

## AN ABSTRACT OF THE THESIS OF

Aram Oganesian for the degree of Doctor of Philosophy in  
Toxicology presented on November 25, 1997. Title: Modulation  
of Chemically-Induced Hepatocarcinogenesis by Indole-3-  
Carbinol: Mechanisms and Species Comparison

Abstract approved: *Redacted for Privacy* \_\_\_\_\_

David E. Williams

There is plenty of evidence from epidemiology studies supporting a link between certain components in the human diet and cancer incidence. It is estimated that 3-4 million annual cases of cancer could be prevented worldwide just by changing dietary habits. In parts of the world where vegetables and fruits constitute a large part of the diet, certain cancer incidences are markedly lower compared to Western countries. In particular, consumption of cruciferous vegetables is negatively associated with occurrence of certain cancers. One of the compounds from crucifers that is implicated in chemoprevention, is indole-3-carbinol(I3C), documented to inhibit tumor formation in several tissues in

rodents, including the mammary tissue. I3C and is currently being evaluated in several clinical trials as a chemopreventive agent against breast cancer in humans. There are, however, some legitimate concerns regarding the use of Pure I3C since, depending upon conditions of administration, I3C can act as a promoter of hepatocarcinogenesis. Evidence is presented here that dietary I3C can promote and/or enhance liver tumor formation in rainbow trout (supporting earlier reports in literature) and the C57BL/6J mouse (enhancement in short-term pre-initiation exposure through lactational transfer, inhibition in a long-term post-initiation feeding study). I3C is also reported to promote in the rat liver model. A major concern associated with dietary I3C supplementation relates to its estrogenic effects as seen in trout and also its ability to induce certain cytochrome P-450s involved in procarcinogen activation. Biological effects of I3C are attributed to its acid condensation products. It was observed in this study that I3C acts through different mechanisms, including the Ah receptor-mediated and estrogenic pathways. Understanding of the role of I3C derivatives in beneficial and/or hazardous effects resulting from dietary exposure will be crucial in evaluating the promise of I3C as a chemoprevention agent. Questions pertaining to the risk/benefit of the use of dietary I3C supplementation for preventing estrogen-related diseases,

without increasing the risk of promotion of hepatocarcinogenesis in humans, may depend on whether the mechanism(s) of action of I3C derivatives in humans is more like the adult mouse or the neonatal mouse, rat and trout.

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**MODULATION OF CHEMICALLY-INDUCED  
HEPATOCARCINOGENESIS BY INDOLE-3-CARBINOL:  
MECHANISMS AND SPECIES COMPARISON**

by  
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## CONTRIBUTION OF AUTHORS

Dr. George Bailey was involved in designing the large trout tumor study (chapter 3) and in the interpretation of the results. Dr. Jerry Hendricks conducted the gross examinations of organs in tumor studies in chapters 2, 3, and 5. He also assisted in the design of the large trout tumor experiment (chapter 3). Dr. Gayle Orner had an input in designing the trout tumor study (chapter 3) and also assisted in procedures involving the initiation with the carcinogen. Dr. Cliff Pereira was responsible in statistical modeling and analysis and he also consulted us in various matters involving experimental designs. Dr. Jan Spitsbergen assessed the viability of tissue slices histologically and also did the gross and histological evaluations of mouse tumors in the study from chapter 6.



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**MODULATION OF CHEMICALLY-INDUCED  
HEPATOCARCINOGENESIS BY INDOLE-3-CARBINOL:  
MECHANISMS AND SPECIES COMPARISON**

Chapter 1

**INTRODUCTION**

Almost on a daily basis, the news media reports on the presence of one chemical or another that is present in the human diet and is claimed to be carcinogenic, or anticarcinogenic. The public is being bombarded with reports that raise both fear and hope. Ever more popular have become numerous dietary supplements mainly originating from plants, and the health food industry is growing faster than ever.

It has been found from epidemiology studies that components of the human diet have some associations with the incidence of certain cancers (1, 2). It is believed that 15-65 % of all cancer occurrences are diet-related (1). For instance, a higher incidence of stomach cancer has been linked to the consumption of salty foods, or lack of intake of fruits and vegetables (3), and a higher occurrence of

colorectal cancer is associated with high intake of fat in the diet (4). Epidemiology studies also demonstrate that, in parts of the world where fruits and vegetables are a large part of the diet, the incidence of certain cancers in the population is lower (5, 6). The American Cancer Society and other health authorities have issued a series of dietary recommendations designed to lower the incidence of cancers believed to be diet-related. It is estimated that 3-4 million cases of cancers worldwide (350-400 thousand in the US) could be prevented annually just by changing dietary habits in the population (6). Although advances in the medical field have significantly improved the rate of cure for some types of cancer, the five year survival rate of cancer patients in the United States was only 51 % in 1992 (7). Not surprisingly, preventative approaches to controlling cancer appear to offer a healthy alternative in decreasing overall mortality in humans who are at high risk for cancer. The Chemoprevention Branch of the Prevention Program, Division of Cancer Prevention and Control, National Cancer Institute, has, as part of their responsibilities, the task to identify and characterize potential candidate compounds for chemoprevention (8). Among several naturally occurring compounds, indole-3-carbinol<sup>1</sup> (I3C<sup>2</sup>) has received serious consideration.

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<sup>1</sup> Synonyms include 3-indolylcarbinol, indole-3-carbinole, indol-3-ylcarbinol, indol-3-ylmethanol, indole-3-methanol, 3-indolemethanol and 3-hydroxymethylindole. Indole-3-carbinol remains widely used and

I3C is a plant secondary metabolite, found in naturally occurring form as a breakdown product of glucobrassicin, the most common member of a class of compounds known as glucosinolates (9). High levels of glucosinolates have been identified in the family of Cruciferae. Representative plants from this family include broccoli, cabbage, cauliflower, Brussels sprouts, etc. Glucobrassicin undergoes enzymatic hydrolysis upon maceration of plant tissue at neutral pH, yielding glucose, sulfate, thiocyanate ion and I3C (Fig. 1.1). In the conditions of lower pH, another pathway yields indole-3-acetonitrile, hydrogen sulfide and elemental sulfur. Both pathways are catalyzed by the plant enzyme myrosinase (thioglucoside glucohydrolase, E.C. 3.2.3.1). Once formed, I3C may undergo condensation into 3,3'-diindolylmethane (I33') or, into ascorbigen in the presence of L-ascorbic acid, found at relatively high levels in cruciferous plants. For experimental purposes, I3C can be commercially obtained from different sources (in this study, from Aldrich Chemical Company, Inc. (Milwaukee, WI)). For the purposes of this thesis, discussion will be limited to I3C and its acid condensation products. For an overview of more details in chemical and biological properties of indole

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referred to in the literature and, for consistency, will be used here.

<sup>2</sup> Abbreviations: Ah, aryl hydrocarbon; I3C, indole-3-carbinol; I33', 3,3'-diindolylmethane; LT, linear trimer, [2-(indol-3-ylmethyl)-indol-3-yl]indol-3-ylmethane); CT, cyclic trimer (5,6,11,12,17,18-hexahydrocyclonona[1,2-*b*:4,5-*b'*:7,8-*b''*][triindole); ICZ, indolo[3,2-*b*]carbazole; ANF,  $\alpha$ -naphthoflavone; BNF,  $\beta$ -naphthoflavone; HCB, hexachlorobiphenyl; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.



glucosinolates, the reader is referred to an excellent review by McDannell et al. (9).

*Beneficial Effects of I3C - the Chemoprevention Promise.*

As early as 1975, Wattenberg and colleagues (10) demonstrated that when administered orally, I3C could induce aryl hydrocarbon hydroxylase (AHH) activity in hepatic and intestinal tissue. This finding resulted in the beginning of the interest in I3C as a possible chemopreventive agent. Later, the promise of I3C was substantiated significantly when it was shown to inhibit mammary tumor formation induced by 7,12-dimethylbenz[a]anthracene (DMBA) in female Sprague-Dawley rats and neoplasia of the forestomach induced by benzo[a]pyrene (BaP) in female ICR/Ha mice (11). I3C possesses characteristics that are very desirable for a potential chemopreventive agent. For example, I3C exhibits very low acute toxicity (12-14) and does not appear to have teratogenic properties (12). The anticarcinogenic activity of dietary I3C in the form of inhibition of tumors and/or DNA-adduct formation has been observed against several classes of environmentally relevant carcinogens, including nitrosamines (15-23), PAHs (11, 24-27), the mycotoxin, aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) (28-33), and the nitroazarene, 4-nitroquinoline 1-oxide (34). It was the demonstration of

protection against estrogen-related tumors (35-37) that suggested the use of I3C as a chemopreventive agent against human breast cancer. In fact, short-term studies with I3C in humans indicated that protection may occur against estrogen-responsive breast cancer development (38, 39). Such capacity to protect against many different carcinogens is a highly desirable feature in a chemopreventive compound, given the diverse etiology of human cancer (1,2). The apparent lack of specificity in protection of various target organs constitutes another desirable characteristic of I3C. Various reports indicate I3C-mediated chemoprotection in the liver (16, 23, 29, 30, 40) forestomach (11) or stomach (27), lung (16, 17, 25), mammary tissue (11, 24, 35), larynx (36), tongue (34), nasal mucosa (17), swim bladder (27) and endometrium (37). Furthermore, chemoprotection from I3C does not seem to be species-specific as it was observed in mice (17, 23, 36, 35), rats (18 34, 22, 33), and trout (15, 27-30, 40). Evidence from mechanistic studies in other species (hamsters, chick embryo and monkey hepatocytes) suggest that similar outcome could occur in them as well (41, 26, 42). There are also encouraging preliminary results in humans (38,39).

### *Concerns Regarding Dietary I3C Exposure*

The enthusiasm associated with the potential of I3C to act as a chemopreventive agent against cancer might be a little premature, as several studies indicate an absence of protection, or even significant adverse effects, with dietary I3C treatment. For example, no protection was observed with a 12 week exposure to 0.5% dietary I3C, following treatment with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), on the incidence of pepsinogen 1-decreased pyloric glands (a suggested preneoplastic marker of stomach tumorigenesis) (43). Pretreatment with dietary I3C in rats initiated with the tobacco-specific nitrosamine 4-(methylnitroamino)-1-(3-pyridyl)-1-butanone (NNK) reduced levels of 7-methylguanine adducts in lung and nasal mucosa DNA, however, it significantly elevated levels of DNA adducts in the liver (16). There is some evidence that, when administered in diet along with nitrites, I3C may have an indirect role in the process of initiation by acting as a mutagen (44, 45).

Despite having wide-range chemopreventive properties against tumorigenesis, I3C has also been implicated in enhancement and/or promotion of carcinogenesis in several animal models. In mouse epidermis, the 12-O-tetradecanoyl phorbol-13-acetate induction of ornithine decarboxylase activity (a marker for tumor promoting activity) was shown

to be elevated by I3C (46). Dietary I3C enhanced tumor incidence in rats when given before, during, and after treatment with the colon specific carcinogen dimethylhydrazine (DMH) (47). Fecal extracts from rats given DMH and I3C were mutagenic in the Ames test, but mutagenicity was not related to metabolites of DMH (48). The importance of the timing of dietary I3C exposure in deciding tumor outcome was demonstrated in the trout tumor model. When given before and during administration of the carcinogens DMBA (27) or AFB<sub>1</sub> (28), I3C reduced the numbers of tumors observed. However, when fed post-initiation, a significant, dose-dependent increase in the number of tumors was seen (27, 49). For AFB<sub>1</sub>, the ability of I3C to enhance or inhibit tumors was nearly equal over a range of doses (14). Promotion of hepatotumorigenesis was also observed in rats fed I3C after initiation (50).

Several I3C acid condensation derivatives (Fig. 1.2) have been shown to mediate toxicity by a mechanism similar to that described for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (51), a known toxic agent and tumor promoter. This is presumed to be due to an ability to bind to the Ah receptor. The most potent agonist among the I3C-related compounds is indolo[3,2-b]carbazole (ICZ) (52, 53). ICZ possesses a binding affinity for the Ah receptor close to that of TCDD and other toxic (carcinogenic and teratogenic) Ah receptor

ligands (54, 55). Like TCDD, ICZ has immunosuppressive, estrogenic and antiestrogenic activities (56). Several other I3C derivatives also bind to the Ah receptor (52, 53, 57, 58).

I3C administration has also been shown to result in toxicity in the form of depletion of glutathione in the liver, elevation of hepatic enzyme levels in plasma, and, at very high dietary levels, I3C also can be neurotoxic (59).

#### *Mechanisms of carcinogenesis modulation by I3C*

Elucidation of the mechanism(s) of action of potential chemopreventive agents is essential in evaluating the benefit of their administration. According to the classification system for chemopreventive compounds suggested by Wattenberg (60), I3C is classified as a blocking agent, meaning it protects against carcinogenesis by preventing the carcinogenic agents from reaching and/or reacting with critical target sites in the cell. Also, under certain conditions, I3C could act as a suppressive agent (22, 34). There are several mechanisms by which a blocking agent could inhibit the manifestation of cancer, including (i) induction of biotransformation enzymes responsible for detoxification pathway(s), (ii) inhibition or inactivation of cytochrome(s) P-450 (CYP) responsible for bioactivation of procarcinogens and (iii) physico-chemical interaction with

carcinogens (i.e. nucleophilic trapping of electrophiles or formation of complex(s)). I3C and/or its acid condensation derivatives are implicated in all three of these mechanisms.

Originally, protective effects seen after dietary I3C administration were thought to be due to the ability of I3C to induce CYP1A1-mediated AHH activity (10, 11). The ability of I3C to induce CYP has been extensively documented at the level of protein and associated catalytic activities (25, 35, 37-39, 41, 42, 47, 53, 58, 59, 61-68) as well as enhanced gene transcription (69, 70). Most studies have focused on induction in the liver and intestinal tissue, however, induction has been reported in other organ sites including kidney (71), lung (41), and colon (69). The most commonly induced CYP isozyme is CYP1A1 (53, 58, 68, 69). *Cyp1a1* is one of the battery of genes whose transcription is activated in the presence of Ah receptor agonists (72). Other P-450 isozymes reported to be induced by I3C and/or derivatives include CYP1A2 (42, 58, 62, 68, 69), CYP2B1 (25, 42, 58, 68, 69) and CYP3A (62, 68). It is proposed that most, if not all, of the induction observed upon dietary I3C administration is due to condensation products rather than I3C itself (25, 53, 65, 73). ICZ has been suggested to be important in enzyme induction since, among I3C-related derivatives, it possesses the highest affinity for binding towards the Ah receptor (54, 55). However, in most studies

it has been found in extremely low concentrations. Other I3C acid condensation derivatives, such as I33' (a major derivative that could also be formed from I3C in aqueous solutions(74)), linear trimer (LT, [2-(indol-3-ylmethyl)-indol-3-yl]indol-3-ylmethane), and cyclic trimer (CT, 5,6,11,12,17,18-hexahydrocyclonona[1,2-b:4,5-b':7,8-b'']triindole) also have P-450 inducing properties (42, 57, 75). The total induction observed upon dietary I3C exposure is certainly the additive effect from all derivatives and thus the relative contribution of each would depend upon their concentration as well as the efficacy.

Another, perhaps more important mechanism for protection, is the ability of I3C to inhibit the binding of carcinogens to DNA in the absence of detectable induction of AHH or other CYP activity in mice (19, 76) or trout(77, 78, 110).

It is important to note that induction of hepatic CYP1A1 and CYP1A2, the most readily induced isoforms by dietary I3C, is implicated in bioactivation of a number of dietary carcinogens (79) including BaP (25) and AFB<sub>1</sub> (32, 111).

Besides phase I enzymes (enzymes introducing functional groups into the chemical structure of xenobiotics) described above, dietary I3C can also induce some phase II enzymes (enzymes responsible for conjugating endogenous ligands with

functional groups introduced in Phase I (80)). Normally, phase II enzymes are involved in detoxifying pathways and are not carcinogen- or tissue-specific (60, 81). Thus, agents inducing only phase II enzymes are more desirable as chemoprevention candidates than those inducing both phase I and II enzymes. Among phase II enzymes, of particular interest are UDP-glucuronosyl transferases (UDPGT), glutathione-S-transferases (GST, conjugates glutathione with carcinogenic electrophiles), epoxide hydrolase (EH, conjugates water with reactive epoxides) and quinone reductase (prevents oxidative cycling and glutathione depletion by reducing quinones). I3C was shown to induce QR (13, 42, 59, 66, 82), UDPGT (13, 26 59), EH (83), and GST (13, 59, 84-86). Of these, induction of GST subunit Yc2 is believed to provide protective effects against AFB<sub>1</sub>-induced carcinogenesis (84). The induction of other phase II enzymes by I3C is not consistent (77, 83, 63, 87) hence their role in I3C protection is not entirely clear.

Dietary I3C inhibited flavin -containing monooxygenase form 1 (FMO) activity and expression in rat liver and intestine (88) and altered the ratio of FMO/CYP. FMOs are involved mainly in detoxifying xenobiotics and their inhibition by I3C is a reason for concern. The simultaneous induction of CYP and repression of FMO by animals fed I3C could have profound effects on the disposition and/or



toxicity of xenobiotics which are substrates for both monooxygenases.

Other proteins are also reported to be affected in response to dietary I3C. Among them, cytosolic UDP-glucose dehydrogenase (required for formation of UDP-glucuronic acid, a cofactor of UDPGT), and microsomal NADPH-cytochrome c-reductase and cytochrome b<sub>5</sub>, all of which were elevated (83). Another enzyme elevated upon I3C administration (gavage) was glutathione reductase (59) which promotes the availability of glutathione. Conversely, reduced activities were observed for superoxide dismutase and glutathione peroxidase; both enzymes are involved in protection against oxidative stress.

Another mechanism of action of I3C derivatives (I33' and CT) in anticarcinogenesis, not related to enzyme induction, is the ability to inhibit mutagenesis which was demonstrated with carcinogen AFB1 *in vitro* (89).

Numerous studies have investigated the possible antioxidant and electrophile scavenging properties of I3C (19-21, 76, 90, 91) and it appears that most of these properties are due to ascorbigen which is formed from I3C and L-ascorbic acid (9, 92). The relative role of I3C as an antioxidant in anticarcinogenesis remains to be evaluated .

In protecting against carcinogenesis in the mammary tissue, the main mechanism is proposed to be the

antiestrogenicity of I3C and/or derivatives which is manifested as an alteration of estrogen metabolism (35, 93-97). However, as is presented in chapter 3, dietary I3C may also have estrogenic activities. Estrogens are reported to promote chemically-induced hepatocarcinogenesis in trout (98) and rats (99-104). In humans, certain estrogens (oral contraceptives) are documented to promote hepatic adenomas and carcinomas (105, 106).

Other possible mechanisms for I3C modulation of carcinogenesis are proposed in the scientific literature. One of them is the reported ability of I3C to increase the activity of the DNA repair enzyme O<sup>6</sup>-mGua-DNA-transmethylase (16). Another possibility involves inhibition of cytosolic steroid binding activity by I3C acid derivatives (86), which might offer protection by interfering with GST-mediated intracellular transport of steroids and carcinogens. Modulation (reversal) of the multidrug resistance (107) is another possible mechanism of action. I33' is capable of inducing apoptosis in human cancer cells (108), suggesting one more possible mechanism for protection.

#### *Other Biological Effects of Dietary I3C*

In addition to tumor modulatory properties, dietary I3C intake is reported to have lowered serum LDL (low density lipoprotein) and VLDL (very low density lipoprotein) levels

(109) which was attributed to the inhibition of acyl-CoA:cholesterol acyltransferase.

### *Summary*

It appears that dietary I3C may act both as a protective and promoting agent in carcinogenesis in various animal models depending on several factors including the timing of administration. When given subsequent to initiation, I3C may act as a promoter whereas, in most cases, pretreatment and co-treatment with the carcinogen resulted in protection. As will be shown in chapter 6, pretreatment with I3C prior to initiation can also enhance tumor outcome. The relative role of the many possible mechanisms involved, however, is not yet completely understood.

Inhibition of CYP bioactivation, induction of CYP detoxification, and induction of phase II enzymes are some of the mechanisms responsible for chemoprotective effects of dietary I3C. Several other possible mechanisms may also play a role. Among them, electrophile and/or radical scavenging, inhibition of intracellular steroid transport and binding, induction of apoptosis, and antiestrogenicity (by mechanisms other than the alteration of estrogen metabolism (i.e. estrogen receptor antagonism)).

Tumor enhancing effects of dietary I3C might be explained by two main candidate mechanisms, bioactivation of

carcinogens through the induction of mainly phase I enzymes, and the potential of some I3C derivatives to alter cellular proliferation by acting as estrogens. Evidence is presented in this work that both of the above mechanisms could be active in explaining the observed promotion of hepatocarcinogenesis in trout and the enhancement in the mouse.

Especially important might be the potential of I3C and/or derivatives in acting as estrogens in humans. Concerns are raised pertaining to the possible risk(s) for promoting hepatic tumors in humans by long-term I3C supplementation, as it is known that certain estrogens (oral contraceptives) promote hepatic tumors in humans.

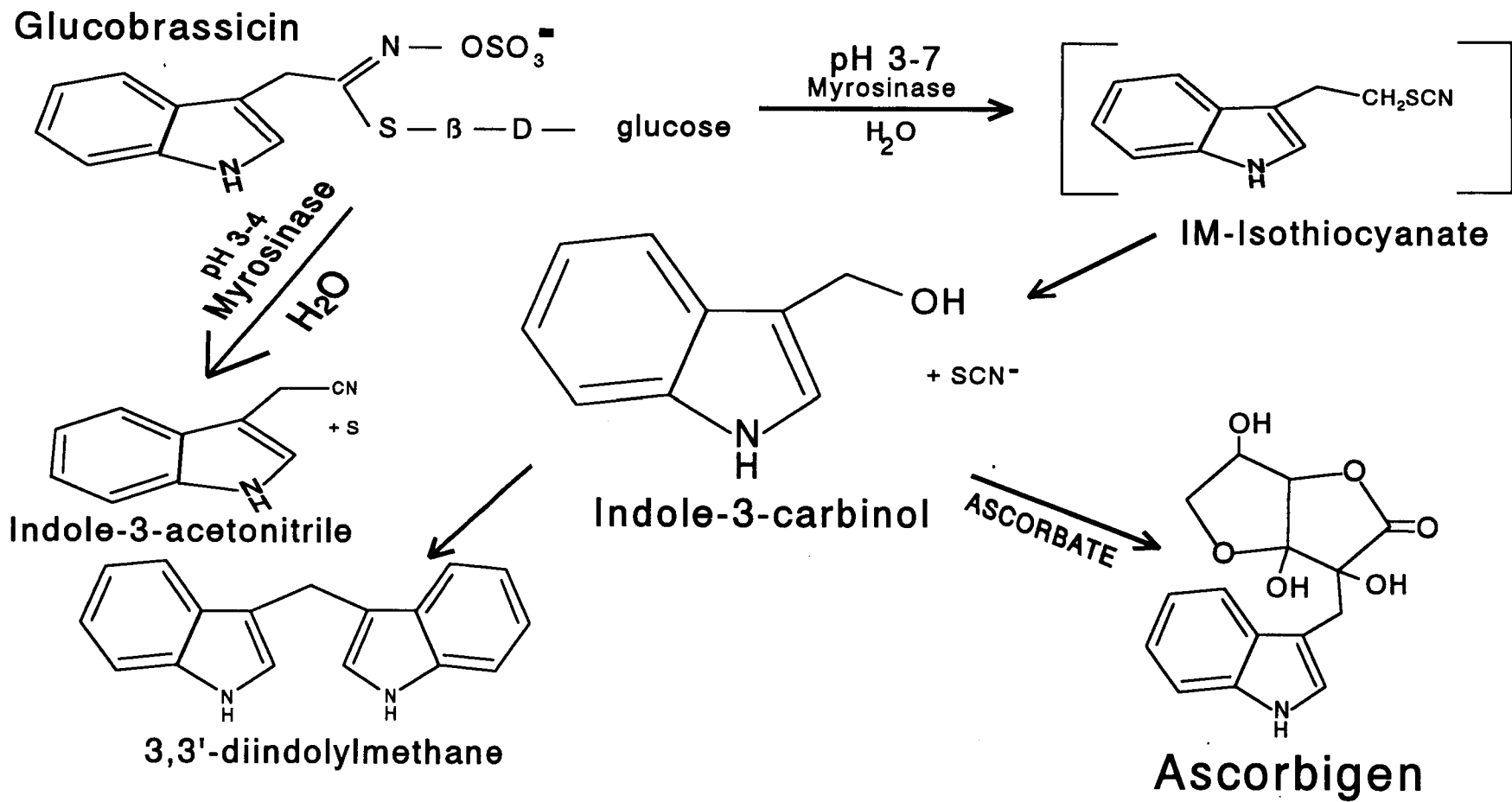


Figure 1.1. Formation of I3C from enzymatic hydrolysis of glucobrassicin in cruciferous vegetables.

## Acid Condensation Products of I3C

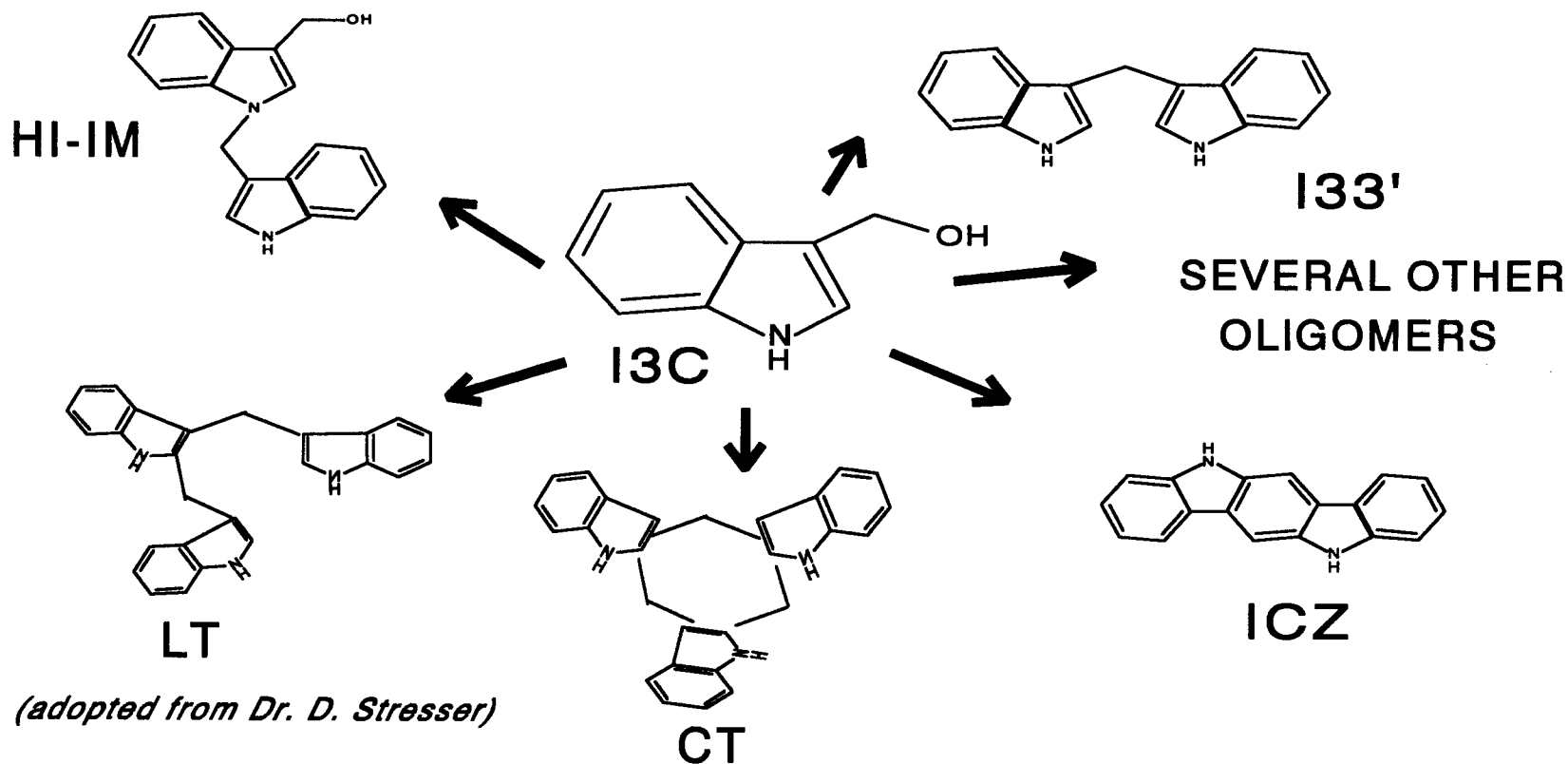


Figure 1.2. **Formation of acid condensation products from I3C.** HI-IM, I33', dimers; LT - linear trimer; CT, cyclic trimer, CT, cyclic trimer; ICZ, indolo[3,2-b]carbazole; I3C, indole-3-carbinol.

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## Chapter 2

**$\alpha$ -NAPHTHOFLAVONE (ANF) ACTS AS A WEAK Ah RECEPTOR  
AGONIST *IN VIVO* AND ANTAGONIST *IN VITRO*  
IN RAINBOW TROUT**

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## ABSTRACT

ANF, a synthetic flavone, is an Ah receptor (AhR) antagonist and partial agonist in mammals, capable of blocking TCDD-mediated biological effects *in vitro* and *in vivo*. Our aim was to study the involvement of the AhR in tumor promotion in the trout model by utilizing ANF as a blocking agent. We report here the results of a tumor study designed to assess the potential for dietary ANF to block promotion of carcinogenesis by dietary  $\beta$ -naphthoflavone (BNF) as a test of whether the latter may be Ah receptor-dependent. The potency of ANF as an Ah receptor antagonist was observed *in vitro* in liver slices. When slices were exposed to ANF before, during, and after BNF was added into medium, CYP1A induction was lower compared to levels seen in slices exposed to BNF alone. These results confirm reports in the literature that ANF can act as an Ah receptor antagonist and block AhR-mediated biological responses. In the tumor study, trout were initiated as fry with 1.5 ppm 7,12-dimethylbenz[a]anthracene (DMBA). Experimental diets, containing 200 ppm ANF, BNF, or both were given after 3 months of control diet. Fish were sampled for liver, stomach and swim bladder tumors at 11 months. Significant promotion of hepatic carcinogenesis was observed for both the BNF (27.5 % tumor incidence versus 8 %) and ANF-fed (18 % versus 8 %) groups. An even greater

enhancement (39% incidence) was observed with a combined ANF-BNF diet (200 ppm each). A short-term *in vivo* experiment was conducted to determine the potency of ANF as an Ah receptor agonist and/or antagonist. Trout were injected *ip* with 0, 0.5, 10, 20, or 40 mg/kg ANF daily for 4 days. All ANF-treated groups were then administered either 0 or 500 ppm BNF in the diet for a period of 1 week. ANF alone induced CYP1A and the groups given dietary BNF exhibited additive responses. The additivity of ANF and BNF in inducing trout CYP1A is consistent with the tumor enhancement data and supports a role for the Ah receptor. Hence, in trout, (as in mammals) ANF can function as a partial agonist and is not a suitable choice for blocking Ah receptor-dependent carcinogenesis in this model.

## INTRODUCTION

The aryl hydrocarbon receptor (Ah receptor) is a cytosolic protein with a basic helix-loop-helix region which mediates many biochemical and toxic responses to a number of xenobiotics. During the Ah receptor-mediated induction of different enzymes a sequence of events takes place (1-5). Foreign chemical inducers, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 3-methylcholanthrene or  $\beta$ -naphthoflavone (BNF) bind to the Ah receptor to form an inducer-receptor complex which then translocates into the nucleus and, after forming a dimer with the Ah receptor nuclear translocator protein, binds to dioxin-responsive elements (DREs) (or xenobiotic-responsive elements (XREs)) triggering the enhanced transcription of various genes including CYP1A1 and CYP1A2. These enzymes are responsible for catalyzing various reactions in drug metabolism and procarcinogen activation (4).

We have been characterizing properties of the Ah receptor in the trout (*Oncorhynchus mykiss*) model as an initial step towards understanding possible Ah receptor mediated tumor promotion. In particular, we are exploring the potential for blocking Ah receptor-dependent promotion by the antagonist  $\alpha$ -naphthoflavone (ANF). The objective of this study was to attempt to use the antagonistic properties of ANF to inhibit Ah receptor-mediated promotion of chemically-induced

carcinogenesis by BNF in the rainbow trout tumor model. Post-initiation dietary BNF is reported to promote chemical carcinogenesis in trout (6). If proven to be able to inhibit promotion by the Ah receptor agonist BNF, ANF could be used as a blocking agent to examine the mechanism(s) of action of other promoters, namely agents believed to act through the Ah receptor pathway, including indole-3-carbinol (I3C) (7, 8).

In addition to being an effective antagonist, ANF can also function as a partial agonist depending on the dose and the conditions of the experiment. Here we analyzed ANF potency as (ant)agonist in both *in vitro* and *in vivo* experimental designs, including a dose-response experiment with both ANF and BNF. As will be shown later, ANF acts as an antagonist of the Ah receptor *in vitro* (liver slices) but as a weak agonist *in vivo* in both short- and long-term (tumor study) experiments.

## MATERIALS AND METHODS

### *Materials*

All chemicals were purchased from Sigma Chem. Co. (St. Louis, MO) unless otherwise noted. Antibody for immunoblotting against CYP1A was generously provided by Dr. Donald Buhler of Oregon State University.

### *Animals and treatments*

Rainbow trout (*Oncorhynchus mykiss*) were hatched and reared at the Oregon State University Food Toxicology and Nutrition Laboratory in 14°C (average temperature) flowing well water.

For the tumor study, approximately 1,600 fry were initiated with 1.5 ppm DMBA by immersion for 5 hours. Sham-exposed embryos were exposed to vehicle alone (0.01 % ethanol) and served as non-initiated controls. After initiation, fry were fed Oregon Test Diet (OTD), a semipurified, casein-based diet (20) for three months, after which trout were divided into experimental treatment groups and fed OTD diets containing 0 or 200 ppm ANF, BNF, or both ANF and BNF (200 ppm each). Once on experimental diets, trout were fed *ad lib* (2.8-5.6 % body weight), five times per week. ANF and BNF were added to the oil portion of the diets during preparation. Diets were prepared biweekly and stored frozen at -20°C until 2-4 days prior to feeding when



diets were allowed to thaw at 4°C. Each treatment group contained 200 fish housed 100 per tank in 100-gallon continuous flow tanks. At 11 months of age trout were sampled for liver, stomach and swim bladder tumors while still sexually immature. Several randomly selected livers from each group were frozen and later analyzed for CYP1A induction.

In a short-term dose-response experiment, 7-8 month old trout were injected *ip* with ANF or BNF daily for four days at 0 (vehicle - dimethylsulfoxide (DMSO)), 0.2, 0.5, 2, 5, 15, 30, or 75 mg/kg. On day 5 fish were sacrificed and livers instantly frozen for preparation of microsomes. A second dose-response study, involving a wider dose range (0.05-75 mg/kg), was carried out for measuring EROD (ethoxyresorufin-O-deethylase) activity. In another experiment, trout were pretreated with *ip* injection of ANF (daily for 4 days), and BNF was thereafter given in the diet at 500 ppm for 1 week, after which trout were sacrificed and livers collected for preparation of microsomes.

*In vitro* dose-response, and ANF-BNF co-, pre-, and post-incubation studies with trout liver slices were conducted with fresh precision-cut slices. Slices were prepared using a Krumdieck tissue slicer and incubated in the Hanks modified salts buffer (supplemented with 1 % BSA and 50 mg/ml gentamicin) containing ANF or BNF at concentrations of 200  $\mu$ M

for ANF and 40  $\mu$ M for BNF for the first 24 h. ANF was administered either with BNF from the start of incubation, or 6 h prior and/or after BNF addition into medium. Slices were incubated under sterile conditions in 95% O<sub>2</sub>/5% CO<sub>2</sub> atmosphere at 14° C for 48 hours, and then homogenized and analyzed for CYP1A protein levels by immunoblotting.

*Preparation of hepatic microsomes and enzyme assays*

Livers were homogenized in 4-5 volumes of ice cold 0.1 M potassium phosphate buffer (pH 7.4) containing 0.15 M KCl, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. Microsomes were prepared by differential centrifugation according to Guengerich (9). The supernatant from a 10,000 g centrifugation of liver homogenate was subjected to centrifugation at 100,000 g. The resulting pellet was washed with potassium pyrophosphate buffer (0.1 M, pH 7.4, 1 mM EDTA, 1 mM PMSF), centrifuged at 100,000 g, and the pellet resuspended in homogenization buffer containing 20 % glycerol to obtain microsomes.

Protein determinations were made according to the method of Lowry *et al.* (10).

The 7-ethoxyresorufin-O-deethylase (EROD) assay was modified from Pohl and Fouts (11) using liver microsomes at 25° C.

The total specific content of cytochrome P-450 was measured spectrophotometrically by the reduced-CO versus oxidized-CO difference spectrum (9).

#### *Electrophoresis and immunoblotting*

CYP1A levels were examined in liver microsomal fractions, prepared as described above. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 8 % acrylamide gels (12) and electrophoretically transferred onto nitrocellulose (Bio-Rad Trans-Blot). Blots were probed with rabbit polyclonal antibodies to trout CYP1A, followed by a horseradish peroxidase-linked secondary antibody. Proteins were detected using an Amersham ECL chemiluminescence kit (Amersham Corp., Arlington Heights, IL). Western blots were scanned on a flatbed HP Scanjet IICx scanner. Densitometry was performed with the public domain software NIH Image, version 1.57 (written by Wayne Rasband at the US National Institutes of Health).

#### *Necropsy and histopathology*

At termination, fish were sacrificed by a combination of deep anesthesia, resulting from an overdose of tricaine methane sulfonate (MS-222), and bleeding, after cutting the gill arches on the left side of the fish. Tissues were inspected for neoplasms under a dissecting microscope. After marking and recording the size and location of all surface

tumors, the livers were fixed in Bouin's solution for 2-7 days. All livers were then cut into one mm slices with a razor blade to retrieve previously marked tumors and to locate any internal, previously unseen tumors. At least one piece of liver from each tumor-bearing fish was then processed by routine histological procedures, and stained with hematoxylin and eosin for histological evaluation (21).

#### *Statistical analysis*

Only fish from initiated groups had tumors. Statistical differences between treatment groups were assessed by logistic regression using GENMOD procedure, SAS System for Windows, version 6.12, SAS Institute, Inc., Cary, NC).

Differences between groups were tested using one-way analysis of variance, followed by the least significant difference multiple comparison test using the statistical software package, Statgraphics (version 5.0), (Statistical Graphics Corporation, Princeton, New Jersey). A probability value of less than 0.05 was considered significant.

## RESULTS

*In vitro*, ANF did antagonize CYP1A induction by BNF in liver slices when administered concurrently with, 6 hours before, or after BNF addition into medium. ANF alone induced CYP1A at weaker levels compared to BNF, but in all cases when co-incubated with BNF, induction was seen at levels significantly lower when compared to BNF-alone samples (Fig. 2.1).

In the short-term dose-response experiment both ANF and BNF exhibited induction of total P-450 in the liver (Fig. 2.2) with BNF exhibiting greater potency. Induction somewhat faded at higher doses (15 mg/kg and higher), perhaps due to toxicity. Mortality (10 %) was observed at 30 and 75 mg/kg.

Induction of EROD activity, which represents CYP1A, was significant only at 2 mg/kg for ANF, whereas, BNF significantly induced across the whole range of treatment doses in a dose-dependent fashion (Fig. 2.3).

The tumor incidences and values of odds ratio versus initiated controls are presented in Table 2-I. Statistically significant promotion was observed for all treatment groups, with the combined ANF and BNF diet exhibiting higher tumor incidence than the group fed BNF only. The incidence of multiple tumors was significantly higher in groups fed BNF and the combined BNF-ANF diet. Hepatic CYP1A levels (measured in

microsomes) were induced in all treatment groups at levels consistent with tumor incidences (Fig. 2.4).

The ability of ANF to act as an agonist or antagonist *in vivo* after short-term exposure was analyzed by measuring CYP1A levels in hepatic microsomes at the conclusion of the experiment involving ANF-pretreatment followed by dietary BNF. Additivity of CYP1A induction was apparent in the treatment group featuring pretreatment with a high dose of ANF (Fig. 2.5). No antagonism by ANF, as evidenced by inhibition of BNF-mediated induction, was detected in this short-term *in vivo* study.

## DISCUSSION

ANF and BNF, both synthetic compounds, have been used in research studying the mechanism(s) of Ah receptor mediated toxicity. BNF binds to the Ah receptor and is used as a classic agonist. ANF, however, was originally proposed as an antagonist of the Ah receptor and is suggested to exhibit antagonism by binding to the Ah receptor and changing the conformation of the protein which prevents it from binding to DREs (13-16).

We are investigating the mechanism(s) of carcinogenesis promotion by indole-3-carbinol, a plant secondary metabolite common in crucifers. Some of the I3C-derived condensation products possess relatively high affinity for the Ah receptor and induce CYP1A (7, 8, 17-19) leading to speculation that promotion by I3C could be due to the Ah-receptor mediated pathway. The choice of ANF as a candidate compound for inhibiting Ah-receptor mediated tumor promotion was made with the hypothesis that if successful, it could be used to test whether carcinogenesis promotion by I3C is indeed mediated through the Ah receptor.

ANF, however, is also known to have weak agonistic activity in mammalian systems under certain conditions (14, 15). This was confirmed in our study in trout. ANF itself did induce CYP1A both *in vivo* after long-term (tumor study) and

short-term exposure, and *in vitro* (liver slices). Dietary ANF also resulted in promotion of hepatocarcinogenesis, however, not as potently as BNF, and when given concurrently with BNF, promotion was enhanced in an apparently additive manner. The antagonism by ANF was observed only *in vitro* in liver slices and at concentration (200  $\mu\text{M}$ ) exceeding that of the agonist BNF (40  $\mu\text{M}$ , derived from a dose-response experiment in slices and shown to be the maximum CYP1A inducing concentration).

Our findings suggest that ANF is not a good choice for an antagonist for blocking promotion of carcinogenesis by Ah agonists (such as BNF). We are currently examining the potential of a reportedly pure AhR antagonist 4'-amino-3'-methoxyflavone, provided by Dr. Stephan Safe (University of Texas) *in vitro* with trout liver slices. If successful, future studies *in vivo* will test the role of the AhR in I3C-dependent promotion of hepatocarcinogenesis in trout.



## ACKNOWLEDGMENTS

We thank the personnel of the OSU Food and Nutrition Laboratory, particularly Dan Arbogast, for taking good care of fish throughout the study.

Table 2-I. Promotion of DMBA-initiated hepatocarcinogenesis by ANF and BNF.  
 Only data from initiated fish are presented (no tumors were observed in  
 vehicle-exposed fish).

Initiation Diet	# fish	tumor incidence(%)	incidence of multiple tumors <sup>1</sup>	odds ratio vs control (95% c.i.)
DMBA <sup>2</sup> control	200	8.0	12.5	1
DMBA ANF	200	18.0*	11.1	2.52 (1.35-4.72)
DMBA BNF	200	27.0*	29.6*	4.36 (2.40-7.93)
DMBA ANF+BNF <sup>3</sup>	192	39.1*	33.3*	6.89 (3.84-12.39)

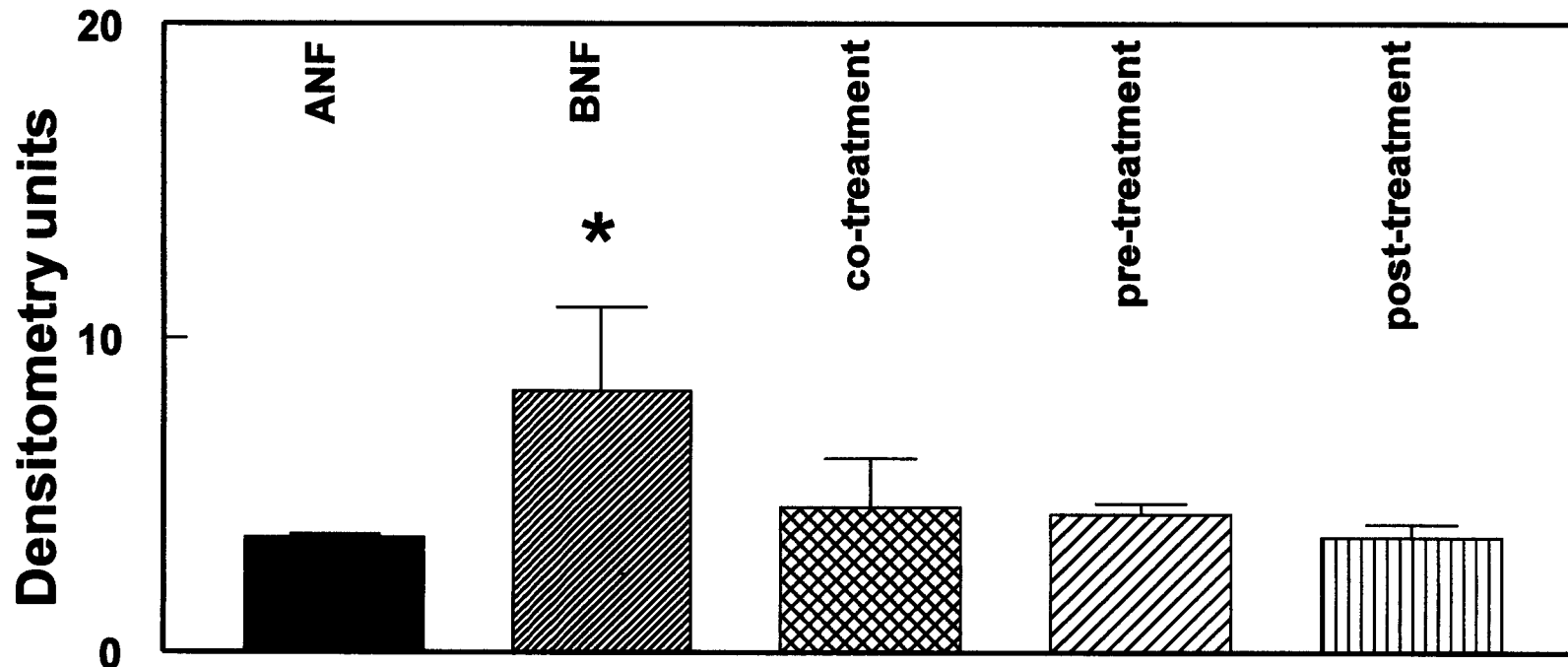
<sup>1</sup> % tumor bearing fish with multiple tumors

<sup>2</sup> initiation with 1.5 ppm DMBA as fry, 5 h immersion

<sup>3</sup> compounds were given in diet at 200 ppm each

\* represents statistically significant difference from positive control (p < 0.05).

## Effects of ANF on CYP1A Induction by BNF in Trout Liver Slices <sup>1</sup>



<sup>1</sup> BNF - 40  $\mu$ M, ANF - 200  $\mu$ M

\* significantly different ( $p < 0.05$ ) from other groups

Figure 2.1. Antagonism of BNF CYP1A induction by ANF *in vitro* in trout liver slices. Inhibition of CYP1A induction by BNF with pre-, co-, and post-treatment with ANF *in vitro* in trout liver slices.

# Total P-450 Induction: Dose-Response

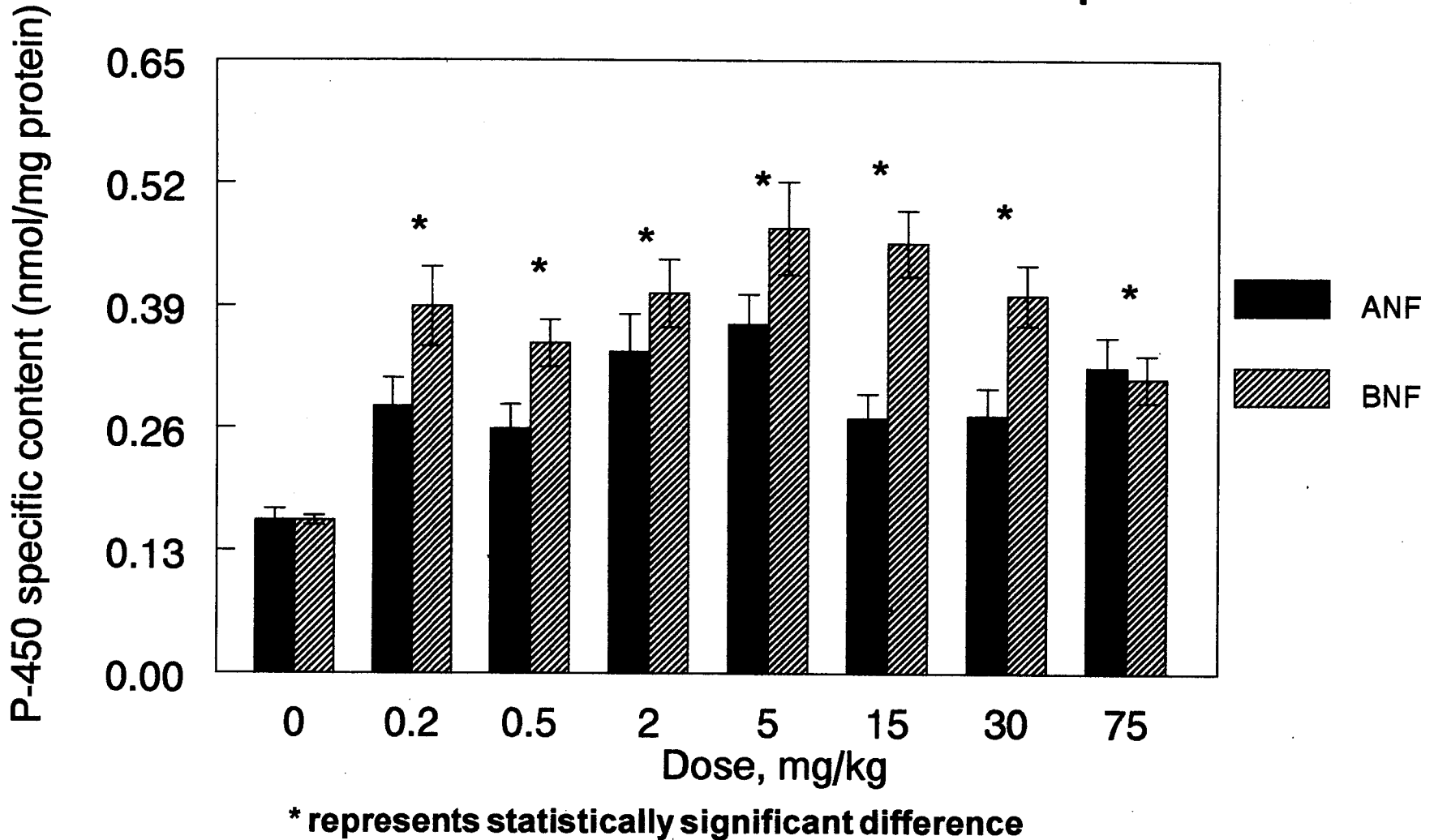


Figure 2.2. Induction of total P-450 *in vivo* in trout by ANF and BNF: dose-response experiment with *i.p.* injection of ANF and BNF.

# EROD Activity: Dose-Response

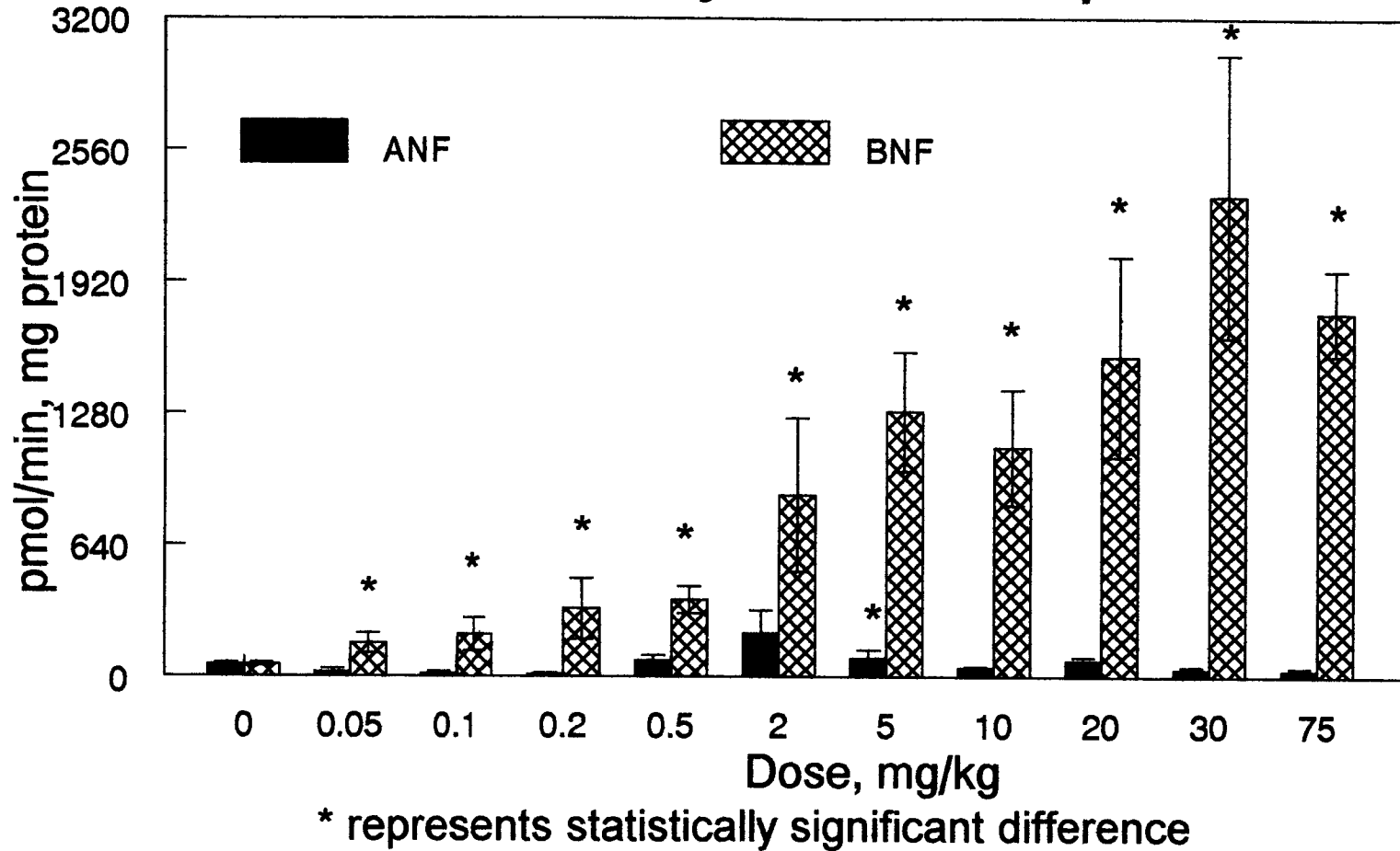
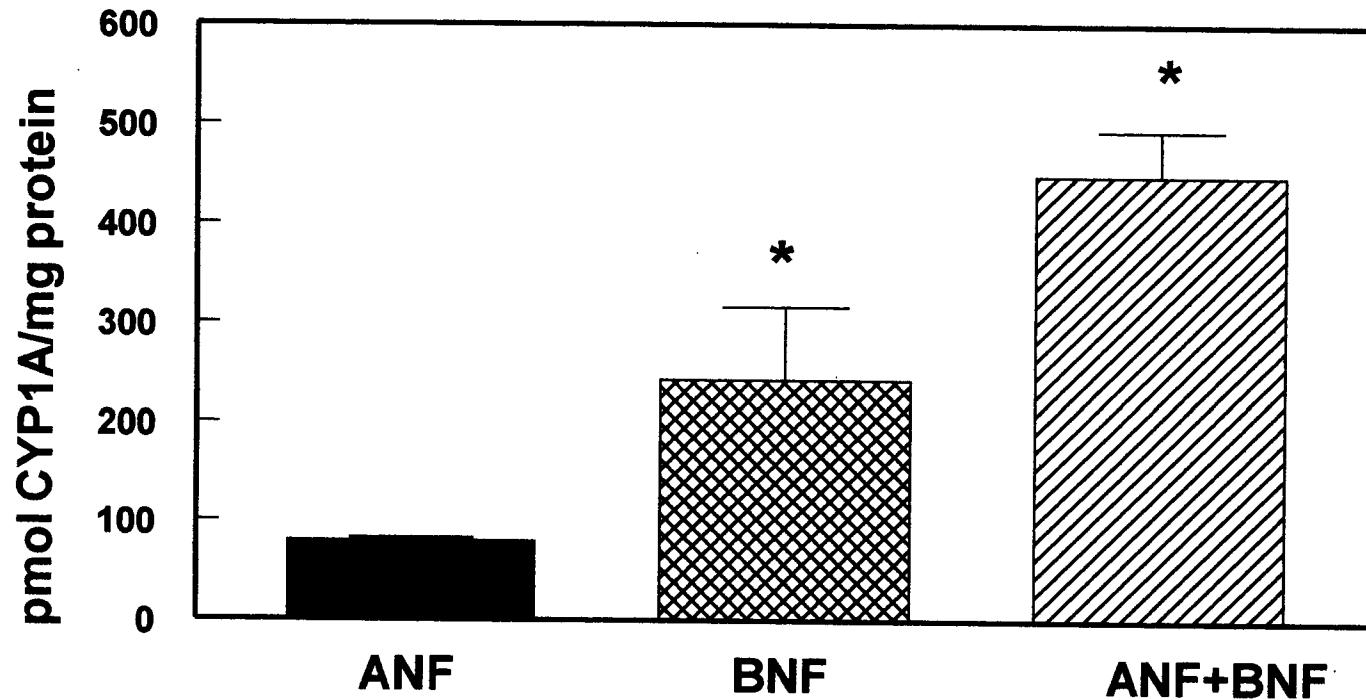


Figure 2.3. Induction of microsomal EROD activity in trout: Dose response experiment of *i.p.* injection with ANF and BNF.

## CYP1A Induction in Tumor Study



\* significantly different ( $p < 0.05$ ) from the ANF group (no induction detected in controls)

Figure 2.4. Induction of hepatic microsomal CYP1A in the trout tumor study. DMBA-initiated trout were fed diet containing 0, or 200 ppm BNF, ANF, or both.

## CYP1A Induction In Vivo: Pretreatment Study

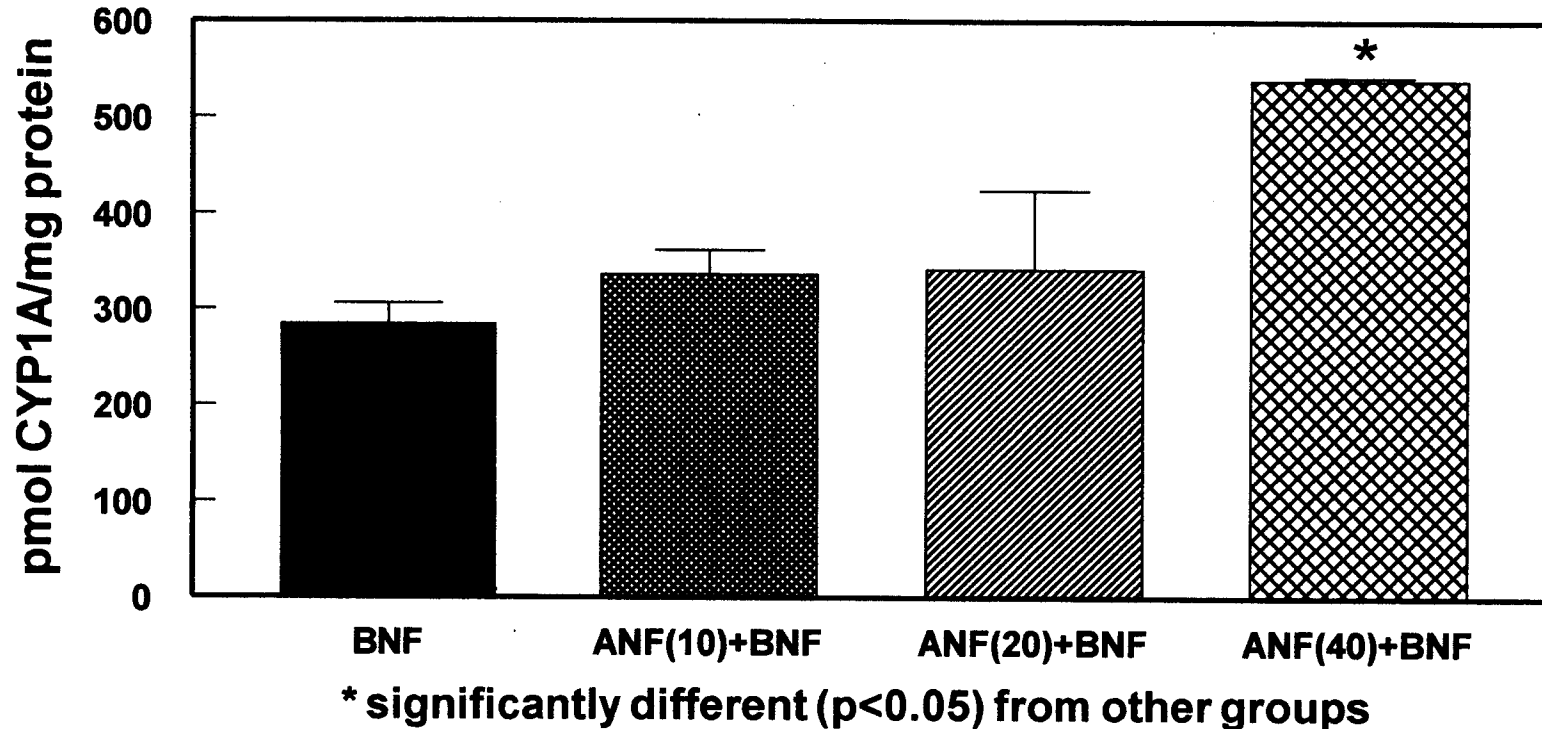


Figure 2.5. Induction of hepatic CYP1A by dietary BNF (500 ppm) after *i.p.* pretreatment with ANF: Weak Ah receptor agonistic properties of ANF and additivity of induction.

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## Chapter 3

**PROMOTION OF AFLATOXIN B1-INITIATED  
HEPATOCARCINOGENESIS IN RAINBOW TROUT BY  
RELATIVELY LOW LEVELS OF DIETARY INDOLE-3-CARBINOL.**

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**ABSTRACT**

Indole-3-carbinol (I3C)\*, a metabolite of glucobrassicin found in cruciferous vegetables, is documented to act as a modulator of carcinogenesis and, depending on timing and dose of administration, it may promote hepatocarcinogenesis in some animal models. In this study we demonstrate that, when given post-initiation, dietary I3C promotes aflatoxin B<sub>1</sub> (AFB<sub>1</sub>)-induced hepatocarcinogenesis in the rainbow trout model at levels as low as 500 ppm. Trout embryos (approximately 9,000) were initiated with 0, 25, 50, 100, 175, or 250 ppb AFB<sub>1</sub> with a 30 min immersion. Experimental diets containing 0, 250, 500, 750, 1000, or 1250 ppm I3C were administered starting at 3 months and fish were sampled for liver tumors at 11-13 months. Promotion at the level of tumor incidence was statistically significant for all dietary levels, except 250 ppm. Relative potency for promotion markedly increased at dietary levels above 750 ppm. We propose that more than one mechanism could be involved in promotion and that both estrogenic and Ah receptor-mediated pathways could be active. The estrogenicity of I3C, measured as its ability to induce vitellogenin (an estrogen biomarker in oviparous vertebrates) was evident at the lowest dietary level (250 ppm), whereas, CYP1A (a P450 isozyme induced through the Ah

receptor pathway) was not induced until dietary levels of 1000 ppm. Therefore, at lower dietary levels, promotion by I3C in this model could be explained by estrogenic activities of I3C acid derivatives, as it is known that estrogens promote hepatocarcinogenesis in trout. Much stronger promotion was observed at high dietary I3C levels (1000 and 1250 ppm), at which levels both CYP1A and vitellogenin were induced.

**\*Abbreviations:** I3C, indole-3-carbinol (3-indolemethanol); AFB<sub>1</sub>, aflatoxin B<sub>1</sub>; OTD, Oregon Test Diet; CYP, cytochrome P450; VG, vitellogenin; MS-222, tricaine methane sulphonate.

## INTRODUCTION

Indole-3-carbinol (I3C), a plant secondary metabolite found in cruciferous vegetables such as broccoli, cauliflower, Brussels sprouts, etc and is available to the general public for purchase as a dietary supplement in various forms over the internet as well as through health food stores and distribution networks.

Dietary I3C has been documented to inhibit tumorigenesis (1-4) in various target organs, including mammary tissue (5), liver (6, 7), endometrium (8), lung (9-12), and other target organs (13, 14) in various animal models and is currently being evaluated in human clinical trials as a potential chemopreventive agent against breast and ovarian cancers (15). The chemopreventive properties of I3C are proposed to occur through several possible mechanisms, including the alteration of estrogen metabolism (16-21). Furthermore, I3C is reported to inhibit glutathione-S-transferase-mediated steroid binding activity (22), act as a scavenger of free radicals (23), modulate the activity of multidrug resistance (24), and alter the expression of various phase I and phase II drug-metabolizing enzymes (20, 25-28) contributing to detoxification of carcinogenic compounds.

Upon dietary intake, I3C undergoes acidic condensation reactions in the stomach, yielding various derivatives

believed to be responsible for its biological effects (29-33). Some of the condensation products of I3C have antiestrogenic as well as estrogenic activities (29) and also possess relatively high affinity for binding to the Ah receptor (34, 35). A major condensation product, the dimer 3,3'-diindolylmethane (I33'), is capable of inducing apoptosis in human cancer cells (36) and is an effective inhibitor *in vitro* of cytochromes P450 (35, 37, 38). Carcinogenesis chemoprevention properties of dietary I3C in most of the models are evident when it is administered concurrently with the carcinogen or prior to initiation. Yet there are reports that, when given after initiation (promotion-progression stage), I3C can enhance carcinogenesis (1, 3, 7). There is also some evidence that I3C may be mutagenic when co-administered in diet along with nitrites (39).

Earlier studies (3, 7) have documented the ability of I3C to promote AFB<sub>1</sub>-initiated hepatocarcinogenesis at relatively high dietary levels (1000 ppm). The objective of this study was to evaluate the tumor promoting properties of I3C at relatively low dietary levels, across a wide range of initiator doses, and to investigate the possibility of a threshold for promotion within these range of doses. We report that I3C promoted significantly in all treatment groups except 250 ppm. We further demonstrate, that the estrogenicity of I3C is evident at the lowest dietary levels at which point CYP1A

was not induced. Perhaps, at the lower dietary I3C treatment groups, the mechanism for hepatocarcinogenesis promotion involves estrogenic pathways, as it is known that estrogens promote chemically-induced hepatocarcinogenesis in the trout (40). The Ah receptor-mediated pathway could play a role in promotion at higher dose treatment groups (above 750 ppm), where the ability of I3C to induce CYP1A was evident. It is documented that certain estrogens (oral contraceptives) are implicated in promotion of hepatic adenomas and carcinomas in humans (41, 42). Based on observations from this study and the relevance of estrogens in human cancer risk, we suggest that dietary I3C supplementation be approached with caution until the mechanism(s) of hepatocarcinogenesis promotion in the trout and rat and the implications for human cancer risk are fully understood.



## MATERIALS AND METHODS

### *Materials*

AFB<sub>1</sub> was purchased from Sigma Chem. Co. (St. Louis, MO). I3C was purchased from Aldrich Chem. (Milwaukee, WI). Rabbit polyclonal antibodies against vitellogenin and CYP1A were generously provided by Dr. Donald Buhler (Oregon State University).

### *Animals and treatments*

Rainbow trout (*Oncorhynchus mykiss*) were hatched and reared at the Oregon State University Food Toxicology and Nutrition Laboratory in 14°C (average temperature) flowing well water. Approximately 9,000 embryos were initiated with 25, 50, 100, 175, or 250 ppb AFB<sub>1</sub> for 30 min. Sham-exposed embryos were exposed to vehicle alone (0.01 % ethanol) and served as non-initiated controls. After hatching, fry were fed Oregon Test Diet (OTD), a semipurified, casein-based diet (43) for three months, after which trout were randomly (within initiator treatment groups) divided into experimental treatment groups and fed OTD diets containing 0, 250, 500, 750, 1000 or 1250 ppm I3C. Once on experimental diets, trout were fed *ad lib* (2.8-5.6 % body weight), five times per week. I3C was added to the aqueous portion of the diets during preparation. Diets were prepared biweekly and stored frozen at -20°C until 2-4 days prior to feeding when

diets were allowed to thaw at 4°C. Each treatment group contained 200-400 fish (larger numbers of fish were used for low-dose initiated groups fed lower levels of I3C in diet) housed approximately 100 per tank in replicate 100-gallon continuous flow tanks. At 11 months of age trout were sampled for liver tumors while still sexually immature. Because of the large number of fish involved, the sampling was carried out over 66 days (all groups were switched to OTD diets during sampling). Blood was drawn from the caudal vein (in order to examine for vitellogenin induction), and, at the start of the sampling period (while trout were still on I3C diets), 10 livers from each group were frozen and later analyzed for CYP1A induction.

#### *Electrophoresis and immunoblotting*

Vitellogenin levels were determined in plasma from trout. CYP1A levels were examined in liver microsomal fractions, prepared as previously described (44). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 8 % acrylamide gels (45) and electrophoretically transferred onto nitrocellulose (Bio-Rad Trans-Blot). Blots were probed with rabbit polyclonal antibodies to salmon vitellogenin (for plasma) and trout CYP1A (liver microsomes) followed by a horseradish peroxidase-linked secondary antibody. Proteins were detected using an Amersham

ECL chemiluminescence kit (Amersham Corp., Arlington Heights, IL). Western blots were scanned on a flatbed HP Scanjet IICx scanner. Densitometry was performed with the public domain software NIH Image, version 1.57 (written by Wayne Rasband at the US National Institutes of Health).

#### *Necropsy and histopathology*

At termination, fish were sacrificed by a combination of deep anesthesia, resulting from an overdose of tricaine methane sulfonate (MS-222), and bleeding, after cutting the gill arches on the left side of the fish. The fish were weighed, livers removed and weighed, and the livers inspected for neoplasms under a dissecting microscope. After marking and recording the size and location of all surface tumors, the livers were fixed in Bouin's solution for 2-7 days. All livers were then cut into one mm slices with a razor blade to retrieve previously marked tumors and to locate any internal, previously unseen tumors. At least one piece of liver from each tumor-bearing fish was then processed by routine histological procedures, and stained with hematoxylin and eosin for histological evaluation. Neoplasms were classified by the criteria of Hendricks *et. al.* (46). The relative numbers of different tumor types were thus based on a random, non-exhaustive sample of all the tumors that occurred. When multiple tumors occurred on a tissue slide, only the largest or the most frequently occurring tumor type was recorded.

### *Statistical analysis*

Tumor incidence and incidence of multiple tumors in 78 tanks containing initiated fish, was modeled by logistic regression, allowing for possible overdispersion (GENMOD procedure, SAS System for Windows, version 6.12, SAS Institute, Inc., Cary, NC). Variation between replicate tanks provided the initial estimated multiplicative dispersion factor. Parsimonious models were chosen based on the results of drop-in-deviance quasi-likelihood F-tests.

Relative potency (RP) for promotion was calculated using  $RP = TDx_i / TDx_0$ , where "x" is the tumor incidence being compared (e.g. 50%),  $TDx_0$  is the dose of AFB<sub>1</sub> needed to yield that tumor incidence in the 0 ppm I3C (initiate control) group, and  $TDx_i$  is the dose of AFB<sub>1</sub> that is required to produce that tumor incidence in the group receiving "i" ppm I3C. Estimates and confidence intervals were generated from logistic regression modeling results ( $\log(\text{relative potency}) = \text{difference between estimated intercepts divided by estimated slope}$ ) with the delta method used to calculate standard errors.

## RESULTS

A dose response was observed for tumor incidence with increasing I3C levels in diet (Table 3-I), Fig. 3.1. The tumor promotional effects of I3C were apparent at all levels across the initiator dose range ( $p < 0.01$ ) except for 250 ppm (significant for only the highest AFB<sub>1</sub> groups, fed 250 ppm I3C). (Table 3-II). Based on these observations, the existence of a threshold for promotion of AFB<sub>1</sub>-hepatocarcinogenesis by dietary I3C could neither be demonstrated, nor discounted

Overall, the variation between replicate tanks was 1.92 times larger than expected under the binomial error assumption indicating significant overdispersion ( $p < 0.0001$ ). The variation was greatly reduced by adding a continuous covariate for day of sampling ( $p < 0.0001$ ). After adjusting for the covariate the remaining variation between replicate tanks was only 1.24 times larger than expected under the binomial error assumption (test for overdispersion,  $p = 0.11$ ). Therefore the conclusions for the more conservative overdispersed model used here will be similar to the conclusions from a model assuming only binomial variation.

The relative potency (RP) or promotion of AFB<sub>1</sub>-hepatocarcinogenesis by dietary I3C is depicted in Fig. 3.2. There is a marked increase in the value for the RP with

dietary I3C increasing above 750 ppm. At levels below 750 ppm promotion is evident, however, with lower potency.

The spectrum of tumor types was the same as previously observed (47), and included malignant and benign neoplasms of hepatocellular, cholangiocellular, or mixed hepato/cholangio origin. There was a difference in this experiment, however, since the hepatocellular carcinoma type was the predominant tumor rather than the mixed carcinoma observed in previous studies (40, 48, 49). Some variability in the relative percentages of the various tumor types occurred, particularly in the lower incidences seen at the low dose levels, but the trend was the same throughout (Table 3-III tumor type summary). The overall averages of tumor occurrences were as follows: hepatocellular carcinoma, 56.5%; mixed hepatocellular-cholangiocellular carcinoma, 29.4%; cholangiocellular carcinoma, 1.2%; hepatocellular adenoma, 8.7%; mixed adenoma, 0.3%; and cholangiocellular adenoma, 3.9%. With the exception of an approximate reversal of the percentages of the first two tumor types, everything else was very consistent with what has been seen previously. No obvious reason for this change in tumor type is apparent.

The potential of I3C to function as an estrogen in trout was assessed by measuring plasma vitellogenin (VG) levels (50). No VG was detected in controls (0 ppm I3C) (Fig. 3.3). For the groups fed 250 ppm I3C, weak VG protein

bands were seen on the film, however, the levels were below the resolution limit for quantification by scanning densitometry. VG bands were readily observed and quantified for the rest of the groups (500 ppm and above). Marked induction was observed for the groups fed 750, 1000 and 1250 ppm I3C.

The induction of CYP1A, a marker for the Ah receptor-mediated mechanism, was apparent only in the highest I3C groups (1000 and 1250 ppm) (Fig. 3.4).

## DISCUSSION

Dietary I3C supplementation for the healthy adult human population has been advanced for the purpose of chemoprevention against estrogen-related diseases (15). However, after it was recognized that long-term dietary I3C may lead to enhancement of hepatocarcinogenesis in rats (1) and trout (3, 7), and the ability of this plant derivative to induce both phase I and phase II enzymes involved in possible procarcinogen activation was documented (25, 26, 35, 51), legitimate concerns were raised regarding possible risks associated with the above approach. Earlier studies documented tumor promotional effects of I3C at relatively high dietary doses (3, 7). One of the objectives of this experiment was to address the potency of post-initiation long-term I3C to act as a promoter of hepatocarcinogenesis. Thus, the inclusion of treatment groups with relatively low dietary I3C exposure (250, 500 and 750 ppm) allowed for determination of a possible threshold below which no significant promotion would be detected. Such contemplation would not be unreasonable since biological effects of dietary I3C are attributed to its acid condensation derivatives that are formed in the stomach upon intake (29-32).



The results are inconclusive regarding the existence of a threshold. The data are consistent with the hypothesis of no treatment effect at 250 ppm (odds ratio vs. Control = 1) and therefore consistent with the existence of a threshold in the range studied. However, the variation in the data is such that the results are also consistent with moderate treatment effects at 250 ppm based on the width of the 95% confidence interval (upper limit odds ratio vs. Control = 1.46, Table 3-II) and on the observed increases in pooled incidences (250 ppm vs. 0 level) at the three highest doses of AFB<sub>1</sub> (Table 3-1). Based on the logistic regression modeling, if a threshold exists at all, it would appear to be below 500 ppm. Further studies focusing on 250 ppm and lower doses of I3C would help to clarify the threshold question.

Similar trend for promotion was observed with respect to the incidence of multiple tumors among tumor bearing fish.

Post-initiation long-term dietary I3C has ambivalent effects depending on the animal model. It promotes hepatocarcinogenesis in the trout and the rat as mentioned above, however, it acts as an inhibitor in the C57BL/6J mouse (6). Possible mechanism(s) for tumor modulation could include Ah receptor-mediated enzyme induction (25, 34, 35) and/or the ability of I3C derivatives to exhibit estrogenic

and/or antiestrogenic properties (29). It is known that estrogens promote chemically-induced carcinogenesis in rats (52-57) and trout (40), but inhibit in the mouse (6, 58, 59). It is unclear how estrogens inhibit liver tumors in mice. The mechanisms of estrogen-related hepatocarcinogenesis promotion in the rat are proposed to involve alterations in the cell cycle with ensuing increased cellular proliferation, and/or the ability of some estrogen metabolites to cause direct and/or indirect DNA damage resulting from reactive oxygen intermediates generated by redox cycling involving catechol metabolites of estrogens (60). The ability of I3C derivatives to induce P450s through the Ah receptor and also to possess estrogenic and/or antiestrogenic activities suggest that either of these mechanisms could be involved in promotion in the trout liver model.

Vitellogenin is the precursor for egg yolk protein, and is found in all oviparous vertebrates (61). Its synthesis is mediated through the estrogen receptor (62) and is required for oocyte maturation. Thus, VG synthesis is a reliable estrogen biomarker in sexually immature trout. We observed in this experiment that, at low-I3C treatment groups, VG synthesis was evident whereas CYP1A was induced only in the 1000 and 1250 ppm groups (Figure 4). Previous studies show Ah agonists to promote chemical hepatocarcinogenesis in trout (63). This may suggest that estrogenicity of I3C may play a

more important role in promotion of chemically-induced hepatic tumors in trout, especially at lower dietary levels. In fact, the slope for the relative potency for promotion increases markedly above 750 ppm (Figure 2). This could be an indication of more than one mechanism being involved in the promotion. Perhaps, at higher I3C levels, both estrogenic and Ah receptor-mediated mechanisms are responsible for the observed promotion, but at lower dose range just the estrogenic pathway is active.

When estrogenic activities of a compound of interest are concerned, trout is a very sensitive model for tumor studies. Estrogens promote liver tumors in trout. This is particularly relevant considering that human and trout estrogen receptors have equal specificity for binding estrogens (64).

If I3C indeed promotes in trout by acting as an estrogen, its proposed supplementation for chemoprevention purposes should be approached with caution, until its mechanism(s) of promotion in trout and rats and the relation to human cancer risk are thoroughly understood.

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Table 3-I. Summary of liver tumor incidence in trout fed I3C (initiated with AFB<sub>1</sub> as embryos by a 30 min immersion).

I3C (p.p.m.)	AFB <sub>1</sub> (p.p.b.)	# fish	% incidence
0	0	200	0.0
0	50	370	6.5
0	100	249	9.2
0	175	400	15.0
0	250	400	20.5
250	0	200	0.5
250	50	387	4.4
250	100	259	11.6
250	175	400	18.8
250	250	400	25.3
500	0	200	0.0
500	50	374	5.1
500	100	285	9.5
500	175	400	23.8
500	250	400	27.5
750	0	200	0.0
750	50	286	9.8
750	100	189	17.5
750	175	300	28.3
750	250	300	32.3
1000	0	200	0.0
1000	25	300	9.7
1000	50	270	18.9
1000	100	180	23.9
1000	175	300	46.0
1250	0	200	0.5
1250	25	300	17.0
1250	50	270	33.0
1250	100	164	53.1
1250	175	300	78.0

Table 3-II. Odds ratio and probability values for promotion of tumor incidence and multiplicity<sup>1</sup>

I3C (ppm)	Tumor incidence Odds ratio vs. control <sup>2</sup> (95% CI <sup>3</sup> )	p-value	Multiple tumors <sup>4</sup> Odds ratio vs. control (95% CI)	p-value
250	1.15 (0.91, 1.46)	0.24	1.43 (0.82, 2.55)	0.22
500	1.30 (1.03, 1.65)	0.027	1.50 (0.87, 2.63)	0.15
750	2.00 (1.57, 2.54)	<0.0001	1.92 (1.13, 3.36)	0.019
1000	4.34 (3.37, 5.60)	<0.0001	3.92 (2.32, 6.820)	<0.0001
1250	13.6 (10.6, 17.6)	<0.0001	8.24 (5.06, 13.9)	<0.0001

<sup>1</sup>The group containing fish fed 1250 ppm I3C is not included since it was omitted from the model involving forced parallelism for exhibiting somewhat higher levels of promotion.

<sup>2</sup>Initiated controls.

<sup>3</sup>Confidence interval.

<sup>4</sup>Incidence of multiple tumors among tumor bearing fish.

Table 3-III. Histological classification of liver tumors in trout fed I3C, initiated as embryos with AFB<sub>1</sub> by a 30 min immersion.

I3C (ppm)	% of total tumors by tumor type <sup>a</sup>					
	HCC	MC	CCC	HCA	MA	CCA
0	45.8	38.5	1.9	7.7	0.0	6.1
250	38.3	41.7	2.1	12.0	0.9	5.0
500	51.3	28.3	1.6	12.1	0.2	6.5
750	66.1	19.1	0.4	9.8	0.8	3.8
1000	68.5	22.4	1.2	6.1	0.0	1.8
1250	68.7	26.4	0.1	4.3	0.0	0.5
overall average	56.45	29.4	1.2	8.7	0.3	3.95

<sup>a</sup> HCC, hepatocellular carcinoma; MC, mixed carcinoma; CCC, cholangiocellular carcinoma; HCA, hepatocellular adenoma; MA, mixed adenoma; CCA, cholangiocellular adenoma.

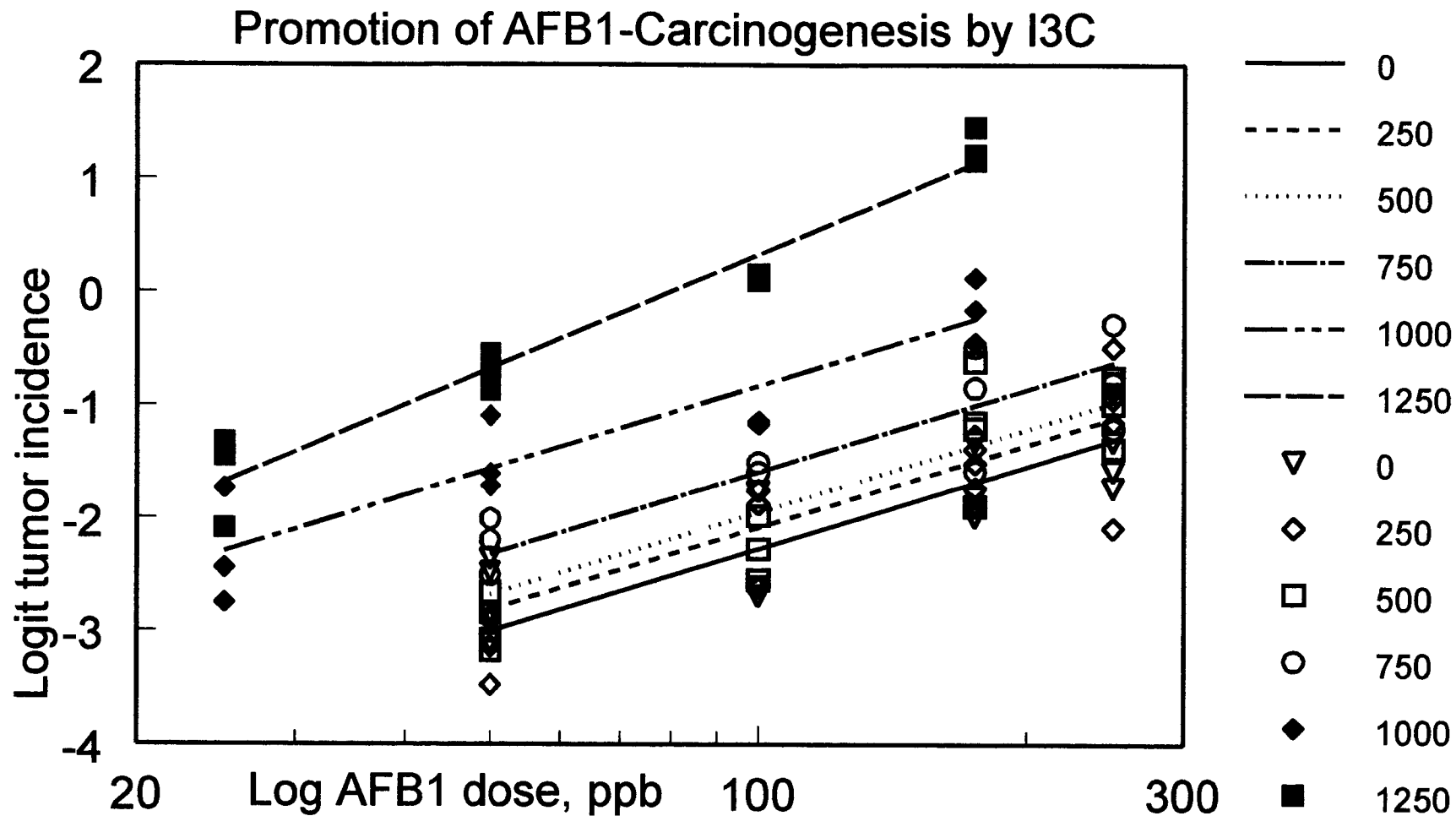


Figure 3.1. Promotion of AFB<sub>1</sub>-induced hepatocarcinogenesis by dietary indole-3-carbinol. Tumor incidence data were obtained by logistic regression with enforced parallelism and with the inclusion of sampling time point in the model. Promotion was significant starting from the 500 ppm group. For 250 ppm, promotion was significant only for the higher dose AFB<sub>1</sub>-initiated groups.



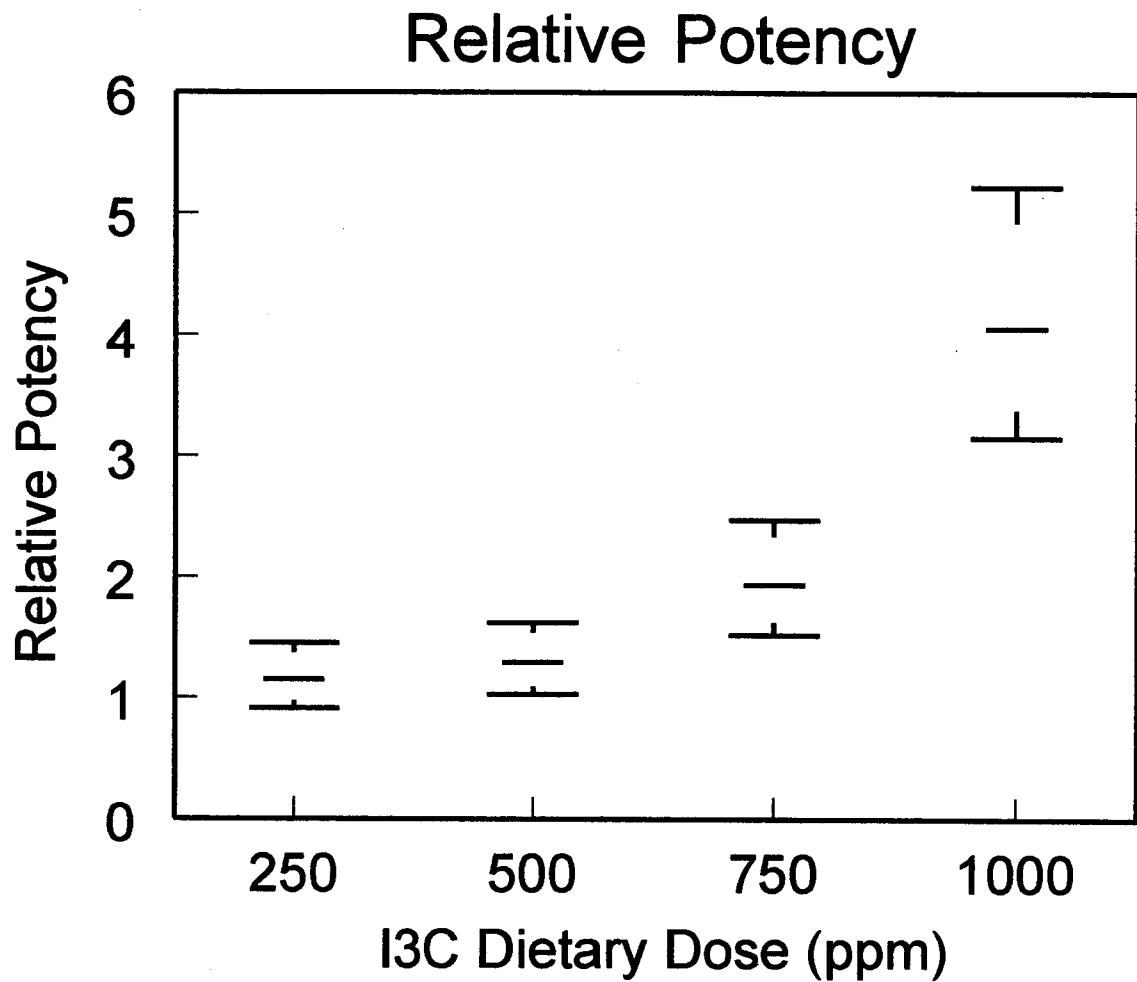


Figure 3.2. Promotional potency of I3C (95% CI), calculated as the ratio of ED<sub>50</sub>s versus positive control across the initiator dose range.

## Induction of Vitellogenin by I3C in the Tumor Study

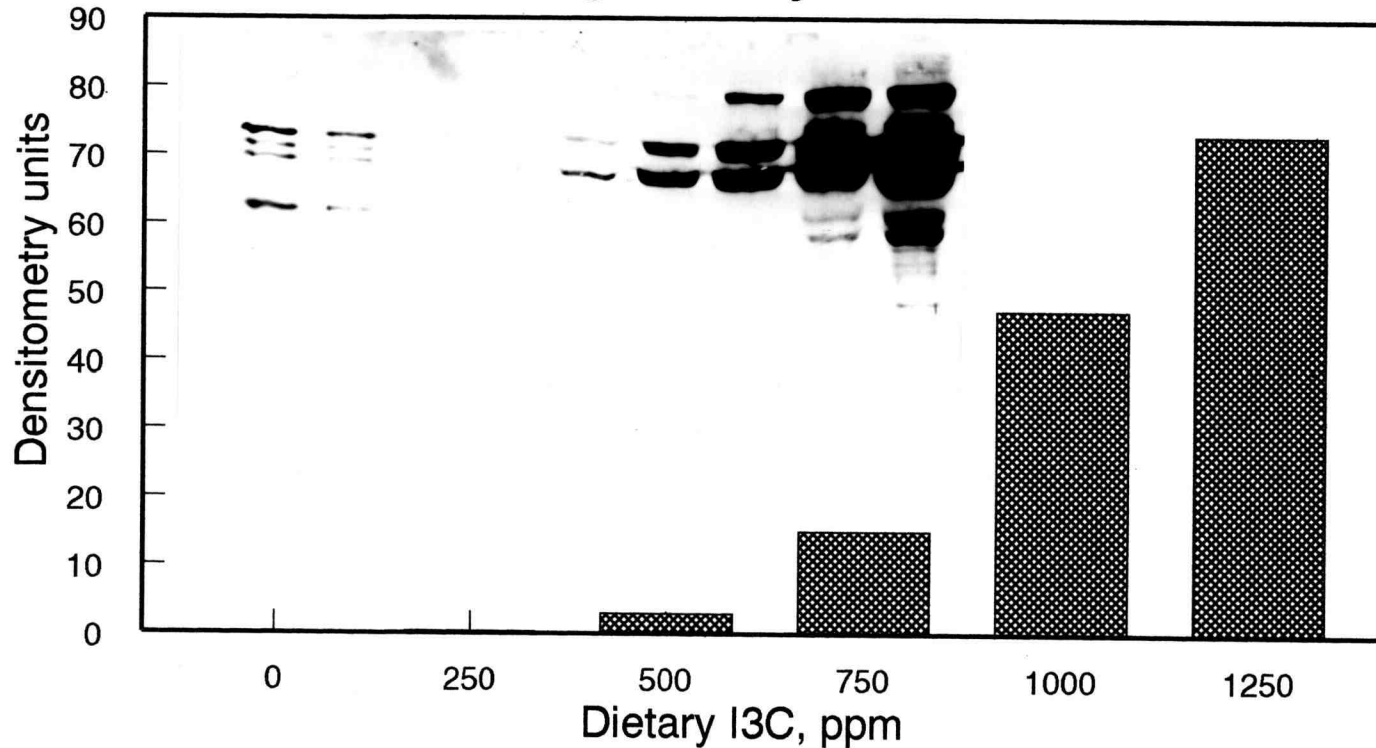


Figure 3.3. Induction of plasma vitellogenin (estrogen biomarker) in trout from the tumor study, fed I3C continuously at 0, 250, 500, 750, 1000, 1250 ppm. Strong induction of vitellogenin is apparent at most dietary levels whereas CYP1A was induced only at highest I3C groups (Fig 3.4) suggesting a more important role for estrogenic properties of I3C in promotion at low dietary levels.

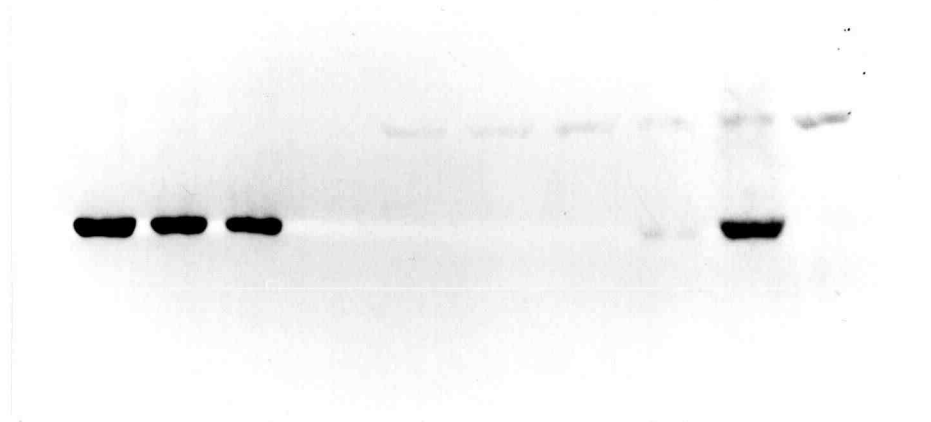


Figure 3.4. Induction of hepatic microsomal CYP1A in trout from the tumor study, fed I3C continuously at 0, 250, 500, 750, 1000, 1250 ppm. Induction is apparent only at highest dietary I3C levels.

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## Chapter 4

**INDUCTION OF CYP1A BY INDOLE-3-CARBINOL, ITS ACID  
CONDENSATION DERIVATIVES AND OTHER Ah  
LIGANDS IN HIGH-PRECISION LIVER SLICES  
FROM TROUT, MOUSE AND RAT**

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## ABSTRACT

I3C is found at relatively high levels in cruciferous vegetables and has been shown to act as a dual modulator of carcinogenesis, depending on species, target organ and conditions of the experiment. It is believed that I3C condensation products are responsible for the *in vivo* effects of I3C administration. One of the candidate mechanisms for tumor promotion by I3C is proposed to be mediation through the aryl hydrocarbon (Ah) receptor. In this study we utilized precision-cut liver slices from rainbow trout (*Oncorhynchus mykiss*), F344 rats and C57Bl/6J mice to analyze various xenobiotics, including I3C and derivatives, for their potency to induce CYP1A1. Slices were incubated for 48 h in Waymouth medium at 34°C (rat, mouse) or 14°C (trout) and viability monitored histologically and by measuring ATP levels. CYP 1A1 protein levels were measured by western blotting. In all three species, ICZ was the most potent inducer among I3C related products (EC<sub>50</sub> 2 µM for trout, 12 µM for rat and 15 µM for mouse slices). When incubated in optimum conditions using the well plate-shaker system, rat, mouse and trout liver slices can be kept viable for at least 48 h and CYP1A1 (1A in trout)

can be induced to levels comparable to those seen in microsomes isolated from animals induced *in vivo*.

Abbreviations: Ah, aryl hydrocarbon; I3C, indole-3-carbinol; I33', 3,3'-diindolylmethane; LT, linear trimer, [2-(indol-3-ylmethyl)-indol-3-yl]indol-3-ylmethane); CT, cyclic trimer (5,6,11,12,17,18-hexahydrocyclonona[1,2-*b*:4,5-*b'*:7,8-*b''*]triindole); ICZ, indolo[3,2-*b*]carbazole; ANF,  $\alpha$ -naphthoflavone; BNF,  $\beta$ -naphthoflavone; HCB, hexachlorobiphenyl; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

## INTRODUCTION

During recent years the utilization of high precision tissue slices has been increasingly employed for *in vitro* metabolism studies (1-3). Tissue slices, to a large degree, maintain the structural integrity of the tissue and thus provide an advantage over cultured cells.

Our laboratory is investigating the mechanism(s) of hepatocarcinogenesis promotion by a plant secondary metabolite, indole-3-carbinol (I3C) which is found at relatively high levels in cruciferous vegetables. It is believed that I3C condensation products are responsible for the *in vivo* effects of I3C administration (4-8). Dietary I3C administration is reported to modulate carcinogenesis and one of its proposed mechanisms of action is via the Ah receptor pathway. This pathway is believed to be the mechanism of TCDD-induced toxicity and tumor promotion (TCDD possesses the highest affinity for binding to the Ah receptor of all known ligands) (9-12). The Ah receptor is a cytosolic protein with a basic helix loop helix structure similar to other transcription factors (10), which binds various xenobiotics of planar stereostructure in a step involving dissociation from two HSP90 subunits. The ligand-bound receptor thereafter translocates into the nucleus and dimerizes with the Ah receptor nuclear translocator protein

(ARNT) (12). The dimer acts as a transcription factor by playing a role in the activation of a cascade of genes downstream from the xenobiotic-response elements (XRE) in the nucleus. These genes include several phase I and II enzymes involved in xenobiotic metabolism. Induction of enzymes of the CYP1A subfamily (particularly, CYP1A1) is considered a marker for an affinity for binding to the Ah receptor (12).

The most common approach for incubation of tissue slices is the so-called dynamic culture system (13). One of the drawbacks of this system concerns the question of availability of test compounds to the cells in slices, since contact with incubation medium is limited. In the well-plate-shaker system (3), slices are submerged into medium at all times and availability of the substrate is not limiting. We here demonstrate that, when incubated in proper conditions using the well-plate shaker system, rat, mouse and trout liver slices can be kept viable for at least 48 h (longer for trout) and CYP1A1 (1A in trout) could be induced at levels comparable to those seen in vivo.



## MATERIALS AND METHODS

### *Materials*

I3C was purchased from Aldrich Chem. (Milwaukee, WI); BNF, ANF, incubation media and supplies from Sigma Chem. Co. (St. Louis, MO) unless otherwise noted; 3,4,5,3'4'5'- and 2,4,5,2'4'5'-hexachlorobiphenyl from AccuStandard (New Haven, CT); ICZ was generously provided by Dr. Leonard Bjeldanes of the University of California at Berkley, and 12-well plates from Fisher Scientific (Santa Clara, CA). Rabbit polyclonal antibody against trout CYP1A was generously provided by Dr. Donald Buhler of Oregon State University. Rabbit-anti rat CYP1A1 antibody was purchased from Oxygene (Dallas, TX).

### *Animals*

Sexually immature rainbow trout (approximately 1 year old) from Oregon State University Food and Nutrition Laboratory were used. Five-six week-old male C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, MA). Six week-old Fisher 344 rats were purchased from Simonsens (Gilroy, CA).

### *Incubation conditions*

All operations with liver slices were performed at maximum sterile conditions at ice-cold temperature until the start of incubation. The microtome compartment of the Krumdieck Tissue Slicer (Alabama R&D, Munford, AL) was

sterilized according to manufacturer's instructions the day prior to preparation of slices. For trout liver slices, the Hanks modified salts buffer was supplemented with 10 mM HEPES, 8 mM sodium bicarbonate, 50 mg/ml gentamicin and 1% BSA, pH 7.4. Rat and mouse liver slices were incubated in the Waymouth MB 752/1 medium, supplemented with 10 mM HEPES, 8 mM sodium bicarbonate, 50 mg/ml gentamicin and 10% fetal calf serum. Media was filter-sterilized using a Gelman Vacu-cap filter (Fisher) into sterile containers and stored at 4°C. The same media was used for transportation of livers, slice preparation and incubation. After animals were sacrificed, livers were collected into ice-cold medium, and slices (250 mm in thickness) prepared from liver tissue cores (8 mm in diameter, prepared with a tissue coring press (Alabama R&D)). Slices were then randomized and distributed into 12-well plates (1 slice per 1 ml of medium per well). Test compounds were added into medium prior to the start of incubation. At 24 h, slices were transferred into new plates with fresh medium. Incubation of slices was conducted at 14°C for trout, and 34°C for rat and mouse slices. The plates were held in tight-seal containers which were gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub> repeatedly several times a day. Containers were kept inside an incubator on an orbital shaker set at 110 rpm. Table 4-I lists all compounds tested and ranges of concentrations used for incubation(s).

### *Viability*

ATP levels were measured in slice homogenates at 0, 24, and 48 h using a method based on spectrophotometric determination of ATP-dependent NADH consumption in an enzyme-linked conversion of 3-phosphoglycerate to 1,3-diphosphoglycerate (Sigma ATP assay kit).

At 0, 12, 24 and 48 h, several slices were fixed in buffered formalin and later evaluated histologically for cell viability. The percentage of viable cells in good histological condition was estimated in randomly chosen sections of slices.

### *Preparation of slice homogenates*

Liver slices were homogenized in 4-5 volumes of ice cold 0.1 M potassium phosphate buffer (pH 7.4) containing 0.15 M KCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 20% glycerol. Homogenates were subjected to a 5,000 g centrifugation for 5 min to remove debris. The resulting supernatant was stored at -80°C until analysis. Protein determinations were made according to the method of Lowry et al. (14).

### *Electrophoresis and immunoblotting*

CYP1A levels were examined in slice homogenates. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15) on 8 % acrylamide and electrophoretically transferred onto nitrocellulose (Bio-Rad Trans-Blot). Blots were probed with

rabbit polyclonal antibodies to trout CYP1A, followed by a horseradish peroxidase-linked secondary antibody. Proteins were detected using an Amersham ECL chemiluminescence kit (Amersham Corp., Arlington Heights, IL). Western blots were scanned on a flatbed HP Scanjet IIcx scanner. Densitometry was performed with the public domain software NIH Image, version 1.57 (written by Wayne Rasband at the US National Institutes of Health).

#### *Statistical analysis*

Differences between groups were tested using one-way analysis of variance, followed by the least significant difference multiple comparison test using the statistical software package, Statgraphics (version 5.0), (Statistical Graphics Corporation, Princeton, New Jersey). A probability value of less than 0.05 was considered significant.  $EC_{50}$  values were calculated by linear interpolation using densitometry data from western blots. Triplicates were used, each replicate representing a pool of 4 slices.

## RESULTS

The viability of slices was assessed histologically, as well as by analyzing ATP levels in slice homogenates. Fig. 4.1 shows changes in ATP levels over time throughout the incubation. Trout slices were the least affected after 48 h of incubation (approximately 80 % of ATP were recovered, versus 70-75 % for rat or mouse slices, compared to levels seen in fresh slices (0 h)). Figs. 4.2a, 4.2b and 4.2c are photographs of representative histological sections taken from trout, rat and mouse liver slices. The estimates of living cells were consistent with ATP observations.

Table 4-II contains EC<sub>50</sub> values for CYP1A1 (CYP1A for trout) induction in trout, rat and mouse liver slices. CYP1A induction by all compounds tested was observed only for trout liver slices. EC<sub>50</sub> values were not calculated for compounds that induced at only one or two concentrations (e.g. LT and CT induced only at highest concentration tested (500 μM)).

Fig. 4.3 represents a western blot of CYP1A induction by some of the compounds incubated with trout liver slices. ICZ exhibited the greatest potency among the I3C derivatives (EC<sub>50</sub>=2μM), and 3,4,5,3'4'5'-HCB and BNF were the most efficacious inducers in trout slices.

Fig. 4.4 represents CYP1A1 induction in rat liver slices. ICZ again was the most potent among I3C derivatives. ANF was an effective inducer at relatively high levels as well. The greatest efficacy for induction was observed by BNF, however, ICZ was a more potent inducer ( $EC_{50}=12 \mu\text{M}$  versus  $38 \mu\text{M}$  for BNF). CYP1A induction in mouse liver slices was reduced compared to trout or rat, although the potency for the strongest inducers were comparable to the rat ( $EC_{50}$ , ICZ= $15 \mu\text{M}$ ; BNF= $34 \mu\text{M}$ ).

Representative western blots are shown in Fig. 4.5, documenting CYP1A1 (1A in trout) induction (dose-response).

Fig. 4.6(a, b) shows a dose-response for CYP1A1 induction by 2,4,5,2'4'5'-HCB in trout and mouse liver slices (no induction was observed in rat slices).

## DISCUSSION

Tissue slices are a convenient tool for studying xenobiotic metabolism and enzyme induction. Although total P-450 levels decrease in the course of incubation of slices, they can still be used for studying the induction of individual CYP isoforms (1, 13).

Ah receptor-mediated induction of P450s is one of the mechanism(s) responsible for toxic events including tumor promotion associated with exposure to certain xenobiotics including dioxins, dibenzofurans, polyhalogenated biphenyls and polycyclic aromatic hydrocarbons (PAHs) (11, 12). In particular, CYP1A1 is believed to be responsible for metabolic activation of procarcinogens of PAH group. These enzymes are also involved in detoxifying pathways for elimination of xenobiotics.

Dietary I3C has been shown to act as a modulator of carcinogenesis in various animal models (16-24). Depending on species, target organ, timing and dose of administration, I3C may either inhibit tumorigenesis (mammary tissue in rodents, mouse liver) or promote (trout, rat liver (long-term exposure), mouse liver (short-term exposure)). Although, transient induction of enzymes of the CYP1A subfamily is implicated in the mechanism(s) of protection against some dietary carcinogens, their persistent expression is one of

the characteristics of toxicity seen in association with exposure to some environmental xenobiotics, including TCDD, and PCBs (11, 12).

Dietary I3C yields various acid condensation products (formed in the stomach) that have been shown to possess affinity for binding to the Ah receptor (4-8). This study utilized the liver slices approach to address the issue of efficacy and potency of various I3C derivatives for induction of CYP1A1 (1A in trout) *in vitro* in comparison with common Ah ligands. The scarce availability and the tendency to undergo physico-chemical breakdown renders *in vivo* studies with some of the I3C derivatives very difficult. The tissue slice technique allows conducting experiments with very small amounts of these compounds. One of the drawbacks of this approach, however, is the difficulties associated with the aqueous solubility of some test compounds at relatively high concentrations.

As expected, the planar HCB isomer (3,4,5,3'4'5'-HCB) and BNF (a proposed strong Ah agonist) induced CYP1A1 (1A in trout) with the greatest efficacy in all models. An interesting observation was made with the non-planar HCB (2,4,5,2'4'5'-HCB) which induced CYP1A1 in mouse and trout slices, but not in the rat, and induction was evident only at low concentrations (Fig. 4.6).



It has been shown that, among all I3C derivatives, ICZ has the highest affinity for binding to the Ah receptor and has been characterized as a TCDD-mimic (7). As we demonstrate here, ICZ indeed induces CYP1A1 (1A for trout) in liver slices very strongly and at relatively low concentrations in all three animal models studied. However, being only one of the minor derivatives of I3C acid condensation, ICZ's relative role in the mechanism(s) of events resulting from dietary I3C *in vivo* is not entirely clear. If ICZ is formed in very minute amounts *in vivo* upon dietary I3C exposure, it is possible that other mechanism(s) of action of I3C derivatives, including documented antiestrogenicity and/or estrogenicity, could be responsible for carcinogenesis modulation in the trout, rat, or mouse.

**ACKNOWLEDGMENTS**

We thank Beth Siddens for purifying LT and CT from I3C acid reaction mixture, Dr. David Stresser for valuable advice and OSU personnel for taking good care of animals involved in this work. This work was supported by US PHS grants ES04766 and ES03850.

Table 4-I. Compounds and incubation concentrations.

Compound	comment	concentration range ( $\mu\text{M}$ )
I3C		0.1-2000
I33'	(dimer of I3C)	0.01-200
LT	(linear trimer)	0.01-500
CT	(cyclic trimer)	0.01-500
ICZ	(indolocarbazole)	0.001-200
ANF		0.01-200
BNF		0.01-80
3,4,5,3'4'5'-HCB		0.01-200
2,4,5,2'4'5'-HCB		0.01-200

Table 4-II EC<sub>50</sub><sup>1</sup> values calculated for CYP1A1<sup>2</sup>  
induction by compounds tested (µM)

compound	trout	rat	mouse
I3C	87	-	-
I33'	6	50	-
LT	-	-	-
CT	-	-	-
ICZ	2	12	15
ANF	37	77	-
BNF	3	38	34
3-HCB <sup>3</sup>	1	10	22
2-HCB <sup>4</sup>	65	-	4

<sup>1</sup> blanks indicate induction was either not detectable or was observed at less than three concentrations tested.

<sup>2</sup> CYP1A for trout

<sup>3</sup> 3,4,5,3'4'5'-HCB

<sup>4</sup> 2,4,5,2'4'5'-HCB

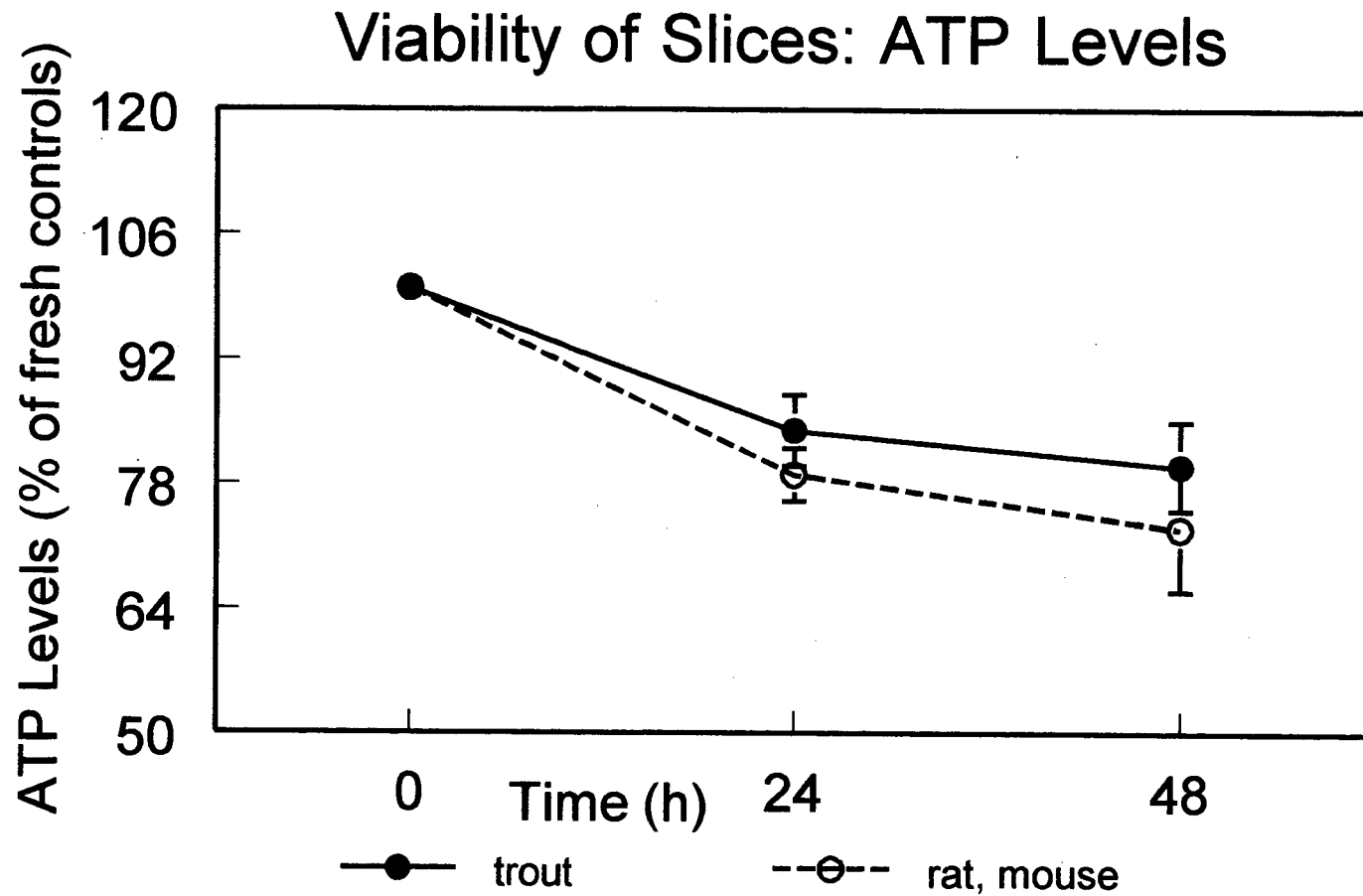
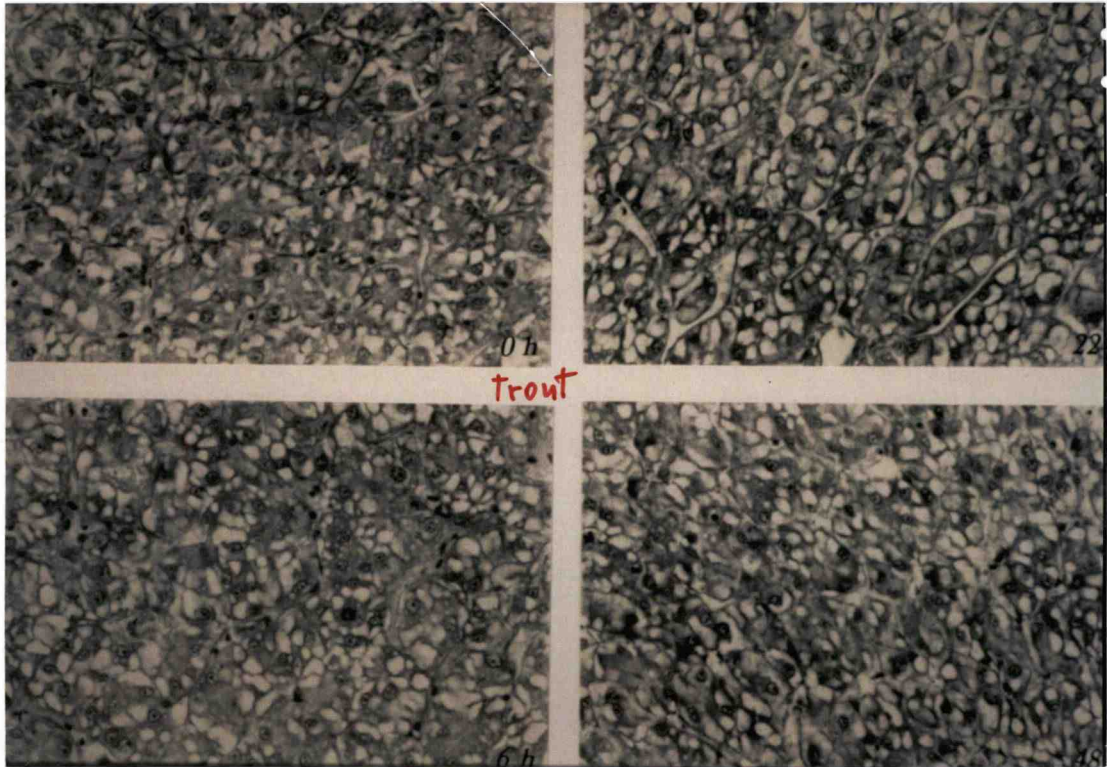
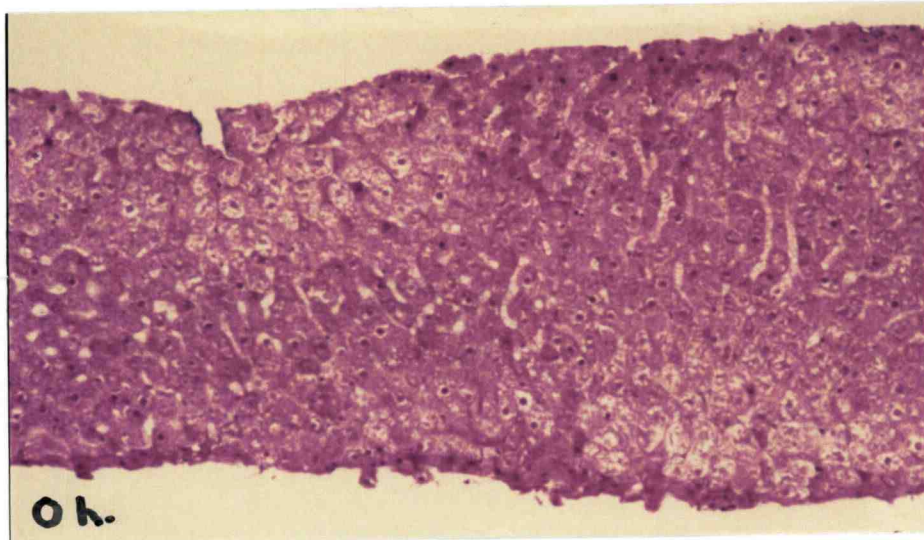


Figure 4.1. Viability of liver slices over the course of 48 h incubation period. Measurement of ATP levels in slice homogenates.

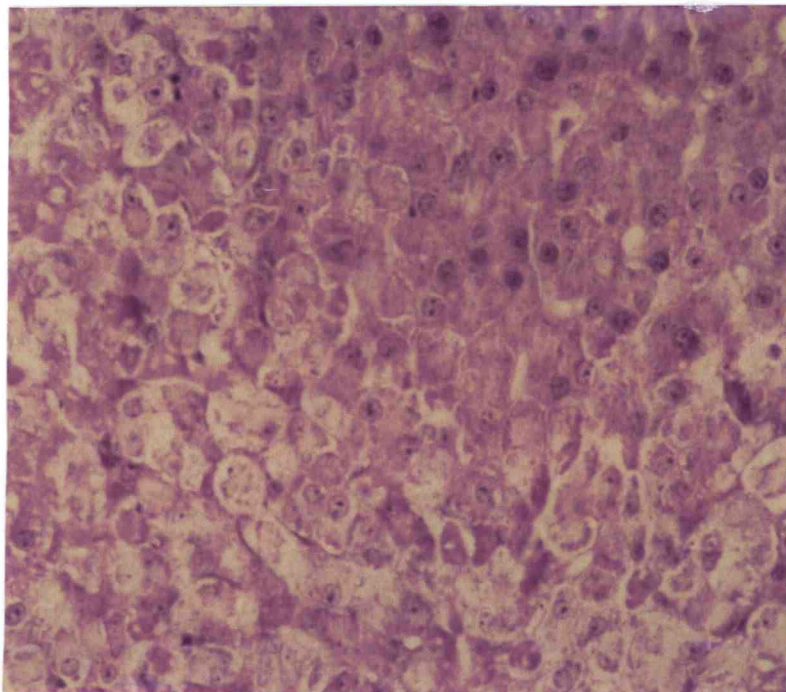


Trout

Figure 4.2. Viability of liver slices over the course of 48 h incubation period: Histological observations: (a) trout; (b) rat; (c) mouse. After 48 h, estimated 80% of cells in trout slices and 70-75% of cells in rat and mouse slices appear viable. (a) Trout.

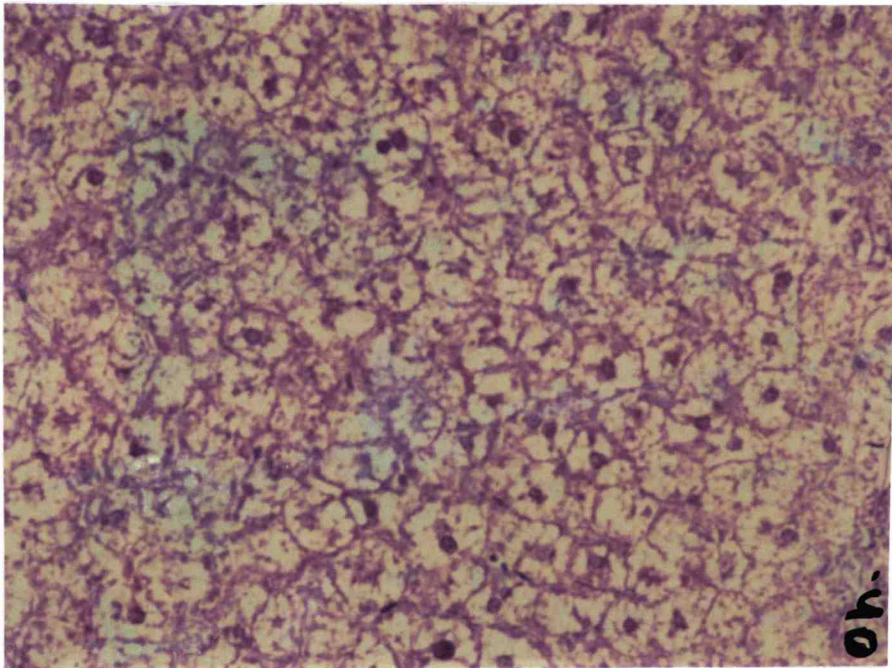


Rat, 0 h

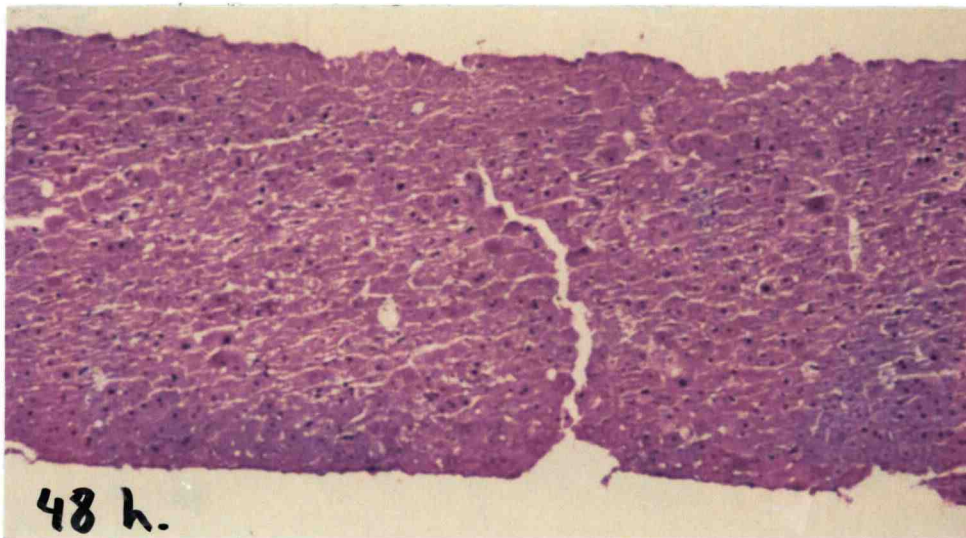


Rat 48 h

Figure 4.2 (b). Viability of liver slices over the course of 48 h incubation period: Histological observations: Rat.



Mouse, 0 h



Mouse, 48 h

Figure 4.2 (c). Viability of liver slices over the course of 48 h incubation period: Histological observations: mouse.



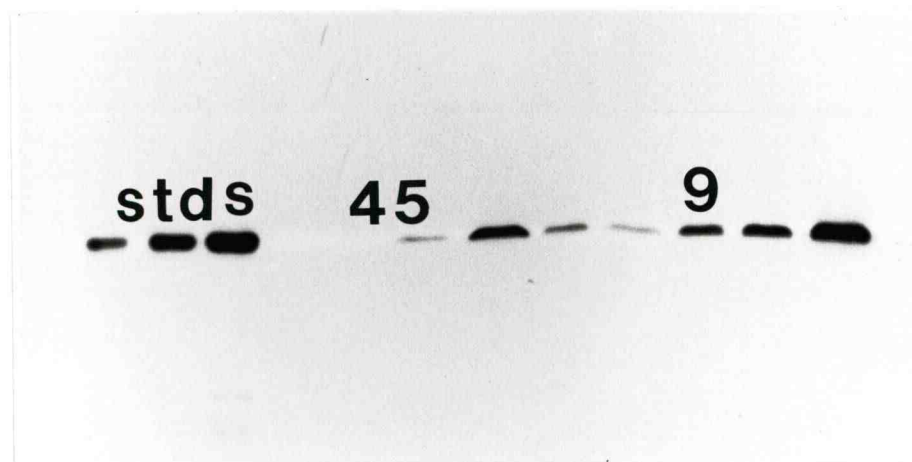
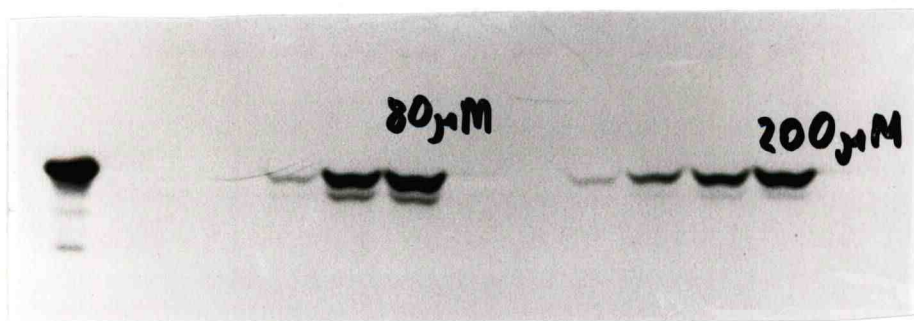


Figure 4.3. **Western blot of CYP1A induction by compounds incubated with trout liver slices.** Left to right: 1-3, purified trout CYP1A (0.1-0.5 pmol); 4, control; 5, ANF; 6, BNF; 7, I3C; 8, I33'; 9, ICZ; 10, 2,4,5,2'4'5'-HCB; 11, 3,4,5,3'4'5'-HCB. All compounds are shown at their highest inducing concentration. Protein amount loaded: 20  $\mu$ g. EC<sub>50</sub> values were calculated from dose-response curves by linear interpolation.

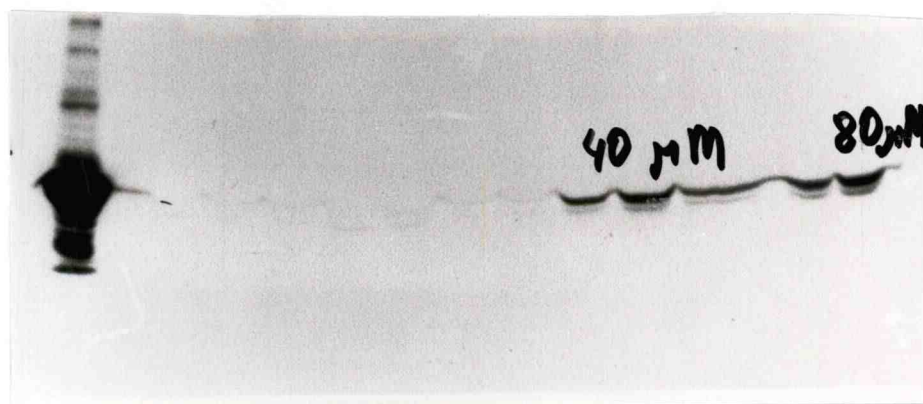


Figure 4.4. **Western blot of CYP1A1 induction by compounds incubated with rat liver slices.** Left to right: 1-3, *in vivo* BNF-induced rat microsomes; 4, BNF; 5, ANF; 6, I3C; 7, control; 8, ICZ; 9, LT; 10, CT; 11, I33'. All compounds are shown at their highest inducing concentration. Protein amount loaded: 20  $\mu$ g.  $EC_{50}$  values were calculated from dose-response curves by linear interpolation.

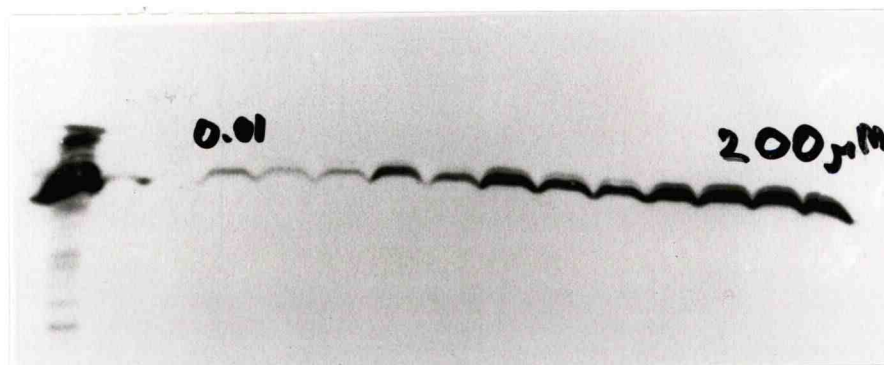


BNF in rat slices

ICZ in rat slices

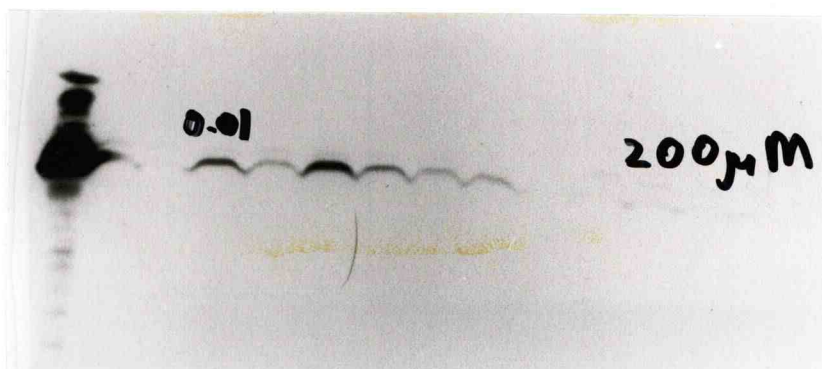


BNF in mouse slices

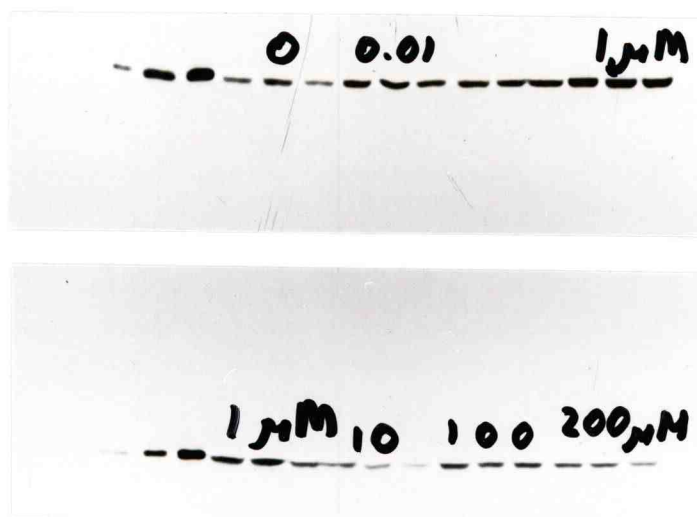


3,4,5,3'4'5-HCB in mouse slices

Figure 4.5. **Representative western blots of dose-response for CYP1A1 induction in precision-cut liver slices.** First well (left) contains BNF-induced (*in vivo*) microsomes. Protein load: 20  $\mu\text{g}$ . Increasing concentrations (Table 4-I) from left to right.



2,4,5,2'4'5'-HCB in mouse liver slices



2,4,5,2'4'5'-HCB in trout liver slices

Figure 4.6. Induction of CYP1A1 (1A in trout) by non-planar 2,4,5,2'4'5'-hexachlorobiphenyl in mouse and trout liver slices: dose-response. Protein load: 20 mg. In both mouse and trout, induction was observed at lower concentrations, fading (trout) at higher concentrations.

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## Chapter 5

**LONG TERM DIETARY INDOLE-3-CARBINOL INHIBITS  
DIETHYLNITROSAMINE-INITIATED HEPATOCARCINOGENESIS IN  
THE INFANT MOUSE MODEL**

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**ABSTRACT**

Indole-3-carbinol (I3C), a natural component from cruciferous vegetables, has been demonstrated to be a modulator of carcinogenesis in various animal models. Along with the promise of I3C as a possible chemopreventive agent for human breast cancer, some concerns have been raised regarding the tumor-promotional potency of this compound in other target organs. In this study we examined the hepatic tumor-modulatory properties of I3C fed to C57BL/6J mice, initiated with diethylnitrosamine (DEN). Infant male mice were initiated with 0, 2 or 5 mg/kg DEN (*ip* injection) at 15 days of age. Mice were weaned nine days later and immediately put on AIN76A semipurified diet (with no antioxidants) containing 0 or 0.15 % (1500 ppm) I3C. In addition, at the age of two months, one group of mice initiated with 2 mg/kg DEN was injected *ip* with a single dose (20 mg/kg) of 3,4,5,3'4'5'-hexachlorobiphenyl (HCB) to serve as a positive control group for promotion. Mice were sampled for hepatic tumors at the age of six or eight months. Each sampled group contained 11-12 mice except the HCB group (9 animals). After 8 months, there was a statistically significant ( $p < 0.0005$ ) inhibition of hepatocarcinogenesis observed for I3C-fed animals initiated with the high dose of DEN. A single injection of HCB at two months of age significantly ( $p = 0.0003$ ) enhanced

hepatocarcinogenesis in mice initiated with 2 mg/kg DEN. There was no statistically significant difference between groups sampled at six months of age. Our observations indicate that long term administration of I3C in the diet inhibits DEN-initiated hepatocarcinogenesis in the infant mouse model.

*Keywords:*

Ah, aryl hydrocarbon; DEN, diethylnitrosamine; I3C, indole-3-carbinol; HCB, 3,4,5,3'4'5'-hexachlorobiphenyl.

## INTRODUCTION

Indole-3-carbinol (I3C) is a hydrolysis product of glucobrassicin, found in cruciferous vegetables such as broccoli, cabbage, and Brussels sprouts. Dietary administration of I3C has been associated with chemoprevention of cancers [1-4] of the endometrium [5], lung [6-9], mammary tissue [10], tongue [11], colon [12] and liver [4,13] in various animal models and is proposed as a chemoprevention agent for use against breast and ovarian cancers in humans [14]. Several possible mechanisms for inhibition of carcinogenesis by I3C have been proposed, one being the alteration of estrogen metabolism [15-20]. In addition, I3C has been reported to inhibit glutathione-S-transferase-mediated steroid binding activity [21], scavenge free radicals [22], modulate the activity of multidrug resistance [23], and alter the expression of various phase I and phase II drug-metabolizing enzymes [24-28] contributing to detoxification of carcinogenic compounds. Upon dietary administration, I3C undergoes condensation reactions in the acidic environment of the stomach. Evidence obtained to date documents that these condensation products, and not I3C itself, are responsible for its biological effects [29-32]. Certain I3C condensation products exhibit relatively high affinity for binding to the Ah receptor [33-36] as well as antiestrogenic and weak

estrogenic activities [29]. Another condensation product, the dimer 3,3'-diindolylmethane (I33'), is a major component of the I3C acid condensation mix formed *in vitro* or *in vivo* [37]. I33' is capable of inducing apoptosis in human cancer cells [38] and is an effective inhibitor *in vitro* of cytochromes P450 [39,40].

In most carcinogenesis models I3C acts as a chemopreventive agent when administered concurrently with the carcinogen or prior to initiation. However, a few studies have reported that, when given after initiation (promotion-progression stage), I3C acts as an enhancer of carcinogenesis [1, 3, 13]. One report concludes that I3C may be mutagenic when administered in diet along with nitrites [41].

In this study we examined the hepatic tumor modulatory potency of I3C in the male C57BL/6J mouse, initiated with DEN. Synthetic estrogens are reported to have liver tumor promoting activities in rats [42,43], however, mice apparently are not susceptible to estrogen-mediated promotion of hepatic carcinogenesis [44]. We investigated whether promotion by I3C is evident in mouse as it is in rat (1) and trout [45]. If I3C enhances tumorigenesis through estrogenic mechanisms, mice may be resistant to these effects.

## MATERIALS AND METHODS

### *Chemicals*

Diethylnitrosamine (DEN) was purchased from Sigma Chemical Co. (St. Louis, MO) and indole-3-carbinol from Aldrich Chemical (Milwaukee, WI).

### *Animals*

All experimental procedures were conducted under NIH guidelines for the care and use of laboratory animals. C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME) as litters, each containing 5 males.

### *Animal Treatments and Diets*

At 15 days of age mice were injected *ip* with 2 or 5 mg/kg DEN in saline. DEN stock concentrations were prepared such that for each g of body weight mice received 10  $\mu$ l stock solution or saline for controls. The average body weight at day 15 was 5 g. Small gauge insulin syringes were used for injection procedures. Nine days after injection mice were weaned and experimental diets started immediately. AIN76A semipurified rat/mouse diet containing no antioxidants (butylated hydroxyanisole or butylated hydroxytoluene) was prepared using components from ICN (Costa Mesa, CA). I3C was added into the diet in dry powdered form. Once in mixtures,

I3C is known to undergo spontaneous breakdown. For this reason the stability of I3C in the AIN76A powdered diet was monitored by solubilizing fractions of the diet and recovering I3C and condensation products using HPLC with a UV-detector. After 7 days at least 80 % of I3C was still in the parent form. I3C diets were prepared every week and stored at 4°C until used.

Animals were divided into ten treatment groups (Table I). Groups 1 and 2 received *ip* 2 mg/kg DEN and control diet; groups 3 and 4 received *ip* 5 mg/kg DEN and control diet; groups 5 and 6 received 2 mg/kg *ip* DEN and 1500 ppm I3C in diet; groups 7 and 8 received 5 mg/kg *ip* DEN and 1500 ppm I3C in diet; group 9 received *ip* saline and 20 mg/kg single *ip* injection of 3,4,5,3'4'5'-hexachlorobiphenyl (HCB) at the age of two months, and received control diet; group 10 received *ip* 2 mg/kg DEN, 20 mg/kg HCB at two months and control diet. Groups 1-3, 5, and 9 consisted of 11 mice, groups 4, 6-8 - 12 mice, and group 10 had 9 animals. Groups 1, 3, 5, and 7 were sampled for liver tumors at six months of age; groups 2, 4, 6, 8-10 - at eight months. Mice were euthanized in a CO<sub>2</sub> chamber according to the 1993 AVMA panel on Euthanasia [46].

In a previous experiment, saline-injected C57BL/6J male mice fed control AIN76A diet (negative control group) showed no tumors and our decision not to include a negative control group in the second study was based on this observation.

### *Animal Husbandry*

Animals were kept at the Oregon State University Laboratory Animal Resources Center. Lactating females with litters were housed in plastic cages with polyurethane covers. Pups remained with the mother until weaning (day 24). Once weaned, mice were housed one per cage, diet given daily, and water and bedding changed twice a week. Diet and water were available *ad libitum*. Clinical observations and body weights for individual mice were recorded weekly.

### *Necropsy*

At the age of six (groups 1, 3, 5, and 7) or eight (the rest of the groups) months, mice were weighed and euthanized by CO<sub>2</sub> asphyxiation. Blood was collected by cardiac puncture, allowed to stand for 1-3 h and then centrifuged to obtain serum. Livers were removed, weighed and examined macroscopically for tumors. Tumors  $\geq 0.5$  mm in diameter were counted. All livers were fixed in Bouins fixative for processing to paraffin sections and microscopic examination. A tumor-free section from each liver was frozen in liquid nitrogen immediately after examination.

### *Statistics*

All data (unless otherwise noted) are presented as mean  $\pm$  SD. Data for treatment groups of interest were compared by

an unpaired, two-tailed t-test for tumor multiplicity and size data, and Fisher's two-tailed exact test for incidence data. All observations with  $p < 0.05$  were considered significantly different.



## RESULTS

### *Body Weight*

Fig. 5.1 shows the body weight gain throughout the study. Body weights were recorded weekly and were not significantly affected negatively by consumption of 1500 ppm I3C in diet. Our visual observations showed that groups fed I3C diet consumed the same amount of food as groups fed control diet. A slowdown of growth was observed for groups 9 and 10 after injection of HCB at two months of age. This trend persisted for both groups throughout the rest of the experiment. By week 33, the average weight for mice from groups 9 and 10 were 33 and 35 g, respectively, versus 37-41 g for mice from the rest of the groups.

### *Liver weight*

There was a significant increase ( $p < 0.05$ ) in the liver somatic index (compared to the rest of groups) for both HCB-treated groups, 7.34 % for group 9 (saline-injected, 20 mg/kg HCB-injected at two months, control diet) and 6.68 % ( $p < 0.05$ ) for group 10 (2 mg/kg DEN, 20 mg/kg HCB) (Table 5-I).

### *Hepatic Neoplastic Findings*

Pale white neoplasms were found macroscopically in the livers of initiated mice (all but group 9). Table 5-I shows the differences between treatment groups with respect to tumor incidence, multiplicity and size. The tumor multiplicity is

also depicted in Figs. 5.2 and 5.3 to illustrate the data for each individual mouse. A 100 % tumor incidence was observed in all groups, except groups 1 (8/11, 2 mg/kg DEN, control diet, sampled at 6 mo.), 6 (9/12, 2 mg/kg DEN, 1500 ppm I3C diet, sampled at 8 mo.), and 9 (uninitiated)., The difference in tumor incidence between these and corresponding treatment groups was not statistically significant ( $p > 0.2$ ). However, a significant inhibition of tumor multiplicity was observed between groups 8 ( $12.5 \pm 11.9$ , 5 mg/kg DEN, 1500 ppm I3C diet, sampled at 8 mo.) and 4 ( $35.4 \pm 15.1$ , 5 mg/kg DEN, control diet, sampled at 8 mo.), Fig. 5.3. The difference in tumor size between these groups ( $0.7 \pm 0.2$  and  $1.1 \pm 0.2$  for 8 and 4, respectively) was statistically significant as well ( $p < 0.0003$ ).

Histologically, hepatocytes of most DEN-treated mice displayed extensive lipidosis, especially in the portal zones. The neoplasms were consistently the same from group to group, and were classified as basophilic, well-differentiated, non-invasive hepatocellular adenomas. Lipid vacuoles were also frequently observed in the neoplastic hepatocytes.

#### *Other observations*

Group 9 (non-initiated, HCB-treated, control diet) was the only group with zero tumor incidence. Four animals (from eleven) from this group had a scruffy skin condition during

the last 2-3 weeks of the study and were treated with tetracycline in the drinking water, by the attending DVM (Dr. Nephi Patton). Two of those four mice had a moderately enlarged spleen. One mouse had a 3 mm cyst on the kidney.

## DISCUSSION

A major objective of this study was to test whether long term post-initiation exposure to dietary I3C promotes hepatocarcinogenesis in mice as it does in rats (Stresser, et al., unpublished data; [1]), and trout [3, 13].

The C57BL/6J mouse was selected because congenic animals from this strain (C57BL/6J(Ahd/d), possessing a defective aryl hydrocarbon (Ah) receptor gene, are available and could be used to demonstrate whether promotion is linked to Ah receptor-mediated mechanisms. On the other hand, if modulation of hepatocarcinogenesis by I3C is mediated through estrogenic mechanisms, inhibition rather than promotion could be expected as it is reported that estrogens normally suppress liver tumor development in mice [44]. An inhibition of DEN-induced hepatocarcinogenesis by ovarian hormones has been reported for the C57BL/6J strain [47]. Several strains of male mice, when treated with 10 ppm ethinyl estradiol, exhibited reductions in the number and size of altered hepatic foci in a two-stage carcinogenesis model [49]. However, it is well established that rats are susceptible to promotion of liver tumors by synthetic estrogens [42, 43, 49]. The tumor modulating effects of I3C may be attributed to estrogenic and antiestrogenic activities of acid condensation products.

In this study, mice initiated with 5 mg/kg DEN as infants and sampled at eight months of age, exhibited statistically significant inhibition of hepatocarcinogenesis when fed 1500 ppm I3C from weaning throughout the duration of the experiment (Fig. 5.2, Table 5-I). The I3C-dependent protection was apparent both at the level of tumor multiplicity and size. For the mice sampled at six months, a single outlier in group 7 (5 mg/kg DEN, 1500 ppm I3C diet, average number of tumors was 9.7) with 53 tumors prevented the demonstration of significant tumor reduction. With the exclusion of this mouse, the difference between this group and the corresponding control would be significant, as well. No statistically significant differences were observed between control and I3C diet groups for mice initiated with low-dose DEN (2 mg/kg) at either six or eight months.

Our finding that 1500 ppm dietary I3C inhibits DEN-initiated hepatocarcinogenesis in C57BL/6J mouse supports the speculation that I3C and its acid condensation derivatives may mediate chemically induced carcinogenesis through estrogen-dependent pathways. The success of the use of dietary I3C supplementation for preventing estrogen-related diseases without increasing the risk of promotion of hepatocarcinogenesis in humans may depend on whether the mechanism(s) of action of I3C derivatives in humans is more like the mouse or the rat and trout.

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Table 5-I. Final body weights, relative liver weights and tumor incidence.

Group	DEN (mg/kg)	Promotion treatment (HCB, mg/kg)	Diet	Necropsy age (months)	Final body wt (g)	Liver wt (% of body wt)	Tumor incidence	Macroscopic hepatic neoplasms (no. $\geq$ 0.5 mm)	Mean size (mm)
1	2		control	6	37.9 $\pm$ 1.9	5.01 $\pm$ 0.50	8/11	3.4 $\pm$ 1.3	0.7 $\pm$ 0.3
2	2		control	8	38.5 $\pm$ 5.1	5.48 $\pm$ 0.91	11/11	4.2 $\pm$ 2.4	1.1 $\pm$ 0.5
3	5		control	6	37.1 $\pm$ 4.3	5.01 $\pm$ 1.05	11/11	16.1 $\pm$ 7.0	0.8 $\pm$ 0.1
4	5		control	8	40.8 $\pm$ 2.4	5.73 $\pm$ 1.18	12/12	35.4 $\pm$ 15.1	1.1 $\pm$ 0.2
5	2		I3C <sup>1</sup>	6	41.0 $\pm$ 3.4	5.54 $\pm$ 1.34	11/11	2.8 $\pm$ 2.6	0.8 $\pm$ 0.4
6	2		I3C	8	38.4 $\pm$ 3.8	5.81 $\pm$ 1.15	9/12	6.9 $\pm$ 6.2	0.9 $\pm$ 0.3
7	5		I3C	6	36.8 $\pm$ 4.1	5.51 $\pm$ 1.20	12/12	9.7 $\pm$ 14.3 <sup>b</sup>	0.8 $\pm$ 0.4
8	5		I3C	8	37.7 $\pm$ 2.3	5.17 $\pm$ 0.66	12/12	12.5 $\pm$ 11.9	0.7 $\pm$ 0.2
9	0	20	control	8	32.4 $\pm$ 6.7 <sup>a</sup>	7.34 $\pm$ 1.6 <sup>a</sup>	0/11	-	-
10	2	20	control	8	35.2 $\pm$ 6.6 <sup>a</sup>	6.68 $\pm$ 1.0 <sup>a</sup>	9/9	18.3 $\pm$ 10.3 <sup>a</sup>	1.2 $\pm$ 0.3

Male C57BL/6J mice received *i.p.* injections of DEN (2 or 5 mg/kg) or saline at 15 days of age. Nine days after injection mice were weaned and fed AIN76A semipurified diet (no BHA or BHT) containing 0 or 1500 ppm I3C. Groups 9 and 10 received *ip* injection of 20 mg/kg HCB at two months of age. Values are mean  $\pm$  SD of 11-12 mice (9 mice for group 10).

<sup>a</sup> - significantly different from corresponding control group

<sup>b</sup> - significantly different from corresponding control (group 3) with the exclusion of one outlier (see text)

<sup>1</sup>1500 ppm

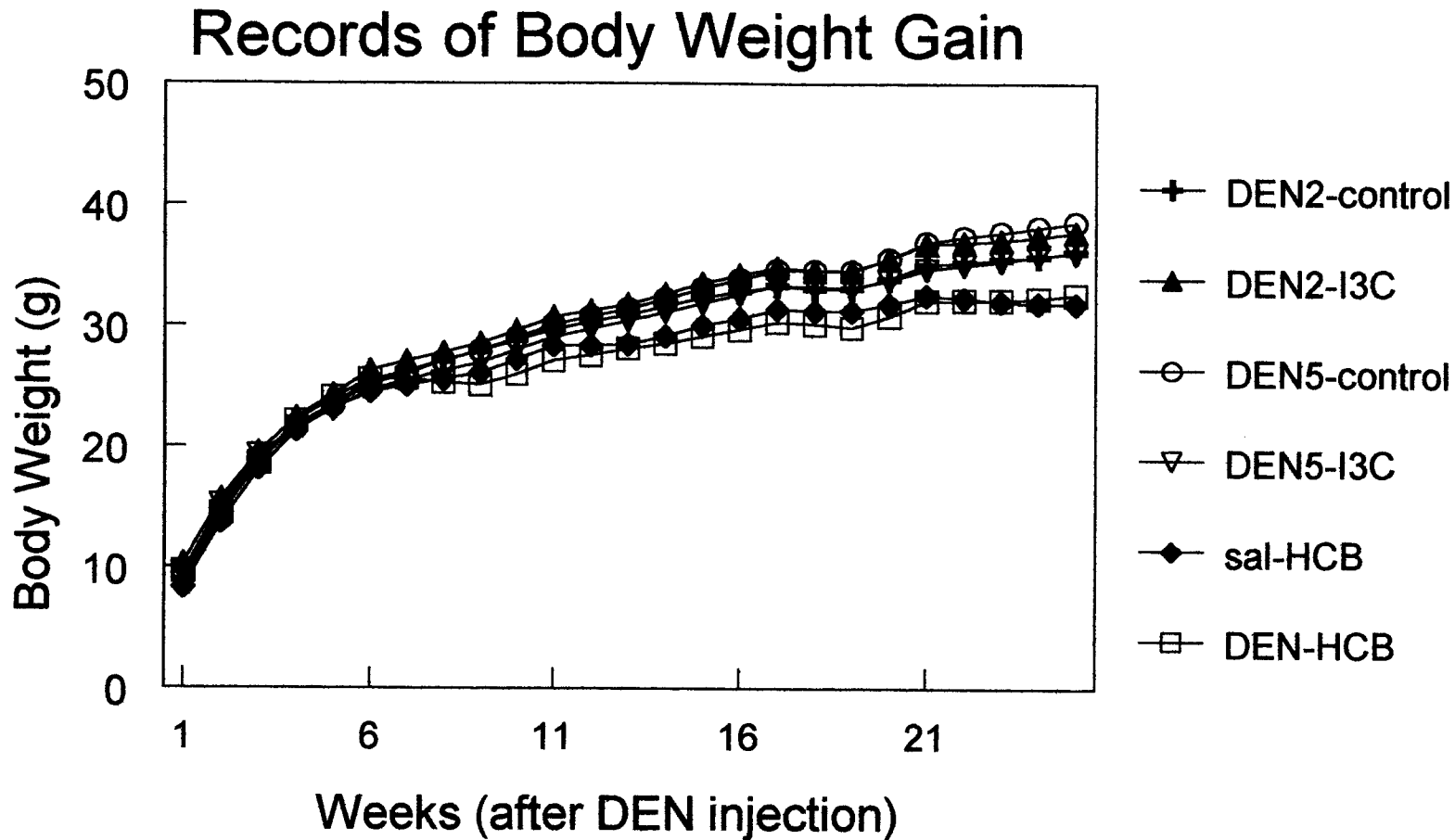


Figure 5.1. **Body weight records of mice from the tumor study.** Mice were treated on day 15 with 0, 2, or 5 mg/kg DEN and fed control or I3C diet (1500 ppm). Two groups were also injected *ip* with a single dose (20 mg/kg) of 3,4,5,3'4'5'-HCB at two months of age. Mice were sampled at six or eight months. (A) Weight records up to six months; (B) weight records from sixth to eighth months.



## Tumor multiplicity in mice at 6 months

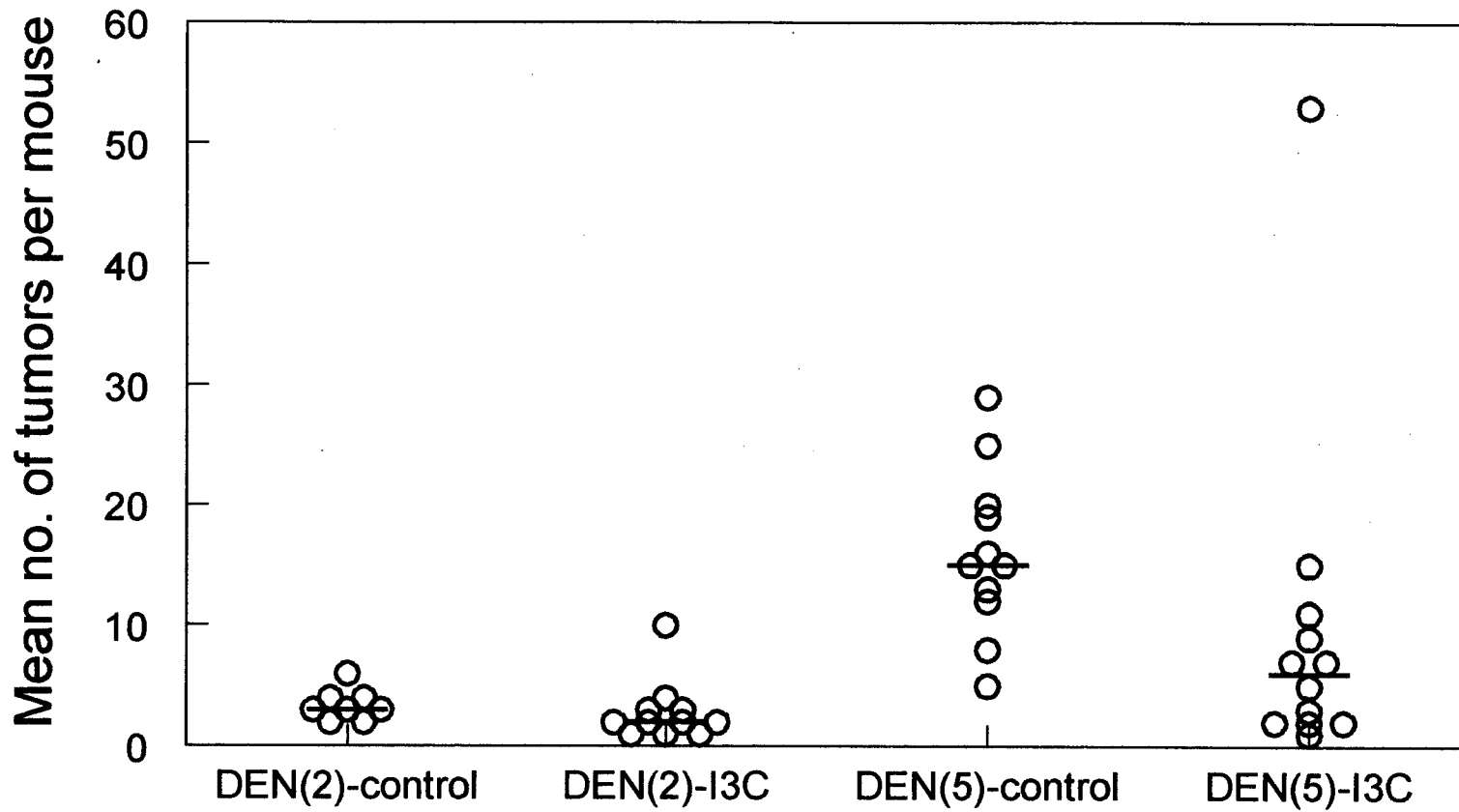


Figure 5.2. Tumor multiplicity data for groups sampled for tumors macroscopically at six months.

## Tumor multiplicity in mice sampled at 8 months

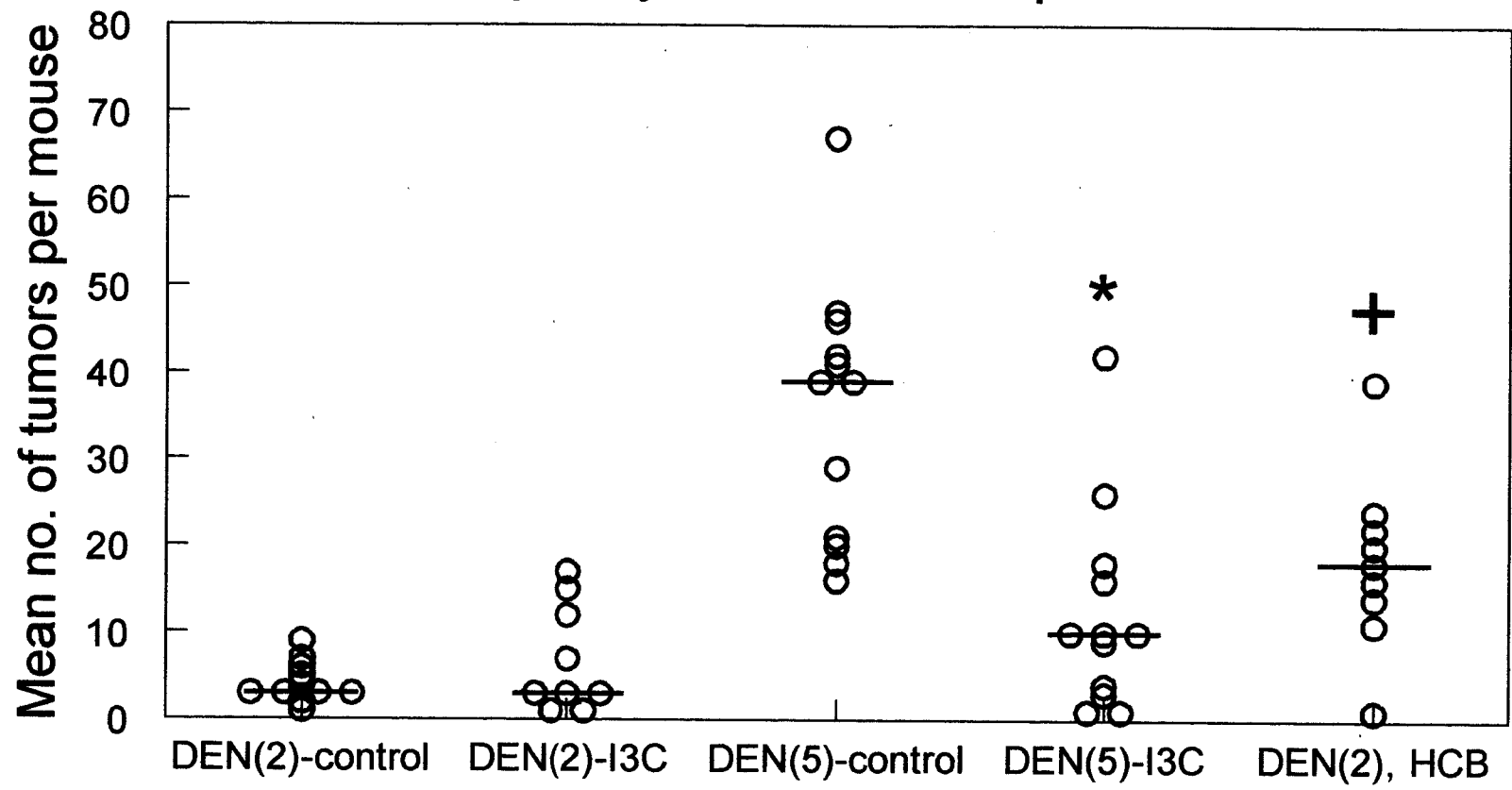


Figure 5.3. Tumor multiplicity data for groups sampled for tumors macroscopically at eight months. \* And + represent statistically significant difference ( $p < 0.05$ ) from the corresponding control group.

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## CHAPTER 6

**ENHANCEMENT OF DIETHYLNITROSAMINE (DEN)-INDUCED  
HEPATOCARCINOGENESIS BY SHORT-TERM LACTATIONAL  
EXPOSURE TO INDOLE-3-CARBINOL (I3C)  
IN THE C57BL/6J MOUSE.**

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**ABSTRACT**

I3C, a plant secondary metabolite found in cruciferous vegetables, inhibits various types of cancer in different animal models, including mammary tumors in rodents and is currently being evaluated in human clinical trials. However, depending on timing of administration, I3C is also capable of acting as a promoter of hepatocarcinogenesis in trout and rats. In this study, lactating female mice were given 0 or 2000 ppm I3C in AIN76A diet, starting on day 8 after birth of litters and until weaning on day 21. Pups from all litters were injected *ip* with 5 mg/kg DEN on day 15. After weaning, pups were fed control diet, sacrificed at 8 months of age and examined for liver tumors. Mice from litters of dams fed I3C had a significantly higher tumor incidence (14/17 versus 4/11 for controls,  $p < 0.001$ ). In a short-term study involving lactating females fed rodent chow, AIN76A or 2000 ppm I3C diet for one week, total P450 levels were significantly elevated in pup livers from both I3C and chow diet groups. EROD activity was induced 3-fold in the pups from I3C litters. We also observed radioactivity in the liver extracts of pups from [<sup>3</sup>H]I3C-gavaged dams, indicating transfer of I3C (or metabolites) through lactation. HPLC analysis of liver extracts showed a peak in all litters, corresponding in retention time to the linear trimer of I3C.

We suggest that enhancement of DEN-initiated hepatocarcinogenesis in pups pre- and co-exposed to I3C condensation product(s) through lactation could be due to induction of various phase I enzymes which could be responsible for procarcinogen activation in this model.

## INTRODUCTION

Indole-3-carbinol, (I3C) a breakdown product of glucobrassicin from cruciferous vegetables has been shown to elicit a number of biological responses upon dietary administration, including chemoprevention of cancers in various animal models (1-9) in several target tissues. It is currently being evaluated as a possible chemopreventive agent for use against certain hormone-dependent cancers in humans (10). Mechanisms for inhibition of tumorigenesis by I3C include the alteration of estrogen metabolism (11-16), inhibition of glutathione-S-transferase-mediated steroid binding activity (17), and to a lesser extent, scavenging of free radicals (18), reversal of multidrug resistance (19), and alteration of the expression of various phase I and phase II drug-metabolizing enzymes (20-24) contributing to detoxification of carcinogenic compounds. Acid condensation derivatives of I3C are formed in the stomach upon dietary administration and are believed to be responsible for its biological effects (25-28). Some of I3C derivatives exhibit relatively high affinity for binding to the Ah receptor (29, 30) as well as antiestrogenic and weak estrogenic activities (25). One of the major condensation products, the dimer 3,3'-diindolylmethane (I33') is capable of inducing apoptosis

in human cancer cells (32) and inhibits cytochromes P450 activity *in vitro* (30, 33).

Chemopreventive properties of I3C are usually reported when it is administered concurrently with the carcinogen or prior to the initiation. However, it was observed that, when given after initiation (promotion-progression stage), I3C can enhance tumorigenesis (1, 3, 7). There is also evidence that I3C may be mutagenic when administered in diet along with nitrites (34).

In an earlier study, we reported that long term post-initiation dietary I3C protected against DEN-initiated hepatic tumors in the male C57BL/6J infant mouse model (6), supporting the hypothesis that long term I3C may have estrogenic effects *in vivo*. Estrogens are reported to have liver tumor promoting activities in rats (35, 36) and trout (37). However, mice are apparently not susceptible to estrogen-mediated promotion of hepatic carcinogenesis (38), and in fact, estrogens are reported to inhibit chemically induced hepatic tumors in certain strains of mice, including C57BL/6J (38, 39). In this study we examined whether short term lactational I3C, administered prior to and shortly after initiation, could alter tumor outcome in the offspring. Specifically, protection was hypothesized since I3C is known to inhibit tumorigenesis when given prior to initiation and concurrently with the carcinogen (3). We report here the unexpected finding that

short-term lactational I3C enhanced liver tumor incidence in C57BL/6J mice and provide evidence for induction of P-450s as a possible factor for enhanced procarcinogen activation in this model.

## MATERIALS AND METHODS

### *Chemicals*

I3C was purchased from Aldrich Chemical (Milwaukee, WI). Diethylnitrosamine (DEN) and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and supplies from Fisher Scientific (Santa Clara, CA), unless otherwise noted. I3C was tritium-labeled at the 5 position using the method of Dashwood *et al* (40). [<sup>3</sup>H]I3C was diluted with unlabeled I3C to an approximate specific activity of 0.1585 Ci/mmol. The radiochemical purity of this compound was shown to be at least 95 % based on HPLC analysis using a Packard Radiomatic radioisotope detector.

### *Animals*

All experimental procedures were conducted under NIH guidelines for the care and use of laboratory animals. C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME) as litters, each containing 5 male pups.

### *Animal Treatments, Diets, and Husbandry*

Starting from day 7, lactating female dams were fed powdered AIN76A semipurified rat/mouse diet, (American Institute of Nutrition, prepared using components from ICN, Costa Mesa, CA) with no antioxidants, and containing 0 or 2000

ppm I3C. I3C was added into the diet in dry powdered form. Once in mixtures, I3C is known to undergo spontaneous breakdown. For this reason the stability of I3C in the AIN76A powdered diet was monitored by HPLC (UV-detector). After 7 days at least 80 % of I3C was still in the parent form. I3C diet was prepared once a week and stored at 4°C until used. On day 15, pups were injected *ip* with 5 mg/kg DEN in saline. DEN stock concentration was prepared such that for each g of body weight, mice received 10 µl stock solution. No liver tumors were seen in control (saline-injected) mice in previous tumor studies of the same duration, and it was decided not to include vehicle-injected control animals in this experiment. The average body weight at day 15 was around 6 g. Small gauge insulin syringes were used for injection procedures. Nine days after injection, pups were weaned and thereafter fed AIN76A control diet until termination of the experiment.

Animals were housed at the Oregon State University Laboratory Animal Resources Center in plastic cages with polyurethane covers at one per cage, diet given daily, and water and bedding changed twice a week. Diet and water were available *ad libitum*. Mice were maintained under controlled conditions of temperature ( $21 \pm 1^\circ\text{C}$ ) and humidity ( $50 \pm 10\%$ ) and a light/dark cycle of 12 h. Clinical observations and body weights for individual mice were recorded weekly.



The design for the short-term experiment following the tumor study was identical, except one more group of lactating mice, fed regular rodent chow diet instead of AIN76A, was added, and on day 15 (initiation day for the tumor study), dams and pups were sacrificed and the livers instant-frozen for further analysis. Four lactating dams fed I3C diet were twice given 100  $\mu\text{Ci}$  [ $^3\text{H}$ ]I3C by gavage (dissolved in DMSO, corn oil suspension) 8 and 4 hours prior to sampling.

#### *Necropsy*

At eight months, mice were euthanized in a  $\text{CO}_2$  chamber according to the 1993 AVMA panel on Euthanasia (41). Livers were examined grossly with a stereomicroscope during necropsy and numbers of neoplasms and pale foci recorded. Livers were fixed in 10% neutral buffered formalin, dehydrated in graded ethanols and embedded in paraffin. Four micrometer sections of liver were stained with H&E. At least 3 pieces of liver, 1 piece taken from each of the 3 main lobes (median, L later and R lateral) of liver, were embedded and sectioned. If gross lesions were evident in the liver, additional pieces of liver containing lesions were embedded. Three sections of each piece of liver embedded were examined. Histologic findings in liver were interpreted according to criteria for rodent liver established by the International Agency for Research on Cancer (42) and the

Society of Toxicologic Pathologists together with the American Registry of Pathology of the Armed Forces Institute of Pathology (43).

*Preparation of hepatic microsomes and enzyme assays*

Livers were homogenized in 4-5 volumes of ice cold 0.1 M potassium phosphate buffer (pH 7.4) containing 0.15 M KCl, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF) (homogenization buffer). Microsomes were prepared by differential centrifugation according to Guengerich (44). The supernatant from a 10,000 g centrifugation of liver homogenate was subjected to centrifugation at 100,000 g. The resulting pellet was washed with potassium pyrophosphate buffer (0.1 M, pH 7.4, 1 mM EDTA, 1 mM PMSF), centrifuged at 100,000 g, and the pellet resuspended in homogenization buffer containing 20 % glycerol to obtain microsomes.

Protein determinations were made according to the method of Lowry et al. (45).

CYP1A1 activity was analyzed by the 7-ethoxyresorufin-O-deethylase (EROD) assay, modified from Pohl and Fouts (46) using liver microsomes at 30° C.

The total specific content of cytochrome P-450 was measured spectrophotometrically by the reduced-CO versus oxidized-CO difference spectrum (44).

*Recovery of radioactivity in liver and HPLC analysis*

Recovery of radiolabel was determined after 2 gavage treatments of 100  $\mu\text{Ci}$  [ $^3\text{H}$ ]I3C 8 and 4 hours prior to sampling. Liver tissues were homogenized in homogenization buffer, 50 ml aliquots digested in BTS-450 tissue solubilizer (Beckman) according to manufacturer's instructions and color reduced with 200  $\mu\text{l}$  of 30% hydrogen peroxide. All samples were stored in the dark overnight prior to quantifying radioactivity using a Beckman LS6500 liquid scintillation counter.

A Beckman ODS 5 $\mu$  4.6 X 250 mm HPLC reverse phase column was used to analyze for radioactive I3C and/or derivatives in liver extracts from dams and pups. Starting conditions were 20% acetonitrile (Solvent A) and 80% Milli-Q water (MQ-H<sub>2</sub>O, Millipore Corp., Bedford, MA) (Solvent B) (31). These conditions were held for 30 sec, before changing to 15% solvent B over the next 29.5 min (linear gradient). This ratio was held for 5 min, then programmed to 0% MQ-H<sub>2</sub>O over the following 5 min, and returned to starting conditions over the next 10 min. The flow rate was 1 ml/min. Metabolites were monitored by radioactivity using a Packard Radiomatic radioisotope detector and liquid flow cell (scintillation cocktail flow rate was 3 ml/min). Liver homogenates (1-2 ml) were extracted three times in three volumes ice cold ethyl acetate containing 0.001% BHT. Liver extracts were evaporated under a gentle flow of nitrogen gas. The yellow-green residue

was centrifuged at 14,000 g for 30 sec and 50-100  $\mu$ l injected into the Beckman ODS 5 $\mu$  4.6 X 250 mm analytical column and detected as described above.

### *Statistics*

Data for treatment groups of interest were compared by an unpaired, two-tailed t-test for tumor multiplicity data, and Fisher's two-tailed exact test for incidence data. All differences in observations with probability value of 0.05 or less were considered statistically significant.

## RESULTS

Tumor incidence and multiplicity data are presented in Table 6-I. Tumor incidence was significantly higher in mice exposed to I3C through lactation ( $p < 0.001$ ). Multiplicity data could not be interpreted clearly since one mouse from the control group exhibited 13 neoplasms, whereas the remaining three mice from the same group had only 1 tumor each. Data on foci of hepatocellular alterations are presented in Table 6-II.

Total P-450 levels, measured in the pup livers from the short-term experiment, are presented in Fig. 6.1. Hepatic microsomal P-450 levels were significantly higher in pups from maternal I3C-exposed litters, as well as litters from the group where dams were fed chow diet. EROD activity, representing CYP1A1, was induced 3-fold in microsomes from pups exposed via lactation to I3C (data not shown).

Radioactivity recovery in dam and pup livers was calculated as a percentage of DPMS (disintegrations per minute) of the original radioactivity ( $^3\text{H}$ ) dose received (200  $\mu\text{Ci}$ ). The recovery at this single time point was  $1.56 \pm 0.32$  % of total dose received in livers from dams and  $0.0091 \pm 0.0012$  % in livers from pups.

Fig. 6.2 is a representative (one litter) HPLC chromatogram of detected radioactivity in dam and pups liver

extract. A peak at around 29-30 min was observed consistently in all four litters from dams receiving [<sup>3</sup>H]I3C. This peak is tentatively identified as the linear trimer (LT) of I3C which eludes under this conditions at about 29 min. Confirmation of the identity of this peak awaits further analysis.

## DISCUSSION

The objective of this study was to test whether short term pre- and co-initiation exposure to I3C through lactational transfer protects against hepatocarcinogenesis in the offspring. We have reported that long-term dietary I3C inhibits hepatocarcinogenesis in the C57BL/6J infant mouse-initiation model. It is generally concluded that, when given prior to initiation and/or concurrently with the carcinogen, I3C elicits protection against chemically-induced carcinogenesis. One of the mechanisms of tumor inhibition relates to the induction of phase I and II isozymes responsible for shifting the carcinogen metabolism towards detoxification. This same mechanism, however, could be responsible for enhancement of initiation if particular P-450 isozymes, responsible for procarcinogen activation (i.e. CYP1A1 and CYP1A2) are induced.

Another candidate mechanism of action by I3C for modulating carcinogenesis involves estrogenic and/or antiestrogenic effects of dietary I3C. Estrogens inhibit chemical carcinogenesis in the mouse liver model (38). Our previous finding of inhibition of DEN-induced hepatocarcinogenesis by long term dietary I3C (6) supports the possibility of estrogenic effects of I3C, especially since in a similar experimental design, I3C promotes in trout and the

rat liver. It is well established that the rat and trout are susceptible to promotion of liver tumors by estrogens (35-37). Furthermore, dietary I3C has been demonstrated to be estrogenic in trout as evidenced by enhanced levels of serum vitellogenin, an estrogen-dependent protein.

In this study, infant mice exposed to I3C through lactational transfer for one week prior to initiation with a single 5 mg/kg *ip* injection of DEN and sampled for hepatic tumors at eight months, exhibited significantly enhanced liver tumor incidence compared to initiated mice from litters with dams fed control AIN76A diet. This is one of the first reports that pretreatment with dietary I3C results in enhancement rather than inhibition of chemically-induced carcinogenesis. Earlier studies report enhancement of tumor outcome only in experimental designs when animals were exposed to dietary I3C in a post-initiation long-term exposure time frame.

Our finding that short-term lactational I3C pre-treatment followed by co-treatment with the carcinogen enhances DEN-initiated hepatocarcinogenesis in the neonatal mice supports the speculation that I3C and/or its acid condensation derivatives may mediate chemically induced carcinogenesis by altering (inducing) P-450s responsible for procarcinogen activation. A future study with the congenic strain of C57BL/6J mouse, possessing a defective aryl hydrocarbon (Ah) receptor gene (C57BL/6J(Ahd/d)), could



address the question of whether or not the mechanism of enhancement in this model is linked to Ah receptor-mediated P-450 induction.

We conclude that the proposed dietary supplementation of I3C in humans for chemoprevention purposes should be approached with caution, until its mechanism(s) of carcinogenesis modulation in different animal models and their relation to human cancer risk are thoroughly understood.

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Table 6-I. Histologic hepatic neoplasia in mice receiving lactational exposure to I3C prior to initiation with DEN

Treatment Group (n litters)	Total Hepatic Neoplasia (%)	Hepatocellular Adenoma (%)	Hepatocellular Carcinoma (%)	Neoplasm Multiplicity <sup>a</sup>
Control (3)	4/11 (36)	2/11 (18)	3/11 (27)	1 (1-13)
I3C (4)	14/17 <sup>b</sup> (82)	11/17 (65)	13/17 (76)	6 (1-11)

<sup>a</sup> median number of neoplasms detected histologically in liver of mice bearing neoplasia (range).

<sup>b</sup> significantly different from control (p < 0.001)

Table 6-II. Foci of hepatocellular alteration in mouse livers

Treatment Group (n litter)	Total Foci (%)	Amphophilic Foci (%)	Eosinophilic Foci (%)	Basophilic Foci (%)	Focus Multiplicity <sup>b</sup>
Control (3)	10/11 (91)	10/11 (91)	2/11 (18)	0/11 (0)	3 (1-10)
I3C (4)	16/17 (94)	16/17 (94)	3/17 (18)	4/17 (24)	3 (1-6)

<sup>b</sup> Average number of foci of cellular alteration detected histologically in liver of mice with foci (range).

## Total P-450 Levels: Short-Term Mouse Study

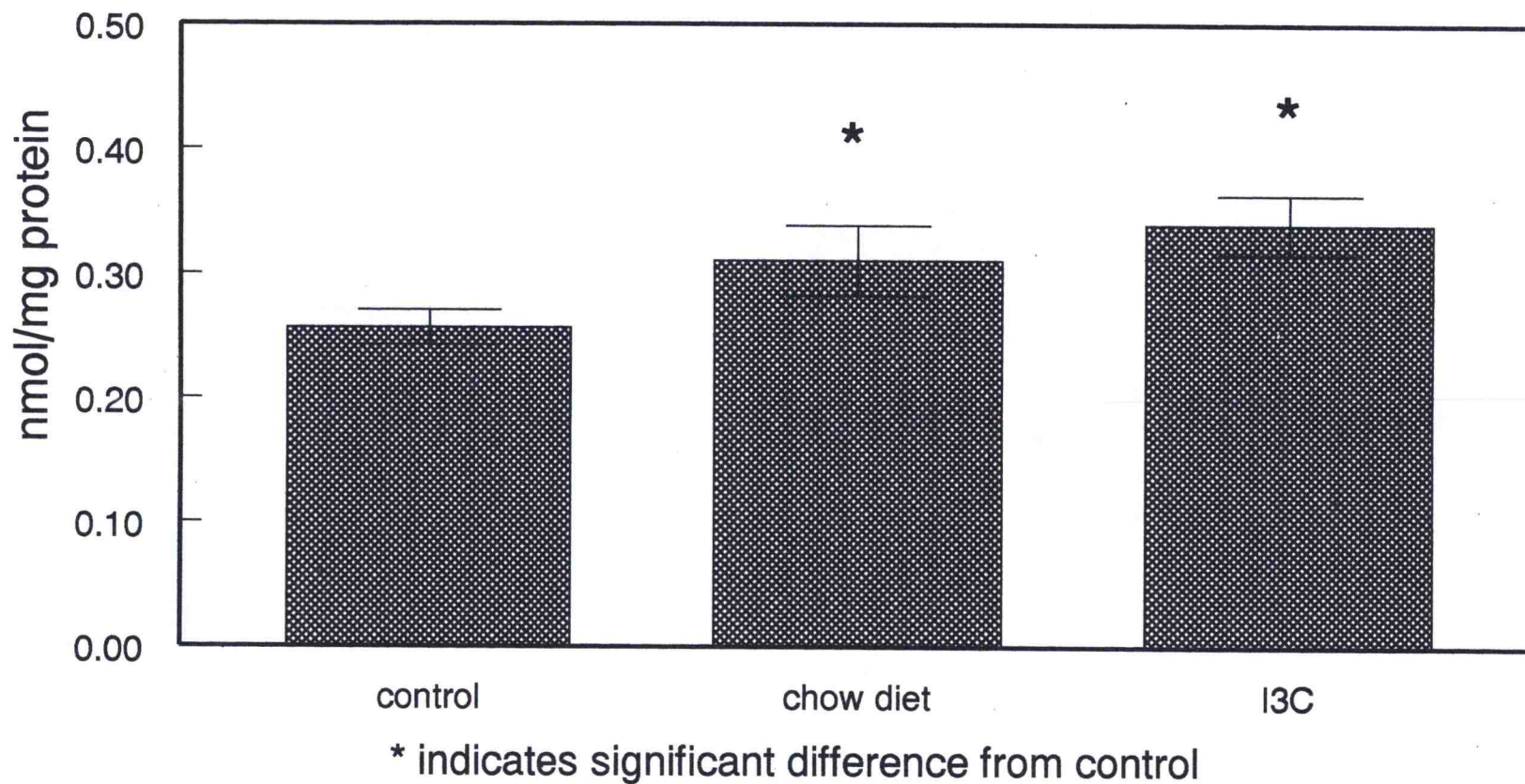


Figure 6.1. Induction of total microsomal P-450 levels in pups exposed to I3C through lactation for seven days. Total P-450 levels were higher in pups from dams fed chow and I3C diet. \* Represent statistically significant difference ( $p < 0.05$ ) from the control diet (AIN76A) group.

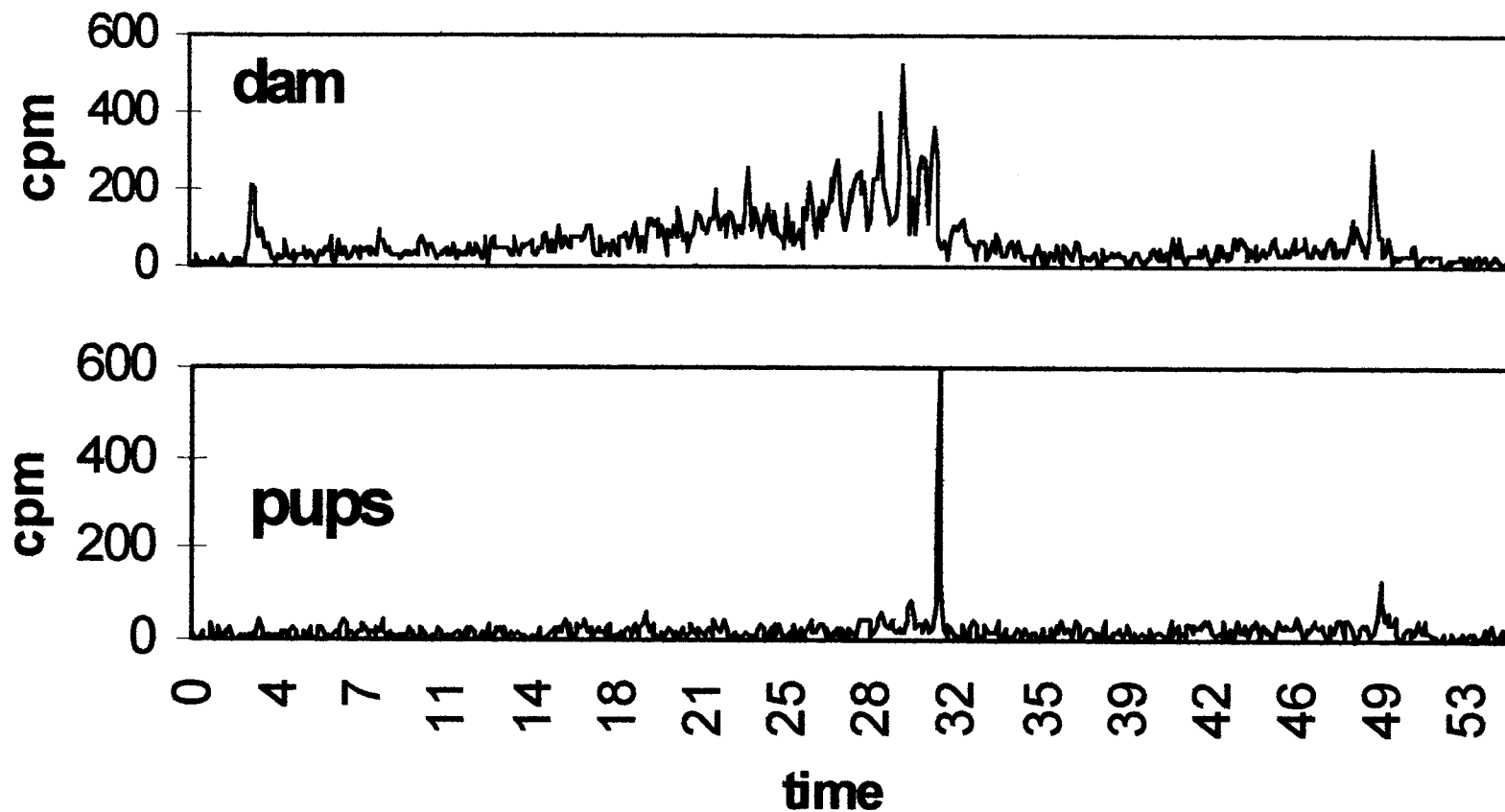


Figure 6.2. A representative HPLC chromatogram of detected radioactivity in dam and pups liver extract. A peak at around 29-30 min was observed consistently in all four litters from dams receiving [ $^3\text{H}$ ]I3C. We propose that this peak corresponds to the linear trimer (LT) of I3C which normally eludes at about 29 min.

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## Chapter 7

**SUMMARY**

Modulation of chemical hepatocarcinogenesis by dietary I3C is linked to several mechanisms. The relevance and/or relative importance of a particular mechanism depends upon the animal species, target organ, and conditions of the experiment, especially the dose and timing of administration. The fact that most biological responses observed after dietary I3C exposure are postulated to be dependent upon one, some or perhaps all of the 15-20 various acid condensation derivatives, complicates the matter even further, since the yield of various derivatives could be expected to vary greatly between species and depending on conditions of dietary administration.

Two of the most important mechanisms for tumor modulation by I3C and/or its acid derivatives are (in no particular order): (i) Ah receptor-mediated enzyme induction and (ii) estrogenic and/or antiestrogenic response(s).

In the 9,000 trout tumor study involving a wide range of the initiator dose (AFB<sub>1</sub>), significant promotion of hepatocarcinogenesis was apparent at dietary levels of I3C as low as 500 ppm (also at 250 ppm, but only at the higher

end of the AFB<sub>1</sub> dose range). The pattern for potency of promotion by I3C suggests a possible role of more than one mechanism involved. Estrogenicity of dietary I3C, measured as its ability to induce plasma vitellogenin, an estrogen biomarker protein, was detected at the lowest treatment levels and increased in a dose-dependent fashion. In contrast, CYP1A levels in trout livers from the same study were induced at just the highest dietary levels (1000 and 1250 ppm), suggesting that Ah receptor pathway while active, is perhaps relevant for explaining the observed promotion at only relatively high dietary levels of I3C.

In the C57BL/6J infant mouse model, long-term dietary I3C inhibited DEN-induced tumor multiplicity, consistent with a hypothesis supporting an estrogenic mechanism(s) of I3C action, as it is known that estrogens inhibit chemically-induced liver tumors in mice. However, in another tumor study involving the same mouse model, designed to test the ability of short-term pre- and co-treatment with dietary (lactational) I3C to protect against hepatocarcinogenesis, resulted in significant enhancement of tumor incidence compared to initiated control animals. In a follow-up experiment, it was seen that P-450 levels in pup livers were markedly induced following one week of lactational I3C exposure, suggesting that induction of xenobiotic metabolizing enzymes responsible for procarcinogen

activation could be an important deciding factor in the tumor outcome, especially for a sensitive model such as the infant mouse. Interestingly, the nursing pup seemed to accumulate I3C condensation products selectively, with the linear trimer apparently absorbed to a much greater extent in the infant liver than other I3C products and/or is excreted/eliminated at a slower rate compared to other derivatives.

Future studies are recommended with I3C addressing the relative role(s) that particular I3C acid condensation derivatives could have in modulating chemical carcinogenesis, particularly, I33', which is the major condensation product of I3C, and the linear trimer which may be selectively accumulated in infant liver through lactation.

The relevance of results obtained from different animal models to human cancer risk will depend on understanding the factors behind observed species differences in response to dietary I3C. For example, if estrogenic effects of long-term dietary I3C can be documented in humans, then concerns could be raised regarding risk(s) associated with possible increase in human liver cancer incidence, since it is known that estrogens promote hepatic adenomas and carcinomas in humans.

In conclusion, there might be a real potential in using I3C for the purpose of cancer chemoprevention. However, before any large-scale supplementation is recommended for humans, a thorough understanding of its mechanisms of action as a carcinogenesis modulator is critical.



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