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MECHANISMS OF ANTI-CARCINOGENESIS BY INDOLE-3-CARBINOL

STUDIES OF ENZYME INDUCTION, ELECTROPHILE-SCAVENGING, AND INHIBITION OF AFLATOXIN B_1 ACTIVATION

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Abstract—The induction of oxidation and conjugation enzymes, the scavenging of carcinogen electrophiles, and the inhibition of aflatoxin B_1 (AFB_1) activation were examined as possible mechanisms of anti-carcinogenesis by indole-3-carbinol (I3C). Liver microsomal 7-ethoxycoumarin *O*-deethylase and 7-ethoxyresorufin *O*-deethylase activities were not induced significantly in rainbow trout fed diets containing 500–2000 ppm I3C for 8 days compared to trout fed the control diet. Furthermore, no detectable changes in the specific contents of cytochrome P-450 isozymes LM_2 and LM_{4b}, as measured by Western-blotting and immunoquantitation, were found in liver microsomes following dietary I3C administration. Dietary I3C had no significant effect on liver microsomal uridine diphosphate-glucuronyltransferase activity, measured using the substrates 1-naphthol and testosterone, or on cytosolic glutathione *S*-transferase activity, measured using the substrate styrene oxide. The ability of I3C or its acid reaction products (RXM; generated by the reaction of I3C with HCl) to act as scavengers for the direct alkylating agent AFB_1-8,9-Cl_2 was examined. Addition of I3C or RXM to *in vitro* incubations did not inhibit the covalent binding of AFB_1-8,9-Cl_2 to calf thymus DNA. Kinetic analyses of microsome-mediated binding of AFB_1 to DNA *in vitro* indicated that RXM inhibited the metabolic activation of AFB_1. RXM increased the apparent K_m for the AFB_1–DNA binding reaction without changing the associated V_{max}; the apparent K_m values at 0, 3.5, 35, and 350 μM RXM were 35, 38, 66, and 86 μM for trout liver microsomes. RXM also inhibited the activation of AFB_1 by rat liver microsomes, but I3C was not an effective inhibitor against AFB_1–DNA binding mediated by either rat or trout liver microsomes. The results of the present study indicate that inhibition of microsome-activated AFB_1 binding to DNA by I3C products may be of significant importance in I3C inhibition of hepatocarcinogenesis in trout and other species. The inhibition of carcinogen activation by I3C is contrasted with the mechanism of anti-carcinogenesis by β-naphthoflavone, which involves induction of xenobiotic metabolizing enzymes.

Cruciferous vegetables contain a variety of compounds that modulate the carcinogenic process [1–5]. One such compound that has received considerable interest is indole-3-carbinol (I3C†), a natural anti-carcinogen found as a glucosinolate in cruciferous vegetables such as broccoli, cauliflower, and cabbage [6]. I3C and the related indoles 3,3′-diindolylmethane and indole-3-acetonitrile were reported a decade ago to inhibit tumorigenesis in rodents exposed to polycyclic aromatic hydrocarbons [7]. In more recent studies, I3C also was found to inhibit aflatoxin B_1 (AFB_1)-induced hepatocarcinogenesis in rats‡ and rainbow trout [8, 9].

It has been suggested that the mechanism of I3C anti-carcinogenesis may be related to an ability to induce cytochrome P-448§ monooxygenase activities [3], perhaps through the involvement of I3C condensation products formed under acid conditions which mimic those in the stomach [10]. However, results of some studies are inconsistent with the proposed relationship between altered monooxygenase activities and I3C inhibition. For example, I3C has been reported to protect against the DNA-damaging effects of orally administered benzo[*a*]pyrene and *N*-nitroso-dimethylamine in mice [11, 12] and AFB_1–DNA binding and hepatocarcinogenesis in trout [8, 13, 14] without appearing to augment hepatic cytochrome P-448 monooxygenase activities.

To explain these apparently conflicting observations on the cytochrome P-448 modifying activities of I3C, Bradfield and Bjeldanes [10] have postulated a role for I3C acid condensation products in which

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† Abbreviations: I3C, indole-3-carbinol; AFB_1, aflatoxin B_1; BNF, β-naphthoflavone; RXM, indole-3-carbinol acid reaction mixture; UDPGT, uridine diphosphate-glucuronyl-transferase; GST, glutathione *S*-transferase; ECOD, 7-ethoxycoumarin *O*-deethylase; EROD, 7-ethoxyresorufin *O*-deethylase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; and DMSO, dimethyl sulfoxide.

‡ Selivonchick DP, Hedstrom O, Oliyai R, Kerkvliet NI and Bailey GS, Dietary modulation of aflatoxin B_1 carcinogenesis. Abstr. No. 48, First Congress of Toxicology in Developing Countries, Buenos Aires, Argentina, 1987.

§ Cytochrome P-448 refers to the major rat liver microsomal cytochrome P-450 isozyme induced by β-naphthoflavone, in accordance with the nomenclature used in the studies cited.

two alternative mechanisms operate: induction of monooxygenase pathways responsible for carcinogen detoxification or inhibition of monooxygenase activation pathways. Shertzer and co-workers [15, 16] have suggested that I3C or its metabolites may be capable of reacting with electrophilic and radical intermediates of carcinogens and toxins, and that this scavenging role, rather than monooxygenase induction, constitutes the central mechanism of I3C inhibition.

We have undertaken the following studies to further examine the possible mechanisms of I3C inhibition of carcinogenesis. The effects of various levels of I3C on a number of trout liver phase I and phase II metabolizing enzymatic activities and specific cytochrome P-450 isozymes have been investigated following dietary administration that was shown to be effective in reducing AFB_1–DNA binding *in vivo*. In subsequent analyses conducted *in vitro*, two other mechanisms were investigated using inhibition of AFB_1–DNA binding as an end-point. First, the postulated scavenging role [15, 16] was investigated both for I3C and acid condensation products of I3C. Second, enzyme kinetics studies of rat and trout liver microsome-mediated AFB_1–DNA binding were initiated in order to investigate a possible mechanism of enzyme inhibition. β-Naphthoflavone (BNF), a synthetic flavone that inhibits AFB_1 carcinogenesis and reduces AFB_1–DNA binding by induction of detoxification enzymes [8], was used as a positive control in this study.

MATERIALS AND METHODS

Materials. [^{3}H(G)]AFB_1 (sp. act. 24 Ci/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA) and checked for purity by TLC and UV spectrophotometry [17]. [^{3}H]AFB_1-8,9-Cl_2 was prepared by the reaction of [^{3}H]AFB_1 and chlorine gas, and purified by reverse-phase HPLC as described by Swenson *et al.* [18]. [7(n)-^{3}H]Styrene oxide (sp. act. 250 mCi/mmol) was obtained from the Amersham Corp. (Arlington Heights, IL), and [glucuronyl-U-^{14}C]uridine diphosphate glucuronic acid (UDPGA, sp. act. 233 mCi/mmol) and [^{125}I]protein A (sp. act. 30 μCi/μg) were from ICN Biochemicals, Inc. (Irvine, CA). I3C, 7-ethyoxyresorufin, and 7-ethoxycoumarin were purchased from the Aldrich Chemical Co. (Milwaukee, WI). SKF 525-A was a gift of the Smith Kline & French Laboratories (Philadelphia, PA). All other chemicals were purchased from the Sigma Chemical Co. (St Louis, MO).

Cytochrome P-450 isozyme LM_{4b} was purified from liver microsomes of trout pretreated with BNF according to the method of Miranda *et al.* [19]. Cytochrome P-450 LM_2, antibodies to purified trout liver cytochrome P-450 isozymes (LM_2-IgG and LM_{4b}-IgG), and rat liver microsomes were prepared as previously described [20, 21].

An acid reaction mixture (RXM) was generated by the addition of I3C to 0.05 N HCl as described by Bradfield and Bjeldanes [10]. RXM was analyzed by C_{18} reverse-phase HPLC using the following mobile phase system: a linear gradient of 10 to 55% acetonitrile in 33 μM potassium acetate, pH 5, in 25 min; isocratic at 55% acetonitrile for 10 min; a linear gradient of 55 to 100% acetonitrile in 15 min; held at 100% acetonitrile for 5 min. Authentic 3,3′-diindolylmethane used as a marker in HPLC analyses of RXM was a gift of Dr L. F. Bjeldanes (University of California, Berkeley, CA).

Animals and diets. Mt Shasta strain rainbow trout were fed a casein-gelatin based semipurified diet [22], or the semipurified diet containing 500 ppm BNF or 500–2000 ppm I3C for 8 days. The fish received no food on day 9 of the pretreatment schedule and were killed on day 10. Three days are required for food to pass from the stomach to the distal regions of the gut [23], and a bolus of food was found in the stomach at termination. For the UDP-glucuronyl-transferase (UDPGT) assay, the fish were fed control, 500 ppm BNF, or 2000 ppm I3C diet for 2 weeks and were not fasted prior to termination. The feeding durations of 2 weeks for UDPGT assay and 8 days for all other enzyme assays were determined experimentally to provide maximum induction by BNF (data not shown), a 3-methylcholanthrene type inducer. The enzyme induction studies were conducted over an 11-week period in which the average body weights of the stock trout increased from 91 to 208 g. The average body weights of treated animals were similar to those of controls at termination.

Preparation of microsomal and cytosolic enzymes. Trout microsomes were prepared from pooled livers (three pools of ten livers each per determination) by differential ultracentrifugation [13]. The final microsomal pellet was resuspended in 0.1 M potassium phosphate, pH 7.25, 20% glycerol, and 1 mM EDTA, and protein concentrations were determined by the method of Lowry *et al.* [24]. Liver microsomes were assayed for enzyme activities immediately after isolation. Aliquots of the microsome suspensions were frozen in liquid nitrogen and stored at −80° until analysis of cytochrome P-450LM_2 and P-450LM_{4b} isozyme contents. The supernatant fraction from the 100,000 *g* centrifugation was frozen in liquid nitrogen and stored at −80° until analysis of glutathione *S*-transferase (GST) activity the following day. Rat liver microsomes were prepared from untreated animals by the method of Guengerich [25].

Enzyme assays. Enzyme assays were carried out at 25°, that is, within range of optimum incubation temperatures of 25–32° for trout enzymatic activities [26]. Microsomal 7-ethoxycoumarin *O*-deethylase (ECOD) activity was determined by the fluorometric method of Srivastava *et al.* [27], and 7-ethoxyresorufin *O*-deethylase (EROD) activity was determined by the method of Burke *et al.* [28]. UDPGT activity was quantified by modifications of the method of Coughtrie *et al.* [29]. The reaction mixture contained 50 mM Tris-maleate, pH 7.4, 10 mM $MgCl_2$, 0.8% digitonin, 2.7 mM [^{14}C]UDPGA (0.25 μCi), 2 mg microsomal protein, and 1 mM 1-naphthol or testosterone as substrate. Radioactive glucuronides were separated from unconjugated [^{14}C]UDPGA by reverse-phase HPLC [30], and radioactivity was measured with a Beckman 171 on-line radioisotope detector. Glucuronide conjugates were identified by HPLC coelution with reference glucuronide standards and by hydrolysis with β-glu-

curonidase. GST activity was measured by the method of James *et al.* [31] using [^{3}H]styrene oxide as substrate, which had been used successfully to measure trout liver GT activity [32].

Cytochrome P-450 quantitation. Total microsomal cytochrome P-450 content was determined by the spectrophotometric method of Estabrook *et al.* [33]. Liver microsomal proteins were separated by SDS–PAGE [34], and the specific contents of cytochrome P-450LM$_2$ and P-450LM$_{4b}$ isozymes were assayed by the Western blotting-immunoquantitation method of Burnette [35].

In vivo *AFB$_1$–DNA binding.* Fish (6–8 g body wt) were fed either the control or the 2000 ppm I3C diet for 8 days, fasted on day 9, and then given [^{3}H]AFB$_1$ in ethanol (2.6 nmol/kg body wt; sp. act. 10.5 Ci/mmol) by i.p. injection on day 10. Livers were removed 24 hr after AFB$_1$ injection, and DNA was isolated from three pools of ten livers per treatment and analyzed as described previously [14].

Binding of AFB1-8,9-Cl$_2$ to DNA. The reaction mixture contained 1 mg calf thymus DNA, 100 μM EDTA, and 0.35 mM I3C or RXM in a final volume of 1.0 ml of 50 μM Na_2PO_4, pH 7.4. After a 2-min preincubation, 0.06 pmol [^{3}H]AFB$_1$-8,9-Cl$_2$ in 20 μl DMSO was added to initiate the reaction, and the mixture was incubated for 60 min. Any residual reaction was stopped by immersion of the incubation tubes into liquid nitrogen. DNA was isolated by phenolic extraction [36], and the specific activity of the isolated DNA was determined by liquid scintillation counting. For analysis of microsome-mediated binding of AFB$_1$ to DNA, the above reaction mixture was altered by the addition of 3 mg microsomal protein and 1 mg NADPH, and AFB$_1$-Cl$_2$ was replaced by 2 nmol [^{3}H]AFB$_1$. The 0.35 mM RXM concentration used in this study was based on extrapolations from the amount of the radioactivity found in the liver of trout 48 hr after oral administration of 40 mg [^{3}H]I3C/kg body weight [23].

Determination of kinetic parameters of AFB$_1$-DNA binding. Detailed analysis of the microsome-mediated binding of AFB$_1$ to DNA was conducted by the method of Williams and Buhler [20]. The reaction mixture, contained in a final volume of 250 μl, was: 150 μg calf thymus DNA, 0.2 mg microsomal protein, 0.05 μmol EDTA, 75 nmol $MgCl_2$, 25 μmol Tris–HCl, pH 8.0, 3.5 to 350 μM RXM (in 10 μl ethanol), and 1.25 to 100 μM [^{3}H]AFB$_1$ (1.08 μCi/sample). The reaction was initiated by the addition of 0.25 μmol NADPH and proceeded for 30 min in the dark at 29° and 37° for trout and rat microsomes, respectively. The reaction tubes were transferred to ice, and 50 μl of 10% SDS was added to terminate the reaction. The upper aqueous phase obtained after phenolic extraction was applied to a Whatman GF/C filter, and the radioactivity retained on the filter after exhaustive trichloroacetic acid and ethanol washes was determined by liquid scintillation counting. The kinetic parameters were obtained by linear regression analyses of the double-reciprocal Lineweaver–Burk plots. The incubation temperature of 29° was selected to compare the present results with previously determined K_m and V_{max} for trout liver microsome-mediated binding of AFB$_1$ to DNA at this temperature [36]. Use of incubation temperatures near the hatchery water temperature of 12° would be expected to give higher K_m and lower V_{max} values, but not to alter the conclusions reached on RXM inhibition.

Statistical analyses. Data were analyzed by one-way analysis of variance, and differences between specific means were compared using Student's *t*-test. Differences with $P < 0.05$ were considered significant.

RESULTS

The effects of dietary I3C treatment on liver microsomal ECOD and EROD activities and total cytochrome P-450 content were examined to explore the possibility that I3C exerts its anti-carcinogenic actions by inducing liver cytochrome P-450-dependent enzymes. In contrast to dietary administration of BNF, which significantly ($P < 0.05$) induced trout liver microsomal ECOD and EROD activities and total cytochrome P-450 content, I3C administration at the dietary levels tested had no significant inductive effect (Fig. 1). Analyses of the specific cytochrome P-450 isozymes LM$_2$ and LM$_{4b}$ by Western-blotting and immunoquantitation provided results that were consistent with data from assays of ECOD and EROD activities and total cytochrome P-450 content. The content of cytochrome P-450LM$_{4b}$ in liver microsomes of BNF-treated fish was markedly higher than that of control fish, but no significant differences were found in the LM$_{4b}$ contents in liver microsomes of I3C-pretreated fish and controls (Fig. 2). The contents of cytochrome P-450LM$_2$, which does not respond to 3-methylcholanthrene-type inducers, were similar among the control, BNF-treated and I3C-treated groups (data not shown).

The effects of BNF and I3C dietary treatments on the activities of liver conjugating enzymes UDPGT and GST are presented in Fig. 3.Gucuronide formation is a major route for elimination of chemical carcinogens in the trout [30], and induction of the conjugating enzyme UDPGT could facilitate the removal of the carcinogen from its sites of action. As shown in Fig. 3, control liver microsomes mediated the formation of glucuronic acid conjugates of 1-naphthol and testosterone in incubations containing [^{14}C]UDPGA. A significant ($P < 0.05$) increase in liver UDPGT activity was found after BNF treatment, whereas I3C treatment did not induce the UDPGT activity. Although glutathione conjugation generally is a minor route of elimination in the trout [37], I3C-induced GST activity has been associated with reduced carcinogen-DNA binding *in vivo* in some rodent species [38]. In the present study, the levels of GST activity in liver cytosolic preparations from control trout were similar to those from BNF-treated and I3C-treated trout.

The 8-day I3C pretreatment schedule was effective in reducing AFB$_1$–DNA binding *in vivo*. Fish fed 2000 ppm I3C prior to AFB$_1$ exposure had markedly lower total liver DNA binding (0.41 ± 0.03 pmol bound/mg DNA) compared to controls (0.98 ± 0.07 pmol bound/mg DNA).

The hypothesis that I3C or one of its products may act as scavengers for electrophilic or radical carcinogen metabolites was tested using AFB$_1$-8,9-

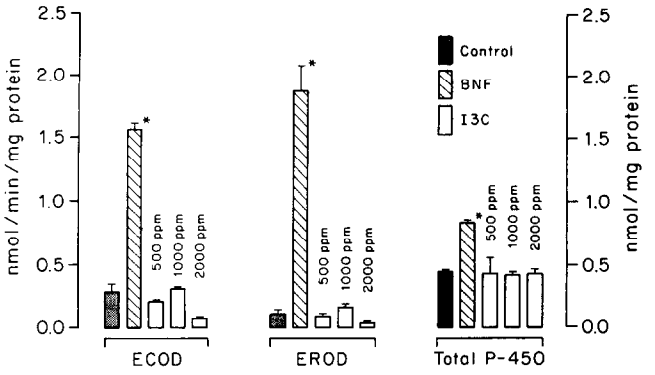

Fig. 1. Liver microsomal monooxygenase activities and total cytochrome P-450 concentration in rainbow trout exposed to control. BNF or I3C diet for 8 days. Each value is the mean ± SD of three pools of ten animals each. Abbreviations: ECOD, 7-ethoxycoumarin *O*-deethylase; and EROD, 7-ethoxyresorufin *O*-deethylase. Key: (*) significantly ($P < 0.05$) different from controls.

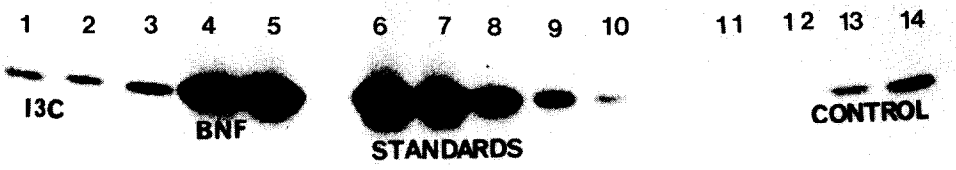

Fig. 2. Western blot of liver microsomal protein isolated from trout fed control, 500 ppm BNF, or 2000 ppm I3C diet. Microsomal protein samples were separated by SDS–PAGE, electrophoretically transferred to nitrocellulose, and probed with anti-trout LM_{4b}-IgG followed by [^{125}I]protein A. Lanes 1, 2, and 3 contained 10 μg microsomal protein from I3C-treated trout; lanes 4 and 5 contained 10 μg microsomal protein from BNF-treated trout; lanes 6, 7, 8, 9, and 10 contained 1.6, 1.2, 0.8, 0.4, and 0.2 nmol purified trout cytochrome P-450LM_{4b}, respectively; lanes 11 and 12 contained 5 μg microsomal protein from untreated trout; and lanes 13 and 14 contained 10 μg microsomal protein from untreated trout.

Cl_2, which is a direct acting model of the putative ultimate carcinogen AFB_1-8,9-epoxide [18], as the source of electrophiles. Covalent binding of AFB_1-8,9-Cl_2 to calf thymus DNA *in vitro* proceeded without the need for metabolic activation (Fig. 4a). Addition of I3C or RXM to the incubation did not inhibit the covalent binding of AFB_1-8,9-Cl_2 to DNA. In subsequent studies (Fig. 4b), covalent binding of reactive AFB_1 metabolites to calf thymus DNA was assayed after substituting AFB_1-8,9-Cl_2 with AFB_1 and liver microsomes as the electrophile-generating system. The covalent binding of AFB_1 metab-

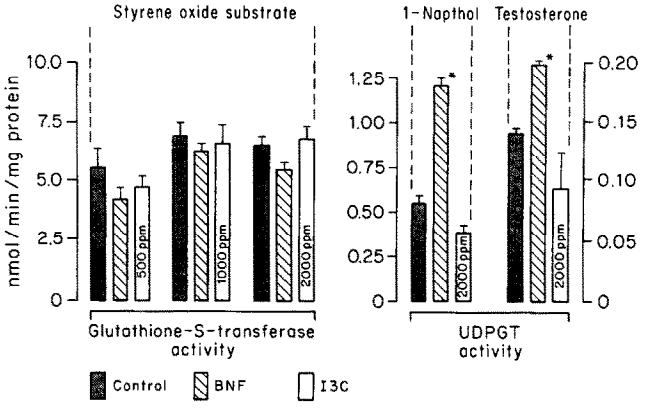

Fig. 3. Liver glutathione *S*-transferase (GST) and uridine diphosphate-glucuronyl-transferase (UDPGT) activities in trout fed control, BNF, or I3C diet for 8 days (GST assay) or 2 weeks (UDPGT assay). Each value is the mean ± SD of three pools of ten animals each. Key: (*) significantly ($P < 0.05$) different from controls.

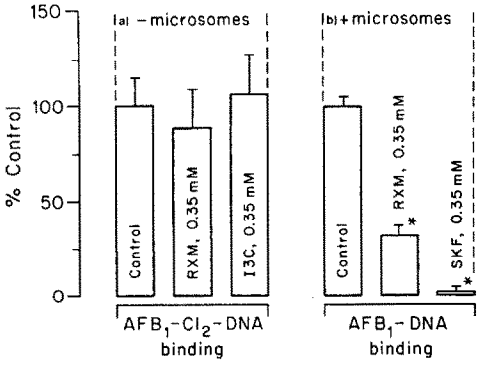

Fig. 4. Effects of addition of I3C, I3C reaction product (RXM), or SKF 525-A on covalent binding of AFB_1-8,9-Cl_2 and AFB_1 to calf thymus DNA *in vitro*. Values are the means ± SD for three incubations. Key: (*) significantly ($P \leq 0.05$) different from controls. Control (100%) covalent binding levels were 26 ± 2 fmol/mg DNA for AFB_1-8,9-Cl_2 (−microsomes) and 132 ± 8 pmol/mg DNA for AFB_1 (+microsomes).

olites to calf thymus DNA was decreased significantly ($P \leq 0.05$) by addition of either 0.35 mM RXM or the P-450 inhibitor SKF 525-A to the incubation mixture (Fig. 4b). Covalent binding of AFB_1 to DNA was not detected in incubations containing boiled microsomes or no added NADPH (data not shown).

Kinetic constants for the microsome-dependent binding of AFB_1 to DNA *in vitro* were determined to examine the effect of RXM on DNA binding (Fig. 5). After incubation with trout liver microsomes, an apparent K_m value of 35 μM and a corresponding V_{max} value of 11.4 pmol AFB_1–DNA adduction per mg protein per min were obtained from double-reciprocal Lineweaver–Burk plots at [^{3}H]AFB_1 concentrations between 1.25 and 100 μM (minus RXM; Fig. 5a). RXM at 3.5, 35, and 350 μM inhibited the activation of AFB_1 mediated by trout liver microsomes, such that at each RXM concentration, a higher [^{3}H]AFB_1 concentration was needed to reach the maximum velocity of the reaction. The presence of RXM in the incubation increased the apparent K_m for the AFB_1–DNA binding reaction without changing the V_{max}; the apparent K_m values at 3.5, 35, and 350 μM RXM were 38, 66, and 86 μM respectively. Replot of the slope of the double-reciprocal plot against inhibitor concentration yielded a hyperbolic curve (Fig. 5a inset), suggesting that inhibition by I3C was of the partially competitive type. In contrast to the inhibitory action of RXM, I3C at 350 μM did not affect the activation of AFB_1 (data not shown). Kinetic constants for the AFB_1–DNA binding reaction also were obtained using rat liver microsomes to determine whether the inhibitory effect of RXM was restricted to the trout model. RXM also inhibited the activation of AFB_1 by rat liver microsomes. The apparent K_m value increased from 10 to 30 μM when 350 μM RXM was added to the 37° control incubation (Fig 5b; V_{max}, 1.4 pmol AFB_1–DNA adduction per mg protein per min).

The reaction of I3C with HCl produces a reaction mixture which consists of several acid condensation products [10]. A preliminary investigation of the I3C acid treatment products was undertaken by reverse-phase HPLC in this study to characterize the individual products from the reaction mixture that may contribute to the observed inhibitory action. Three major UV absorbing peaks were detected (Fig. 6), and these were identified tentatively by positive fast atom bombardment mass spectrometry as the 3,3′-diindolylmethane salt and two cyclic trimers of 3-methylindole. The identities of the compounds present in the three major UV absorbing peaks corresponded to the structures of acid condensation products reported by Bradfield and Bjeldanes [10].

DISCUSSION

The inhibition of AFB_1-induced hepatocarcinogenesis in trout by I3C has been associated with attenuated AFB_1–DNA binding in the target organ [14, 39]. Protection against carcinogen–DNA binding potentially may result from one or more of three mechanisms: (a) inhibition of carcinogen activation pathways, (b) induction of detoxification pathways,

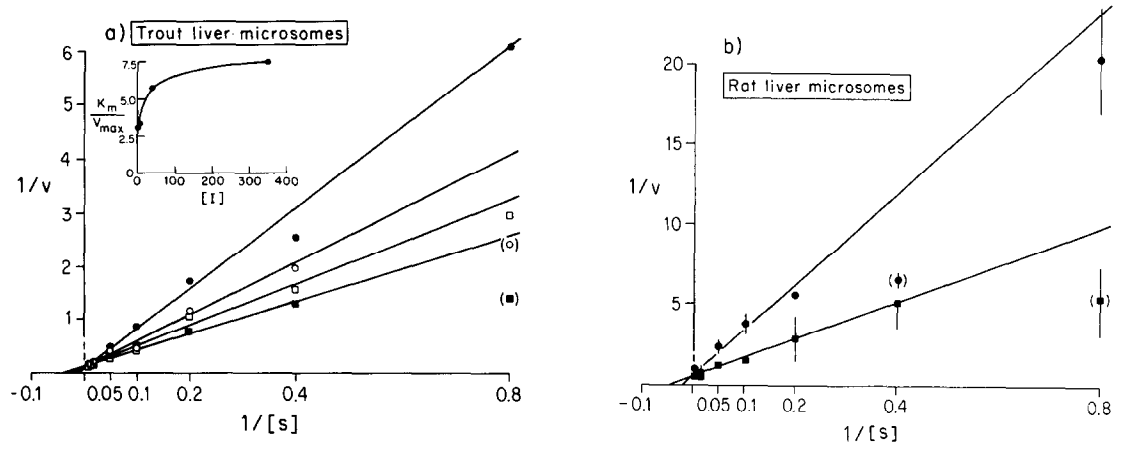

Fig. 5. Double-reciprocal plots of the inhibition of microsome-mediated AFB_1-DNA binding (a, trout liver microsomes; b, rat liver microsomes). Microsomes were incubated with 1.25 to 100 μM [^{3}H]AFB_1 in the presence of 0 (■), 3.5 (□), 35 (○) or 350 (●) μM I3C reaction mixture. V, reaction rate (pmol AFB_1–DNA adduction per mg protein per min); $[S]$, substrate AFB_1 concentration (μM); $[I]$, inhibitor RXM concentration. Figure 5a inset, replot of data in Fig. 5, K_m/V_{max} as a function of $[I]$.

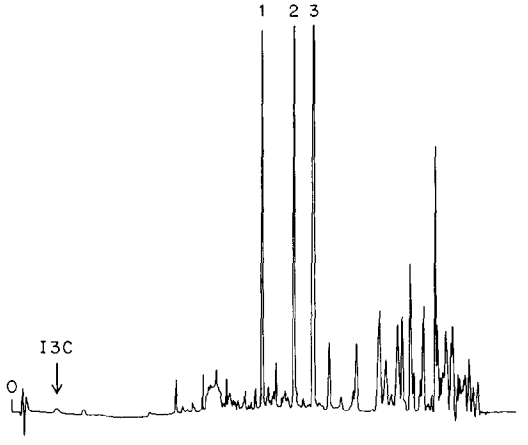

Fig. 6. HPLC elution profile (UV at 280 nm) of an I3C reaction mixture sample generated by the addition of I3C to 0.05 N HCl. The three major UV absorbing peaks were identified tentatively by positive fast atom bombardment mass spectrometry (3-nitrobenzyl alcohol carrier) as the 3,3′-diindolylmethane salt (peak 1, $[M + H]^+ = 246$) and two cyclic trimers of 3-methylindole (peak 2, $[M + H]^+ = 386$; peak 3, $[M + H]^+ = 382$).

and (c) electrophile/radical scavenging via direct carcinogen–inhibitor interactions.

In the rainbow trout, two principal cytochrome P-450 monooxygenase pathways are involved in the metabolism of AFB_1. Cytochrome P-450LM_2, a constitutive isozyme with high activity toward AFB_1, forms the putative ultimate carcinogen AFB_1-8,9-epoxide as the major metabolite, while cytochrome P-450LM_{4b}, a BNF-inducible isozyme with lower overall activity toward AFB_1, produces the less potent carcinogen AFM_1 as the major metabolite [20]. In addition to cytochrome P-450-dependent metabolism, AFB_1 is reduced to aflatoxicol (AFL) by cytosolic enzymes in the trout liver. Previous studies have demonstrated that I3C at a dietary level of 500 ppm was ineffective in inducing liver cytochrome P-450-dependent monooxygenase activities in the trout [13]. Only minor changes in *in vivo* AFB_1 phase I metabolism were found in trout prefed 2000 ppm I3C [39], and this was reflected in recent studies where quantitative changes only were observed in AFB_1–DNA adduct profiles from control and I3C-treated animals [14]. The lack of an inductive effect at 500–2000 ppm I3C on both liver cytochrome P-450 monooxygenase activities and specific cytochrome P-450 isozyme contents found in the present study confirms and extends previous findings in trout, and indicates that induction of the cytochrome P-450-dependent monooxygenases is unlikely to be the central mechanism of I3C inhibition of AFB_1 hepatocarcinogenesis in this species.

I3C has been shown in some rodent species to induce cytochrome P-448 monooxygenases, and Bradfield and Bjeldanes [10] have postulated a mechanism of I3C inhibition based on altered carcinogen metabolism resulting in lower production of electrophilic metabolites. However, inductive effects in the liver were obtained only in rats fed extremely high dietary levels of 5000 and 7500 ppm I3C [40]. The absence of detectable cytochrome P-450 induction in liver in the present study at I3C dietary levels that have been shown to inhibit hepatocarcinogenesis [9] and attenuate AFB_1–DNA binding *in vivo* ([14; present results) further indicates that other mechanisms may be in operation in I3C anti-carcinogenesis. The preabsorption metabolism by intestinal monooxygenases, which are induced by I3C dietary levels as low as 50 ppm, has been suggested to be a possible mode of inhibitor action in rats [41]. Although there has been no direct demonstration of an effect of intestinal enzyme induction on AFB_1 uptake and distribution to liver, such induction in extrahepatic tissues would be consistent with our previous observation of decreased plasma and hepatic levels of AFB_1 [39].

Dietary I3C has been shown to increase the level of AFL-M_1-glucuronide in the bile of fish exposed to AFB_1 [39]. Higher production of biliary AFL-M_1-

glucuronide may result from induction of liver UDPGT-activities or from elevated substrate AFL-M_1 production. The UDPGT substrate AFL-M_1 is produced by the hydroxylation of AFL or the reduction of AFM_1. Results of the present study show that I3C has no detectable inductive effect on trout liver UDPGT activity, as measured by the substrates 1-naphthol and testosterone. Attempts to measure this activity using AFL as the substrate revealed only very low activity *in vitro*.

Although enhancement of glutathione conjugation, a minor pathway in trout, potentially could contribute to the detoxification of AFB_1, the results of the present and other studies [37, 42] indicate that trout liver GST is not induced by treatment with the anti-carcinogens I3C, BNF, polychlorinated biphenyl, or butylated hydroxyanisole.

The possible scavenging of electrophilic or free radical intermediates of chemical carcinogens by I3C or its reaction products appears not to be of functional significance in the inhibition of AFB_1–DNA binding. In the present study, neither I3C nor RXM at various concentrations prevented the *in vitro* DNA binding of AFB_1-8,9-Cl_2. AFB_1-8,9-Cl_2 is expected to form a resonance-stabilized carbonium ion like that derived from AFB_1-8,9-epoxide, and Wood *et al.* [43] have demonstrated that the major adducts formed in the reaction of AFB_1-8,9-Cl_2 with calf thymus DNA are essentially identical to the DNA adducts formed from AFB_1 *in vivo* and *in vitro*. This, together with the failure to detect any AFB_1-I3C adduct *in vitro* or *in vivo* [39], does not support the idea of electrophile scavenging by I3C or its reaction products.

The present kinetic analyses of microsome-mediated AFB_1–DNA binidng indicate that I3C reaction products produced under acidic conditions *in vitro* act as inhibitors in the metabolic activation of AFB_1. Although it has not been determined which of the RXM components are active in inhibiting AFB_1 activation, HPLC profiles of I3C products extracted from trout liver 24–72 hr after oral administration of I3C [23] contained certain I3C products which also were found in RXM in the present study. The I3C-associated inhibition of AFB_1 activation may increase the percentage of the AFB_1 metabolized by competing pathways to the less potent carcinogens AFM_1 and AFL. This hypothesis is supported by data showing increased AFM_1 production and depressed AFB_1–DNA binding in isolated hepatocytes from I3C-pretreated fish [39]. As a result of inhibiting the AFB_1 activation pathway, the previously observed increased recovery of AFL-M_1-glucuronide in the bile of I3C-treated trout [39], therefore, may reflect the increased production of AFL-M_1 and its precursor AFM_1 rather than the induction of cytochrome P-$450LM_{4b}$ or UDPGT. The inhibition by I3C or AFB_1 activation was not a peculiarity of the trout metabolic system. The present finding that I3C products also inhibited rat liver microsome-mediated AFB_1 activation suggests that this mechanism may contribute to I3C anti-carcinogenesis in mammalian species.

In summary, several possible mechanisms of I3C anti-carcinogenesis were examined in the present study, and the results indicate that the inhibition by I3C products of microsome-activated AFB_1 binding to DNA may be of significant importance in I3C inhibition of hepatocarcinogenesis in trout and other species. The mechanism of inhibition by I3C products differs from the anti-carcinogenic mechanisms of BNF; although both I3C and BNF reduce AFB_1–DNA binding *in vivo*, the effects of BNF are mediated in part by induction of cytochrome P-$450LM_{4b}$ and consequently a greatly enhanced production of the less potent carcinogen AFM_1 [8].

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REFERENCES

1. Stoewsand GS, Babish JG and Wimberly HC, Inhibition of hepatic toxicities from polybrominated biphenyls and aflatoxin B_1 in rats fed cauliflower. *J Environ Pathol Toxicol* **2**: 399–406, 1978.
2. Boyd JN, Babish JG and Stoewsand GS, Modification by beet and cabbage diets of aflatoxin B_1-induced rat plasma α-foetoprotein elevation, hepatic tumorigenesis, and mutagenicity of urine. *Food Chem Toxicol* **20**: 47–52, 1982.
3. Wattenberg LW, Inhibition of neoplasia by minor dietary constituents. *Cancer Res* (Suppl) **43**: 2448S–2453S, 1983.
4. Aspry KE and Bjeldanes LF, Effects of dietary broccoli and butylated hydroxyanisole on liver-mediated metabolism of benzo[*a*]pyrene. *Food Chem Toxicol* **21**: 133–142, 1983.
5. Whitty JP and Bjeldanes LF, The effects of dietary cabbage on xenobiotic metabolizing enzymes and binding of aflatoxin B_1 to hepatic DNA in rats. *Food Chem Toxicol* **25**: 581–587, 1987.
6. McDanell R, McLean AEM, Hanley AB, Heaney RK and Fenwick GR, Chemical and biological properties of indole glucosinolates (glucobrassicins): a review. *Food Chem Toxicol* **26**: 59–70, 1988.
7. Wattenberg LW and Loub WD, Inhibition of polycyclic aromatic hydrocarbon-induced neoplasia by naturally occurring indoles. *Cancer Res* **38**: 1410–1413, 1978.
8. Nixon JE, Hendricks JD, Pawlowski NE, Pereira CB, Sinnhuber RO and Bailey GS, Inhibition of aflatoxin B_1 carcinogenesis in rainbow trout by flavone and indole compounds. *Carcinogenesis* **5**: 615–619, 1984.
9. Dashwood RH, Arbogast DN, Fong AT, Pereira C, Hendricks JD and Bailey GS, Quantitative interrelationships between aflatoxin B1 carcinogen dose, indole-3-carbinol anti-carcinogen dose, target organ DNA adduction and final tumor response. *Carcinogenesis* **10**: 175–181, 1989.
10. Bradfield CA and Bjeldanes LF, Structure–activity relationships of dietary indoles: a proposed mechanism of action as modifiers of xenobiotic metabolism. *J Toxicol Environ Health* **21**: 311–323, 1987.
11. Shertzer HG, Protection by indole-3-carcinol against covalent binding of benzo[*a*]pyrene metabolites to mouse liver DNA and protein. *Food Chem Toxicol* **21**: 31–35, 1983.

12. Shertzer HG, Indole-3-carbinol protects against covalent binding of benzo[*a*]pyrene and *N*-nitrosodimethylamine metabolites to mouse liver macromolecules. *Chem Biol Interact* **48**: 81–90, 1984.
13. Eisele TA, Bailey GS and Nixon JE, The effect of indole-3-carbinol, an aflatoxin B_1 hepatocarcinoma inhibitor, and other indole analogs on the rainbow trout hepatic mixed function oxidase system. *Toxicol Lett* **19**: 133–138, 1983.
14. Dashwood RH, Arbogast DN, Fong AT, Hendricks JD and Bailey GS, Mechanisms of anti-carcinogenesis by indole-3-carbinol: detailed *in vivo* DNA binding dose–response studies after dietary administration with aflatoxin B1. *Carcinogenesis* **9**: 427–432, 1988.
15. Shertzer HG and Tabor MW, Nucleophilic index value: implication in the protection by indole-3-carbinol from *N*-nitrosodimethylamine cyto and genotoxicity in mouse liver. *J Appl Toxicol* **8**: 105–110, 1988.
16. Shertzer HG, Berger ML and Tabor MW, Intervention in free radical mediated hepatotoxicity and lipid peroxidation by indole-3-carbinol. *Biochem Pharmacol* **37**: 333–338, 1988.
17. Loveland PM, Coulombe RA, Libby LM, Pawlowski NE, Sinnhuber RO, Nixon JE and Bailey GS, Identification and mutagenicity of aflatoxin-M_1 produced by metabolism of aflatoxin B_1 and aflatoxicol by liver fractions from rainbow trout (*Salmo gairdneri*) fed β-naphthoflavone. *Food Chem Toxicol* **21**: 557–562, 1983.
18. Swenson DH, Miller JA and Miller EC, The reactivity and carcinogenicity of aflatoxin B_1-2,3-dichloride, a model for the putative 2,3-oxide metabolite of aflatoxin B_1. *Cancer Res* **35**: 3811–3823, 1975.
19. Miranda CL, Wang J-L, Henderson MC and Buhler DR, Purification and characterization of hepatic steroid hydroxylases from untreated rainbow trout. *Biochim Biophys Acta*, in press.
20. Williams DE and Buhler DR, Purified form of cytochrome P-450 from rainbow trout with high activity toward conversion of aflatoxin B_1 to aflatoxin B_1-2,3-epoxide. *Cancer Res* **43**: 4752–4756, 1983.
21. Williams DE and Buhler DR, benzo[*a*]pyrene-hydroxylase catalyzed by purified isozymes of cytochrome P-450 from β-naphthoflavone-fed rainbow trout. *Biochem Pharmacol* **33**: 3743–3753, 1984.
22. Sinnhuber RO, Hendricks JD, Wales JH and Putnam GB, Neoplasma in rainbow trout, a sensitive animal model for environmental carcinogenesis. *Ann NY Acad Sci* **298**: 389–408, 1977.
23. Dashwood RH, Uyetake L, Fong AT and Bailey GS, The *in vivo* disposition of the natural anti-carcinogen indole-3-carbinol after p.o. administration to rainbow trout. *Food Chem Toxicol*, in press.
24. Lowry, OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
25. Guengerich FP, Microsomal enzymes involved in toxicology-analysis and separation. In: *Principles and Methods of Toxicology* (Ed. Hayes AW), pp. 609–634. Raven Press, New York, 1982.
26. Pedersen MG, Hershberger WK and Juchau MR, Metabolism of 3,4-benzpyrene in rainbow trout (*Salmo gairdneri*). *Bull Environ Contam Toxicol* **12**: 481–486, 1974.
27. Srivastava SP, Seth PK and Mukhtar H, 7-Ethoxycoumarin *O*-de-ethylase activity in rat brain microsomes. *Biochem Pharmacol* **32**: 3657–3660, 1983.
28. Burke MD, Prough RA and Mayer RT, Characteristics of a microsomal cytochrome P-448 mediated reaction. *Drug Metab Dispos* **5**: 1–7, 1976.
29. Coughtrie MWH, Burchell B and Bend JR, A general assay for UDPglucuronosyltransferase activity using polar amino-cyano stationary phase HLPC and UDP[U-^{14}C]glucuronic acid. *Anal Biochem* **159**: 198–205, 1986.
30. Loveland PM, Nixon JE and Bailey GS, Glucuronides in bile of rainbow trout (*Salmo gairdneri*) injected with [^{3}H]aflatoxin B_1 and the effects of dietary β-naphthoflavone. *Comp Biochem Physiol* **78C**: 13–19, 1984.
31. James MO, Fouts JR and Bend JR, Hepatic and extrahepatic metabolism, *in vitro*, of an epoxide (8-^{14}C-styrene oxide) in the rabbit. *Biochem Pharmacol* **25**: 187–191, 1976.
32. Eisele TA, Coulombe RA, Pawlowski NE and Nixon, JE, The effects of route of exposure and combined exposure of mixed function oxidase inducers and suppressors on hepatic parameters in rainbow trout (*Salmo gairdneri*). *Aquat Toxicol* **5**: 211–226, 1984.
33. Estabrook RW, Peterson J, Baron J and Hildebrandt AG, The spectrophotometric measurement of turbid suspensions of cytochromes associated with drug metabolism. *Methods Pharmacol* **2**: 303–350, 1972.
34. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227**: 680–685, 1970.
35. Burnette WN, "Western blotting". Electrophoretic transfer of proteins from sodium dodecyl sulfate–polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem* **112**: 195–203, 1981.
36. Bailey GS, Williams DE, Wilcox JS, Loveland PM, Coulombe RA and Hendricks JD, Aflatoxin B1 carcinogenesis and its relation to DNA adduct formation and adduct persistence in sensitive and resistant salmonid fish. *Carcinogenesis* **9**: 1919–1926, 1988.
37. Valsta LM, Hendricks JD and Bailey GS, The significance of glutathione conjugation for aflatoxin B_1 metabolism in rainbow trout and coho salmon. *Food Chem Toxicol* **26**: 129–135, 1988.
38. Sparnins VL, Venegas PL and Wattenberg LW, Glutathione *S*-transferase activity: enhancement by compounds inhibiting chemical carcinogenesis and by dietary constituents. *J Natl Cancer Inst* **68**: 493–496, 1982.
39. Goeger DE, Shelton DW, Hendricks JD and Bailey GS, Mechanisms of anti-carcinogenesis by indole-3-carbinol: effect on the distribution and metabolism of aflatoxin B_1 in rainbow trout. *Carcinogenesis* **7**: 2025–2031, 1986.
40. Babish JG and Stoewsand GS, Effect of dietary indole-3-carbinol on the induction of the mixed-function oxidase of rat tissue. *Food Cosmet Toxicol* **16**: 151–155, 1978.
41. Bradfield CA and Bjeldanes LF, Effect of dietary indole-3-carbinol on intestinal and hepatic mono-oxygenase, glutathione *S*-transferase and epoxide hydrolase activities in the rat. *Food Chem Toxicol* **22**: 977–982, 1984.
42. Goeger DE, Shelton DW, Hendricks JD, Pereira C and Bailey GS, Comparative effect of dietary butylated hydroxyanisole and β-naphthoflavone on aflatoxin B1 metabolism, DNA adduct formation, and carcinogenesis in rainbow trout. *Carcinogenesis* **9**: 1793–1800, 1988.
43. Wood ML, Smith JRL and Garner RC, Structural characterization of the major adducts obtained after reaction of an ultimate carcinogen aflatoxin B_1-dichloride with calf thymus DAN *in vitro*. *Cancer Res* **48**: 5391–5396, 1988.