a substantial reduction in sucrose uptake would be expected if non-specific masking by CHL

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[11]Pratt, M. M., Reddy, A. P., Hendricks, J. D., Pereira, C., and Bailey, G. S. Modulation of dibenzo[*a,l*]pyrene-initiated liver, stomach, and swim bladder tumor response by dietary chlorophyllin in rainbow trout. In preparation for submission, 2003.

was occurring to a significant extent, but this was not evident.

The fact that non-specific masking was not observed lends considerable support to the hypothesis that CHL is a blocking agent that acts primarily by a mechanism of complex formation with the carcinogen. The results reported in this thesis are largely consistent with that mechanism. Protection against DBP-initiated DNA adduct formation and final tumor response was not detectably dependent on DBP dose, suggesting a reduced uptake of carcinogen in a CHL dose-dependent fashion. Unlike the studies with AFB_1 against CHL, molecular dosimetry analysis in this study with DBP gave no consistent indication of other, CHL dose-dependent mechanisms of protection. Similarly, there was no change in tumor phenotype with the incorporation of CHL into the dietary treatments, again suggesting exposure to lower levels of carcinogen. A very slight change in the ratio of stomach DNA adducts is suggestive of another, cellular mechanism, but this change was small in relation to the reduction in total adducts.

At dietary doses comparable to that of dietary concentrations of chlorophyll, CHL was found to be highly protective against DBP-initiated tumorigenesis in the three target organs evaluated. Additionally, evaluation of the clinical CHL preparation echoed that response, whereas the placebo tablets, coated with a green dye, had little to no effect in reducing DNA binding and tumor response. The amount of green dye used in the placebo tablets and its chemical relationship to CHL were not investigated as part of these studies. Discerning the nature of the dye

and any potential protective activity it possesses would be an interesting follow-up study. The results reported here and the lack of a placebo effect noted in the clinical trial conducted in China (2) indicates the binders, which are present in both the Derifil and, to a greater extent, the placebo tablets, are largely inactive toward DBP. CHL, the derivative of chlorophyll, continues to demonstrate its effectiveness as a blocking agent. It is possible that natural chlorophyll may have similar protective effects in dietary quantities, though further experimentation is necessary before this is known.

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