Indole-3-carbinol, but not its major digestive product 3,3’-diindolylmethane, induces reversible hepatocyte hypertrophy and cytochromes P450

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Abstract

Indole-3-carbinol (I-3-C) and 3,3’-diindolylmethane (DIM) have been shown to reduce the incidence and multiplicity of cancers in laboratory animal models. Based on the observation that I-3-C induced hepatocyte hypertrophy when administered orally for 13 weeks to rats, a treatment and recovery study was undertaken to test the hypothesis that the induction of hepatocyte hypertrophy and cytochrome P450 (CYP) activity by I-3-C are adaptive, reversible responses. Additionally, we directly compared the effects of I-3-C to those of its principle metabolite DIM. Rats were treated orally for 28 days with 2 doses of I-3-C (5 and 50 mg I-3-C/kg body weight/day) and DIM (7.5 and 75 mg DIM/kg body weight/day) and then one-half of the animals were not treated for an additional 28 days. Organ weights, histopathology, and the CYP enzyme activities of 1A1/2, 2B1/2, 2C9, 2D6, 2E1, 3A4, and 19 A were measured both after treatment and after recovery. Oral administration of 50 mg I-3-C/kg body weight/day to rats for 28 days significantly increased liver weights and CYP enzyme activities. The effects in males were more pronounced and persistent after recovery than the effects in females. The increased organ weights returned to control values after treatment. Conversely, DIM did not alter liver weights and had no effect on CYP activities after the 28-day treatment. Some changes in CYP activities were measured after the DIM recovery period but the magnitudes of the changes were considered biologically insignificant. The results show that I-3-C, but not DIM, induces reversible adaptive responses in the liver.

Keywords: Indole-3-carbinol; 3,3’-diindolylmethane; Cancer prevention; Cytochromes P450

Introduction

Indole-3-carbinol (I-3-C) and 3,3’-diindolylmethane (DIM) were reported to be chemopreventive for dimethylbenzanthracene (DMBA)-induced, rodent mammary cancer in the 1970s (Wattenberg and Loub, 1978). I-3-C and DIM, found in Brassica vegetables, such as broccoli, cabbage, and Brussels sprouts, are formed by the plant enzyme, myrosinase, acting on glucurobassicin (Bradfield and Bjeldanes, 1987). In addition to inducing cytochrome (CYP) enzymes responsible for DMBA metabolism, oral I-3-C was reported in 1990 to be a potential breast cancer preventive agent affecting estrogen metabolism in humans (Michnovic and Bradlow, 1990). Subsequently, modulation by I-3-C of estrogen-stimulated proliferation in cells infected with human papillomavirus led to its evaluation in the treatment of recurrent respiratory papillomatosis (Newfield et al., 1993; Coll et al., 1997; Rosen and Bryson, 2004) and in cervical intraepithelial neoplasia (Bell et al., 2000). Additionally, in animal models of cancer, I-3-C has been reported to prevent breast (Grubbs et al., 1995), endometrial (Kojima et al., 1994), cervical (Jin et al., 1999), and lung cancers (El Bayoumy et al., 1996). However, the ability of I-3-C and its
gastric products to induce CYP enzymes and activate the aryl hydrocarbon receptor (AhR) has made the use of I-3-C controversial because in vivo studies also have shown that oral I-3-C can promote as well as block carcinogenesis (Pence et al., 1986; Stoner et al., 2002; Yoshida et al., 2004).

DIM is a biologically active dimer of I-3-C that has also been shown to influence estrogen metabolism in women (Dalessandri et al., 2004) and is the only measurable analyte in plasma after I-3-C administration (Arneson et al., 2001). DIM is a stable compound and has shown efficacy in a preclinical breast cancer prevention model (McDougal et al., 2000). Interestingly, DIM has also been reported to bind as an antagonist to the androgen receptor and to have efficacy in vivo in mice injected with prostate tumor cells (Le et al., 2003). DIM also selectively antagonizes the activation of the AhR (Hestermann and Brown, 2003).

Based on the publications supporting the efficacy of I-3-C to prevent endocrine-associated cancers and on a lack of reported toxicities in clinical use, the Division of Cancer Prevention (DCP) of the National Cancer Institute (NCI) initiated projects to develop I-3-C as a potential pharmaceutical. In a 13-week toxicity study in rats, I-3-C was found to increase liver weights and to enlarge centrlobular hepatocytes; the histological features of enlarged hepatocytes with pale cytoplasm were considered to be consistent with the adaptive induction of smooth endoplasmic reticulum (Levine, 1995). In the studies reported here, we tested the hypothesis that the induction of cytochromes P450 and Phase II enzymes in rats by I-3-C correlate with hepatic hypertrophy over a 28-day period of treatment and that both are reversible after a 28-day period without treatment; thus, representing adaptive, rather than toxic, effects of I-3-C on the liver (Williams and Iatropoulos, 2002). Additionally, we directly compared the effects of orally administered I-3-C, and the numerous oligomeric products it forms in the stomach (Grose and Bjeldanes, 1992; Stresser et al., 1995; Anderton et al., 2003), with the effects of the orally administered, single chemical entity DIM on these endpoints.

**Methods**

**Test article.** I-3-C (supplier lot no. A80481) was purchased by the NCI from Sabinsa Corp., Edison, NJ. Compound identity was confirmed by GC–MS and the purity was determined by HPLC to be 99.08%. Formulated DIM (BioResponse-DIM®, supplier lot no. 21699) was provided by the NCI in collaboration with BioResponse, LLC, Boulder, CO. The DIM was a microencapsulated formulation and DIM content was determined by HPLC, using a reference standard, to be 26.6% DIM by weight. Both I-3-C and DIM were stored refrigerated, ambient humidity, and protected from light.

**Animals.** For the 13-week toxicity study at the University of Illinois at Chicago, IL, male and female CD® Virus Antibody Free (VAF) rats (Charles River Breeding Laboratories, Kingston, NY) were singly housed in polycarbonate cages with Anderson bed-o’cobs® bedding (Heinold, Kankakee, IL) at 64–79 °F, 30–70% room humidity, and 14/10 light/dark cycle. The animals were approximately 7 weeks old and weighed 294–378 g (males) and 156–209 g (females) at dosing initiation.

For the 28-day treatment and recovery study at Southern Research Institute, Birmingham, AL, male and female CD® [Crl: CD(SD)Br] rats (Charles River Breeding Laboratories, Raleigh, NC) were singly housed in plastic shoebox-type cages at 68–77 °F, 40–82% room humidity, and 12/12 light/dark cycle. The animals were approximately 6 weeks old and weighed 184–240 g (males) and 130–171 g (females) at dosing initiation.

Both facilities were AAALAC Intl.-accredited according to the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and the studies were conducted in compliance with Good Laboratory Practices. Animals were assigned randomly to treatment groups based on body weight. Certified Rodent Chow No. 5002 (PMI Feeds Inc., St. Louis, MO) and tap water were provided ad libitum.

**Dosing formulations.** For the 13-week study the vehicle was 2% aqueous sodium carboxymethylcellulose (CMC). For the 28-day study, the vehicle for the I-3-C was 0.5% CMC and the vehicle for the microencapsulated DIM was sterile water. Dosing formulations of I-3-C were prepared every 4 days and stored at 2–8 °C, homogenized daily prior to dosing, allowed to warm to room temperature before administration, and stirred continuously while the dosing procedure was ongoing. Dosing formulations of DIM were prepared every 14 days and stored and handled similarly to I-3-C. The concentrations and stability of the dosage formulations were confirmed by HPLC prior to the beginning and during the course of the study. All concentrations were within 10% of theoretical, except in the first and third DIM mixes and the dose volumes were slightly adjusted from 5 ml/kg body weight to deliver the desired doses.

**Study designs.** The dosages in the 13-week study were based on a 28-day range finding study in which 0, 20, 60, 200, 600, and 2000 mg I-3-C/kilogram body weight/day (mg/kg bwt/day) were administered to 5 animals/sex/group. Hepatotoxicity as evidenced by clinical chemistry and/or organ weight increases was seen at the 4 highest dose levels and 20 mg I-3-C/kg bwt/day was considered at or near the no observed adverse effect level. In the 13-week study, 20 animals/sex/group were dosed once daily with 0, 4, 20, and 100 mg I-3-C/kg bwt/day for 13 weeks by gavage (10 ml/kg bwt/day). The amount administered was based on the most recently measured body weight. Body weight measurements, food consumption calculations, and physical exams were conducted weekly. All animals were observed daily for
clinical signs of toxicity. Indirect ophthalmoscopy was conducted on all animals prior to study initiation and on the last 10 survivors/sex/group during week 13.

In weeks 4 and 12 of the 13-week study, blood samples were collected for hematology and clinical chemistry measurements from the first 10 animals/sex/group (anesthetic: CO$_2$:O$_2$, 70%:30%) from the orbital sinus. Hematology parameters were measured using a Sysmex K1000 Hematology Analyzer and included: erythrocyte count, hemocrit, hemoglobin, leukocyte count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelet count, erythrocyte morphology, and reticuloocyte count. Clinical chemistry parameters were measured using a Boehringer Mannheim/Hitachi 704 and included: alanine aminotransferase, aspartate aminotransferase, albumin, globulin ratio, total bilirubin, BUN/creatinine ratio, calcium, chloride, cholesterol, creatinine, glucose, inorganic phosphorus, potassium, sodium, total protein, triglycerides, urea nitrogen. Blood samples collected at scheduled terminal necropsy from the vena cava from 10 animals/sex/group were used to measure coagulation parameters using an MLA, Inc. Electra 700 Automatic Coagulation Timer (activated partial thromboplastin time, prothrombin time, and fibrinogen).

Animals found dead or sacrificed moribund were necropsied. All other animals were euthanized by CO$_2$ asphyxiation and necropsied 1 day after the last dose in week 13. The necropsy procedure was a thorough and systematic examination and dissection of the viscera and carcass, and collection, weighing (organs marked with *) and fixation of the following tissues/organs: adrenal glands, aorta, brain*, cecum, colon, duodenum, epididymides, esophagus, eyes, femur with marrow, gross lesions, heart*, ileum, jejunum, kidneys*, liver*, lungs/bronchi*, mesenteric lymph node, mammary gland, ovaries/fallopian tubes, pancreas, pituitary, prostate, mandibular salivary gland, sciatic nerve, seminal vesicles, skeletal muscle, skin, spinal cord, spleen, sternum with marrow, stomach, testes*, thymus, thyroid/parathyroid*, tissue*, trachea, urinary bladder, uterus (corpus and cervix), and vagina. All tissues and organs collected at necropsy were examined microscopically in the vehicle control and high-dose groups. The livers were subsequently examined microscopically in the other dose groups. Tissue changes received a severity grade where: 1 = minimal, 2 = mild, 3 = moderate, and 4 = marked. Mean severity scores for each change were determined by dividing the sum of the severity scores by the number of tissues examined in that group.

In the 28-day treatment and recovery study, 20 animals/sex/dose were administered vehicle (0.5% CMC in sterile water), 5 or 50 mg I-3-C/kg bwt/day, or 7.5 or 75 mg DIM/kg bwt/day by gavage (5 ml/kg bwt) for 28 days. On day 29, one-half of the animals in each dose group were sacrificed and the other one-half were administered vehicle for the next 28 days and then sacrificed. The doses of I-3-C were based on the toxicities observed in the 13-week oral toxicity study. The doses of DIM were conservatively based the work of De Kruijf et al. (1991) showing that at pH 4, such as in the postprandial stomach, the conversion of I-3-C to DIM and a linear trimer was 30–40%; thus, a dose of 50 mg I-3-C/kg bwt/day to a fed animal might produce an intragastric dose of approximately 20 mg DIM/kg bwt/day. Therefore, a high dose of 75 mg/kg bwt/day of the formulated DIM was used, which provided DIM at 20 mg/kg bwt/day based on the 26.6% DIM content of the formulation. The amount administered was based on the most recently measured body weight. Body weight measurements, food consumption calculations, and physical exams were conducted weekly. All animals were observed daily for clinical signs of toxicity.

In weeks 4 and 8 of the 28-day study and at least 4 days prior to sacrifice, blood samples were collected for hematology and clinical chemistry measurements from all animals scheduled for sacrifice (anesthetic: CO$_2$:O$_2$, 70%:30%) from the orbital sinus. The same hematology parameters as in the 13-week study were measured using a Technicon H1 Hematology Analyzer. Clinical chemistry parameters were measured using a Boehringer Mannheim/Hitachi 911 and included: alanine aminotransferase, aspartate aminotransferase, albumin, globulin ratio, total bilirubin, BUN/creatinine ratio, calcium, chloride, cholesterol, creatinine, glucose, total protein, triglycerides, urea nitrogen, gamma glutamyl transferase, and 5'-nucleotidase.

Animals found dead or sacrificed moribund were necropsied. All other animals were euthanized by CO$_2$ asphyxiation and necropsied on days 29 (treatment) and 57 (recovery groups). The necropsy procedure was a thorough and systematic examination and dissection of the viscera and carcass, and collection, weighing (organs marked with *) and fixation of the following tissues/organs: adrenal glands*, aorta, brain*, carotid arteries, colon, duodenum, epididymides, esophagus, eyes, femur with marrow, gross lesions, heart*, ileum, jejunum, kidneys*, liver*, lungs/bronchi*, mesenteric lymph node, mammary gland, ovaries/fallopian tubes, pancreas, pituitary, prostate, mandibular salivary gland, sciatic nerve, seminal vesicles, skeletal muscle, skin, spinal cord, spleen, sternum with marrow, stomach, testes*, thymus, thyroid/parathyroid*, tissue*, trachea, urinary bladder, uterus (corpus and cervix), and vagina. All tissues and organs collected at necropsy were examined microscopically in the vehicle control, high-dose groups, and in the target organs in the low- and mid-dose groups. Additionally, at sacrifice, the prostate, uterus, and liver from the 5 lowest numbered surviving animals/sex/treatment group were rinsed in ice-cold saline and frozen in cryovials in liquid nitrogen for subsequent enzyme analysis.

Microsomes and cytosol from the prostate, uterus, and liver were prepared by the calcium chloride aggregation method of Schenckman and Cinti (1978). Cytochrome P450 (CYP) activities were measured in microsomes. CYP family 1A1/2 enzyme activity was measured using the substrate 7-ethoxyresorufin as described by Chang and Waxman (1999). CYP2B1/2 activity was measured using 7-pentoxyresorufin as described by Lubet et al. (1985). CYP2C9 was...
assayed using 7-methoxy-4-trifluoromethylcoumarin; CYP2D6, using 3-[2-(N,N-diethyl-N-methylamino)ethyl]-7-methoxy-4,4-methylcoumarin; CYP3A4, using 7-benzoyl-4-(trifluoromethyl)-coumarin (Genetest technical bulletin, 2000). CYP2E1 was assayed using 4-nitrophenol hydroxylase activity (Chang et al., 1998) and CYP19 A was assayed using tritium release from tritiated androstenedione (Lephart and Simpson, 1991). Phase II enzyme activities were measured in cytosol. UDP-glucuronosyltransferase was measured by the method of Winsies (1969) using the substrate 4-nitrophenol. Glutathione-S-transferase activity was measured using 1-chloro-2,4-nitrobenzene conjugation with glutathione (Habig et al., 1974); glutathione reductase, by the method of Racker (1995); NADPH:quinone reductase, following 2,6-dichloroindophenol reduction (Benson et al., 1980); and aflatoxin B1 aldehyde reductase, by the method of Judah et al. (1993). Protein was measured by modification of the method of Lowry et al. (1951).

**Statistical analyses.** For each sex, analysis of variance tests were conducted on body weight, food consumption, clinical pathology data, organ/body weight and organ/brain weight ratios, and enzyme activity data. If a significant F ratio was obtained (P < 0.05), Dunnett’s t test was used for pair-wise comparisons to the control group.

**Results**

In rats administered 100 mg I-3-C/kg bwt/day orally by gavage for 13 weeks, the absolute weights of the kidneys, liver, and spleen were increased (P < 0.05) in the males; in the females, only the liver weights were increased (Table 1). The final body weights of the treated groups were not statistically different from those of the control group. The organ to body weight ratios of the kidneys, liver, and spleen of the males administered both 20 and 100 mg I-3-C/kg bwt/day were increased (P < 0.05). Among the females, the organ to body weight ratios of the kidneys and livers in the 100 mg I-3-C/kg bwt/day group and only the livers in the 20 mg I-3-C/kg bwt/day group were increased (P < 0.05). The liver to brain weight ratios of both the males and females treated with 20 and 100 mg I-3-C/kg bwt/day were statistically increased (P < 0.05). The liver was identified as having a histopathological response to treatment with 100 mg I-3-C/kg bwt/day (Fig. 1). Among the males, hepatocyte hypertrophy occurred in 16 of 20 animals, with a group mean severity score of 0.95; no other groups were affected. Among the females, 2 of 20 animals were affected with a mean group severity score of 0.10; no other groups were affected. In some animals hepatocyte necrosis was observed but the incidence was low and not ascribed to treatment. Minimal, but significant (P < 0.05), elevations in total bilirubin were seen at week 12 in the females treated with 20 and 100 mg I-3-C/kg bwt/day (0.15 ± 0.03 mg/dl in both groups versus 0.11 ± 0.02 mg/dl in the control group) and in the males treated with 100 mg I-3-C/kg bwt/day at week 4 (0.17 ± 0.09 mg/dl versus 0.11 ± 0.02 mg/dl in the control group). The kidneys and spleen of the rats treated with 100 mg I-3-C/kg bwt/day were not identified as being histologically different from the control group and were not examined in the other treatment groups. No effects of treatment on body weight, food consumption, hematology, clinical chemistry, or ophthalmology occurred. On the basis of the marginal increases in relative organ weights in the animals treated with 20 mg I-3-C/kg bwt/day without any associated histopathology observations and minor bilirubin elevations, the no adverse effect level was considered 20 mg I-3-C/kg bwt/day.

Following 28 days of treatment with I-3-C and formulated DIM, the liver weights of the rats treated with 50 mg I-3-C/kg bwt/day were significantly increased (P < 0.05) in the males (+14.9%) and in the females (+13.0%). The adrenal weights in the females were also increased (+17.8%, P < 0.05). Mean body weights of the treated groups were not different from the weights of the control groups. No other significant organ weight changes were observed in any of the other I-3-C- and DIM-treated rats. After a 28-day recovery period, the liver weights in the 50 mg I-3-C/kg bwt/day group were not different from the control group, indicating that the effect of I-3-C was reversible. A decrease (−20.7%, P < 0.05) in the adrenal weights of the males previously treated with 7.5 mg DIM/kg bwt/day compared to the controls was also observed but attribution to treatment could not be made, based on no findings at the higher dose and occurrence in 1 sex. No histological lesions were identified in any organ at either time point.

Treatment of rats orally for 28 days with I-3-C and formulated DIM differentially altered liver activities of cytochrome P450 enzymes. In the male rats (Table 2), treatment with 50 mg I-3-C/kg bwt/day significantly

<table>
<thead>
<tr>
<th>Dose (mg/kg bwt/day)</th>
<th>0</th>
<th>4</th>
<th>20</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M (SD)</td>
<td>F (SD)</td>
<td>M (SD)</td>
<td>F (SD)</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.38 ± 0.56</td>
<td>2.24 ± 0.25</td>
<td>4.46 ± 0.41</td>
<td>2.23 ± 0.23</td>
<td>4.69 ± 0.38</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19.96 ± 3.86</td>
<td>9.54 ± 1.44</td>
<td>19.45 ± 2.64</td>
<td>9.49 ± 1.15</td>
<td>21.55 ± 2.34</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.93 ± 0.09</td>
<td>0.49 ± 0.09</td>
<td>0.98 ± 0.15</td>
<td>0.51 ± 0.10</td>
<td>1.02 ± 0.12</td>
</tr>
</tbody>
</table>

Weights in grams, mean ± SD (n = 17–20).

* P < 0.05.
elevated levels of CYP 1A1/2 (8.3-fold), 19 A (4.1-fold), 3A4 (2.8-fold), and 2C9 and 2E1 (1.6- and 1.7-fold) in the livers, compared to levels in the livers from vehicle-treated rats. Enzyme activities of CYP 2B1/2 and 2D6 were not significantly altered by treatment, although CYP 2B1/2 was significantly elevated in the females (see below). The low- and high-dose DIM treatments and the 5 mg I-3-C/kg bwt/day treatment did not significantly alter any of the liver enzyme activities in the males. After the 28-day recovery period, some of the liver enzyme activity levels in male rats previously treated with 50 mg I-3-C/kg bwt/day were significantly elevated, in comparison to the sample activities from the vehicle-treated rats (Fig. 2). Enzyme activity levels of CYP 1A1/2, 2B1/2, and 2E1 in the recovery groups were not different from activities in the control group.

In the female rats (Table 3), treatment with 50 mg I-3-C/kg bwt/day significantly elevated levels of CYP 1A1/2 (12.3-fold), 3A4 (5.8-fold), and 2C9 (2.8-fold), and 2B1/2 and 2E1 (2.5- and 1.7-fold) in the livers, compared to levels in the livers from vehicle-treated rats. Enzyme activities of CYP 19 A and 2D6 were not altered by treatment. The low and high DIM treatments and the 5 mg I-3-C/kg bwt/day treatment did not consistently alter any of the liver enzyme activities, although treatment with 5 mg I-3-C/kg bwt/day elevated CYP 2B1/2 (1.3-fold) and 7.5 mg DIM/kg bwt/day elevated CYP 1A1/2 (1.4-fold). After the 28-day recovery period (data not shown), the liver enzyme activities of CYP 3A4 were slightly decreased in the females previously treated with DIM compared to controls: 0.3-fold ($P < 0.05$) and 0.6-fold ($P < 0.001$) lower than controls after 7.5 and 75 mg DIM/kg bwt/day, respectively. CYP 19 A was slightly increased ($P < 0.05$) in the females previously treated with 5 mg I-3-C/kg bwt/day (1.3-fold) and 7.5 mg DIM/kg bwt/day (1.2-fold).

Samples were also collected from the uterus and prostate of the female and male rats and analyzed for CYP 3A4, 19 A, and 2E1. Table 4 shows the activity levels in the vehicle-treated males and females. Treatment with I-3-C and DIM did not alter CYP3A4 (data not shown). In females treated with 75 mg DIM/kg bwt/day, CYP19 A activity was elevated by treatment ($0.171 \pm 0.070 \text{ pmol/min/mg protein}, P < 0.01$) compared to vehicle-treated control. Activities of CYP 2E1 were significantly higher in the uteri and prostates of the vehicle-treated rats than the activities in the liver. Treatment with 7.5 mg DIM/kg bwt/day slightly elevated CYP2E1 activity in the female uterus ($394 \pm 74 \text{ pmol/min/mg protein}, P < 0.05$), compared to the vehicle-treated control. No differences from control were seen after the recovery period.

Activities of the phase II enzymes UDP-glucuronosyl transferase (UDP-GT), glutathione-S-transferase (GST), glutathione reductase (GR), NADPH-quinone reductase (QR), and aldehyde reductase (AR) were measured in the cytosols of samples from the liver, uterus, and prostate.

### Table 2

Phase I enzyme activities in livers of male rats administered indole-3-carbinol and diindolylmethane orally for 4 weeks

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CYP1A1/2</th>
<th>CYP2B1/2</th>
<th>CYP2C9</th>
<th>CYP2D6</th>
<th>CYP2E1</th>
<th>CYP3A4</th>
<th>CYP19A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.038 ± 0.015</td>
<td>0.030 ± 0.021</td>
<td>1400 ± 400</td>
<td>253 ± 122</td>
<td>11 ± 4</td>
<td>650 ± 310</td>
<td>0.344 ± 0.109</td>
</tr>
<tr>
<td>I-3-C (5 mg)</td>
<td>0.041 ± 0.007</td>
<td>0.023 ± 0.012</td>
<td>1080 ± 470</td>
<td>244 ± 86</td>
<td>9 ± 3</td>
<td>270 ± 220</td>
<td>0.309 ± 0.109</td>
</tr>
<tr>
<td>I-3-C (50 mg)</td>
<td>0.317** ± 0.083</td>
<td>0.056 ± 0.038</td>
<td>2290* ± 537</td>
<td>353 ± 70</td>
<td>19* ± 7</td>
<td>1830* ± 910</td>
<td>1.420* ± 0.733</td>
</tr>
<tr>
<td>DIM (7.5 mg)</td>
<td>0.038 ± 0.012</td>
<td>0.019 ± 0.013</td>
<td>1260 ± 540</td>
<td>266 ± 119</td>
<td>10 ± 4</td>
<td>460 ± 250</td>
<td>0.291 ± 0.193</td>
</tr>
<tr>
<td>DIM (75 mg)</td>
<td>0.044 ± 0.008</td>
<td>0.027 ± 0.014</td>
<td>1690 ± 440</td>
<td>333 ± 76</td>
<td>12 ± 4</td>
<td>930 ± 380</td>
<td>0.288 ± 0.142</td>
</tr>
</tbody>
</table>

*a Dosages of indole-3-carbinol (I-3-C) and diindolylmethane (DIM) given as mg/kg bwt/day.

*b Values are mean ± SD ($n = 5$).

* $P < 0.05$.

** $P < 0.001$. 

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Fig. 1. (a) Liver from a terminal sacrifice male treated with 100 mg I-3-C/kg bwt/day for 13 weeks. Centrilobular hypertrophy and hepatocyte necrosis are illustrated. Magnification = 40×. (b) Liver from a terminal sacrifice male treated with vehicle for 13 weeks. No significant lesion present. Magnification = 40×.
Treatment with DIM did not alter phase II enzyme activities in the livers of either the males or the females after 28 days of treatment (data not shown). After the 28-day recovery period, a single, significant ($P < 0.05$) elevation in GST activity was observed in the female rats treated with 7.5 mg DIM/kg bwt/day versus vehicle-treated control (1004 ± 111 nmol/min/mg protein vs. 795 ± 138 nmol/min/mg protein). Treatment with I-3-C did alter liver phase II enzyme activities. Treatment with 50 mg I-3-C for 28 days caused significant elevations in UDP-GT activity in the livers of both the males (14.7 ± 2.1 nmol/min/mg protein versus 8.5 ± 5.0 nmol/min/mg protein in the vehicle-treated controls, $P < 0.05$) and the females (13.6 ± 1.8 nmol/min/mg protein versus 3.0 ± 1.1 nmol/min/mg protein in the vehicle-treated controls, $P < 0.001$). Treatment with 50 mg I-3-C for 28 days also caused significant elevations in NADPH-quinone reductase (QR) activity in the females (361 ± 44 nmol/min/mg protein versus 248 ± 84 nmol/min/mg protein in the vehicle-treated controls, $P < 0.05$). After the recovery period, the only change measured in the females was in those previously treated with 5 mg I-3-C/kg bwt/day in which QR activity was elevated (317 ± 58 nmol/min/mg protein versus 246 ± 30 nmol/min/mg protein in the vehicle-treated controls, $P < 0.05$).

Phase II enzyme activities in the uterus and prostate showed occasional, significant effects of treatment but no patterns of modulation were apparent. In females treated with 75 mg DIM/kg bwt/day, aldehyde reductase and GST were increased ($P < 0.05$) above the vehicle-treated control (64 ± 31 and 118 ± 38 nmol/min/mg protein, respectively, versus 21 ± 8 and 73 ± 13 nmol/min/mg protein in the controls). In the prostate, GST was slightly decreased ($P < 0.05$) in the males treated with 7.5 mg DIM/kg bwt/day (97 ± 10 versus 131 ± 14 nmol/min/mg protein in the controls) and QR was slightly elevated ($P < 0.05$) in the males treated with 50 mg I-3-C/kg bwt/day (10 ± 4 versus 8 ± 2 nmol/min/mg protein in the controls).

**Discussion**

In a 13-week toxicity study of I-3-C, rats were administered vehicle, 4, 20, or 100 mg I-3-C/kg bwt/day orally by gavage. The rats administered 100 mg I-3-C/kg bwt/day developed hepatocyte hypertrophy and the incidence and severity was greater in the males than in the females. Plasma levels of I-3-C were not measured in this study and it is possible that the pharmacokinetic profiles differ between sexes. The no adverse effect level was 20 mg I-3-C/kg bwt/day. These results are consistent with other studies of I-3-C.

In a study of I-3-C reported by Stoner et al. (2002), rats administered 2000 parts/million (ppm) I-3-C in the diet for

**Table 3**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Enzyme activity (pmol/min/mg protein)</th>
<th>CYP1A1/2</th>
<th>CYP2B1/2</th>
<th>CYP2C9</th>
<th>CYP2D6</th>
<th>CYP2E1</th>
<th>CYP3A4</th>
<th>CYP19A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.046 ± 0.011</td>
<td>0.006 ± 0.001</td>
<td>0.040 ± 0.040</td>
<td>0.163 ± 0.101</td>
<td>0.7 ± 0.3</td>
<td>0.40 ± 0.30</td>
<td>0.151 ± 0.080</td>
<td></td>
</tr>
<tr>
<td>I-3-C (5 mg)</td>
<td>0.061 ± 0.011</td>
<td>0.008* ± 0.001</td>
<td>0.062 ± 0.032</td>
<td>0.168 ± 0.73</td>
<td>1.0 ± 0.4</td>
<td>0.52 ± 0.19</td>
<td>0.184 ± 0.031</td>
<td></td>
</tr>
<tr>
<td>I-3-C (50 mg)</td>
<td>0.568*** ± 0.055</td>
<td>0.015** ± 0.004</td>
<td>1.10* ± 0.30</td>
<td>2.07 ± 0.31</td>
<td>12* ± 0.2</td>
<td>2.30** ± 0.30</td>
<td>0.188 ± 0.036</td>
<td></td>
</tr>
<tr>
<td>DIM (7.5 mg)</td>
<td>0.066* ± 0.014</td>
<td>0.005 ± 0.001</td>
<td>0.066 ± 0.13</td>
<td>2.07 ± 0.13</td>
<td>10 ± 0.4</td>
<td>2.2 ± 0.13</td>
<td>0.204 ± 0.026</td>
<td></td>
</tr>
<tr>
<td>DIM (75 mg)</td>
<td>0.056 ± 0.025</td>
<td>0.004 ± 0.001</td>
<td>0.070 ± 0.40</td>
<td>2.16 ± 0.49</td>
<td>8 ± 0.3</td>
<td>0.20 ± 0.10</td>
<td>0.187 ± 0.059</td>
<td></td>
</tr>
</tbody>
</table>

* Dosages of indole-3-carbinol (I-3-C) and diindolylmethane (DIM) given as mg/kg bwt/day.

** Values are mean ± SD (n = 5).

* $P < 0.05$.

** $P < 0.001$. 

Fig. 2. CYP activities in livers of males 28 days after treatment with (a) 5 mg I-3-C, (b) 50 mg I-3-C, (c) 7.5 mg DIM, and (d) 75 mg DIM/kg bwt/day versus vehicle-treated control. **$P < 0.05$, based on mean ± SD (n = 5) per group.
23 weeks developed GST-P positive foci in the liver. GST-P positive foci have been associated with an increased risk of cancer. However, the dietary dose used in this study was approximately equivalent to 200 mg I-3-C/kg bwt/day administered orally by gavage and exceeded the no adverse effect level identified in our 13-week study by 10-fold. Therefore, the GST-P positive foci in the liver were likely to be the result of a toxic dose of I-3-C. This is consistent with our data in which the 100 mg I-3-C/kg bwt/day oral dose resulted in centrilobular hypertrophy and, at least in a few animals, some areas of necrosis. In another study reported by Leibelt et al. (2003), a dose of 50 mg I-3-C/kg diet/day (or 50 ppm) administered for 12 months significantly increased the liver weight/body weight ratio but did not cause histopathological changes in the liver. This dietary dose is approximately equivalent to 5 mg I-3-C/kg bwt/day administered orally and is consistent with our data from the 4 mg I-3-C/kg bwt/day dose group in which no adverse effects were found.

In order to differentiate between the potentially adaptive response of the liver to low doses of I-3-C and its potential toxicity at higher doses, we conducted a treatment and recovery study of I-3-C and its main metabolite DIM. As reviewed by Williams and Iatropoulos (2002), characteristics of adaptive effects on liver cell function and proliferation include not compromising organismal viability, being potentially beneficial on structure or function, resulting in an enhanced capacity to respond to stress, and being reversible; whereas, toxic effects can be lethal at the cellular or organismal level, can impair function or structure, can diminish the capacity to respond to stress, and can be irreversible.

Rats were administered 50 and 5 mg I-3-C/kg bwt/day for 28 days and then allowed a 28-day recovery period without treatment. Administration of 50 mg I-3-C/kg bwt/day for 28 days increased liver weights; males and females were equally affected. Twenty-eight days after treatment with 50 mg I-3-C/kg bwt/day, the liver weights of the previously treated rats were not different from those of the vehicle-treated control rats, indicating reversibility of the hepatic hypertrophy induced by I-3-C. Treatment with 5 mg I-3-C/kg bwt/day did not affect the liver weight. I-3-C (Safe, 2001) and its digestive products, including the linear trimer (Chang et al., 1999) and indolocarbazole (Chen et al., 1995), are known to be aromatic hydrocarbon (Ah) receptor agonists and hepatic hypertrophy is associated with Ah receptor agonists (Williams and Iatropoulos, 2002). We also evaluated the treatment and recovery effects of DIM, the major oligomeric product of I-3-C. Treatment of rats for 28 days with 7.5 and 75 mg DIM/kg bwt/day did not affect liver weights. DIM binds the Ah receptor but does not activate gene transcription (Hestermann and Brown, 2003) and did not, in this study, induce changes in liver weights.

Consistent with the reversible induction of hepatocyte hypertrophy by I-3-C, treatment of rats with 50 mg I-3-C/kg bwt/day also significantly increased CYP activities in the liver as an adaptive response. These results, and particularly the 12.3-fold increase in CYP 1A1/2 in the female rats, are consistent with the hypothesis that induction of CYP enzymes by I-3-C facilitates estrogen 2-hydroxylation and elimination as a mechanism of breast (Michnovic et al., 1997; Grubbs et al., 1995), endometrial (Kojima et al., 1994), and cervical (Jin et al., 1999) cancer prevention. However, other investigators (Ritter et al., 2001; Yoshida et al., 2004) have also reported increased estrogen 4-hydroxylation, which has been associated with carcinogenesis. Neither the 2 DIM treatments (7.5 and 75 mg DIM/kg bwt/day) nor the 5 mg I-3-C/kg bwt/day treatment consistently altered any of the liver CYP enzyme activities. Thus, during the treatment phase of this study, only 50 mg I-3-C/kg bwt/day caused liver hypertrophy and CYP enzyme induction. Leibelt et al. (2003) similarly found that I-3-C increased total CYP activity in the liver more than did DIM but did not report increased liver weights in the I-3-C-treated groups. In that dietary study, changes in CYP 1A2, 1B1, and 3A2 were also detected by immunoblotting. In the current study, oral gavage was chosen as the route of administration in order to model the human route and functional assays of CYP activity were performed.

After the 28-day recovery period, several of the liver enzyme activity levels in the males previously treated with 50 mg I-3-C/kg bwt/day were significantly elevated. Additionally, liver enzyme activity elevations, which were not observed during treatment with 5 mg I-3-C/kg bwt/day, were observed after the recovery period in these males. None of the alterations seen at 28 days persisted in the females after the 28-day recovery period, except that a slight increase in CYP 19 A was observed in the females previously treated with 5 mg I-3-C/kg bwt/day (1.3-fold). Therefore, the effects of treatment with I-3-C on liver CYP enzyme activities were more pronounced and persistent in the males than in the females; however, the magnitude of the CYP elevations after recovery would not be considered biologically significant.

After recovery from treatment with DIM, which did not alter CYP activities during treatment, several significant alterations were observed in the liver CYP activities of the male rats in comparison to the liver enzyme activities of the vehicle-treated rats. In the females, the liver enzyme activities of CYP 3A4 and CYP 19 A were slightly decreased and increased, respectively. Therefore, the male rats were more sensitive to treatment with DIM than the females and enzyme activity changes occurred after treatment, although in neither sex were liver weights affected. The fold changes were not biologically significant but do suggest that DIM can affect liver enzyme activities.

In addition to liver tissue, samples were also collected from the prostate and uterus. The uterus was evaluated as an endocrine surrogate for mammary tissue which was not sufficiently abundant to evaluate. Prostate and uterus were evaluated because DIM and I-3-C, respectively, have been reported to effect animal models of prostate and breast cancer incidence. Activities of CYP 3A4 and 19 A were lower in prostate and uterus than in liver. Treatment with
DIM slightly increased uterine CYP 19 A (approximately 3-fold) and CYP 2E1 activity (approximately 1.7-fold). The effect of DIM on uterine CYP 19 A suggests that DIM might alter local estrogen formation. These effects were reversed to control levels after the recovery period.

Phase II enzyme activities were also evaluated in liver, prostate, and uterus after both treatment and recovery. The only consistent observation was that I-3-C treatment elevated UDP-GT in the female liver 4.5-fold and in male liver, 1.7-fold. Activity of QR in liver was also elevated by treatment with I-3-C in females, 1.5-fold. Changes during the recovery period were not consistent across sex and, while statistically significant in some instances, were small in magnitude and unlikely to be biologically significant. No patterns of modulation in phase II enzymes were observed in samples from the prostate and uterus. Thus, treatment with I-3-C appeared to alter phase II enzyme activities more than DIM but the effects were not maintained after the recovery period.

In summary, the safety of I-3-C as a potential cancer preventive agent has been controversial (Stoner et al., 2002). At high doses (approximately 200 mg/kg bwt/day), toxic responses occur. However, at lower doses, the responses appear to be adaptive. Administration of 50 mg I-3-C/kg bwt/day to rats for 28 days (greater than the no observed adverse effect level of 20 mg/kg bwt/day in a 13-week study) resulted in an adaptive response in the liver characterized by increased liver weights, hepatocyte hypertrophy, and increased CYP enzyme activities. These effects were largely reversed 28 days after treatment. A lower dose of I-3-C (5 mg/kg bwt/day) did not cause adaptive changes in the liver. Similarly, DIM at doses of 7.5 and 75 mg/kg bwt/day did not cause adaptive responses in the liver. Since DIM has been reported to have cancer preventive and therapeutic activity in animal models (McDougall et al., 2000; Le et al., 2003; Chen et al., 1995; Nachshon-Kedmi et al., 2004; Chang et al., 2005), it appears likely that DIM is acting through other mechanisms than I-3-C. Using computer-assisted molecular design based on I-3-C and DIM, Jong et al. (2004) recently reported synthesis of an AKT inhibitory molecule. The effects of DIM on androgen signaling and the AKT pathway warrant further study.

I-3C, DIM, and other indoles designed from them have the potential to be effective in cancer prevention and treatment. In current phase I clinical studies, dosages of I-3-C and DIM between 100 and 800 mg are being investigated for safety, pharmacokinetics, and CYP modulation. Further preclinical safety studies and controlled clinical trials will be necessary to define the risk–benefit relationships of these compounds.

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