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The Anticarcinogen 3,3′-Diindolylmethane is an Inhibitor of Cytochrome P-450

D. M. Stresser, L. F. Bjeldanes, G. S. Bailey, and D. E. Williams*

Toxicology Program, Marine/Freshwater Biomedical Sciences Center, Department of Food Science and Technology, Oregon State University, Corvallis, OR 97331-6602 (D.M.S., D.E.W., G.S.B.); Department of Nutritional Sciences, University of California, Berkeley, CA 94720 (L.F.B.)

ABSTRACT: Dietary indole-3-carbinol inhibits carcinogenesis in rodents and trout. Several mechanisms of inhibition may exist. We reported previously that 3,3′-diindolylmethane, an in vivo derivative of indole-3-carbinol, is a potent noncompetitive inhibitor of trout cytochrome P450 (CYP) 1A-dependent ethoxyresorufin *O*-deethylase with K_i values in the low micromolar range. We now report a similar potent inhibition by 3,3′-diindolylmethane of rat and human CYP1A1, human CYP1A2, and rat CYP2B1 using various CYP-specific or preferential activity assays. 3,3′-Diindolylmethane also inhibited in vitro CYP-mediated metabolism of the ubiquitous food contaminant and potent hepatocarcinogen, aflatoxin B_1. There was no inhibition of cytochrome *c* reductase. In addition, we found 3,3′-diindolylmethane to be a substrate for rat hepatic microsomal monooxygenase(s) and tentatively identified a monohydroxylated metabolite. These observations indicate that 3,3′-diindolylmethane can inhibit the catalytic activities of a range of CYP isoforms from lower and higher vertebrates in vitro. This broadly based inhibition of CYP-mediated activation of procarcinogens may be an indole-3-carbinol anticarcinogenic mechanism applicable to all species, including humans. © 1995 John Wiley & Sons, Inc.

KEY WORDS: **Indole-3-Carbinol, 3,3′-Diindolylmethane, Aflatoxin B_1, Cytochrome P-450, CYP1A, CYP2B1.**

INTRODUCTION

Indole glucosinolates are found in high concentrations in the Cruciferae family of vegetables (1), whose members include cabbage, brussels sprouts, broccoli, and cauliflower. Glucobrassicin is the most abundant of all glucosinolates and, after enzymatic hydrolysis by the enzyme myrosinase, yields indole-3-carbinol (I3C),[1] glucose, and isothiocyanate anion. When fed to experimental animals, I3C has been shown to possess potent tumor-modulating properties, in most cases inhibiting tumor incidence (2–4), but with some protocols, I3C enhances tumor incidence (5,6) or preneoplastic lesions (7). The primary breakdown product of I3C in aqueous solution is a dimer of I3C, 3,3′-diindolylmethane (I33′) (Figure 1) (8), and I33′ can be prepared in high yield by simply refluxing I3C in neutral solution (9). Under acidic conditions, as found in the stomach, I3C quickly and irreversibly condenses with itself to yield, in addition to I33′, several other oligomeric derivatives as major products (10,11). Thus, I33′ has been found as a major product present in vivo after oral administration of I3C, whereas I3C was not detected (12,13) or was found in much lesser amounts (14). When aflatoxin B_1 (AFB_1) was coinjected with I33′ or I3C into rainbow trout embryos, a profound reduction in hepatic AFB_1-DNA binding and tumor incidence was observed (15), but only in animals given I33′. Wattenberg and Loub (16) observed that oral administration of I3C or I33′ to rats inhibited mammary tumor formation induced by 7,12-dimethylbenz[*a*]anthracene in female Sprague–Dawley rats and neoplasia of the forestomach induced by benzo[*a*]pyrene in female ICR/Ha mice. These data show that I33′ itself can be anticarcinogenic and that I33′ may be a major factor in the anticarcinogenicity observed with orally administered I3C.

Several anticarcinogenic mechanisms may exist for I33′. Previous studies have shown that oral (17,18) or i.p. (19) administration of I33′ can induce hepatic CYPs

*Present address: Worcester Foundation for Biomedical Research, Shrewsbury, MA 01545.

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Address correspondence to Dr. David E. Williams, Department of Food Science and Technology, Oregon State University, Corvallis, OR 97331-6602.

[1]**Abbreviations:** I3C, indole-3-carbinol; I33′, 3,3′-diindolylmethane; AFB_1, aflatoxin B_1; CYP, cytochrome P-450; QR, quinone reductase; UDPGT, uridine diphosphate glucuronosyl transferase; GST, glutathione *S*-transferase; PB, phenobarbital; BNF, β-naphthoflavone; AFM_1, aflatoxin M_1; AFG_1, aflatoxin G_1; AFM_1, aflatoxin M_1; AFQ_1, aflatoxin Q_1; AFP_1, aflatoxin P_1; PROD, pentoxyresorufin *O*-depentylase; EROD, ethoxyresorufin *O*-deethylase; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; BHA, butylated hydroxyanisole; EI, electron impact; PAH, polycyclic aromatic hydrocarbons; DBA, dibenz(*a*,*h*)anthracene.

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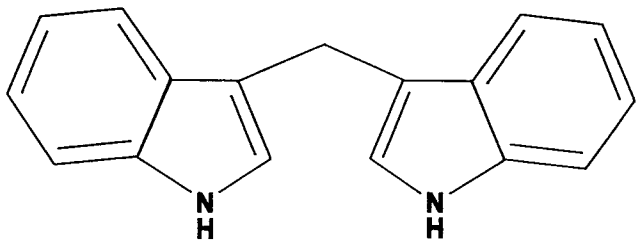

FIGURE 1. Structure of 3,3'-diindolylmethane (I33'), a primary acid condensation product of I3C.

1A1 and 1A2 or associated activities in rats. CYP1A[2] protein or activity can also be induced in trout embryos microinjected with I33' (20), or in primary cultures of rat and monkey hepatocytes exposed to I33' in the culture media (21,22). It has been suggested that this induction is responsible for altered carcinogen metabolism leading to reduced tumor incidence (23). In addition, administration of I33' to rats was found to induce CYP1A2 and microsomal estradiol 2-hydroxylation when given i.p. (19) and 4-androstenedione metabolism, when given i.p. or p.o. (24). Enhancement of estradiol 2-hydroxylation or 4-androstenedione metabolism are suggested as mechanisms responsible for the antiestrogenic effects of I3C given orally (19,24). Induction of phase II enzymes, such as quinone reductase (QR), uridine diphosphate glucuronosyl transferase (UDPGT), and glutathione *S*-transferase (GST), is an established mechanism of protection against carcinogenesis (25,26). Treatment of rat or monkey hepatocytes in primary cell culture with I33' resulted in induction of QR and UDPGT (12,22), but not GST (21,22). However, administration of I3C orally (and therefore also I33' and other I3C oligomers) to trout or mice failed to induce monooxygenases (27–29), GST, or UDPGT activity (28,30), yet protection against carcinogen-DNA binding or tumorigenicity was observed. It was recently observed that I33' could inhibit mutagenesis induced by AFB_1 8,9-epoxide in the *Salmonella* mutagenesis assay, suggesting a role for direct electrophile trapping as a means of protection (31).

CYP comprises a superfamily of enzymes that have various endo- and xenobiotics as substrates (32). In general, CYPs oxidize xenobiotics to more polar, nontoxic products; however, activation to carcinogenic metabolites is sometimes a sequela (33). When tested in vitro, I33' exhibited inhibition of CYP activity (20,24,34,35). To further characterize the effects of I33' as an in vitro inhibitor, we examined inhibition of rat and human CYP activities. In addition, we examined the potency of I33' to modulate the in vitro oxidative metabolism of the ubiquitous food contaminant and potent hepatocarcinogen AFB_1 (36). Limited studies on in vitro microsomal metabolism of [^{3}H]-I33' were also performed. Our results show that, in addition to trout, I33' is a potent nonspecific in vitro inhibitor of rat and human-CYP activities. In all three species, inhibition constants are in the low to submicromolar range. In vivo levels of I33' after administration of an anticarcinogenic dose of I3C to rat or trout are near inhibition constant values suggesting that, under these conditions, I33' should be an inhibitor of CYP in vivo as well. Thus, I33' inhibition of CYP-mediated activation may be an additional mechanism of anticarcinogenesis that could apply to all animal species, including humans.

[2]Berndtson and Chen (68) have recently isolated a second member of the trout CYP1A subfamily, induced by 3-methylcholanthrene and designated CYP1A2. Trout CYP1A2 is 96% identical in sequence to trout CYP1A1. This high sequence identity precludes us from positively identifying the purified protein as CYP1A1 and verifying the specificity of polyclonal antibodies raised against this protein. Therefore, in this article, we use the designation trout CYP1A.

MATERIALS AND METHODS

Animals

Male Fischer 344 rats were obtained as weanlings from Simonsen's (Gilroy, CA) and housed at the Laboratory Animal Resource Center at Oregon State University. Rats were maintained on a AIN-76A semipurified diet (U.S. Biochem. Corp., Cleveland, OH) and received diet and drinking water ad libitum. To induce CYP1A, rats received BNF (40 mg/kg) suspended in approximately 0.5 mL of corn oil, by i.p. injection for 4 consecutive days, were starved on day 4, and sacrificed on day 5. To induce CYP2B1, rats received drinking water containing 0.1% PB for seven consecutive days, were starved on day 7, and sacrificed on day 8 by CO_2 asphyxiation.

Chemicals

Ethoxyresorufin, pentoxyresorufin, and resorufin were obtained from Molecular Probes Inc. (Eugene, OR). Aflatoxins B_1, Q_1, M_1, and G_1, cytochrome *c*, NADPH, and BNF were obtained from Sigma Chemical Co. Ltd (St. Louis, MO). Aflatoxin B_1 8,9-epoxide-glutathione conjugate was a gift of Dr. David Eaton of the University of Washington. Aflatoxin B_1 8,9-epoxide was a gift of Dr. Thomas Harris of Vanderbilt University. I33' was synthesized according to the method of Leete and Marion (9) in a neutral solution and shown to be pure by HPLC. Acetanilide, 3-OH acetanilide, 4-OH acetanilide, and I3C were purchased from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were purchased from Sigma. The method of Dash-

wood *et al.* (37) was used to tritium-label I3C at the 5 position. The [^{3}H]-I3C was diluted with cold carrier to an approximate specific activity of 270 mCi/mmol. An acid reaction mixture of products originating from [^{3}H]-I3C was generated according to the method of Bjeldanes *et al.* (38). We isolated [^{3}H]-I33' from this mixture by HPLC using a Beckman ODS 5μ 4.6 × 250 mm analytical column (Palo Alto, CA). Starting solvent concentrations were 20% acetonitrile and 80% Milli-Q water (Millipore Corp., Bedford, MA). These conditions were held for 30 seconds before increasing to 85% acetonitrile over the next 29.5 minutes (linear gradient). After elution for 5 minutes with 85% acetonitrile, the most hydrophobic products were eluted by increasing to 100% acetonitrile over the following 5 minutes, holding for an additional 5 minutes, and then returning to starting conditions over the next 10 minutes. The mobile phase flow rate was 1 mL/min. Metabolites were monitored by UV absorbance at 280 nm using a Shimadzu SPD-6AV spectrophotometer detector (Kyoto, Japan). The retention time for I33' under these conditions was routinely 24.4 minutes.

Preparation of Microsomal and Cytosolic Enzymes

Microsomes were prepared from rat liver by differential centrifugation according to the method of Guengerich (39). Cytosol from BHA-induced mice, used to quantify production of AFB_1 8,9-epoxide, was a gift from Dr. David Eaton. Protein concentrations of cytosol and microsomes were determined according to the method of Lowry *et al.* (40). Control, CYP1A1, CYP1A2, and microsome products, prepared from a human lymphoblastoid cell line, were purchased from Gentest (Woburn, MA). CYP 1A1 microsome products were prepared from cells treated with dibenz(*a,h*)anthracene (DBA) that greatly induces the low levels of natively expressed CYP1A1. Any CYP1A2 present in the CYP1A1 microsome product that might be induced (as occurs in hepatic tissue following exposure to CYP1A1 inducers) is estimated at less than 10% the content of CYP1A1 (Dr. C. Crespi, Gentest Corp., personal communication). CYP1A2 microsome product was prepared from cells transfected with human CYP1A2 cDNA. Microsomes from the native, uninduced cell line that expresses only low levels of CYP activity (CYP1A1) were used as control for CYP1A2-mediated acetaniline 4-hydroxylase assay.

Enzyme Assays

The EROD assay was conducted essentially as described by Burke *et al.* (41), but modified to include $MgCl_2$ (5 mM) and bovine serum albumin (1.6 mg/mL) in the reaction buffer as suggested by Pohl and Fouts (42) to enhance activity. EROD determinations were performed using liver microsomes from BNF-treated rats, or microsomes from a DBA-induced human lymphoblastoid cell line. The assay was conducted at 37°C.

The PROD assay was conducted according to the method of Burke *et al.* (41), at 37°C using microsomes from PB-treated rats. For both EROD and PROD, the slight quenching of fluorescence by I33' was compensated for by including the inhibitor in the standard curve solutions at the concentrations used in the assay.

Cytochrome *c* reductase activity, which we used as an indirect measure of microsomal NADPH-cytochrome P450 reductase, was determined according to the method of Yasukochi and Masters (43).

The metabolism of AFB_1 was determined essentially as described by Monroe and Eaton (44) using liver microsomes from BNF-treated rats. This assay allows simultaneous quantification of hydroxylated metabolites and AFB_1 8,9-epoxide by trapping the latter as a stable glutathione conjugate. In the presence of mouse cytosol, trapping efficiency of the epoxide as the conjugate has been reported to be greater than 99% (44). I33' was added to the reaction mixture at final concentrations of 10, 50, or 100 μM in DMSO (final DMSO concentration was 2% v/v) and preincubated at 37°C for 5 minutes before initiating the reaction with AFB_1. The final reaction mixture included 1 mg/mL microsomal protein, 3 mg/mL BHA-induced mouse cytosolic protein, 16 or 124 μM AFB_1, 5 mM GSH, 1 U/mL glucose-6-phosphate dehydrogenase, 5 mM glucose-6-phosphate, and 1 mM $NADP^+$ in a buffer containing 190 mM sucrose, 60 mM potassium phosphate, 80 mM Tris, 15 mM NaCl, 5 mM KCl, and 4 mM $MgCl_2$, pH 7.6 in a reaction volume of 250 μL. After 10 minutes [within the linear range for metabolite production (45)], the reaction was terminated by the addition of 50 μL 2 M acetic acid and 10 μL methanol containing the internal standard. The mixture was then frozen for at least 2 hours. The frozen mixture was then subjected to centrifugation at 14,000 *g* for 4 minutes at ambient temperature to thaw the mixture and to pellet the precipitated protein. Metabolites were resolved by HPLC on a 4.6 × 250 mm, C_{18} Econosphere cartridge column (Alltech Associates, Deerfield, IL) and detected by UV absorption at 362 nm. Quantification of metabolites was achieved with a Shimadzu Chromatopac integrator (Kyoto, Japan) using an AFB_1 standard curve and AFG_1 as an internal standard to correct for recovery. The mobile phase consisted of a combination of 0.1% ammonium phosphate, pH 3.5 (solvent A) and 95:5 methanol:THF (solvent B). From 0 to 2 minutes, the concentration of solvent B was increased from 10 to 24%, then further increased to 38% solvent B at 13 minutes 60% at 16 minutes, and 90% at 17 minutes. At 20

minutes, the mobile phase was returned to starting conditions over a period of 5 minutes. All changes in the composition of the mobile phase were accomplished using a linear gradient. The flow rate was kept constant at 1.0 mL/min. The concentration of the AFB_1 stock solution (prepared in DMSO) was determined by UV absorbance spectrophotometry using an extinction coefficient of 21.8 mM^{-1} cm^{-1}. The final concentration of AFB_1 in the assay was calculated by adding a known amount of an AFB_1 stock solution. When mouse cytosol was included in the absence of microsomes, no AFB_1 8,9-epoxide-GSH conjugate was detected.

CYP1A2-mediated 4-hydroxylation of acetanilide was conducted essentially as described by Liu *et al.* (46). The final reaction mixture contained 0.1–3.0 mM acetanilide, 0–100 μM I33′ delivered in DMSO (final concentration DMSO 1% v/v), 1 U/mL glucose-6-phosphate dehydrogenase, 10 mM glucose-6-phosphate, 0.5 mM NADPH, 50 mM potassium phosphate (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, and 0.5 mg/mL microsomal protein in a volume of 250 μL. Samples were preincubated at 37°C for 2 minutes before initiating the reaction with microsomes. Control incubations excluded either the NADPH-regenerating system or microsomes, or contained the control microsome product described in the Preparation of Microsomal and Cytosolic Enzymes section. The reaction was terminated at 45 minutes with the addition of 0.1 μg 3-OH acetanilide (internal standard) in 10 μL acetone and 0.3 mL ice-cold ethyl acetate. The ethyl acetate extract (3 × 0.3 mL) was evaporated under a stream of N_2 and resuspended in 50 μL of HPLC mobile phase (initial conditions). The 4-hydroxy metabolite was quantified by HPLC (40 μL injection volume) with a Shimadzu Chromatopac integrator using 3-OH acetanilide as an internal standard to correct for recovery and a 4-OH acetanilide standard curve. Metabolites were separated using a binary gradient consisting of 5:17 acetonitrile:methanol (solvent A) and water (solvent B). Initial conditions were 22% solvent A:78% solvent B. This solvent ratio was changed linearly to 23.5% solvent A:76.5% solvent B over 3 minutes, then changed to 45% solvent A:55% solvent B over the next 2 minutes. This solvent ratio was held for 5 minutes before returning to initial conditions over the following 2 minutes. The metabolites were detected at a wavelength of 254 nm using a Shimadzu SPD-6AV detector. The column used was an Econosil (Alltech Assoc., Deerfield, IL) C_{18} 5μ 250 × 4.6 mm held at 40°C. The flow rate was constant at 1 mL/min.

We used BNF-treated rat microsomes to examine the microsomal metabolism of [^{3}H]-I33′. Carrier-free, tritium-labeled I33′ (0.5 μCi) was added in 29 μL ethanol to microcentrifuge tubes and reduced to dryness under a stream of argon. Radioinert I33′ in 2 μL DMSO was added so that the final concentration in the assay was 100 μM. Other components were added so that their final concentrations were 1 U/mL glucose-6-phosphate dehydrogenase, 10 mM glucose-6-phosphate, 0.5 mM NADPH, 10 mM potassium phosphate (pH 7.4), 1 mM $MgCl_2$, and 0.2 mM EDTA. Following incubation at 37°C for 2 minutes, 0.1 mg microsomal protein was added to initiate the reaction. The final volume of the incubation was 100 μL. Incubations in the absence of microsomes, in the presence of heat-inactivated microsomes, or lacking an NADPH-regenerating system were run concurrently as controls. After 30 minutes, the reaction was terminated by the addition of 100 μL cold acetonitrile, and the mixture was cooled on ice for 30 minutes prior to centrifugation at 10,000 *g* for 10 minutes at 4°C. The mixture was frozen until analysis of the supernatant by HPLC under the conditions described previously for the isolation of [^{3}H]-I33′. Metabolites were monitored at a wavelength of 280 nm and by radioisotope detection using an on-line Beckman M171 radioisotope detector (liquid cell) using 3a70B liquid scintillation cocktail (Research Products International, Mount Prospect, IL) at a flow rate of 2 mL/min.

Mass Spectrometry

Low-resolution electron-impact (EI) mass spectral analysis was performed on a Finnigan model 4023 quadrupole mass spectrometer upgraded with a model 4500 source and a Varian model 3400 gas chromatograph at the Environmental Health Sciences Center at Oregon State University. The mass spectrometer is controlled by a Galaxy 2000 data system (LGC Co., San Jose, CA). Chromatography was performed using an Alltech (Deerfield, IL) SE-54 column (10 m × 0.25 mm i.d. with a 0.25 μm coating) operated at injector pressures of 3 psi. EI mass spectra were obtained at an electron energy of 70 eV at a source temperature of 140°C. To facilitate GC/MS analysis, active hydrogens on the indole or alcohol of the putative hydroxylated metabolite of I33′ were silylated by dissolving the anhydrous sample in 20 μL of anhydrous pyridine followed by 20 μL of *N*-methyltrimethylsilytrifluoroacetamide solution (Pierce Biochemical Co., Rockford, IL). The septum-topped vial was heated at 60°C for 0.5 hour prior to injection onto the GC (usually 4 μL).

Kinetic Analysis

K_{ii} and K_{is} values were determined by nonlinear regression using computer analysis (47). Data were fit to a competitive or noncompetitive inhibition model, based on visual examination of $1/S$ vs. $1/v$ plots. Inhibition was deemed either noncompetitive or com-

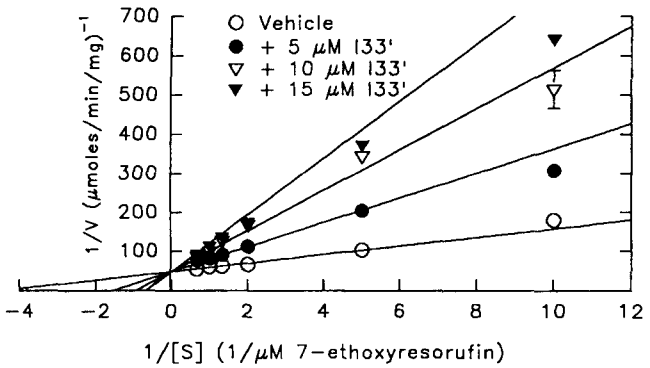

FIGURE 2. Lineweaver–Burke plot of inhibition of rat liver microsomal EROD by 3,3'-diindolylmethane (I33'). Incubations were performed as described in the Materials and Methods section and included 5 μg/mL protein. Points represent the means of three determinations ±SE. V_{max} = 20.3 ± 6.0 nmol/min/mg protein; K_m = 205 ± 24 nM; K_i = 2.2 ± 0.2 μM I33'.

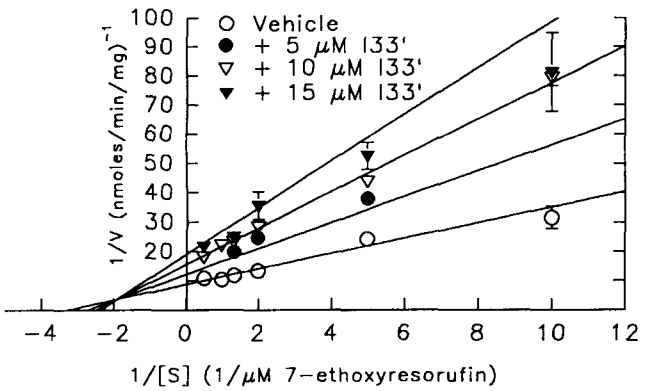

FIGURE 3. Lineweaver–Burke plot of inhibition of human CYP1A1-catalyzed EROD by 3,3'-diindolylmethane (I33'). Incubations were performed as described in the Materials and Methods section and included 50 μg protein/mL. Points represent means of three determinations ±SE or means of two determinations ±range. V_{max} = 114 ± 7.0 pmol/min/mg protein; K_m = 301 ± 50 nM; K_{is} = 7.4 ± 2.0 μM I33'; K_{ii} = 13 ± 2.7 μM I33'.

petitive, respectively, when both the slope and intercept, or the slope only, were found to be a function of inhibitor concentration [nomenclature of Cleland (48)]. We found no evidence of uncompetitive inhibition, in which the intercept only is a function of inhibitor concentration. In some cases, the correct model choice was not unequivocal since computer-drawn regression curves reflect experimental error.

Statistics

Data for inhibition of AFB_1 metabolite production and inhibition of cytochrome *c* reductase were analyzed by one-way analysis of variance, and differences between specific means were compared using least significant difference. Differences with $P < 0.05$ were considered significant.

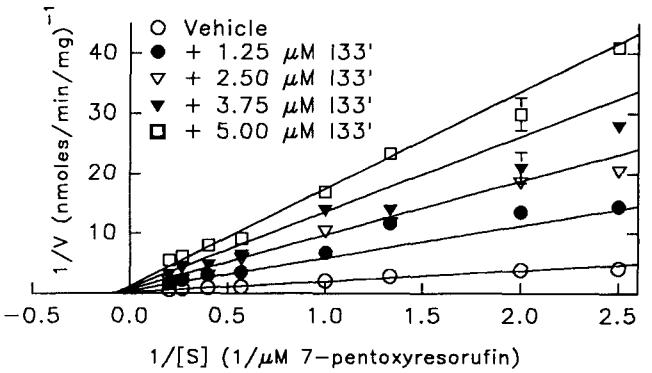

FIGURE 4. Lineweaver–Burke plot of inhibition of rat liver microsome-catalyzed PROD by 3,3'-diindolylmethane (I33'). Incubations were performed as described in the Materials and Methods section and included 75 μg/mL protein. Points represent means of 3–5 determinations ±SE or means of two determinations ±range. In limited instances, only one data point was obtained. V_{max} = 4.06 ± 0.68 nmol/min/mg protein; K_m = 7.3 ± 1.6 μM; K_{is} = 0.62 ± 0.08 μM; K_{ii} = 1.2 ± 0.60 μM I33'.

RESULTS

In Vitro Inhibition of EROD and PROD

In a recent study, we found I33' to be a potent noncompetitive inhibitor of trout liver microsomal EROD, with a K_{is} value of 2.7 ± 0.4 μM I33' and a K_{ii} value of 13.7 ± 2.2 μM I33' (35). With rat microsomes (Figure 2), inhibition appeared competitive and a K_i value of 2.2 ± 0.2 μM I33' was determined. Ethoxyresorufin *O*-deethylase is catalyzed primarily by CYP1A in trout (49) and CYP1A1 in rat (50) when animals have been induced by BNF or similar inducer. I3C (100 μM) had a much weaker, though measurable, inhibitory effect on resorufin production (data not shown); however, inhibition due to I33' (formed from decomposition of I3C in an aqueous medium) could not be ruled out, and no attempt was made to determine K_i values for this compound.

Figure 3 shows I33' inhibition of human CYP1A1-mediated EROD. The activity of these microsomes was relatively low compared with BNF-induced animals [V_{max} = 114 pmol/min/mg protein vs. 600 pmol/min/mg protein (trout) and 20,300 pmol/min/mg (rat)]. Though these data are somewhat imprecise, the fitted lines are more compatible with noncompetitive inhibition and yield a K_{is} value of 7.4 ± 2.0 μM I33' and a K_{ii} value of 13 ± 2.7 μM I33'.

Figure 4 depicts I33' noncompetitive inhibition of rat liver microsomal PROD, an activity associated with

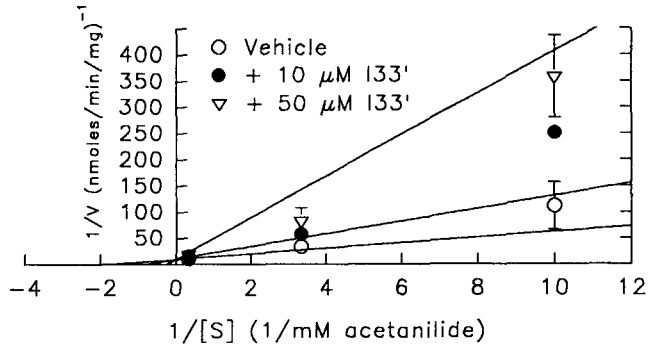

FIGURE 5. Lineweaver–Burke plot of inhibition of human CYP1A2–catalyzed 4-hydroxylation of acetanilide by 3,3'-diindolylmethane (I33'). Incubations were performed as described in the Materials and Methods section and included 0.5 mg/mL protein. Points represent the means of two determinations ± range. V_{max} = 95 ± 19 pmol/min/mg protein; K_m = 500 ± 208 μM; K_i = 7.6 ± 4.1 μM I33'.

CYP2B1. Kinetic constants were determined to be K_{is} = 0.62 ± 0.08 μM I33' and K_{ii} = 1.2 ± 0.60 μM I33'.

Inhibition of Acetanilide 4-Hydroxylase

Figure 5 shows the inhibition of expressed human CYP1A2-mediated acetanilide 4-hydroxylase at concentrations of 10 and 50 μM I33'. While there is clear evidence for inhibition, the data intersects are inadequate to suggest inhibitory mechanism unequivocally. Competitive inhibition was judged to best fit the data, and a K_i value of 7.6 ± 4.1 μM I33' was determined. No acetanilide 4-hydroxylase activity was observed in incubations containing control microsomes that express only a low level of CYP1A1 activity (see the Materials and Methods section).

Inhibition of In Vitro AFB_1 Metabolism

Figures 6A and 6B depict the results of an experiment assessing inhibition of in vitro metabolism of AFB_1 by I33' using BNF-induced rat liver microsomes. Inhibition was examined at two concentrations (16 and 124 μM) of AFB_1 and three concentrations (10, 50, and 100 μM) of I33'. At the low concentration of AFB_1, the velocity of CYP1A-catalyzed conversion of AFB_1 to AFM_1 (51) was inhibited 14, 32, and 46% at the three concentrations of inhibitor tested (Figure 6A). Inhibition of AFB_1 8,9-epoxide-glutathione conjugate (i.e., AFB_1 8,9-epoxide) formation occurred in a nearly identical concentration-dependent manner (9, 31, and 47% inhibition) (Figure 6A).

At the high concentration of AFB_1 used (124 μM), I33' inhibition of AFM_1 production was not as strong as that seen at the lower concentration. Inhibition was 11, 14, and 29% at 10, 50, and 100 μM I33', respectively

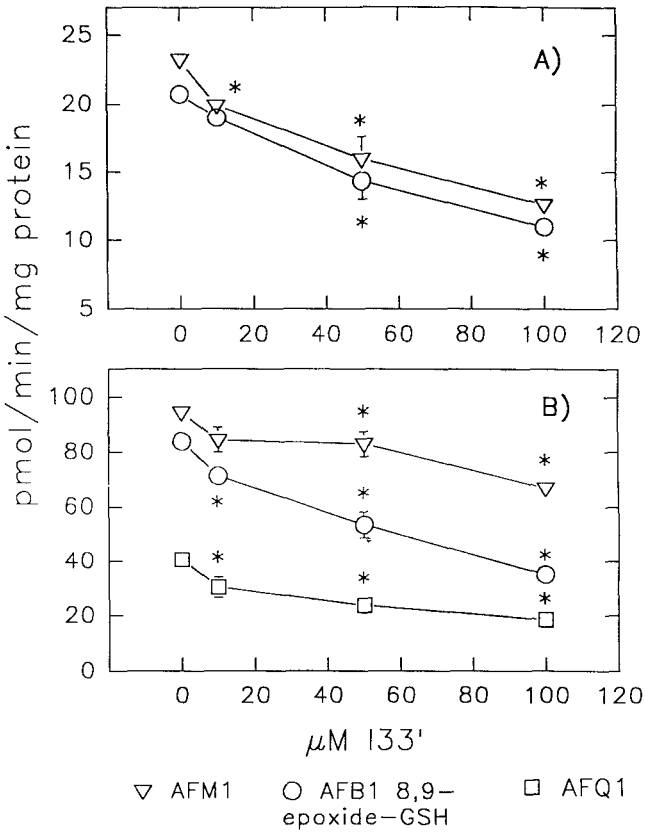

FIGURE 6. Inhibition of BNF-induced rat microsomal aflatoxin M_1 (AFM_1), aflatoxin Q_1 (AFQ_1), or aflatoxin B_1 8,9-epoxide-GSH conjugate formation by 3,3- diindolylmethane (I33'). The assay was performed as described in the Materials and Methods section using an AFB_1 substrate concentration of 16 μM (A) or 124 μM (B). Control activity in (A) was 23.2 ± 0.6 pmol AFB_1 8,9-epoxide-GSH conjugate/min/mg protein and 20.8 ± 0.5 pmol AFM_1/min/mg protein. The production of AFQ_1 at this concentration of substrate could not be reliably quantified. Control activity in (B) was 94.6 ± 1.0 pmol AFM_1/min/mg protein, 84.0 ± 2.5 pmol AFB_1 8,9-epoxide-GSH conjugate/min/mg protein, and 40.5 ± 1.0 pmol AFQ_1/min/mg protein. Values represent the means of three determinations ± SE. Bars marked by * are significantly different than control ($P < 0.05$).

(Figure 6B). In contrast to the findings at the lower concentration of AFB_1, the degree of inhibition of AFB_1 8,9-epoxide-glutathione conjugate production at the higher concentration was nearly twice that of AFM_1 (Figure 6B). Calculated noncompetitive inhibition constants for AFB_1 8,9-epoxidation were K_{is} = 138 ± 43 μM I33', K_{ii} = 52 ± 11 μM. Competitive inhibition was apparent with AFM_1 formation, and the calculated K_i value was 128 ± 24 μM I33'. At this concentration of AFB_1, we were able to accurately quantify production of AFQ_1. Its production, mediated by CYP3A, was inhibited 17, 42, and 54% at the three concentrations of I33' used (Figure 6B). Other peaks, representing AFP_1 and two unidentified metabolites, also showed inhibition, but peak areas could not be reliably quantified.

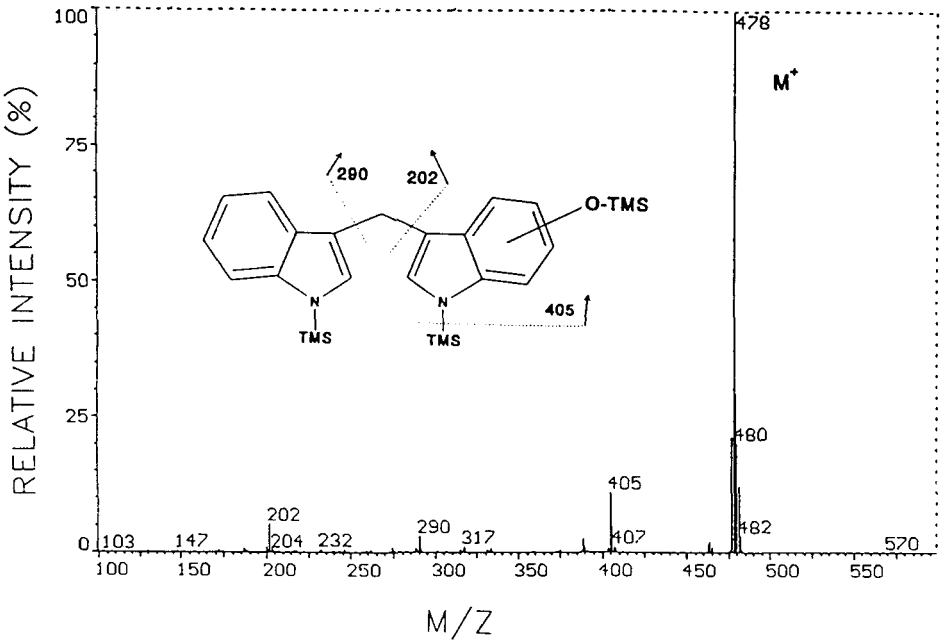

FIGURE 7. Mass spectra of a 3,3-diindolylmethane (I33') metabolite isolated by HPLC from a 30 minute incubation of rat liver microsomes in the presence of an NADPH-regenerating system and 100 μM [3-H]I33'. The m/z ratio is consistent with a trimethylsilated derivative of monohydroxylated I33'.

Inhibition of AFB_1 8,9-epoxide-glutathione conjugate formation by I33' was not observed when AFB_1 8,9-epoxide was added in the presence of increasing concentrations of mouse cytosol (data not shown).

I33' (100 μM) had no apparent effect on the ability of PB-induced rat liver microsomes to reduce cytochrome *c* (data not shown), indicative of lack of inhibition of NADPH-cytochrome P-450 reductase.

Metabolism of [^{3}H]-I33'

When microsomes from BNF-treated rats were incubated with 100 μM [^{3}H]-I33' in the presence of NADPH for 30 minutes, a major metabolite with an HPLC retention time of 18.7 minutes was produced at the rate of 49 $\pm$ 13 pmol/min/mg protein. Electron-impact mass spectral analysis of this peak revealed a compound possessing an m/z ratio of 478, and a fragmentation pattern consistent with a trimethylsilated derivative of monohydroxylated I33' (Figure 7). Mass spectral data for the *N*-trimethylsilyl derivative of the putative hydroxylated I33': m/z 479 (86), 478 (100, M^+), 405 (15), 333 (1.4), 290 (4.2), 202 (8.3). The fragmentation pattern did not permit the assignment of the position of the putative hydroxyl moiety.

DISCUSSION

Inhibition of CYP Isoform-Specific Reactions

According to Wattenberg (25), chemopreventive agents, compounds that prevent the occurrence of cancer, may be classified on the basis of mechanism of action. One category is inhibitors of enzymes, such as CYP, which activate procarcinogens. Results presented here and elsewhere (35) show that I33', a nonenzymatic product of I3C, is a potent inhibitor of reactions catalyzed preferentially by CYP1A1/CYP1A2 (trout, rat, or human), CYP2B1, and CYP3A (rat), enzymes known to activate carcinogens. In liver microsomes from BNF-induced rats, EROD activity is attributed primarily to CYP1A1, with a minor contribution from CYP1A2 (50). CYP1A is also responsible for EROD activity in trout (49,52). Human CYP1A1 is essentially the only CYP enzyme present in the microsomes from the lymphoblastoid cell line, and therefore the inhibition of EROD by I33' is due to inhibition of this isoform. We also found I33' to strongly inhibit the activity of human CYP1A2. Expression of CYP1A2 is largely confined to hepatic tissue in humans (53), whereas CYP1A1 appears to be expressed only in extrahepatic tissues (53–55). Inhibition of CYP1A proteins by I33' in these tissues would be expected to protect against carcinogens that are CYP1A-activated, including PAHs, heterocyclic amines (33,54,56), and the mycotoxin, AFB_1 (57).

In hepatic microsomes from PB-induced rat, CYP2B1 is most probably responsible for the high level of PROD activity observed (41). This isoform can activate a number of carcinogens (33), including 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (58). Though present only in low levels in liver, it is constitutively expressed in mammalian lung (59,60). Potent inhibition of CYP2B might therefore contribute to the

I3C inhibition of NNK-induced lung tumorigenesis in mice (3).

We also examined the ability of I33′ to inhibit activation of the hepatocarcinogen AFB_1, a common contaminant of human food supplies. As shown previously, the contributions of the various CYP enzymes to AFB_1 microsomal metabolism vary with initial substrate concentration (45,57,61), particularly in animals pretreated with I3C or BNF (61). However, in the rat it appears that primarily CYP2C11 (62,63) and CYP3A (63) contribute to activation reactions. CYP1A and 3A are associated with detoxication reactions that form AFM_1 (64) and AFQ_1 (65), respectively.

Failure of I33′ to inhibit NADPH-cytochrome P-450 reductase, at up to 100 μM I33′, is consistent with inhibition of catalytic activity via interaction with the hemoprotein component of CYP. Indeed, we observed that I33′ is a substrate of a microsomal and NADPH-dependent enzyme, forming a putative monohydroxylated form of I33′, consistent with a typical CYP-catalyzed product.

I33′ Inhibition of CYP as a Mechanism of Protection against AFB_1 Carcinogenesis

Dietary I3C is a potent inhibitor of AFB_1 carcinogenesis in the trout (2) and rat.[3] A decrease in hepatic in vivo AFB_1-DNA binding is associated with induction of specific phase I and phase II AFB_1-metabolizing enzymes by I3C in the rat (61,66), but not in trout (28), suggesting that other mechanisms apply for the latter species. Takahashi *et al.* (31) have recently shown inhibition of trout microsome-catalyzed AFB_1-DNA binding by I33′ in vitro, and we extend those results to the rat by an alternate method, showing inhibition of formation of the major genotoxic AFB_1 metabolite, AFB_1 8,9-epoxide. It is possible that the decrease in epoxide formation is attributable to nucleophilic trapping of the epoxide by I33′. However, this occurrence seems unlikely under these conditions, given the AFB_1 8,9-epoxide trapping efficiency of mouse cytosol in the presence of glutathione (44). Rather, I33′ may inhibit in vivo CYP activation of AFB_1 in the rat, as was concluded for the trout model (15). This mechanism of protection could occur in addition to induction of GST (66). Because I33′ inhibits human CYP1A2, oral administration of I33′ may offer a means of inhibiting CYP1A2 activation of AFB_1 (57). However, interpretation of our data is not unequivocal because I33′ also inhibits phase I detoxication pathways (i.e., AFM_1 and AFQ_1 formation). In addition, under conditions of sustained feeding, I3C (and, therefore, possibly I33′) enhances in vitro production of AFB_1 8,9-epoxide, as well as AFM_1 and AFQ_1, in rats (61). Dietary I3C has been shown to induce CYP1A2- and CYP3A-associated estradiol 2-hydroxylation in humans (67), suggesting that the possibility of enhancement of AFB_1 activation may also occur. In vivo studies are necessary to fully comprehend the balance between the I33′ inhibition and induction of both AFB_1 activation and detoxication pathways.

Although hitherto we have not discussed I33′ bioavailability, it is known that I33′ is a major component of both trout and rat liver extract following oral gavage or dietary administration (12–14). Six hours after oral gavage of an anticarcinogenic dose of 1.0 mmol I3C/kg body weight, I33′ liver concentrations were estimated to be 4–6 μM, whereas levels found in trout 48 hours after oral gavage of an anticarcinogenic dose of 0.27 mmol I3C/kg body weight were found to be approximately 70 μM. These levels are in the range of I33′ inhibition constants calculated for EROD, PROD, or acetanilide 4-hydroxylase, but are below that calculated for AFB_1 oxidation reactions, despite, in some cases, the similarity of enzymes catalyzing the reaction (i.e., rat CYP1A1 for both AFB_1 9a-hydroxylase and EROD). One explanation for this apparent anomaly is the fact that, in contrast to most enzymes, CYPs exhibit broad substrate specificity. Thus, affinity of ethoxyresorufin and AFB_1 for the CYP1A1 catalytic site may be substantially different, resulting in unequal inhibition constants calculated for the same inhibitor, I33′. Nevertheless, significant inhibition of AFB_1 8,9-epoxidation is observed using I33′ concentrations as low as 10 μM. I33′ inhibition of AFB_1 or other carcinogen activation should participate in the overall I3C-mediated anticarcinogenic mechanism, the degree of which would depend on many variables, including the extent of CYP induction and the concentration of I33′ and carcinogen present at the site of activation.

[3]D. M. Stresser, R. Oliyai, N. I. Kerkvliet, R. C. Bender, O. R. Hedstrom, L. Baecher-Steppan, G. S. Bailey, and D. P. Selivonchick, manuscript in preparation.

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