Single-Dose and Multiple-Dose Administration of Indole-3-Carbinol to Women: Pharmacokinetics Based on 3,3′-Diindolylmethane

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Abstract

We have completed a phase I trial in women of the proposed chemopreventive natural product indole-3-carbinol (I3C). Women received oral doses of 400, 600, 800, 1,000, and 1,200 mg I3C. Serial plasma samples were analyzed by high-performance liquid chromatography-mass spectrometry for I3C and several of its condensation products. I3C itself was not detectable in plasma. The only detectable I3C-derived product was 3,3′-diindolylmethane (DIM). Mean Cmax for DIM increased from 61 ng/mL at the 400-mg I3C dose to 607 ng/mL following a 1,000-mg dose. No further increase was observed following a 1,200-mg dose. A similar result was obtained for the area under the curve, which increased from 329 ng·h/mL at the 400-mg dose to 3,376 h·ng/mL after a 1,000-mg dose of I3C. Significant interindividual quantitative variation was seen in plasma DIM values within each dosing group, but the overall profiles were qualitatively similar, with no quantifiable DIM before dosing, tmax at ~2 h, and DIM levels near or below 15 ng/mL (the limit of quantitation), by 24 h. Different results were obtained for 14 subjects who received a 400-mg dose of I3C after 8 weeks of twice-daily I3C dosing. Although the predose sampling occurred at least 12 h after the last known ingestion of I3C, 6 of 14 subjects exhibited Cmax for DIM in their predose plasma. Despite this high initial value, plasma DIM for all subjects decreased to near or below the limit of quantitation within the 12-h sampling period. Possible reasons for this disparity between apparent t1/2 of DIM and the high predose values are discussed. (Cancer Epidemiol Biomarkers Prev 2006;15(12):2477–81)

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, ultimately yielding 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 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 observation of a chemopreventive effect of I3C in a range of species and organ sites supports the further study and development of this compound.

Although most animal studies with I3C and carcinogen treatment have shown chemopreventive effects (1-10), some experimental models have instead pointed to a tumor-promoting effect of this compound (11-14). In contrast to the reported chemopreventive effects of I3C, the apparent promoting effects of I3C are highly dependent on the species, carcinogen, target organ, and exposure regimens employed (12, 13). Moreover, the I3C doses required for promotion, particularly in liver, are doses that elicit substantial toxicity. This suggests that the observed tumor promotion results from enhanced proliferation necessary to restore homeostasis following this toxic effect (15). The determination of nontoxic, well-tolerated I3C doses for humans, thus, is a key step in the development of this compound as a possible chemopreventive agent.

We have carried out a phase I trial of I3C in humans to further this development, examining both the pharmacokinetics and the effects of I3C. 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 (16, 17), we chose to carry out this phase I study in a population of women at elevated risk for breast cancer.

The initial task in this study was to determine the tolerability of single doses of I3C and to assess the pharmacokinetics of the compound at those doses. Preliminary reports of I3C administration to subjects and patients indicated that daily doses up to 400 mg were well tolerated (18, 19), which we thus adopted as our initial single dose, with escalation of up to 1,200 mg I3C. This was followed by a multiple-dose study, in which women ingested 200 mg twice daily and then 400 mg I3C twice daily, each for a period of 4 weeks. Pharmacokinetic variables for I3C were determined for each dose from subjects in the single-dose study and for the final 400-mg dose from subjects in the 8-week study.

Pharmacokinetic characterization of I3C following oral administration is complicated by the instability of this compound in acidic environments (20). As a result, we developed a high-performance liquid chromatography-mass spectrometry method for the detection and quantitation of the parent compound and a major product of its acid-catalyzed condensation, 3,3′-diindolylmethane (DIM). We report here on...
our characterization of the pharmacokinetics of I3C-derived species in humans after either single doses or multiple doses of I3C. Our findings are discussed with regard to the fate of I3C in humans and in relation to the observed biological effects of this proposed chemopreventive agent (21).

Materials and Methods

Materials. I3C capsules were provided by the Division of Cancer Prevention, National Cancer Institute. Capsules were distributed by McKesson Bioservices under contract to the National Cancer Institute. I3C and the internal standard indole-3-ethanol were purchased from Sigma-Aldrich (St. Louis, MO) and DIM was provided by BioResponse LLC (Boulder, CO). All organic solvents were of high-performance liquid chromatography grade and were products of Fisher Scientific (St. Louis, MO).

Subjects. Twenty-four healthy, nonsmoking women between 23 and 58 years of age were recruited for the single-dose study. For the determination of I3C pharmacokinetics following chronic I3C administration, 14 healthy, nonsmoking women between 20 and 58 years of age with an elevated risk of breast cancer were studied. Breast cancer risk was defined as a Gail score of ≥1.67 (22) or by other family history of breast cancer. Premenopausal subjects in the chronic administration group were not taking oral contraceptives, and one postmenopausal woman was not taking hormone replacement therapy. Nonsmoking status for all subjects was confirmed by a urine cotinine test. All women had a negative pregnancy test before administration of I3C. They were instructed not to consume cruciferous vegetables for 1 week before I3C administration for the single-dose study and during the entire course of the chronic study. All documents and procedures, including informed consent for all subjects, were reviewed and approved by the Institutional Review Boards of the University of Kansas Medical Center.

Dosing and Sampling Procedures. All subjects came to the clinic after fasting overnight. First morning urine samples were collected for analysis of clinical chemistries, cotinine, and hCG. An i.v. line was placed and blood was obtained for clinical chemistries and for baseline analysis for I3C and I3C-derived compounds. Subjects were then administered I3C capsules orally with 200-mL water. Fasting was continued for an additional 2 h to allow for absorption. Blood was collected in heparinized tubes at 1, 2, 4, and 8 h after dosing for the initial 400-mg single dose, and at 1, 2, 4, 8, 12, and 24 h after dosing for the remainder of the single-dose study. Sampling was done at 1, 2, 4, 8, and 12 h after dosing for the chronic administration study. For all studies, plasma was prepared within 30 min of collection by centrifugation and was stored below −70°C. All plasma samples were analyzed within 4 months of collection.

For the single-dose study, subjects were seen in six groups of four subjects. Each subject received an unblended dose of I3C equal to 0.5 of the daily multiple-dose study for I3C up to 1,200 mg. Twenty-four healthy, nonsmoking women between 20 and 58 years of age with an elevated risk of breast cancer were studied. Breast cancer risk was defined as a Gail score of ≥1.67 (22) or by other family history of breast cancer. Premenopausal subjects in the chronic administration group were not taking oral contraceptives, and one postmenopausal woman was not taking hormone replacement therapy. Nonsmoking status for all subjects was confirmed by a urine cotinine test. All women had a negative pregnancy test before administration of I3C. They were instructed not to consume cruciferous vegetables for 1 week before I3C administration for the single-dose study and during the entire course of the chronic study. All documents and procedures, including informed consent for all subjects, were reviewed and approved by the Institutional Review Boards of the University of Kansas Medical Center.

Analytic Procedures. Aliquots of thawed plasma (1.0 mL) were mixed with 300 ng of indole-3-ethanol as an internal standard, and analytes were isolated by liquid-liquid extraction with diethyl ether. Extracts were evaporated to dryness, reconstituted in 200-μL acetonitrile, and then analyzed by reverse-phase high-performance liquid chromatography-mass spectrometry. Analysis of 50-μL aliquots of each sample was done by chromatography with a Metachem Inertsil octadeyl silane column (5 μm, 250 × 4.6 mm; Varian Instrument Co., Walnut Creek, CA). Elution used methanol and 30 mmol/L ammonium acetate, with a linear gradient from 30% to 96% methanol over 15 min, followed by an additional 10 min at 96% methanol. Flow rate was 1.0 mL min⁻¹, with 200 μL min⁻¹ delivered to the mass spectrometer. Mass spectrometry used a Micromass Quattro I in the positive ion electrospray mode, with a source temperature of 170°C and a cone voltage of 15 V. Selected ion monitoring was done at m/z 130 (DIM), 148 (I3C), 162 (indole-3-ethanol), and 263 (hydroxylated DIM). These ions are M + H for most analytes; however, greater sensitivity for DIM was obtained by monitoring its major fragmentation product m/z 130. The limit of quantitation for all analytes was estimated to be 15 ng/mL plasma, and the limit of detection for I3C and DIM was 1.0 ng/mL. The high limit of quantitation for DIM was due to an interfering endogenous compound present in blank plasma.

Pharmacokinetic Modeling. All calculations using WinNonLin v. 4.0 (PharSight; Mountain View, CA) used a noncompartmental model. Plasma DIM levels below the limit of quantitation were set to 0 and area under the curve (AUC) was calculated using the linear trapezoidal interpolation.

Results

Two types of adverse events were noted in some of the subjects in the single ascending dose study. Headaches, which were reported by 3 of 24 women, did not seem to be temporally related to I3C ingestion and were not consistent in location nor related to dose. Gastrointestinal distress, in contrast, seemed to be dose dependent and dose limiting. Nausea and vomiting occurred in 2 of 20 women, one after ingesting a single-dose of 800 mg and the other after 1,200 mg. Three other women reported mild gastrointestinal distress. Thus, although most subjects had no gastrointestinal complaints after a single-dose of I3C up to 1,200 mg, 25% (5 of 20) did have gastrointestinal symptoms at ≥600 mg. Those subjects with gastrointestinal symptoms spontaneously recovered with no long-term effects. There were no other adverse events by history, physical examination, or laboratory tests. We noted that the batch of I3C used for the single ascending dose study had a strong and foul odor. A new batch of I3C without the odor was employed for the daily multiple-dose study. No adverse events related to study agent were reported in the multiple-dose study.

Initial development of the high-performance liquid chromatography-mass spectrometry assay for I3C and its condensation products used authentic standards for I3C and DIM and used indole-3-ethanol as the internal standard. Standard reference compounds for indolo-[3,2-b]-carbazole and for the trimeric condensation products were not available; thus, I3C was incubated in 0.05 mol/L HCl to form these other products, the reaction mixture was neutralized, and then extracted and analyzed as described. Selected ion monitoring included m/z 257 for indolo-[3,2-b]-carbazole and m/z 376 and 388 for trimeric products. These preliminary studies provided retention times for these products. Analyses of serial plasma samples from subjects dosed orally with 400 mg I3C showed no detectable indolo-[3,2-b]-carbazole, trimeric products, or hydroxylated DIM; thus, these ions were deleted from monitoring for analysis of subsequent samples.

Analysis of all plasma samples employed selected ion monitoring of the most abundant ions observed for the detection and quantitation of I3C (m/z 148), DIM (m/z 130), and the internal standard indole-3-ethanol (m/z 162). No detectable I3C was found in any plasma sample regardless of I3C dose or sampling time. This complete absence of detectable parent compound indicates that, if present, I3C concentration...
in plasma was <1 ng/mL at all sampling times. In contrast, DIM was detected in plasma from all dosed subjects and was present above the limit of quantitation of 15 ng/mL in most samples.

Mean plasma DIM concentrations with time are shown for each single-dose level of I3C (Fig. 1) and mean values for pharmacokinetic variables from all subjects are presented in Table 1. Both C_max and AUC increase with increasing I3C dose up to 1,000 mg; with no further increase seen at the 1,200-mg level. The change in these variables between the 600 and 1,000 mg is markedly nonlinear, with dose-normalized values 2- to 4-fold higher than were calculated for the lower doses of I3C. The half-life for DIM also increases with increasing I3C dose, but not as dramatically as did C_max and AUC.

The observed pharmacokinetics of DIM following chronic I3C administration, again presented as mean plasma concentration (n = 14) at each time, is shown in Fig. 2, with calculated variables for the group presented in Table 1. As shown in Table 1, the C_max and AUC of DIM from this final 400-mg I3C dose in chronically dosed subjects are very close to those values determined for the single acute 400-mg dose of I3C. The t_max, however, occurs earlier. This results from 6 of 14 subjects having their highest DIM levels in their predose plasma. Of the remaining eight subjects, only one showed baseline DIM below the limit of detection; for the remaining individuals, their baseline plasma DIM was 77 ± 11% of their respective C_max levels. Despite these high predose plasma levels, 8 of 14 subjects showed a drop in plasma DIM after 12 h to at or below limit of quantitation (i.e., 15 ng/mL), and the remaining subjects had a mean decline in plasma DIM level to 37% of their C_max at 12 h. The observed elimination kinetics yielded a t_1/2 of 6.0 h after the chronic daily dosing period with I3C, a value marginally higher than the values of 4 to 5 h determined in the single-dose study (Table 1). It is noteworthy that the six subjects who exhibited C_max in predose plasma did not differ from the remaining eight subjects in AUC or t_1/2. Moreover, only two of the six subjects with high initial plasma values for DIM showed quantifiable DIM in their plasma after 12 h, similar to the four of eight remaining subjects with DIM quantified in their final plasma sample. In summary, C_max and AUC for DIM following a 400-mg I3C final administration after 4 to 5 weeks of daily treatment with I3C are comparable to those values measured for the initial 400-mg dose of this agent, with a dramatic difference in t_max for some individuals and a minor change in t_1/2. Possible reasons for this change in t_max are discussed.

### Discussion

The maximum tolerated single-dose of I3C, based on results of the single ascending dose study, was estimated to be 400 mg. Gastrointestinal distress occurred in some subjects at doses of ≥600 mg. This adverse effect, however, may not be related to I3C itself. The batch of capsules provided for the single ascending dose study were noted to have a foul odor, similar to that of 3-methylindole. This impurity could have resulted from the preparation of I3C, and thus the amount would be proportional to the study agent. It seems possible that the nausea could have resulted from the impurity and its odor, rather than from I3C itself, and that the exposure to the impurity would increase in parallel to the I3C dose. The multiple-dose study was done with a subsequent batch of I3C that did not have any odor; with doses of up to 400 mg twice daily, we did not have any reports of gastrointestinal distress. We conclude from the results of the multiple-dose study that I3C is well tolerated at doses up to 400 mg twice daily.

Assessing the pharmacokinetics of oral I3C was expected to be complicated by the reactivity of this molecule. Previous works have characterized the kinetics of I3C consumption and the products formed in aqueous media (20, 23). The oligomerization of I3C is acid catalyzed, with the reaction rate and the resultant product distribution highly pH dependent. Ingestion of I3C by fasting individuals would be expected to result in rapid consumption of the parent compound; however, the complete absence of detectable I3C in plasma was not expected.

Our results differ qualitatively from those reported by Anderton et al. (24) following I3C administration to mice. They found that after oral administration of 250 mg/kg I3C, the parent compound was the first and the most abundant analyte in plasma, with t_max at 0.25 h and an AUC of ~1,600 h ng/mL. DIM was the most next abundant, with t_max at 2 h and C_max of ~750 ng/mL. In addition, detectable levels of a linear trimere of I3C and of [1-(3-hydroxymethyl)]-indolyl-3-indolylmethane,
Methods.

preparation, and analysis were done as described in Materials and

after dosing with 400 mg I3C. Subjects had been taking 400 mg I3C
dosing with I3C. Data are mean plasma values for subjects (n = 14)

Figure 2. Mean plasma concentrations of DIM following multiple
dosing with I3C. Data are mean plasma values for subjects (n = 14)
after dosing with 400 mg I3C. Subjects had been taking 400 mg I3C
twice daily for a period of 4 weeks before this dose. Sampling,
preparation, and analysis were done as described in Materials and

a previously unreported dimeric product, were reported. This
difference in circulating compounds between those observed
in an animal model and the results reported here for human
ingestion of I3C is striking, but may be explained based on the
I3C doses, I3C chemistry, and species differences in gastric
physiology. As noted, the oligomerization of I3C is sensitive
both to pH and to I3C concentration (20). The dose of I3C
administered to mice (i.e., 250 mg/kg) is 10 to 40 times higher
than the body weight–normalized doses administered to our
human subjects, resulting in a proportional skewing of I3C
gastric concentration. In regard to acidity, the gastric pH of
rodents (25, 26) is markedly higher than that of humans (27),
resulting in a slower consumption of I3C and a different
distribution of products than would be expected at the pH of
the human stomach (20, 23). All subjects for I3C pharmaco-
kINETIC studies had been fasting, ensuring a highly acidic gastric
environment that would favor rapid consumption of I3C.

Finally, gastric emptying time of the mouse is less than that in
the human. This longer exposure of I3C to far more reactive
conditions in human subjects, as compared with the studies in
mice, will result in far less I3C being available for absorption.

These qualitative and quantitative differences in the pharma-
cokinetics of I3C between rodents and humans underscore the
pitfalls in species extrapolation for this compound and support
the importance of the detailed study of this proposed chemo-
preventive agent in humans.

The acid-catalyzed condensation of I3C may produce a
range of dimeric, trimeric, and higher oligomeric products (20),
even though an earlier report noted only the conversion of I3C
to DIM (23). Our studies following a 400-mg dose of I3C revealed
no condensation product other than DIM in human plasma.
The absence of authentic standards for indolo-[3,2-b]-carbazole
and the higher oligomeric products precluded precise quan-
tification of those other products; however, they were detected
in the extract of our acid-catalyzed model reaction when
analyzed by high-performance liquid chromatography-mass
spectrometry but were not detected in the plasma of subjects
receiving a single 400-mg dose of I3C. It is possible that indolo-
[3,2-b]-carbazole and the higher oligomers were formed from
I3C following ingestion, but that the amounts in plasma were
below our limits of detection. Whether the absence of
detectable products in plasma reflects a lack of production of

those compounds, as in the model studies of Arnao et al. (23),
or if they are actually formed from I3C in the stomach but are
not absorbed sufficiently to generate detectable levels in
plasma is not known at this time.

The single-dose pharmacokinetics of DIM, derived from oral
administration of I3C, showed a significant interindividual
variability. This could reflect effects on both absorption and
distribution but could also result from variability in gastric
conditions and the resultant formation of DIM. Beyond this
interindividual variability, it was clearly shown that the
kinetics of DIM were not a linear function of I3C dose. Dose-
normalized values for C\text{\textsubscript{max}} and AUC for the 400 and 600 mg
doses of I3C were comparable, but those transformed values
from the 800, 1,000, and 1,200 mg doses were increased by 2.0-, 4-, and 3-fold, respectively. This deviation from linearity most
likely represents saturation of a dominant step in the
disposition of DIM. Saturation of biotransformation could
result in the observed deviation from linear pharmacokinetics;
however, DIM metabolites have not been reported from in vivo
studies. Staub et al. (28) reported the formation of
hydroxylated DIM sulfates by human breast cancer cells in
culture, but the relevance of this finding to intact organism
is not known. It is noteworthy that Anderton et al. did not
observe any DIM metabolites in either tissues or plasma of
mice dosed with I3C (24) or DIM (29).

Absorption and excretion are additional pharmacokinetic
steps where saturation might occur. Previous reports state that
the bioavailability of DIM is low (29). Staub et al. (28) reported
extensive passive uptake of DIM by cultured human cells,
supporting simple diffusion as the means of cellular uptake.
In contrast, DIM bioavailability is greatly enhanced by
formulations that include inhibitors of gut efflux transporters
(30, 31). If these efflux transporters were saturated by
absorbed DIM, then efflux rate would remain constant while
uptake by diffusion would continue to increase with dose. The
net result from concentration-dependent uptake by diffusion
coupled to saturable efflux would be increased absorption of
DIM and subsequent increases in plasma levels and AUC
beyond a linear relationship with the dose of I3C adminis-
tered. This mechanism provides a plausible explanation for
our observations.

The observed pharmacokinetics of DIM following a chronic
dosing period with I3C are surprising and cannot be fully
explained. Although the C\text{\textsubscript{max}} and AUC for this dosing are
nearly identical to the values obtained for the single acute 400-
mg dose of I3C, and the t\text{1/2}\text{obs} is only modestly increased, the
observed high plasma DIM values before dosing and the effect
of that measurement on t\text{max}\text{obs} are problematic. Subjects were
instructed to take their doses of I3C with meals, so presumably
the previous dose of I3C would have been ingested at least
12 h before the drawing of the baseline blood sample for the
pharmacokinetic study. Ingestion of that previous dose of I3C
with a meal would slow gastric emptying, thus delaying
absorption, which would shift the plasma concentration-time
curve, but it would not seem that this would be sufficient to
result in the high plasma DIM values observed for most
subjects in their baseline samples. The high initial plasma
level also cannot be explained solely based on bioaccumulation.
The t\text{1/2}\text{obs} observed for DIM after the last dose of I3C, following
chronic twice-daily administration, is increased to 6.0 h. Given
the twice-daily dosing regimen, the multiple-dose C\text{\textsubscript{max}} for
DIM would be estimated to be ~34% higher than from the
single dose at steady state. Assuming this degree of bio-
accumulation, the baseline plasma sample for the final I3C
dose pharmacokinetics determination would have to be drawn
within 2 h of t\text{max}\text{obs} for the previous dose to contain the observed
concentration of DIM. This seems highly unlikely and is
further complicated by the high variability in baseline DIM
levels for these subjects. Three subjects showed exceptionally
high DIM t\text{1/2}\text{obs levels (117-150 ng/mL). Perhaps a more complete
The relationship between these findings in humans (current work and ref. 21) and those observed in rats (15), where oral DIM and a modest increase in DIM production or uptake of this compound is maximal at the production or uptake of this compound is maximal at the 1,000-mg dose. In addition, nonlinear increases in DIM Cmax and AUC were observed at I3C doses >600 mg. With the exception of an unexplained high predose plasma level for DIM and a modest increase in t1/2, the pharmacokinetic variables for a 400-mg dose of I3C following chronic twice-daily dosing with this compound were not different from those values determined in naïve subjects taking their first dose of I3C. This suggests that neither extensive bioaccumulation nor enhanced clearance due to enzyme induction occurs with I3C. These data have been used to define dosing schedules for further human studies with I3C, which showed changes in biomarkers consistent with chemoprevention (21). Moreover, they suggest that the biological activities of I3C administered orally to humans cannot be attributed to the parent compound but rather to DIM and perhaps other oligomeric derivatives. The relationship between these findings in humans (current work and ref. 21) and those observed in rats (15), where oral I3C but not oral DIM induced CYP1A1/2, remains to be defined.

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