A Phase I Study of Indole-3-Carbinol in Women: Tolerability and Effects

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Abstract

We completed a phase I trial of indole-3-carbinol (I3C) in 17 women (1 postmenopausal and 16 premenopausal) from a high-risk breast cancer cohort. After a 4-week placebo run-in period, subjects ingested 400 mg I3C daily for 4 weeks followed by a 4-week period of 800 mg I3C daily. These chronic doses were tolerated well by all subjects. Hormonal variables were measured near the end of the placebo and dosing periods, including determination of the urinary 2-hydroxyestrone/16α-hydroxyestrone ratio. Measurements were made during the follicular phase for premenopausal women. Serum estradiol, progesterone, luteinizing hormone, follicle-stimulating hormone, and sex hormone binding globulin showed no significant changes in response to I3C. Caffeine was used to probe for cytochrome *P*450 1A2 (CYP1A2), *N*-acetyltransferase-2 (NAT-2), and xanthine oxidase. Comparing the results from the placebo and the 800 mg daily dose period, CYP1A2 was elevated by I3C in 94% of the subjects, with a mean increase of 4.1-fold. In subjects with high NAT-2 activities, these were decreased to 11% by I3C administration but not altered if NAT-2 activity was initially low. Xanthine oxidase was not affected. Lymphocyte glutathione *S*-transferase activity was increased by 69% in response to I3C. The apparent induction of CYP1A2 was mirrored by a 66% increase in the urinary 2-hydroxyestrone/16α-hydroxyestrone ratio in response to I3C. The maximal increase was observed with the 400 mg daily dose of I3C, with no further increase found at 800 mg daily. If the ratio of hydroxylated estrone metabolites is a biomarker for chemoprevention, as suggested, then 400 mg I3C daily will elicit a maximal protective effect. (Cancer Epidemiol Biomarkers Prev 2005;14(8):1953–60)

Introduction

Cruciferous vegetables and specific compounds they contain have been shown to modulate carcinogenesis in animals and humans (1-10). Among these compounds is glucobrassicin (3-indolylmethyl glucosinolate). This compound is hydrolyzed by the endogenous plant enzyme myrosinase, releasing indole-3-carbinol (I3C). I3C has been shown to have pronounced chemopreventive effects against development of both spontaneous (4, 6) and chemically induced (1-3, 5, 7-10) tumors in rats, mice, and trout. These reports involved the use of polycyclic aromatic hydrocarbons (1, 7), nitrosocompounds (3, 5, 7, 8), heterocyclic aromatic amines (10), and aflatoxin (2) as initiating agents. In addition, chemopreventive effects of I3C were reported against tumor development in mammary gland (1, 4, 7), liver (2, 3), lung (5, 8), cervix (6, 9), and gastrointestinal tract (1). This range of species, causes, and organ sites showing a chemopreventive effect of I3C supports the further study and development of this compound. The project reported here, a phase I trial of I3C in humans, provides a further step in this development. Because the chemopreventive effects of I3C were observed in mammary gland and because I3C has been proposed as a potential chemopreventive agent for breast cancer in particular (11, 12), we chose to carry out this phase I study in a population of women at elevated risk for breast cancer.

Several targets and mechanisms have been proposed for the chemopreventive activity of I3C, and these have been investigated both *in vitro* and in animals. Among the mechanisms proposed for chemoprevention by I3C are alteration of carcinogen metabolism (3), alteration of estrogen metabolism (4), endocrine disruption (13-15), general membrane/antioxidant effects (16), modulation of signal transduction pathways (17, 18), and effects on apoptosis (19-21). Given the practical limitations of a phase I study in healthy subjects, and with consideration for etiologic factors favoring the development of breast cancer, we have chosen to focus on the first three of these proposed mechanisms. Each can be studied readily by noninvasive means in human subjects. Activities of some carcinogen- and estrogen-metabolizing enzymes can be assessed using a probe drug approach, whereas other enzymes may be characterized *ex vivo* using subjects' lymphocytes. Alterations of estrogen metabolism may be directly assessed by quantifying urinary metabolites. Finally, endocrine disruption can be studied at a biochemical level by determining circulating levels of hormones and related proteins and functionally by following estrous in premenopausal subjects. Modulation of activities of carcinogen-metabolizing enzymes is considered relevant to the development of many chemically induced cancers. The focus on estrogen metabolism and on endocrine modulation, however, is of particular relevance for the development of hormone-dependent cancers, such as breast cancer.

Whether one mechanism or others are ultimately proven responsible, sufficient data have been obtained in animal and human studies to warrant further definition of the tolerability and effects of orally administered I3C in humans. We have addressed these issues in a study using subjects from a high-risk breast cancer cohort. Our findings and their implications for I3C as a chemopreventive agent are presented here.

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Materials and Methods

Materials. I3C capsules, each containing 200 mg I3C, and matching placebos were provided by the Division of Cancer Prevention, National Cancer Institute. Capsules were distributed by McKesson Bioservices under contract to the National Cancer Institute. All organic solvents were high-performance liquid chromatography (HPLC) grade and were products of Fisher Scientific (St. Louis, MO), as were 2,6-dichlorophenol-indophenol and all buffers and salts. A sample of 5-acetylamino-6-formylamino-3-methyluracil was generously provided by Nestec Research Centre (Lausanne, Switzerland), and levallorphan tartrate was donated by Hoffman La Roche (Nutley, NJ). Ficoll-Paque was purchased from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). Kits for cortisol immunoassay were obtained from DRG Diagnostics (Mountainside, NJ). All other substrates, cofactors, enzymes, detergents, and standards were purchased from Sigma-Aldrich (St. Louis, MO).

Subjects and Treatments. Healthy, nonsmoking women between 18 and 65 years of age with an elevated risk of breast cancer were recruited. Breast cancer risk was defined as a Gail score of ≥1.67 (22) or by other family history of breast cancer. Nonsmoking status was confirmed by a urine cotinine test. Premenopausal subjects were not taking oral contraceptives, and postmenopausal women were not taking hormone replacement therapy. All women had a negative pregnancy test before administration of I3C. They were instructed not to consume cruciferous vegetables during the course of the study. All documents and procedures were reviewed and approved by the institutional review boards of the University of Kansas Medical Center.

All subjects proceeded through the three sequential treatment phases of this single-blind study. In the first phase, subjects received two bottles, each containing placebo capsules, and were instructed to ingest one capsule from each bottle at breakfast and at dinner. After 4 weeks under this regimen, they then were issued one bottle containing placebo capsules and a second bottle containing 200 mg I3C capsules. Subjects again were instructed to ingest one capsule from each bottle at breakfast and at dinner for a daily dose of 400 mg I3C. In the final phase, each subject received two bottles, each containing 200 mg capsules of I3C, and ingested two capsules with meals in the morning and evening for a daily dose of 800 mg I3C. At the end of both the placebo and high-dose I3C dosing phases, subjects were instructed to fast overnight and the next morning to swallow their placebo or I3C dose in the clinic under supervision. This was followed immediately by probe drug administration and sampling. Premenopausal subjects were tested during the follicular phase of their cycle. In addition to allowing intraindividual examination of the dose dependency of I3C effects, this dose escalation regimen also enabled us to assure that the lower I3C dose was well tolerated before it was increased to 800 mg daily.

Probe Drug and Sampling Procedures. First morning urine samples were collected and aliquots were taken for analysis of estrone and cortisol hydroxylation. Aliquots for estrone metabolism were treated with 2 mg/mL ascorbate. Blood was obtained for assessment of circulating biomarkers [estradiol, progesterone, luteinizing hormone, follicle-stimulating hormone, sex hormone binding globulin, thyroid-stimulating hormone, insulin-like growth factor binding protein (IGFBP)-1, and IGFBP-3], for clinical chemistries, and for lymphocyte preparation. Lymphocytes were isolated by Ficoll centrifugation (46). All processing of blood was done within 30 minutes of acquisition. Lymphocytes were stored frozen at −80°C for up to 6 months before cytosol preparation and analysis. Subjects, who had fasted overnight, then were administered their morning dose of placebo or I3C and the probe drugs (100 mg caffeine and 30 mg dextromethorphan) orally with 200 mL water (23). Fasting was continued for an additional 2 hours to allow for absorption of the probe drugs. Urine was collected between 0 and 4 hours after dosing and aliquots were taken for dextromethorphan and metabolite analysis. Urine collected from 4 to 5 hours after dosing was acidified with ascorbate (2 mg/mL) and aliquots were taken for caffeine and metabolite analysis. Urine aliquots were stored at −80°C for up to 4 months before analysis.

Analytic Procedures. Immunoassays for 2-hydroxyestrone, 16α-hydroxyestrone, and 6β-hydroxycortisol were done by Dr. Daniel Sepkovic (Institute for Biomedical Research, Hackensack University Medical Center, Hackensack, NJ; ref. 24). Enzyme phenotyping was done using caffeine and dextromethorphan as probe drugs. Samples of urine for caffeine analysis were centrifuged and filtered and then analyzed by reverse-phase HPLC based on the procedure of Kashuba et al. (25). Specific analytes were 1-methylurate, 1-methylxanthine, 1,7-dimethylurate, 3,7-dimethylxanthine, 5-acetylamino-6-formylamino-3-methyluracil, and caffeine. Activities of cytochrome *P*450 1A2 (CYP1A2), flavin monooxygenase-3 (FMO3), *N*-acetyltransferase-2 (NAT-2), and xanthine oxidase were defined by the ratio of urinary caffeine and caffeine metabolites as described previously (25, 26). CYP1A2 activity was defined by the ratio (1-methylurate + 1-methylxanthine + 5-acetylamino-6-formylamino-3-methyluracil)/1,7-dimethylurate, FMO3 activity by 3,7-dimethylxanthine/caffeine, NAT-2 activity by 5-acetylamino-6-formylamino-3-methyluracil/(1-methylxanthine + 1-methylurate + 5-acetylamino-6-formylamino-3-methyluracil), and xanthine oxidase activity by 1-methylurate/(1-methylxanthine + 1-methylurate). Samples for dextromethorphan metabolism were processed and analyzed by HPLC using the procedure of Kashuba et al. (27). CYP2D6 activity was defined by the ratio of parent drug dextromethorphan to its metabolite dextrorphan. Analytes in the caffeine and dextromethorphan assays, which were not detected by HPLC or were below the limit of detection, were given values of 0 in the calculation of metabolite ratios. Lymphocytes were prepared by centrifugation of heparinized blood through Ficoll at 400 × *g* for 40 minutes. Cytosols were prepared from lymphocytes as 100,000 × *g* supernatants following solubilization with 0.08% digitonin (46) and used for *ex vivo* measurement of enzyme activities. NADPH:quinone oxidoreductase (NQO1) was assayed using the spectrophotometric method (46) with menadione as the initial electron acceptor. Duplicate assays were done using 2,6-dichlorophenol-indophenol, proposed as a more specific NQO1 substrate, in place of menadione (28). Total glutathione *S*-transferase (GST) activity was measured spectrophotometrically by the method of Gupta et al. (29), with 1-chloro-2,4-dinitrobenzene as the substrate.

Statistical Analysis. Each of the biomarkers of interest can be treated as a continuous variable analytically. Descriptive statistics on the median levels and variability of final biomarker levels from baseline visit to final visit were developed separately for each dosing level. Because this is a repeated-measures design with increasing dose, Friedman's test was used to assess if there were any effects of dose on each biomarker. Biomarkers that were measured at three dosing levels were first globally compared, and if there was a significant global difference, pairwise comparisons between the dosing levels were done. Measured values for the bimodal activities (i.e., NAT-2 and CYP2D6) were not only globally compared but then analyzed separately by group.

Results

A total of 19 subjects were enrolled in this study. One subject was dropped from the study at the end of the placebo period for noncompliance, and one subject voluntarily withdrew from

the study during the 400 mg daily dosing period. The latter subject complained of an exacerbation of preexisting joint pain. This adverse event was judged as unlikely to be treatment related, but the subject withdrew from the study and her data were not included in our analyses. Overall, we observed that daily I3C at both 400 and 800 mg daily was well tolerated by our subjects. There were no serious adverse events or trends related to ingestion of I3C (Table 1) and none of the adverse events, except an episode of bronchospasm, led to discontinuation. The single exception was a woman who had an unprovoked asthma attack requiring inhaled bronchodilators after >8 weeks on I3C, including 4 weeks at the highest, 800 mg dose. This subject subsequently admitted to a prior history of asthma, which she had denied at the time she was enrolled. Because this subject had completed all procedures for measurement of I3C effects, her data are included here.

Numerous endocrine markers and endocrine-related functions have been proposed as targets for modulation by I3C. Accordingly, the serum levels of estradiol, progesterone, luteinizing hormone, follicle-stimulating hormone, sex hormone binding globulin, and thyroid-stimulating hormone were measured for all subjects during the placebo run-in and at the end of both 400 and 800 mg daily I3C dosing periods. Serum levels of IGFBP-1 and IGFBP-3 also were determined for all subjects during the placebo period and at the end of the high-dose I3C period. In addition, all subjects measured their basal temperature daily throughout the study to assess effects of I3C on the estrous cycle. Except for two slightly elevated serum progesterone concentrations, all baseline endocrine tests were within normal limits. No significant changes or trends related to I3C administration were observed in serum levels of estradiol, progesterone, luteinizing hormone, follicle-stimulating hormone, sex hormone binding globulin, thyroid-stimulating hormone, IGFBP-1, IGFBP-3, or basal temperature cycles (data not shown).

Examination of I3C effects on the activities of carcinogen- and steroid-metabolizing enzymes used probe drugs, endogenous substrates, and *ex vivo* approaches. The probe drugs employed were caffeine and dextromethorphan. Analysis of caffeine and its specific metabolites in urine was accomplished by reverse-phase HPLC. The ratios of those analytes provide validated measures for three enzyme activities (CYP1A2, NAT-2, and xanthine oxidase) and a more controversial measure of a fourth enzyme activity (FMO3). The metabolite ratios showed that 16 of 17 subjects exhibited increased CYP1A2 activity, with the remaining subject showing no change (Fig. 1A). The mean ± SD of the individual ratios of I3C-treated to placebo CYP1A2 activity was 5.06 ± 4.98, a highly significant difference ($P < 0.0001$).

Assessment of NAT-2 activity revealed a bimodal distribution of metabolite ratios, with a node occurring at a ratio of ~0.4 (Fig. 1B), very similar to the distribution reported previously (25). This resulted in the classification of 11 of these subjects as ''fast'' acetylator phenotype, whereas 6 subjects were ''slow'' acetylators. Data derived from each acetylator subgroup were analyzed separately. For the fast acetylators, one subject showed an increase in activity following I3C treatment, whereas six subjects showed decreased activity. The mean ± SD of the individual I3C:placebo ratios was 0.82 ± 0.26. This small reduction in activity resulting from I3C treatment in fast acetylators showed statistical significance ($P = 0.012$). Among the slow acetylators, two showed increased NAT-2 activity following I3C, whereas four subjects showed decreased activity. The mean ± SD of the activity ratios was 1.12 ± 1.03, which was not significant.

The results of xanthine oxidase phenotyping are shown in Fig. 1C. Five subjects showed increased activity following I3C, one showed decreased activity, and the majority showed no change following treatment. The mean ± SD of the activity ratios was 1.09 ± 0.25. This was not a statistically significant change ($P = 0.1435$).

Phenotyping of FMO3 using caffeine is supported by some reports (26) but not by all (30). Our findings for a preliminary characterization of I3C effects on this enzyme are reported here (Fig. 1D). Thirteen of 17 subjects showed a decrease in apparent FMO3 activity in response to I3C treatment. The mean ± SD of the individual ratios of I3C-treated to placebo activities is 0.40 ± 0.56, resulting in $P = 0.02$.

Phenotyping for CYP2D6 was based on analysis of dextromethorphan metabolism (Fig. 2). The ratio of parent drug (dextromethorphan) to the CYP2D6-dependent metabolite dextrorphan was determined. This enzyme exhibits a dimorphic distribution, such that those with dextromethorphan/dextrorphan < 0.3 are classed as extensive metabolizers (27) and those with a ratio above 0.3 as poor metabolizers. Fifteen subjects were extensive metabolizers and 7 of these showed decreased CYP2D6 activity in response to I3C, 7 showed an increase, and 1 was unchanged. The one poor metabolizer in this subject pool exhibited ~18% lower activity following I3C treatment. Analysis of samples from one other subject did not yield interpretable data for this assay. The mean ± SD for the individual activity ratios for CYP2D6 activity is 1.40 ± 1.25. No statistically significant I3C effect on CYP2D6 activity was detected.

Metabolism of endogenous substrates also was examined. Cortisol is converted to 6β-hydroxycortisol by CYP3A. The ratio of this product to parent steroid in urine samples was determined during the placebo period and at the end of both the low-dose I3C treatment (i.e., 400 mg daily) and the high-dose (800 mg daily) treatment periods. Data shown in Fig. 3A and B provide an assessment of effects of I3C on this CYP3A activity. Individual data are shown (Fig. 3A) for 16 subjects. Of these, 4 showed an increase in the metabolite/parent ratio at low-dose I3C, 7 showed a decrease, and 5 showed no change. After the high-dose I3C treatment, 7 of the subjects showed an increased ratio, 4 showed a decrease, and 5 showed no change. One subject did not excrete measurable levels of cortisol, precluding the calculation of the metabolite/parent ratio. Compiling all ratio data for each dose generated I3C-treated/placebo means and SDs of 0.97 ± 1.17 for the low-dose treatment period and 1.18 ± 0.54 for the high-dose period. Neither treatment resulted in a statistically significant change in this measure of CYP3A activity.

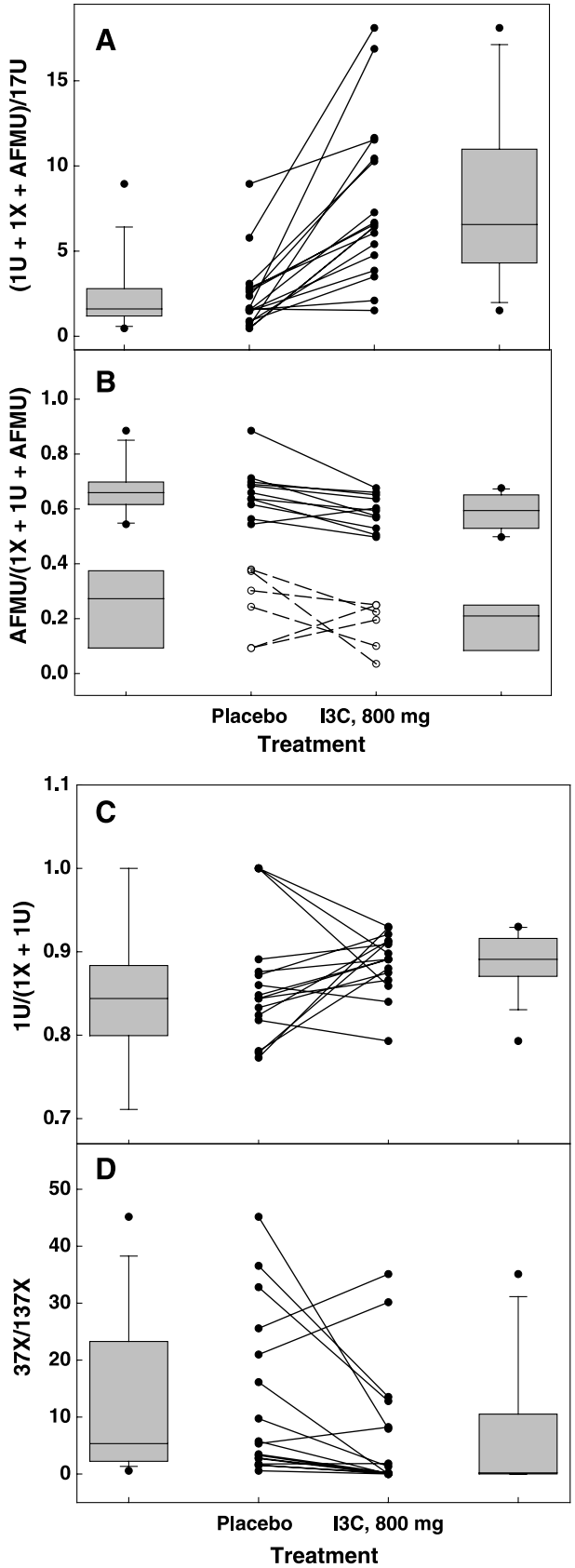
Estrone hydroxylation also was measured as a possible biomarker for I3C effect. The ratio of excreted 2-hydroxyestrone to 16α-hydroxyestrone has been proposed as an indicator of breast cancer susceptibility (31) and for breast

Table 1. Reported adverse events, multiple dose I3C, and placebo

Adverse event	Placebo	400 mg I3C	800 mg I3C
Abdominal discomfort, bloating, nausea, vomiting	6 (35)	7 (41)	3 (18)
Asthma	—	—	1 (6)
Breast tenderness	3 (18)	—	—
Chest pain	1 (6)	—	—
Constipation	2 (12)	—	—
Diarrhea/loose stools	—	2 (12)	1 (6)
Dizziness	1 (6)	1 (6)	1 (6)
Headache	3 (18)	1 (6)	2 (12)
Musculoskeletal complaints	1 (6)	4 (24)	2 (12)
Rash	—	3 (18)	1 (6)
Sciatic nerve pain	1 (6)	—	—
Sharp pain behind right ear	—	1 (6)	—
Upper respiratory symptoms	1 (6)	3 (18)	4 (24)

NOTE: Values denote the number of individuals who complained of a class of adverse events during each dosing period of the study followed by the percentage of all subjects who expressed that complaint. The total number of subjects was 17.

cancer prognosis (32). These endogenous metabolites were measured in urine of subjects during all three periods of the study. After treatment for 4 weeks with 400 mg I3C daily, 14 subjects showed increased ratios of 2-hydroxyestrone/16α-hydroxyestrone, 1 showed a decrease, and 2 showed no change



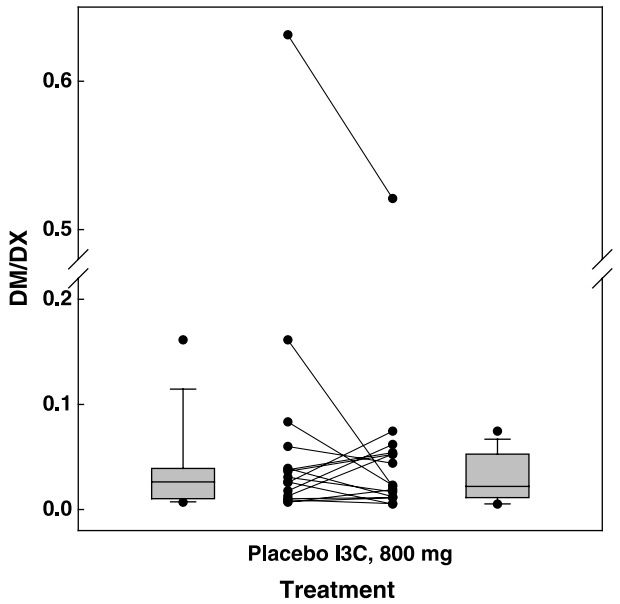


Figure 2. CYP2D6 phenotyping with dextromethorphan. Enzyme activity is reported as the validated ratio of dextromethorphan (*DM*)/dextrorphan (*DX*) determined based on the procedure of Kashuba et al. (27). Line graphs represent values for each individual during placebo phase and after ~4 weeks of 800 mg I3C daily. Box plots show the median and the 5th, 25th, 75th, and 95th percentiles for all subjects for each dosing regimen as well as outlying values.

(Fig. 4A). After high-dose (800 mg daily) I3C, 14 subjects showed an increased ratio relative to the placebo period and 3 were unchanged. Interestingly, the means and SDs of the I3C-treated/placebo ratios were 1.65 ± 0.81 and 1.67 ± 0.66, respectively, for the low-dose and high-dose treatments (Fig. 4B). The changes in 2-hydroxyestrone/16α-hydroxyestrone elicited by both I3C doses were statistically different from the placebo value ($P < 0.0001$), but the results at the two different doses did not differ from each other. This suggests that the maximal effect on estrone hydroxylation occurs at or below 400 mg I3C daily.

The final two biomarkers of effect were assessed using *ex vivo* assays. Lymphocytes were isolated from whole blood and cytosols prepared by standard techniques (46). Total GST activity was determined spectrophotometrically using 1-chloro-2,4-dinitrobenzene as the substrate (29). Comparison of the individual activity values for the placebo period versus the high-dose I3C treatment showed that 13 subjects exhibited increased activity, 1 showed a decrease, and 3 did not change (Fig. 5). Compiling these values resulted in a mean and SD for the ratio of I3C-treated/placebo of 1.69 ± 1.15. This increase in GST was significant ($P = 0.002$).

Figure 1. Enzyme phenotyping using urine caffeine and caffeine metabolites. **A.** CYP1A2 phenotyping. **B.** NAT-2 phenotyping. **C.** Xanthine oxidase phenotyping. **D.** FMO3 phenotyping. Quantitation of excreted caffeine and specific metabolites was obtained based on the HPLC procedure of Kashuba et al. (25), and enzyme activities were inferred from the validated metabolite ratios listed. Details are provided in the text. Line graphs represent values for each individual during placebo phase and after ~4 weeks of 800 mg I3C daily. Box plots show the median and the 5th, 25th, 75th, and 95th percentiles for all subjects for each dosing regimen as well as outlying values. *1U,* 1-methylurate; *1X,* 1-methylxanthine; *AFMU,* 5-acetylamino-6-formylamino-3-methyluracil; *17U,* 1,7-dimethylurate; *37X,* 3,7-dimethylxanthine; *137X,* caffeine.

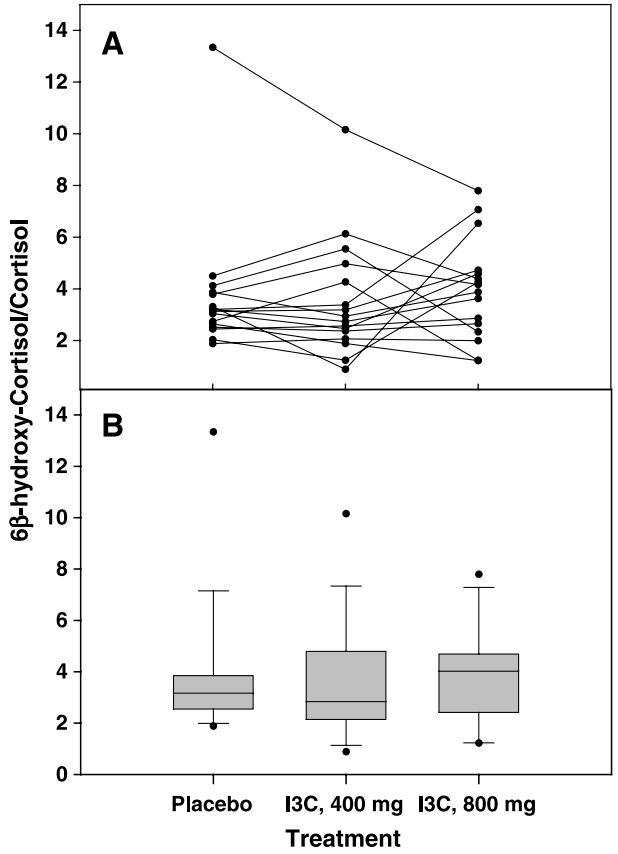


Figure 3. CYP3A4 phenotyping from cortisol 6β-hydroxylation. Concentrations of cortisol and 6β-hydroxycortisol in urine were measured by immunoassay. **A.** Individual product ratios. Line graphs represent values for each individual during placebo phase and after ~4 weeks of 400 mg I3C daily and a subsequent 4 weeks of 800 mg I3C daily. **B.** Box plots show the median and the 5th, 25th, 75th, and 95th percentiles for all subjects for each dosing regimen as well as outlying values.

Analysis of NQO1 activity was problematic. Data shown (Fig. 6) are from a standard NQO1 assay using menadione as the initial electron acceptor (46). It is noted that these activities for all subjects showed only minimal inhibition by the inclusion of 100 μmol/L dicoumarol. An additional assay for NQO1 activity was therefore done using 2,6-dichlorophenol-indophenol as a more specific electron acceptor for NQO1 (28). The results, however, were virtually identical to those obtained with menadione, including the lack of inhibition by dicoumarol (data not shown). The inability to block the reaction with dicoumarol indicates that the activity measured is not classic NQO1. Due to this lack of clear identification of the enzyme responsible for this result, we simply refer to this activity as menadione reductase. Individual subject data showed that high-dose I3C elicited apparent increases in this menadione reductase in 6 subjects, decreases in 9 subjects, and no change in 2 subjects. The mean and SD of the ratios of I3C-treated/placebo menadione reductase activities was 1.03 ± 0.37, a nonsignificant change.

Discussion

Previous reports on chemoprevention by I3C in animal models, and supporting studies *in vitro*, have suggested several possible mechanisms for this effect. The most general mechanisms have invoked membrane stabilization and antioxidant effects of I3C (16), alterations in signal transduction pathways related to growth factors (17, 18), and effects on apoptosis (19-21). Other proposed targets and mechanisms for this chemoprevention are more specific for given carcinogens and target tissues and derive from effects on carcinogen metabolism (3), estrogen metabolism (4), and endocrine disruption (13-15). Given the restrictions on experimental approaches for a phase I study, we focused on markers relevant for carcinogen and estrogen metabolism and endocrine disruption.

One approach to the noninvasive assessment of xenobiotic- and steroid-metabolizing enzyme activities is based on the use of probe drugs. Caffeine and its metabolites provide validated measures of the activity of CYP1A2, NAT-2, and xanthine oxidase. The most striking I3C-induced alteration in these activities was the increase in CYP1A2. This is a key enzyme in the metabolism of carcinogens, especially aromatic amines (33), and also plays a role in estrogen metabolism (34). The magnitude of the increase in CYP1A2 activity suggests that significant alterations in metabolism of other CYP1A2 substrates should result, as was seen in the hydroxylation of estrone (see below). NAT-2 activity also showed an increase, although only in fast acetylators. Although this increase was statistically significant, the magnitude of the increase does not support any biological significance for this effect. Xanthine oxidase activity was not altered by I3C treatment.

We also present data for caffeine metabolism, which focuses on the activity of FMO3, and these data indicate a 50% mean

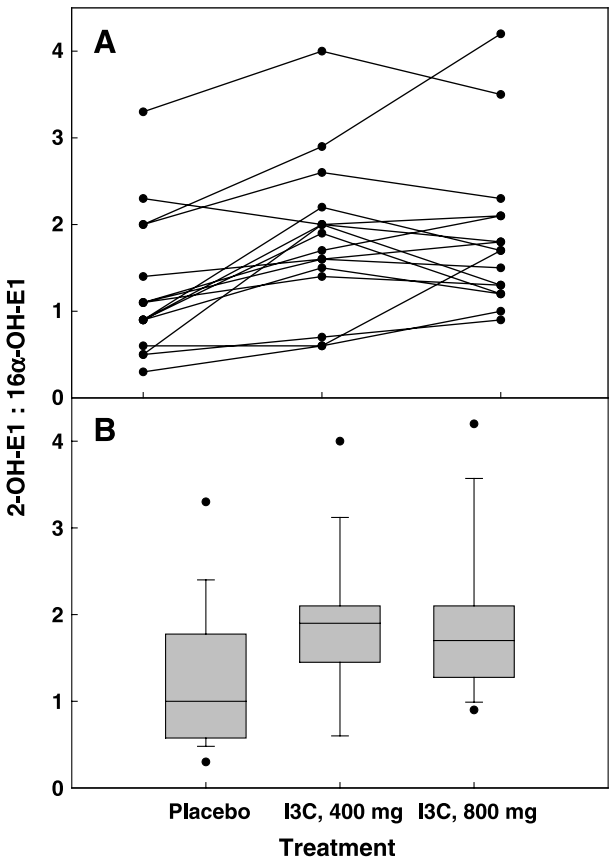


Figure 4. Effect of I3C on estrone hydroxylation products. Concentrations of 2-hydroxyestrone (*2-OH-E1*) and 16α-hydroxyestrone (*16α-OH-E1*) in urine were measured by immunoassay. **A.** Individual product ratios. Line graphs represent values for each individual during placebo phase and after ~4 weeks of 400 mg I3C daily and a subsequent 4 weeks of 800 mg I3C daily. **B.** Box plots show the median and the 5th, 25th, 75th, and 95th percentiles for all subjects for each dosing regimen as well as outlying values.

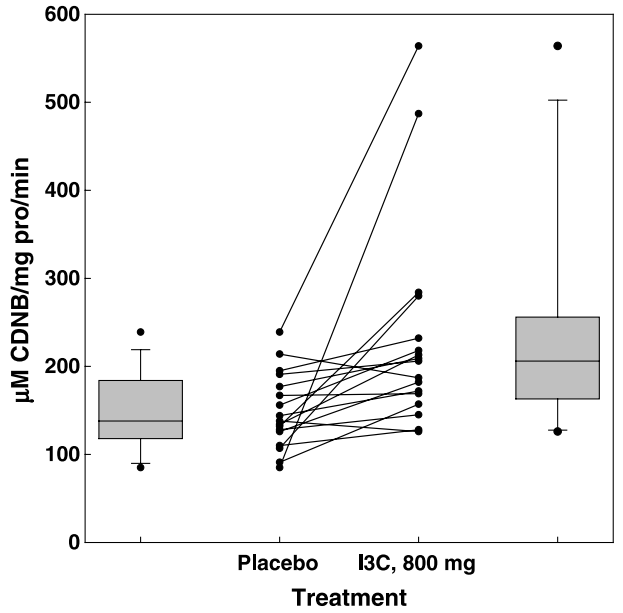


Figure 5. Effect of I3C on GST activity. Total GST activity in lymphocyte cytosols was determined with 1-chloro-2,4-dinitrobenzene (*CDNB*) as substrate using the method of Gupta et al. (29). Line graphs represent values for each individual during placebo phase and after ~4 weeks of 800 mg I3C daily. Box plots show the median and the 5th, 25th, 75th, and 95th percentiles for all subjects for each dosing regimen as well as outlying values.

decrease in this activity. The simultaneous induction of CYP1A2 and suppression of FMO is a hallmark of I3C actions in rodents (35) and has been suggested to occur in humans (36). As we note, the use of caffeine as a probe for FMO3 activity *in vivo* has been challenged (30). We thus report our finding as suggestive, but not conclusive, evidence for suppression of FMO3 activity by I3C in humans. Definitive evidence could be provided by examination of the metabolism of a more specific endogenous probe substrate, namely trimethylamine, and its FMO3-dependent product, trimethylamine-*N*-oxide (30, 36).

CYP2D6 also is involved in xenobiotic and steroid metabolism. Although not reported to be inducible, inhibition of CYP2D6 is known. We probed the activity of this enzyme using dextromethorphan and its *O*-demethylation to dextrorphan. Our subjects showed no effect of I3C treatment on CYP2D6 activity.

Specific metabolites of two endogenous substrates also were employed to assess additional targets for biological effects of I3C. CYP3A4 is a key enzyme for xenobiotic metabolism (33) as well as a known estrogen hydroxylase (34), supporting its potential importance with regard to breast cancer chemoprevention. Conversion of cortisol to 6β-hydroxycortisol is a diagnostic reaction for CYP3A4 activity, with the ratio of metabolite to parent in urine an accepted measure of CYP3A4 activity. Admittedly, cortisol metabolism may be more sensitive for detection of induction than of inhibition (37). Our measurement of the 6β-hydroxycortisol/cortisol ratio showed no evidence for modulation of CYP3A4 by I3C. In rats, I3C has been reported to induce CYP3A4 activity; however, the daily dose of I3C administered was 250 to 500 mg/kg, and the increase in rat hepatic CYP3A activity was only 80% above the control level (38). The much higher dose of I3C given to rats, when coupled with the modest increase elicited in CYP3A activity, argues against this enzyme as a key target for modulation by I3C. Our data indicate that this modulation is unlikely to occur at doses administered to humans.

The second endogenous substrate we studied was estrone. We specifically measured the ratio of two of its metabolites, 2-hydroxyestrone and 16α-hydroxyestrone. Although this metabolite ratio lacks the specificity for characterization of particular enzyme activities, its inclusion in the present study is derived from its proposed value as either a diagnostic or a prognostic indicator for breast cancer prevention (31, 32). Accordingly, we determined the effect of I3C treatment on the ratio of urinary 2-hydroxyestrone/16α-hydroxyestrone. We found that this metabolite ratio was significantly increased after 4-week treatment with both doses of I3C, showing a mean increase of 66%. The hepatic 2-hydroxylation of estrogens in humans is catalyzed in part by CYP1A2, which we showed to be increased 4-fold by I3C treatment, but CYP3A4 also may be involved (34). CYP3A4 also has been proposed to be the major estrogen 16α-hydroxylase in humans (34). The lack of quantitative correlation between the measured effects of I3C on CYP1A2 and CYP3A4 activities and the change in the urinary 2-hydroxyestrone/16α-hydroxyestrone underscores the problems with specificity of this marker for given enzyme activities. The observed shift in metabolite ratio of steroid sex hormones, and findings that this was associated previously with either a chemopreventive effect (4, 31) or a positive prognosis (32) for breast cancer, supports this shift as a biomarker for I3C effect and one that suggests chemopreventive activity in humans.

Our results show that a maximal shift in this biomarker was obtained at the 400 mg daily dose of I3C. If this is indeed a biomarker of chemoprevention by I3C, then there is no support for the use of higher daily doses. It should be noted that in a previous human study, with I3C doses of 200 and 400 mg daily for 4 weeks, both dose levels caused an increase in urinary 2-hydroxyestrone/16α-hydroxyestrone, with a more pronounced effect at the higher dose (39). These same daily doses of I3C have been reported to elicit a significant therapeutic response in patients with recurrent respiratory papillomatosis (40) and in the control of cervical interstitial neoplasia (39).

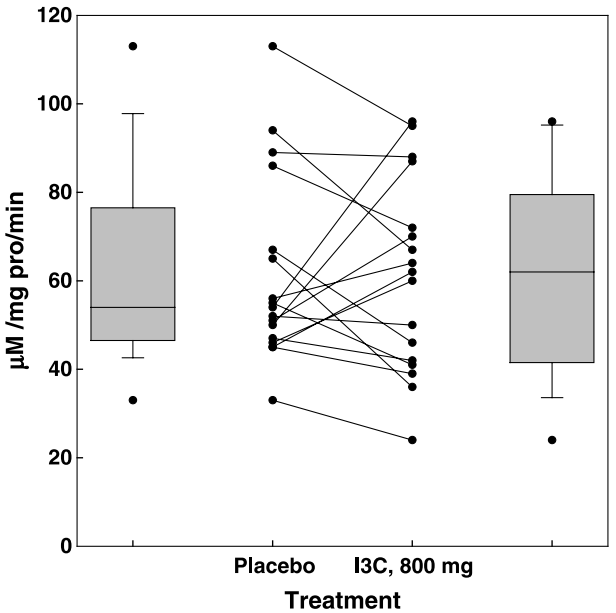


Figure 6. Effect of I3C on menadione reductase activity. Total menadione reductase (NQO1-like) activity in lymphocyte cytosols was determined using the method of Gordon et al. (46). Line graphs represent values for each individual during placebo phase and after ~4 weeks of 800 mg I3C daily. Box plots show the median and the 5th, 25th, 75th, and 95th percentiles for all subjects for each dosing regimen as well as outlying values.

We chose to extend this dose response by examining the effects of a higher dose (i.e., 800 mg daily). The apparent similarity of the I3C dose response for the reported modulation of these two disease states (39, 40) with our dose response for modulation of the 2-hydroxyestrone/16α-hydroxyestrone ratio supports further study of this agent and its actions.

Possible effects of I3C on two other enzymes of interest were examined using an *ex vivo* approach. Lymphocyte cytosols, prepared from samples obtained during the placebo phase and when taking high-dose I3C, were used to assess activities of GST and NQO1. These enzymes have been studied previously and characterized as key targets for chemoprevention (41, 42). Results for GST activity were clear cut, showing a 69% increase in mean activity, with a measurable increase as a result of I3C treatment in all subjects. Although the magnitude of the increase does not approach that seen for CYP1A2 activity, it still suggests a pharmacologically significant effect of the treatment.

The proposed importance of NQO1 as a contributor to the actions of either chemopreventive or chemotherapeutic agents (43) supports a critical appraisal of measuring NQO1 activity in human lymphocytes. Although we found no consistent effect of I3C administration on reductase activity, the interpretation of the apparent absence of effect remains unclear. Our initial assays using menadione as the acceptor substrate failed to show inhibition by the inclusion of 100 μmol/L dicoumarol, an inhibitor diagnostic for NQO1 activity. Hodnick and Sartorelli (28) reported a less specific reductase activity in human lymphocytes, not inhibited by dicoumarol, which will readily reduce menadione bisulfite. These authors suggested the use of 2,6-dichlorophenol-indophenol as a more specific NQO1 substrate. We found no difference when 2,6-dichlorophenol-indophenol was used as the electron acceptor—the reductase activity still was not significantly inhibited by dicoumarol. In view of the importance of NQO1, we have chosen to report our findings but to describe them as menadione reductase activity rather than the more specific but apparently less accurate designation of NQO1.

Rodent studies have clearly shown an ability of I3C to induce hepatic CYP1A2, GST, and NQO1 activities and to suppress FMO activity (35, 38, 44, 45). These specific enzyme changes are accompanied by a marked increase in the 2-hydroxyestrone/16α-hydroxyestrone ratio. These markers respond at doses of I3C, which show potent chemopreventive actions as well, demonstrating clear association between these effects, even if causal relationships cannot be unequivocally ascribed. In the present study, we have examined similar markers of effect in women at elevated risk of breast cancer. We find comparable effects of I3C administration on CYP1A2, GST, and FMO3 activities as well as a similar shift in the 2-hydroxyestrone/16α-hydroxyestrone ratio. The lack of effect on menadione reductase activity in lymphocytes could result either from dominance of an enzyme other then NQO1 in these cells, from a difference in tissue specificity in the induction of NQO1, or from a difference between humans and rodents in this aspect of their response to I3C. Further studies are needed to help resolve this inconsistency in findings, such as characterization of the menadione reductase activity measured, as well as determining the validity of lymphocytes as a surrogate for liver for studying NQO1 activity in humans.

In summary, daily administration of I3C at doses of 400 and 800 mg was well tolerated by subjects in this study. Of great importance is that these dose levels produced significant changes in the activities of at least two xenobiotic- and steroid-metabolizing enzymes and also markedly altered the ratio of hydroxylated estrone metabolites in a manner consistent with chemoprevention. These changes induced by I3C mirror those seen in animal studies where I3C also exhibited a profound chemopreventive effect. If these changes in enzyme activity and steroid metabolism are true biomarkers, or at least predictive, of a chemopreventive effect, then 400 mg I3C daily seems to be a safe and effective supplement for women.

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