Evaluation of Chronic Dietary Exposure to Indole-3-Carbinol and Absorption-Enhanced 3,3’-Diindolylmethane in Sprague-Dawley Rats

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Indole-3-carbinol (I3C) and 3,3'-diindolylmethane (DIM) are naturally occurring dietary components found in cruciferous vegetables. In the stomach, I3C forms condensation products including DIM. I3C and DIM are marketed as dietary supplements, but little is known about the safety of long-term exposure. Rats were fed either control diet, 1 or 10× the current human dose of absorption-enhanced DIM or 5–7× the maximal recommended dose of I3C. Experimental diets were fed continuously for 3 or 12 months or 2 months followed by control diet for 1 month. Results at 3 or 12 months were similar in most respects. No significant differences between groups were found in blood chemistry. A general decrease in serum enzyme levels in male rats was observed, perhaps indicative of a protective effect. Males fed I3C exhibited higher serum levels of 25-hydroxy-vitamin D3 (25OH-D3). There were no observable differences grossly or histologically between groups, although a high number of hyaline casts were found throughout the kidneys of all animals. In both sexes total hepatic CYP levels were significantly induced by I3C, but not by either dose of DIM. Induction of CYP1A1 and CYP1A2 in liver and CYP1A1 in colon was detected for both sexes fed I3C and the high dose of DIM. CYP3A2 was induced in females fed I3C or the high dose of DIM; males were induced with I3C, but not DIM. No induction of CYP1B1 in the colon was observed in either sex. Long-term exposure to DIM produced no observable toxicity, and comparison to I3C indicates that DIM is a markedly less efficacious inducer of CYP in the rat at doses relevant to human supplementation.

Key Words: indole-3-carbinol; 3,3-diindolylmethane; CYP1A1/1A2; CYP 3A2; bone density; 25-OH vitamin D3; clinical chemistry; histopathology; rat.

Indole-3-carbinol (I3C) and 3,3’-diindolylmethane (DIM) are naturally occurring plant alkaloids formed by the hydrolysis of indole glucosinolate (glucobrassicin), found in significant concentrations in cruciferous vegetables such as broccoli and Brussels sprouts (Bradfield and Bjeldanes, 1987; McDonell et al., 1988; Preobrazhenskaya et al., 1993; Slominski and Campbell, 1987). Furthermore, I3C is an unstable compound that undergoes rapid oligomerization in the acid environment of the stomach to form dimers, trimers, tetramers, and several other higher order condensation products (Bjeldanes et al., 1991; Bradfield and Bjeldanes, 1987; Leete and Marion, 1953; Wortelboer et al., 1992). The major product formed in vitro (Spande, 1979) and in vivo after oral administration (Dashwood et al., 1989; Stresser et al., 1995) is DIM.

Both I3C and DIM are marketed as dietary supplements and are under investigation as potential chemopreventive agents. I3C requires conversion to these acid condensation products in order to be chemopreventive against a wide variety of carcinogens and is especially effective when administered before or during the initiation phase of carcinogenesis (Bradlow et al., 1999; Murillo and Mehta, 2000; Shertzer and Senft, 2000). DIM inhibits aflatoxin B1-DNA binding in trout (Dashwood et al., 1994) and mammary tumor growth in the rat (Chen et al., 1998; Wattenberg and Louh, 1978).

Among the hypothesized mechanisms of chemoprevention of I3C and DIM is their ability to modulate xenobiotic metabolizing enzymes and induce estrogen metabolism. When administered through the diet in short-term studies, I3C and DIM induce a number of phase I enzymes in liver and colon, including cytochrome P450 (CYP) 1A1, CYP1A2, and CYP 3A (Bonneseen et al., 2001; Horn et al., 2002; Jellinck et al., 1993; Stresser et al., 1994; Vang et al., 1990). Increased activity of phase I drug-metabolizing enzymes can protect against some carcinogens by increasing their rate of oxidative metabolism to less toxic metabolites (He et al., 2000; Park and Bjeldanes, 1992; Stresser et al., 1994; Xu et al., 1997); however, an increase in activity of certain CYP isozymes could enhance carcinogenicity of some chemicals by increasing their rate of bioactivation (Ioannides and Parke, 1993).

I3C- and DIM-dependent alterations of the monooxygenase systems also raise concerns relative to their potential adverse effects on drug/xenobiotic metabolism. Studies have shown differential metabolism of tamoxifen and nicotine by liver microsomes from rats fed I3C (Katchamart et al., 2000). I3C induction of CYPs in the 1 family markedly enhances estradiol metabolism. CYP1A1 and 1A2 catalyze the 2-hydroxylation of
β-estradiol (E₂), whereas CYP1B1 is an effective E₂-4-hydroxylase (Hayes et al., 1996). Both of these metabolites are catechol estrogens and represent potential toxic metabolites. Evidence suggests that 4-OH-E₂ is the more reactive and toxic metabolite (Newbold and Liehr, 2000).

Preliminary evidence to date indicates that DIM may be a safer alternative to I3C, as it is relatively stable in acid and does not undergo further condensation reactions, preventing the formations of toxic metabolites such as indolo[3,2-b]carbazole (ICZ), a potent aryl hydrocarbon receptor agonist (Bjeldanes et al., 1991) and potential promoter of hepatocarcinogenesis in the rat (Herrmann et al., 2002). DIM induces apoptosis and inhibits growth of human cancer cells (Chen et al., 2001; Ge et al., 1996; Hong et al., 2002; Leong et al., 2001) and inhibits estrogen-dependent mammary tumorigenesis in rats (McDougal et al., 2001). However, due to poor bioavailability of DIM, its use as a supplement has been limited. The objective of this study was to evaluate the biological effects of long-term exposure to rats of an absorbable formulation of pure DIM, [BioResponse DIM® (Indolplex®)] at the current maximal human dose (Anderton et al., in press; U.S. Patent #6,086,915; Zeligs et al., 2002). In addition, a pharmacological dose 10 times the current human dose was tested for toxicity and as a comparison to a similar and previously studied dose of I3C.

**MATERIALS AND METHODS**

**Chemicals.** Indole-3-carbinol was obtained from Sigma-Aldrich Co. (Milwaukee, WI). 3,3’-Diindolylmethane was provided in a bioavailable formulation (BioResponse-DIM®) by BioResponse, LLC (Boulder, CO). The DIM content was independently verified by HPLC (Eurofins-Alpha Laboratories, Petaluma, CA).

**Animals.** One hundred forty Sprague-Dawley rats (70 of each sex) were purchased from Simonsens (Gilroy, CA) at 4 weeks of age. After a 1-week acclimation period, animals were randomly divided into 10 different treatment groups, each containing seven rats of each sex (Table 1). Rats were housed individually in hanging metal wire cages at the Laboratory Animal Resource Center, Oregon State University, and maintained at 22°C and 40 to 60% humidity on a 12-h light/dark cycle. Both tap water and powdered semisynthetic diet were available ad libitum throughout the study. Groups 1 and 7 received only control (AIN-76A) diet. The diet for groups 2, 5, and 8 was supplemented with I3C to levels providing a dose of 50 mg/kg/day. The diets for groups 3, 4, 6, 9, and 10 provided 6.6 mg/kg/day or 66 mg/kg/day. 

**TABLE 1**

<table>
<thead>
<tr>
<th>Group Duration Supplement Daily dose</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 3 months — None</td>
<td>7 7 7</td>
<td></td>
</tr>
<tr>
<td>2 3 months I3C</td>
<td>50 mg/kg</td>
<td>7 7</td>
</tr>
<tr>
<td>3 3 months DIM</td>
<td>2 mg/kg</td>
<td>7 7</td>
</tr>
<tr>
<td>4 3 months DIM</td>
<td>20 mg/kg</td>
<td>7 7</td>
</tr>
<tr>
<td>5 2 months I3C</td>
<td>50 mg/kg</td>
<td>7 7</td>
</tr>
<tr>
<td>6 2 months DIM</td>
<td>20 mg/kg</td>
<td>7 7</td>
</tr>
<tr>
<td>7 12 months — None</td>
<td>7 7</td>
<td></td>
</tr>
<tr>
<td>8 12 months I3C</td>
<td>50 mg/kg</td>
<td>7 7</td>
</tr>
<tr>
<td>9 12 months DIM</td>
<td>2 mg/kg</td>
<td>7 7</td>
</tr>
<tr>
<td>10 12 months DIM</td>
<td>20 mg/kg</td>
<td>7 7</td>
</tr>
</tbody>
</table>

**Note.** Four-week-old Sprague-Dawley rats were obtained commercially, acclimated for 1 week and then randomly assigned to one of 10 treatment groups. The housing, preparation of diet and experimental details are described in Materials and Methods.

**Blood collection and analysis.** Blood was collected from the abdominal aorta while rats were anesthetized under 4% isoflurane with 0% at a flow of 2 l/min. Blood was stored at 4°C for 2 h and then spun for 20 min at 11,000 r.p.m. to isolate serum. One aliquot of serum was sent to the Texas Veterinary Medical Diagnostic Laboratory (College Station, TX) where a small animal clinical chemistry analysis was performed utilizing a Hitachi 911 Clinical Analyzer (Roche, Indianapolis, IN). A second aliquot was sent to the Animal Health Diagnostic Laboratory (Lansing, MI), where circulating 25-hydroxyvitamin D₃ (25-OH-D₃) levels were determined using a commercial radioimmunoassay from DiaSorin (Stillwater, MN) and testosterone levels were determined (males only) using a commercial radioimmunoassay from Diagnostic Products Corporation (Los Angeles, CA).

**Bone density analysis.** Three carcasses of each sex, from groups 1, 2, and 4 were sent on ice to the University of Colorado Health Science Center (William E. Huffer, M.D., Denver, CO). The carcasses were equilibrated to 4°C, and the proximal knee joint with approximately one half of the distal femur and proximal tibia were removed and fixed for 24 h in absolute methanol, also at 4°C. The specimens were then embedded in glycol methacrylate and sectioned on a rotary microtome with a D-profile carbon-tungsten steel knife at 5 μm. The sections were stained by the von Kossa technique (Bills et al., 1971) with a hematoxylin and eosin counter-stain to demonstrate general histology and calcified bone and osteoid, and for tartrate resistant acid phosphatase to demonstrate osteoclasts on bone surfaces. Measurements of the
Histopathology. Tissues were fixed in 10% neutral buffered formalin and processed on a LXS300 Tissue Processor (Fisher Scientific). Sections were cut at 4–5 microns and stained on the S/P Automatic Slide Stainer GLX with Haematoxylin (Gill-3, Shandon Inc., Pittsburgh, PA) and Eosin Y (alcoholic, Shandon Inc., Pittsburgh, PA). Slides were coveredlipped with Shur/Mount (Triangle Biomedical Sciences, Durham, NC).

Microsome preparation and total CYP. Liver samples were homogenized with a Brinkmann Polytron (Westbury, NY) into three volumes of cold homogenization buffer (10 mM potassium phosphate [pH 7.5] containing 0.15 M potassium chloride, 1 mM EDTA, and 0.1 mM phenylmethylsulfonylfluoride [PMSF]). Microsomes were prepared by ultracentrifugation according to Guengerich (1989), and protein levels were determined by the method of Lowry et al. (1951). The total liver microsomal CYP content was quantified by the CO versus CO-reduced difference spectra (Omura and Sato, 1964) on a Cary 300 UV-Vis spectrophotometer (Varian, Walnut Creek, CA).

Colon lysate preparation. Colonies were rinsed with phosphate buffered saline (PBS) to remove mucus and then scraped with the back of a scalpel blade to remove cells. Cells were lysed in a modified RIPA buffer [1× PBS, 1.0% Igepal CA-630, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS) with 1 tablet of Complete-Mini protease inhibitor cocktail (Roche, Indianapolis, IN) added for every 10 ml of buffer] and then sheared by passing through a 23-gauge needle. Sheared cells were incubated 45 min on ice after the addition of 0.574 mM PMSF (1:100 from an isopropanol stock) and then spun at 10,000 r.p.m. for 10 min at 4°C. The supernatant was removed, protein concentration determined by the method of Lowry et al. (1951), and stored at −80°C until analysis.

Electrophoresis and immunoblotting. Microsomal and lysate proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and electrophoretically transferred to nitrocellulose membrane (Towbin et al., 1979). The membranes were incubated with goat antibodies recognizing both rat CYP1A1 and CYP1A2 or CYP3A2 (Gentest, Woburn, MA), and probed with rabbit anti-goat secondary antibody conjugated with horseradish peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, MD) or rabbit antibody raised against rat CYP1B1 (Gentest, Woburn, MA), and probed with goat anti-rabbit secondary antibody also conjugated to horseradish peroxidase (Gentest, Woburn, MA). The blots were visualized by a chemiluminescence detection kit (New England Nuclear, Boston, MA) and densitometry was performed using an HP Scanjet IIcx flatbed scanner and NIH Image software version 1.61/ppc (public domain, National Institutes of Health).

Statistical analysis. Males and females were analyzed separately. When assumptions were reasonably satisfied, treatments were compared using one-way ANOVA and all pairwise comparisons with Tukey’s multiple comparison adjustment. When outliers or other non-normality were indicated, simple transformations, such as logarithmic, were examined. When the problem was nontransformable, nonparametric rank tests (Kruskal-Wallis) were used. In that case, pairwise comparisons were adjusted for multiple comparisons using the global permutation distribution, after first determining that there were no problems with extreme heteroscedasticity or badly unbalanced sample sizes (Westfall et al., 1999). When preliminary analysis indicated normality but heterogeneity of variance, a general mixed model allowing heterogeneity of variance was fit by residual maximum likelihood (REML) with Tukey adjusted pairwise comparisons. The denominator degrees of freedom for testing were adjusted by the method of Kenward and Rogers (SAS, 1999). Statistical analyses were conducted using SAS version 8.2 (Cary, NC). Within SAS/STAT the GLM, Mixed, Npar1way, and Multtest procedures were used.

RESULTS

Body and Tissue Weights

There were no significant differences in diet consumption or weight gain between groups. The body weights in both sexes tended to be lower in the I3C-fed group, but the analysis of weights at the time of sacrifice revealed no significant differences between treatments (p = 0.23 and 0.44, ANOVA, for males and females, respectively, Fig. 1). There were also no treatment-related effects on raw tissue weights in either sex, although I3C, but not DIM, significantly increased the liver somatic index (LSI) in males at both the 3- and 12-month time points (Table 2). In the group fed I3C for 2 months followed by control diet for 1 month, the LSI was lower relative to the group fed I3C for 3 months, but the difference was not statistically significant (Table 2). Both sexes fed I3C had increased LSI after 12 months relative to controls, but only the increase in males was large enough to be statistically significant after multiple comparison adjustment (p = 0.0023 t-test [Table 2] and p = 0.086 Wilcoxon for males and females, respectively). The high dose of DIM marginally induced LSI in males after 12 months (p = 0.042, Tukey-adjusted t-test), but not in females.

Clinical Chemistry, 25OH-D₃, and Testosterone Levels

The results of the clinical chemistry panel with female rats did not demonstrate any treatment related effects after 3 or 12 months of exposure, with the exception of phosphorus in females fed the high DIM diet (3.17 mg/dl compared to 4.20 in controls, adjusted p value = 0.0279, data not shown). In males, neither I3C nor DIM elevated biomarkers of tissue damage, and in fact, a general decrease in the levels of serum enzymes after 12 months of experimental diet administration was observed in male rats (data not shown). Serum aspartate aminotransferase (AST) levels in males were significantly lower in male rats fed I3C or either dose of DIM for 12 months (p < 0.013, for each Tukey-adjusted comparison). A heterogeneous variance mixed model was used for analysis because rats in the control group exhibited greater variation than the other groups. Although alanine aminotransferase (ALT) levels at 12 months were also lower in these same rats, the difference, relative to controls, was not found to be statistically significant, due to high variability in the control group (p > 0.11, all adjusted pairwise comparisons). Alkaline phosphatase (ALP) serum levels were reduced in male rats fed I3C (p = 0.010) and the high dose of DIM (p = 0.004). The only significant reduction in creatinine kinase (CK) was in the male rats fed the high dose of DIM (p = 0.010). At 12 months there were no significant differences between control and treatment groups in either sex for any of the remaining serum chemistry measurements (total protein, albumin, calcium, glucose, BUN, globulins, GGT, cholesterol, amylase, bilirubin, creatinine).
Serum 25-OH-D₃ levels were increased ($p = 0.0371$ and $0.0031$ for males and females, respectively) about 50% by dietary I3C in both sexes after 12 months. At 3 months only the males exhibited elevated serum levels of 25-OH-D₃. Again, the I3C diet increased levels by about 50%. The high dose of DIM also appeared to increase 25-OH-D₃ in serum, but the change was not significant.

Serum testosterone levels in males were not altered by test diets. E₂ levels were unchanged by I3C or DIM at both the 3- and 12-month time points (data not shown).

Histopathology

No significant differences between groups of either sex at 12 months was noted upon necropsy or following histopathology. Most notably, no effects were seen in hormone-responsive tissues such as prostate in male rats and ovaries in female rats, and no toxicities were indicated in treatment groups as compared to controls in the liver (Figs. 2 and 3). There were large numerous hyaline casts found in the kidney tubules. The appearance of such casts...
are common in rats with age (Lord and Newberue, 1990), and no treatment-related differences were evident (data not shown).

**CYP Levels**

The total hepatic CYP of the male and female rats at 12 months is shown in Figure 4. A significant increase in total CYP was observed in the I3C supplemented groups of both sexes. DIM did not significantly induce total hepatic CYP in either sex.

As previously demonstrated by our laboratory and others (Bjeldanes et al., 1991; Bradfield and Bjeldanes, 1987; Katchamart et al., 2000; Larsen-Su and Williams, 1996; Mannon et al., 1997; Stresser et al., 1994; Wortelboer et al., 1992), I3C effectively induces CYP1A1 and 1A2 levels in liver microsomes following oral administration. After 3 months, CYP1A1/1A2 was induced 20- and 44-fold in rat liver microsomes from males and females, respectively. DIM also significantly induced CYP1A1/1A2 but to a much lesser degree (two- and tenfold for males and females, respectively, data not shown). Immunoquantitation of CYP1A1 and CYP1A2 in liver of rats at 12 months is shown in Figure 5A (males) and Figure 5B (females). Relative band densities of hepatic CYP1A1 were increased 82-fold ($p < 0.001$) and 16-fold ($p = \ldots$)

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**TABLE 2**

Liver Somatic Index in Males at 3 and 12 Months

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>LSI (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3-month control</td>
<td>3.58 ± 0.25</td>
</tr>
<tr>
<td>2</td>
<td>3-month I3C</td>
<td>4.00 ± 0.45*</td>
</tr>
<tr>
<td>3</td>
<td>3-month low DIM</td>
<td>3.58 ± 0.15</td>
</tr>
<tr>
<td>4</td>
<td>3-month high DIM</td>
<td>3.66 ± 0.25</td>
</tr>
<tr>
<td>5</td>
<td>2-month I3C/1 month control</td>
<td>3.74 ± 0.26</td>
</tr>
<tr>
<td>6</td>
<td>2-month DIM/1 month control</td>
<td>3.65 ± 0.26</td>
</tr>
<tr>
<td>7</td>
<td>12-month control</td>
<td>2.51 ± 0.18</td>
</tr>
<tr>
<td>8</td>
<td>12-month I3C</td>
<td>2.93 ± 0.08*</td>
</tr>
<tr>
<td>9</td>
<td>12-month low DIM</td>
<td>2.65 ± 0.23</td>
</tr>
<tr>
<td>10</td>
<td>12-month high DIM</td>
<td>2.82 ± 0.24*</td>
</tr>
</tbody>
</table>

*Indicates statistical significance at $p < 0.05$.

**Note.** LSI = Liver weight/body weight x 100. The results are the mean (± SD).

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**FIG. 2.** Photomicrographs of haematoxylin stained liver from male (A, C, and E) and female (B, D, and F) sections from control (A and B), I3C (C, D), and DIM (high dose) (E and F) treated rats at the 12-month time point. The preparation of tissues was as described in the Materials and Methods. Bar = 50 μm.
FIG. 3. Photomicrograph of haematoxylin stained prostate (A, C and E) and ovary (B, D, and F) sections from control (A and B), I3C (C and D), and DIM (high dose) (E and F) treated rats at the 12-month time point. The preparation of tissues was as described in the Materials and Methods. Bar = 50 μm.

FIG. 4. Total hepatic microsomal CYP levels in male and female rats after 12 months of dietary exposures. The bars represent group averages ± SE. The asterisk indicates $p < 0.05$. 
in male rats fed diets containing I3C or the high dose of DIM, respectively. CYP1A1 was not detected in the livers of control female rats, but band densities were induced to levels ninefold higher in I3C supplemented animals than in the DIM treated group (Fig. 5). The band densities of hepatic CYP1A2 were elevated approximately 40-fold ($p < 0.001$) in both male and female rats given I3C but only tenfold in rats fed diet containing the high dose of DIM (Fig. 5). In the colon, CYP1A1 band densities were induced ten- and eightfold (males) and eight- and threefold (females) in the I3C and DIM groups, respectively (Fig. 6). No evidence was seen for CYP1B1 induction in the colon of either sex following administration of I3C or DIM for 12 months. After 12 months, in females exposed to I3C, CYP3A2 band densities were increased five- and twofold with I3C and DIM, respectively (Fig. 7). I3C induced the CYP 3A2 band density almost twofold in male rats, but no induction was observed with DIM exposure (Fig. 7).

**Bone Density**

Qualitative and histomorphometric analysis of methacrylate-embedded undecalcified cancellous and cortical bone from mature Sprague-Dawley rats showed that, in comparison to the control group, the I3C and DIM diets were not associated with any additional adverse effects on bone density, structure, or turnover. All three groups had cancellous osteoporosis more severe than that observed in control rats from previous studies where other diets were utilized. This osteoporosis was more severe in male than female rats (data not shown).

**DISCUSSION**

The doses tested in the study were based on the current maximal human dose of DIM of 2 mg/kg, which was provided by a daily dose of 6.6 mg/kg formula weight of BioResponse-DIM® (Indoloplex®). Twenty mg/kg DIM was provided by a daily dose of 66 mg/kg BioResponse-DIM® to give an expo-
sure 10 times higher than the current human dose, to amplify any potential toxicity not apparent at the lower dose. The I3C exposure utilized (50 mg/kg/day) a dose that was shown in previous studies to result in levels of DIM in human blood complimentary to the level observed when exposed to 66 mg/kg/day BioResponse-DIM (Arneson et al., 2001; Zeligs et al., 2002) and that represents approximately 5–7 times the daily dose recommended by commercial suppliers of I3C supplements.

The body weights and organ weights did not suggest any chronic treatment-related toxicity, with the possible exception of liver. A previous study showed that after a 15-day treatment of 0.5% I3C in the diet of rats, the liver somatic index was significantly increased from 4.4% in controls to 6.4% (Manson et al., 1997). The smaller but chronic doses of I3C and DIM in this study also resulted in a significant increase in the LSI. A 3-month oral administration of I3C to rats at doses of 4, 20, or 100 mg/kg/day also demonstrated an increased LSI (at the 20 and 100 mg/kg/day doses). In that same study, histopathological changes in liver were observed at the 100 mg/kg/day dose, as well as a decrease in testes weight at all doses (NCI, 1996). These differences may be related to the method of administration (diet versus gavage). This increase in LSI observed in our study with I3C or DIM correlates with the degree of CYP induction that was also observed, as I3C was a more efficacious inducer of CYP and also had a greater effect on LSI when

**FIG. 6.** Western blot (inset) and densitometry of CYP1A1 in colonic lysates from male and female rats administered control diet, I3C, or the high dose of DIM for 12 months. Lysate proteins (80 μg) from rat colon were resolved by SDS-PAGE, blotted onto nitrocellulose and probed with goat antibody to rat CYP1A1 as described in Materials and Methods. Lanes 1–3 are from rats fed control diet; lanes 4–7 from rats fed I3C; lanes 8–10 (male, top inset) and 8–11 (female, bottom inset) from rats fed the high dose of DIM and lanes 11–13 (top inset) or 12–14 (bottom inset) were 1.0, 0.5, and 0.25 pmol, respectively of CYP1A1 standard.

**FIG. 7.** Western blot (inset) and densitometry of CYP3A2 in liver microsomes from male (top inset) and female (bottom inset) rats administered control diet, I3C or the high dose of DIM for 12 months. Microsomal proteins (24 μg) were resolved by SDS-PAGE, blotting onto nitrocellulose and probed with goat antibody to rat CYP3A2 as described in Materials and Methods. Lanes 1–3 (male) and 1–2 (female) are from rats fed control diet; lanes 4–7 (male) and 3–7 (female) are from rats fed I3C diet; lanes 8–11 are from rats fed the high dose of DIM; and lanes 12–14 are 1.0, 0.5 and 0.25 pmol, respectively of purified CYP3A2.
compared to DIM. I3C significantly enhanced LSI in males after 3 or 12 months. The high dose of DIM also increased LSI but only after 12 months. The increase was not as marked as with I3C and was seen only in males. We have no explanation for the effect of sex on LSI.

Clinical chemistry panels failed to uncover any significant differences between control, I3C, and DIM treated rats that would indicate toxicity. Conversely, the significant reduction in creatinine kinase (CK) in male rats fed DIM, and the significant reduction in alkaline phosphatase (ALP) and aspartate aminotransferase (AST) in rats fed I3C or DIM could indicate possible protective effects against age-related tissue damage. This may be explained by the antioxidant and electrophilic scavenging properties described for I3C and DIM (Arnao et al., 1996; Fong et al., 1990; Shertzer and Senft, 2000; Shertzer et al., 1988). Again, this significant reduction in the serum enzyme markers was only evident in male rats after 12 months of dietary exposures. With one exception, no other significant alterations in serum chemistry following 12 months of exposure to I3C or DIM was evident. In previous work some acid condensation products of I3C have been shown to lower serum LDL/VLDL cholesterol levels in mice (Dunn and LeBlanc, 1994), resulting from the inhibition of acyl-CoA:cholesterol acyltransferase (ACAT). Treatment with I3C or DIM failed to provide cholesterol-lowering effects in this study. The dietary indoles did not alter serum levels of testosterone in males or E2 in females. The fact that there were no significant changes in testosterone levels in this study may not be surprising, considering the individual variation normally observed in testosterone levels in rats (Overpeck et al., 1978) and that 750 mg/kg of I3C were needed to cause a significant effect in shorter-term studies (Wilson et al., 1999). Clinical trials with I3C have documented reductions in urinary E2 levels in both men and women concurrent with an increase in the 2-OH-E2/16a-OH-E2 ratio (Michnovicz et al., 1997) and absorption-enhanced DIM also increased this ratio in a pilot clinical study (Zeligs et al., 2002).

The basis for investigation of both the 25-OH-D3 levels and bone density stem from reports of individuals with low 25-OH-D3 and a decrease in bone density while on I3C. The concern regarding vitamin D3 is that enzyme induction in the colon or liver could influence levels of this vitamin/hormone, which in turn could effect bone density. It has also been shown that bone density can be influenced by estrogen metabolism, especially CYP1A-dependent 2-hydroxylation (Leelawattana et al., 2000). There appears to be greater bone density with lower ratios of 2-OH/ 16a-OH estrogen levels in postmenopausal women, implying that increasing this ratio with I3C or DIM could result in lower bone density. No effect on bone density was observed in this study following I3C or DIM exposure. DIM had no significant effects on 25-OH-D3 levels; however, significant increases were observed in males fed I3C for 3 or 12 months and in females fed I3C for 12 months.

The absence of data indicating toxicity in the chemistry panel and other blood work was confirmed by the histopathological examination. Other than the increase in hyaline casts in the kidney, no apparent lesions were observed in any of the tissues examined. The appearance and severity of this kidney pathology increased with age but was not treatment related.

The induction of CYP isoforms observed in this study are mostly consistent with data from previous acute or subchronic studies. The 24-, 3-, and 4- fold induction of CYPs 1A1, 1A2, and 3A1/2, respectively, in male Fischer 344 rats consuming 0.2% I3C in their diet for 7 days (Stresser et al., 1994) can be compared to the 82-, 40-, and 2- fold inductions observed in male Sprague-Dawley rats in this study after administration of a similar dose of I3C over a much longer time.

Whereas direct toxicity by long-term exposure to I3C and DIM is not evident in this study, the induction of CYPs, especially those of the 1A subfamily could be a cause for concern, given the role of these enzymes in activation of polycyclic aromatic hydrocarbons such as benzo[a]pyrene and aromatic amines such as 4-aminobiphenyl or PhIP. The induction of CYP 3A may also be significant, as this subfamily contributes to the metabolism of 60% of all clinically relevant drugs (Guengrich, 1999). The dampened induction of CYPs seen with DIM exposure may result in fewer drug interactions with DIM supplementation, when compared to I3C. When all endpoints in this study are considered in the comparison between I3C and DIM, the differences can be contributed to a magnified effect in the increased liver somatic index, total CYP, and induction of specific CYPs in the I3C treated group. The higher efficacy/potency of I3C is expected and related to the fact that in the acidic conditions of the stomach after oral exposure, I3C becomes a complex mixture of not only DIM but more than 20 different I3C-derived compounds, all having pharmacological/toxicological effects, such as possessing different affinities for the Ah receptor. One of these compounds, ICZ, binds to the Ah receptor with an affinity similar to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). DIM has been shown to be relatively more stable in acid and does not robustly undergo further condensation reactions.

The data from this study confirms results from short-term studies indicating that both I3C and DIM are relatively nontoxic compounds. Furthermore, these results confirm earlier long-term feeding studies in other models, including the rainbow trout and the same strain of rat used in the present study, that I3C is not a complete carcinogen (Dashwood et al., 1991; Oganesian et al., 1999; Stoner et al., 2002). A concern, however, with the prolonged use of I3C for cancer chemoprevention is its potential for promotion of liver neoplasms. I3C is an effective promoter of liver cancer in the trout (Dashwood et al., 1991; Oganesian et al., 1999) and recent studies in a multiorgan model (female Sprague-Dawley rats initiated with 7,12-dimethylbenz[a]anthracene for breast, aflatoxin B1 for liver and azoxymethane for colon) demonstrate that long-term post-
initiation with dietary I3C could provide some chemoprotection in breast and colon, but not without a significant increased risk for liver neoplasms (Stoner et al., 2002). The long-term post-initiation effects of I3C in hepatocarcinogenesis are not consistent across species, as this treatment with C57 black mice, initiated at 15 days of age with diethylnitrosamine, provided significant protection (Oganesian et al., 1997). The mechanism(s) of I3C tumor modulation need to be established in these models in order to assess the risk to human health.

Previous work from our laboratory utilizing the rainbow trout has shown both I3C and DIM to be estrogenic (Shilling and Williams, 2000; Shilling et al., 2001). The estrogenicity of I3C is a likely mechanism by which I3C promotes hepatocarcinogenesis in trout (Oganesian et al., 1999). We have not yet tested DIM in trout as a tumor promoter. DIM is primarily an anti-estrogen in mammalian systems (Chen et al., 1998; McDougal et al., 2001). We hypothesize that this difference may be a function of species-specific DIM metabolism. We have preliminary evidence that CYP-dependent hydroxylation of DIM is required in trout to elicit estrogenicity (Shilling et al., 2001).

Also confirmed were the alterations observed in the monoxygenase system that could be important in carcinogen bioactivation/detoxication and potential adverse effects on drug metabolism. The results from this study suggest that DIM is a markedly less efficacious inducer of CYP in the rat, but further studies are required to investigate the effects of both I3C and DIM on carcinogenesis, metabolism, and human health.

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