Biochemical Pharmacology, Vol. 58, pp. 1047-1055, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00166-5

In Vitro and *In Vivo* Inhibition of Human Flavin-Containing Monooxygenase Form 3 (FMO3) in the Presence of Dietary Indoles

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ABSTRACT. The effect of consumption of glucosinolate-containing Brussels sprouts on flavin-containing monooxygenase functional activity in humans was investigated in 10 healthy, male, non-smoking volunteers. After a 3-week run-in period, 5 volunteers continued on a glucosinolate-free diet for 3 weeks (control group), and 5 others consumed 300 g of cooked Brussels sprouts per day (sprouts group). Human flavin-containing monooxygenase activity was measured by determining the levels of urinary trimethylamine and trimethylamine *N*-oxide. In the control group similar trimethylamine to trimethylamine *N*-oxide ratios were observed, while in the sprouts group the trimethylamine to trimethylamine *N*-oxide ratios were increased 2.6- to 3.2-fold, and thus flavin-containing monooxygenase functional activity was decreased significantly. To investigate the molecular basis for the *in vivo* inhibition of functional human flavin-containing monooxygenase activity, *in vitro* studies were carried out examining the effect of acid condensation products of indole-3-carbinol, anticipated to be formed after transit of Brussels sprouts through the gastrointestinal system, on the prominent cDNA-expressed human flavin-containing monooxygenase form 3 enzymes. Two indole-containing materials were observed to be potent inhibitors of human flavin-containing monooxygenases, having K_i values in the low micromolar range. The results suggested that acid condensation products expected to be formed upon transit of Brussels sprouts materials through the gastrointestinal system were potent competitive inhibitors of human flavin-containing monooxygenase form 3 enzymes. The findings indicate that daily intake of Brussels sprouts may lead to a decrease in human flavin-containing monooxygenase activity, and this may have consequences for metabolism of other xenobiotics or dietary constituents. BIOCHEM PHARMACOL **58**;6:1047-1055, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. human flavin-containing monooxygenase; indole-3-carbinol-related inhibitors; trimethylamine N-oxygenation inhibitors; dietary indoles

Chemical constituents present in vegetables of the Cruciferae family (i.e. cabbage, broccoli, cauliflower, Brussels sprouts, and others) have been shown to be chemoprotective agents in experimental animal models of cancer [1-7]. Although a large number of compounds occur naturally in fruits and vegetables (e.g. isothiocyanates, flavonoids, terpenes, retinoids, dithiolanes, and many others), it is the indole class of chemical that has been implicated as a potential chemopreventive agent in humans [8, 9], although some dietary indoles can promote carcinogenesis [10, 11].

I3C‖ is an autolysis product of 3-indolemethyl glucosinolate that has been reported to be present in Brussels sprouts at levels as high as 0.5 to 3.2 mmol/kg [12], although others have observed considerably less 3-indolemethyl glucosinolate to be present [13]. Administration of I3C to experimental animals before carcinogen exposure reduces the incidence of neoplasia and the formation of covalent adducts of carcinogens with DNA [1, 2, 4, 14-16]. However, I3C is not indefinitely stable after transit through the acid contents of the stomach. HPLC analysis of I3C in the presence of aqueous acid showed that a complex mixture of

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Received 28 September 1998; accepted 7 January 1999.

‖ *Abbreviations*: I3C, indole-3-carbinol; HI-IM, 1-(3-hydroxymethyl)indolyl-3-indolymethane; ICZ, indole[3,2-*b*]carbazole; I33′, 3,3-diindolylmethane; LT, 2-(indol-3-ylmethyl)-3,3′-diindolylmethane; CT, 5,6,11,12,17,18- hexahydrocyclonona[1,2-*b*:4,5-*b*′:7,8-*b*′′]triindole; CYP, cytochrome P450; TMA, trimethylamine; TMA *N*-oxide, trimethylamine *N*-oxide; FMO MBP, flavin-containing monooxygenase maltose binding fusion protein; FMO3, flavin-containing monooxygenase form 3; and BSTFA, bis(silyl) trifluoroacetamide.

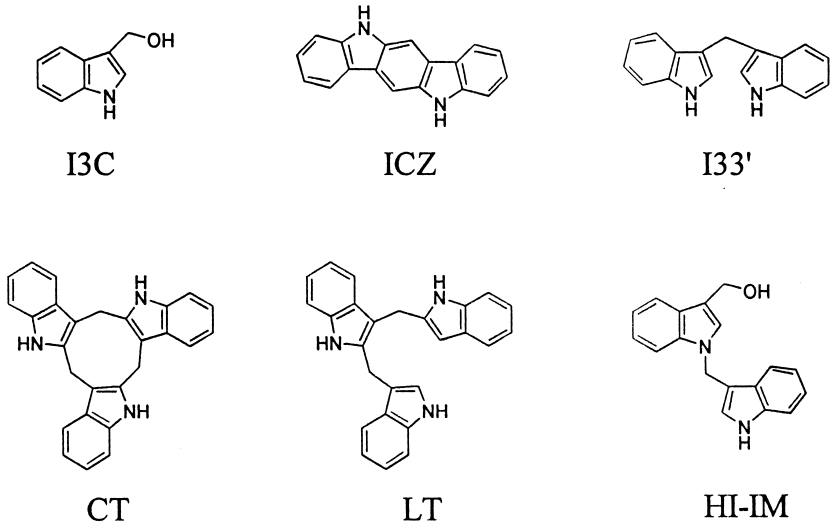

FIG. 1. Chemical structures of a few of the acid condensation products formed after indole-3-carbinol (I3C) goes through the acid contents of the stomach or upon treating I3C with hydrochloric acid.

products is formed [17]. Thus, treatment of I3C with 1 N hydrochloric acid and HPLC analysis after neutralization showed that HI-IM, ICZ, I33′, LT, and CT (Fig. 1) were among the prominent products formed, although the molar yields were in the range of 2-6% of the original I3C. Apparently, the potency of I3C as a chemopreventive agent is related to the ability of I3C (or more likely a gastric acid condensation product) to induce the enzymes involved in the metabolism and excretion of carcinogens, because in rodents oral administration of even very low levels of I3C is effective at increasing CYP1A1, epoxide hydrolase, quinone reductase, glutathione *S*-transferase, and glutathione reductase [4, 18-21].

In human volunteers administered I3C, increased estradiol 2-hydroxylase activity was observed [22]. Individuals consuming Brussels sprouts or other *Brassica* vegetables showed large increases in CYP-dependent oxidative metabolism of phenacetin and antipyrine [23], increased glucuronidation of acetaminophen [24], and increased glutathione transferase activity [25-27]. In rodents, administration of I3C has been shown to induce CYP [21] and glutathione transferase levels [28] and decrease FMO activity [29], although to our knowledge this has not been investigated in humans.

The aim of this study was to examine whether the presence of indoles found in Brussels sprouts could modulate human FMO activity *in vivo* and *in vitro*. Recent studies examining the major components of Brussels sprouts, singularly and together, suggested that indole-3-carbinol was the major component responsible for induction of phase I (CYP1A) and, to a lesser extent, phase II (quinone reductase, glutathione reductase, and glutathione transferase) drug-metabolizing enzymes in rats [30]. The present results indicate that potent inhibitors of human FMO3 activity are present in glucosinolate-containing Brussels sprouts that decrease human FMO3 activity both *in vivo* and *in vitro*.

In this study, we report that human volunteers administered a diet of Brussels sprouts for 3 weeks showed a pronounced inhibition of human FMO3 activity. In addition, treatment of I3C in the presence of hydrochloric acid (i.e. conditions that produce acid condensation products similar to those observed after transit of I3C through the gastrointestinal system [31]) produced acid condensation products that resulted in human FMO3 inhibition *in vitro*. Purification of the active agents has led to the identification of potent human FMO3 inhibitors.

MATERIALS AND METHODS

Chemicals

Chemicals, reagents, buffers, and solvents used in this study were of the highest purity available from commercial sources. TMA, TMA *N*-oxide, and I3C were obtained from the Aldrich Chemical Co. All other solvents and reagents were obtained from Fisher Scientific, Inc. All the compounds of the NADPH-generating system were obtained from the Sigma Chemical Co.

Instruments

GC–MS was performed with Micromass Trio 2000 Quadrupole GC–MS in the electron impact ionization mode. The mass spectrometer was interfaced to a Hewlett Packard 5890 gas chromatograph. For data handling, Windows-based Micromass MassLynx® and MaxEnt software were used with a 100 MHz 486 personal computer. Sampling was done with a Hewlett Packard 7673A Autosampler. Samples were introduced to the column using splitless injection, at an electron energy of 70 eV and a source temperature of 280°. The carrier gas was oxygen-free helium. The initial temperature was 60°; it was increased at a rate of 20°/min to 300° and held at 300° for 10 min. Chromatography was done with an HP-1 column (12 m × 0.2 mm i.d.), with a 0.33-μm film thickness. As described previously [31], trimethylsilylation of active hydrogens on the indoles allowed efficient GC–MS analysis.

Human Treatments

Ten healthy non-smoking male Dutch volunteers (mean age 24 ± 1 years) all consumed glucosinolate-free foods for the first 3 weeks of the study. During the intervention period, they were assigned randomly to a cross-over design experimental protocol as described previously [25]. During a subsequent 3-week period, 5 of the volunteers continued on a glucosinolate-free diet (control group), and the other 5 volunteers consumed 300 g/day of cooked Brussels sprouts (sprouts period). Total daily energy intake and diet were controlled as described before [25-27]. Urine samples were collected as total 24-hr samples in aliquots, frozen immediately in liquid nitrogen, and stored at −80°. Formal approval by the TNO Medical Ethical Committee was obtained prior to the start of the project. No adverse effects on thyroid, liver, or kidney function were observed in the volunteers after Brussels sprouts consumption.

In Vivo *and* In Vitro *Assays*

Human urinary TMA and TMA *N*-oxide concentrations were determined by a GC and HPLC assay with evaporative light-scattering detection, respectively, as described previously [32]. Creatinine concentrations were determined at the TNO Nutrition and Food Research Institute.

Assays for human FMO3 activity were done as previously described [33]. The human FMO3 enzyme used was a maltose binding fusion protein (i.e. FMO3-MBP) [34] that possessed 10-([*N*,*N*-dimethylaminopentyl]-2-trifluoromethyl)phenothiazine tertiary amine *N*-oxygenase activity. For kinetic studies, standard reaction mixtures contained 0.5 mM $NADP^+$, 0.5 mM glucose-6-phosphate, 1 IU of glucose-6-phosphate dehydrogenase, 20-30 μg of cDNA-expressed human FMO3-MBP, 1.2 mM diethylenetriaminepentaacetic acid, 50 mM potassium phosphate buffer (pH = 8.4), and 10-([*N*,*N*-dimethylaminopentyl]-2-trifluoromethyl)phenothiazine. For inhibition studies, either an aliquot of a methanolic solution containing I3C or an acid condensation product of I3C, or an equivalent amount of methanol was used. For K_m and V_{max} determinations, the concentration of 10-([*N*,*N*-dimethylaminopentyl]-2-trifluoromethyl)phenothiazine substrate was varied from 5 to 100 μM. For K_i determinations, the inhibitor concentration was varied from 5 to 200 μM in a final incubation volume of 0.25 mL. The analysis of reaction mixtures was done by HPLC as described previously [33, 34].

HPLC Analysis of I3C and Acid Condensation Products

Acid condensation products of I3C were prepared by treating 22.6 mg (0.15 mmol) of I3C in 630 μL of methanol:water (1:1, v/v) with 35.3 μL (35.3 μmol) of 1.0 N HCl with exhaustive mixing for 1 min. The sample was neutralized with 2.9 mL of 0.25 N ammonium hydroxide: tetrahydrofuran (1:1, v/v), centrifuged at 10,000 *g* to remove any particulate material, and stored in amber vials to protect the products from light. The reaction products were subjected immediately to preparative HPLC that was done with a C_{18} Waters Radial-Pak Cartridge interfaced to a Rainin Rabbit PH PHX Pump, a Versa Monitor Variable Wavelength Detector (Varex Corp.) set at 280 nm and a Hewlett Packard 3396 series II integrator. Starting eluent conditions consisted of 80% water (solvent A) and 20% acetonitrile (solvent B). This condition was held for 15 min before changing to 15% solvent A over the next 15 min (linear gradient). The ratio was held for 7 min, and then programmed to 100% solvent B for 10 min (linear gradient). This condition was held for an additional 5 min, and then returned to the starting conditions over the next 10 min. The flow rate was 1 mL/min. Under these chromatography conditions, the retention times for 13C and acid condensation products were I3C (7.5 min), HI-IM (20 min), and I33′ (33 min). Using this preparative HPLC method, each acid condensation product formed (i.e. approximately 30 fractions) was collected individually, immediately evaporated to dryness under a stream of argon, and stored in the dark at −80° or immediately assayed as an inhibitor of human FMO3-MBP (Fig. 2).

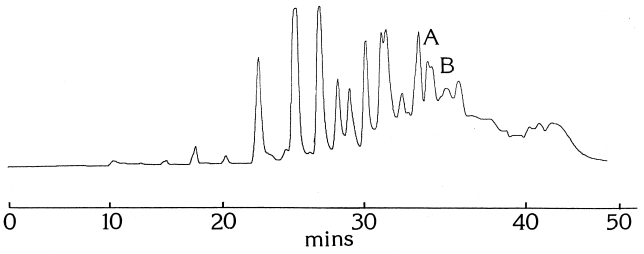

FIG. 2. Reverse phase HPLC of the acid condensation products of indole-3-carbinol. The structural characterization of A and B is described in the text.

Statistics

Mean values of different treatments were compared by standard statistical software packages. The Brussels sprouts-treated group was compared with the control group at the beginning and after the dietary intervention. For comparison of data when the groups had equal numbers of data points (N = 5), Student's *t*-test was used [35]. When comparisons were made between groups with unequal numbers of data points, the Cochran *t*-test was used [36].

RESULTS

Human Urinary TMA Levels

The ratio of TMA to TMA *N*-oxide is a selective functional indication of the activity of human FMO3. Under standard dietary conditions, a normal adult converts 91-99% of TMA to TMA *N*-oxide. The 24-hr urinary TMA and TMA *N*-oxide ratios in all of the human volunteers administered the control diet and the Brussels sprouts diet were determined and are shown in Fig. 3. Panel A shows the results of the run-in control (day C-11), the interven-

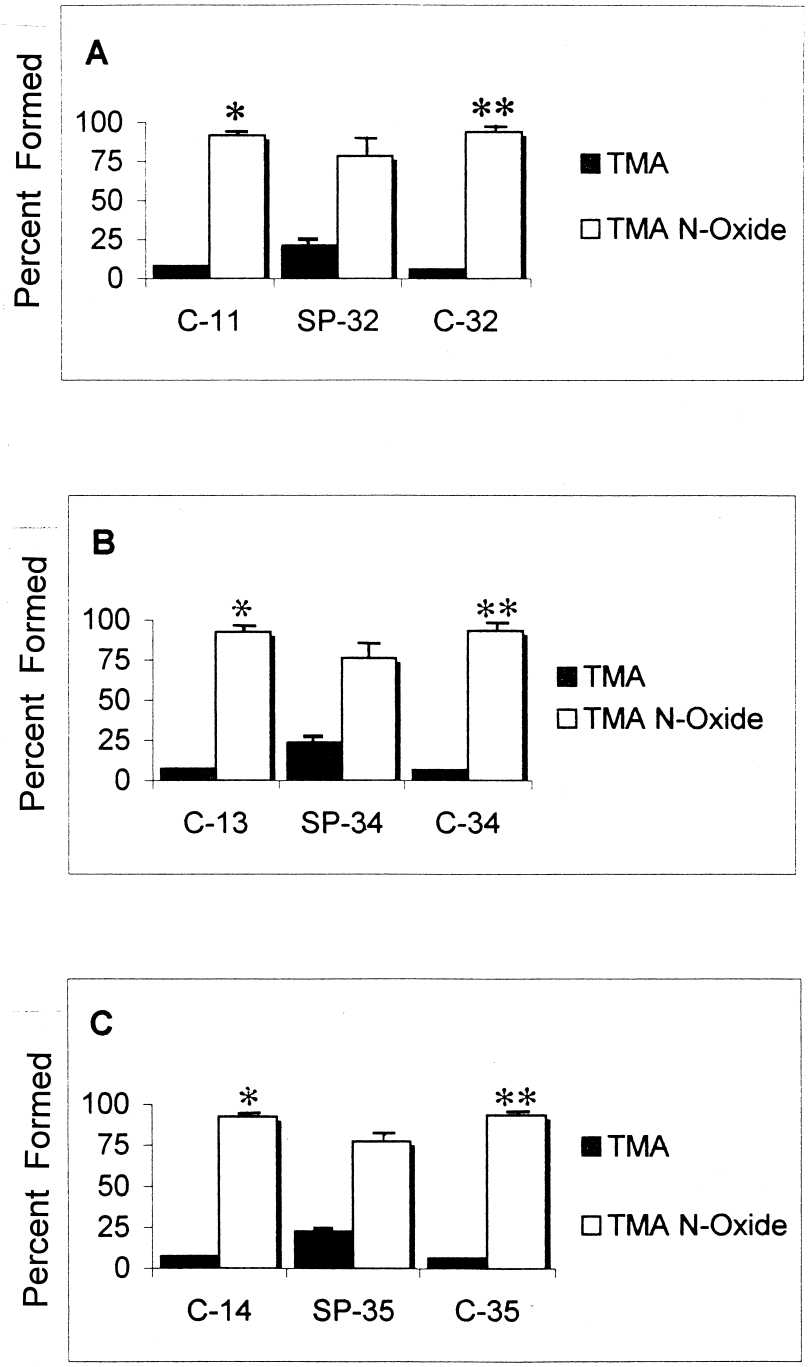

FIG. 3. Bar graphs of the percent of TMA and TMA *N*-oxide formed as a function of diet. As described in the text, each panel represents the 1-day average (± SD) of TMA and TMA *N*-oxide levels in the urine of 10 healthy, male, non-smoking volunteers on 3 separate days (i.e. analyzed on days C-11, C-13, or C-14). Five individuals continued on a glucosinolate-free diet after a 3-week period [i.e. analyzed control group (C) on days C-32, C-34, and C-35], while the other 5 consumed 300 g of cooked Brussels sprouts per day [i.e. analyzed sprouts group (SP) on days SP-32, SP-34, and SP-35]. Student's *t*-test showed that the TMA to TMA *N*-oxide ratio for the Brussels sprouts diet was increased significantly ($P < 0.05$) compared with control groups at the beginning (*) and after the intervention (**) period.

tion period Brussels sprouts-treated (day SP-32), and the intervention control (day C-32) groups. Panel B shows the results of the run-in control (day C-13), the intervention period Brussels sprouts-treated (day SP-34), and the intervention control (day C-34) groups. Panel C shows the results of the run-in control (day C-14), the intervention period Brussels sprouts-treated (day SP-35), and the intervention control (day C-35) groups. Three control days and 3 intervention days (3 weeks later) were chosen as test days to examine for the variability in the data and the longevity of the response. In the run-in period, no significant difference ($P > 0.05$) was observed between the TMA to TMA *N*-oxide ratios (i.e. mean ± SD, 8.1 ± 0.2: 91.9 ± 2.5, 7.5 ± 0.3: 92.5 ± 3.2, and 7.6 ± 0.1: 92.4 ± 1.6 for days 11, 13, and 14, respectively). The TMA to TMA *N*-oxide ratio for the run-in control group (N = 10) was not significantly different from the control group (N = 5) during the intervention period (i.e. 6.1 ± 0.2: 93.9 ± 3.4, 6.6 ± 0.3: 93.4 ± 4.3, and 6.4 ± 0.2: 93.6 ± 2.3 for days 32, 34, and 35, respectively). In contrast with the control groups, the TMA to TMA *N*-oxide ratios of all 5 individuals were increased in the Brussels sprouts-treated group

during the dietary intervention period. Thus, after 3 weeks on the Brussels sprouts diet, the mean ± SD TMA to TMA *N*-oxide ratio was 21.3 ± 3.0: 78.7 ± 11, 23.8 ± 2.9: 76.2 ± 9.4, and 22.5 ± 1.0: 77.5 ± 4.7 for days 32, 34, and 35, respectively. Compared with controls, two individuals showed a marked increase in unmetabolized TMA (i.e. 5.6- to 6.4-fold), while the TMA to TMA *N*-oxide ratios for the other 3 individuals were elevated by a factor of 1.5 to 3.5. For the individuals examined, the Brussels sprouts dietary regimen resulted in a significant increase in the TMA: TMA *N*-oxide ratios compared with the TMA:TMA *N*-oxide ratios in the control groups.

Because TMA *N*-oxide formation has been shown to be formed exclusively by human FMO activity [32] and because human FMO3 is the prominent FMO present in human liver, we investigated the role of acid condensation products of I3C as inhibitors of human FMO3 *in vitro*. Maltose binding fusion proteins of human FMO3 (i.e. human FMO3-MBP) were used because the enzyme is considerably more stable than the non-fusion protein and provides reliable kinetic results [34]. Previous studies have shown that the human *FMO3* Glu 158 and the human *FMO3* Lys 158 genes are present in the Caucasian population in approximately a 53:47 allelic ratio [32]. Thus, human FMO3 Glu 158 is viewed as the wild-type enzyme, and FMO3 Lys 158 is a major polymorphic alloenzyme.

Indole-3-Carbinol Acid Condensation Products

Treatment of I3C with 1.0 N HCl gave acid condensation products providing a model system of I3C products that have been shown to be present in animals after transit of I3C through the gastrointestinal tract [31]. This system was utilized to give sufficient quantities of I3C acid condensation products to evaluate as putative inhibitors of human FMO3. As shown in Fig. 2, a preparative HPLC chromatogram of acid condensation products arising from treatment of I3C with 1.0 N HCl gave approximately 30 products. Minor alterations of the reaction mixture afforded differing amounts of products, but the data of Fig. 2 were representative of a number of chromatograms run under similar conditions. In the presence of a mixture of I3C acid condensation products, $K_{m\ app}$ values obtained from double-reciprocal plots of 10-([*N*,*N*-dimethylaminopentyl]-2-trifluoromethyl)phenothiazine *N*-oxygenation velocity versus substrate concentration showed that the mixture was acting as an apparent competitive inhibitor. From Dixon analysis, the K_i values of neutralized I3C acid condensation products for *N*-oxygenation of 10-([*N*,*N*-dimethylaminopentyl]-2-trifluoromethyl)phenothiazine were 60 and 125 μM for human FMO3-MBP Lys 158 and FMO3-MBP Glu 158, respectively.

To determine the actual agent(s) present in the I3C acid condensation mixture that inhibited human FMO3, the individual preparative HPLC fractions were evaluated immediately after extractive isolation as inhibitors of human FMO3-MBPs at approximately equimolar inhibitor concentrations (i.e. approximately 2 μg/incubation) on the basis of HPLC peak UV-vis absorptivity at 280 nm (i.e. the extinction coefficient at 280 nm was 1.21×10^4, 1.82×10^4, and 1.82×10^4 M^{-1} cm^{-1} for I33′, LT, and CT, respectively). HPLC fractions with retention times of approximately 24-25 min, 30-32 min, and 34-35 min (Fig. 2) consistently inhibited human FMO3 with percent inhibition values of 45-65% under the experimental conditions employed. On the basis of these results that were confirmed by several similar experiments, highly inhibitory fractions of four preparative HPLC runs were pooled and re-examined for human FMO3 inhibitory activity. In all cases examined, the inhibitory activity of the initially identified fractions was confirmed, and the fractions were judged to be highly purified (i.e. greater than 90%) on the basis of HPLC analysis. These highly purified materials were taken forward for individual inhibition kinetic and structural identification studies.

TABLE 1. Kinetic constants for the inhibition of human FMO3-MBP by indole-3-carbinol and purified acid condensation products*

Inhibitor	K_i values (μM)	
	FMO3-MBP Glu 158	FMO3-MBP Lys 158
Unknown **A**, I33'	11.6	0.8
Unknown **B**, LT	6.9	1.2
Indole-3 carbinol	7.5	13.4

*The kinetic constants were calculated from initial velocity measurements by the HPLC procedure described in Materials and Methods. For human FMO3-MBP Glu 158 and FMO3-MBP Lys 158, 20 and 30 μg of protein, respectively, were used in 50 mM potassium phosphate buffer, pH 8.4. The results are the averages of 2-3 determinations, with an estimated error of less than 10%.

Inhibition of Human FMO3-MBP

With the purified inhibitory I3C acid condensation products in hand, kinetic constants for apparent inhibition of 10-([*N*,*N*-dimethylaminopentyl]-2-trifluoromethyl)phenothiazine *N*-oxygenation in the presence of human FMO3-MBP Glu 158 and human FMO3-MBP Lys 158 were calculated from the rate of 10-([*N*,*N*-dimethylaminopentyl]-2-trifluoromethyl)phenothiazine *N*-oxygenation at variable substrate concentrations using the HPLC procedure previously described [34]. The $K_{m\ app}$ and V_{max} values obtained from double-reciprocal plots of 10-([*N*,*N*-dimethylaminopentyl]-2-trifluoromethyl)phenothiazine *N*-oxygenation velocity versus substrate concentration all showed that the inhibitors were apparent competitive inhibitors. Kinetic analysis for 10-([*N*,*N*-dimethylaminopentyl]-2-trifluoromethyl)phenothiazine *N*-oxygenation employing Dixon plots (i.e. plots of the reciprocal of the velocity versus the inhibitor concentration) using concentrations with a range of 5-200 μM inhibitor gave the K_i values listed in Table 1. The results indicated that human FMO3-MBP

Lys 158 activity was considerably more sensitive to inhibition by I3C acid condensation products than was activity of human FMO3-MBP Glu 158. In addition, I3C also inhibited both human FMO3-MBP Glu 158 and Lys 158, but with similar K_i values (Table 1).

Structural Characterization of I3C-Related Inhibitors of Human FMO3

The structural characterizations of the most active purified acid condensation products from preparative HPLC runs (Fig. 2) as inhibitors of human FMO3 10-([*N*,*N*-dimethylaminopentyl]-2-trifluoromethyl)phenothiazine *N*-oxygenation were determined by NMR, UV-vis, GC–MS, and HPLC co-elution studies. First, the structures of authentic acid condensation products were determined by high-field ^{1}H-NMR as previously described [31]. Next, UV-vis and HPLC co-elution studies showed that the acid condensation products were the same as the previously characterized materials. Finally, GC–MS studies confirmed that the anticipated structure agreed with the spectral characterization data. Direct insertion probe electron impact or liquid chromatography–mass spectrometry analysis yielded poor quality data or meaningless results. However, GC–MS of trimethylsilylated derivatives provided ready detection of compounds with the anticipated ions. Thus, in the presence of BSTFA, unknown **A** gave prominent molecular ions at *m/z* 390/391 ($M^+/M^+ + 1$), 389, 317/318, 245/246, 217, and 202. The GC–MS data were consistent with the structure of I33′ that had prominent molecular ions at *m/z* 390/391 ($M^+/M^+ +1$), 389, 318/317, 301, and 246/245. The di-trimethylsilylated derivative of 133′ has a molecular ion at *m/z* 390, the mono-trimethylsilylated derivative of 133′ has a molecular ion at *m/z* 318, and the underivatized 133′ has a molecular ion at 246 *m/z*. The purity of unknown **A** was judged to be approximately 93% on the basis of analytical HPLC. It is possible that a minute amount of LT was also present in unknown **A** on the basis of data from the GC–MS experiment. This may have arisen during the GC–MS experiment itself, however. Unknown **B** was also subjected to GC–MS analysis in the presence of BSTFA. Prominent molecular ions at *m/z* 403, 402, 401, 387, 329, 313, 202, 189, and 147 were observed. The mass spectrum was consistent with the structure of LT that gave prominent ions at *m/z* 591/592 ($M^+/M^+ + 1$), 403, 402, 401, 387, 329, 313, 247, 202, 189, and 147 under similar GC–MS conditions. Although the molecular ion for the tri-trimethylsilylated derivative (anticipated to be present in very small amounts) at *m/z* 591 was not present, a major fragment at *m/z* 402 dominated the spectrum. The purity of unknown **B** was approximately 91% on the basis of analytical HPLC. Thus, the most active human FMO3-MBP inhibitors present in the I3C acid condensation mixture were LT and I33′ (Fig. 1).

DISCUSSION

It has been amply demonstrated that diet influences the incidence of human cancer and especially tumors of the gastrointestinal system [37]. In addition to a vast number of dietary carcinogens, there are also data indicating that anticarcinogens exist in fruits and vegetables, and these agents have been associated with a decreased risk of cancer [38-40]. From animal studies, one mechanism of anticarcinogenesis has been suggested to involve induction of detoxication enzyme systems [4, 18, 20, 21, 27, 40]. Thus, elevated levels of CYP or glutathione transferases can, in principle, protect animals from procarcinogens by increasing the metabolism, conjugation, and excretion of these materials to non-cancer-causing compounds.

The FMO is a prominent enzyme that detoxicates nucleophilic heteroatom-containing dietary constituents and xenobiotics by converting N-, S- and P-containing compounds to *N*-, *S*-, and *P*-oxides that are excreted readily [41, 42]. Previous studies in rodents showed that FMO activity was decreased after administration of I3C [29]. Thus, microsomes isolated from rats administered I3C by oral gavage showed decreased hepatic FMO activity. In the same experiment, increased administration of I3C by oral gavage showed an increase in CYP-mediated metabolism [29]. To date, however, no information about the effects of I3C or dietary indole constituents on the FMO detoxication system in humans has been reported.

After consumption of Brussels sprouts, a significant decrease in the urinary profile of the human FMO3 marker metabolite, TMA *N*-oxide, was observed. Previously, it was shown that in humans, TMA *N*-oxide was produced exclusively by FMO [43, 44], and the TMA to TMA *N*-oxide ratio has been established as a urinary biomarker of human FMO3 activity [32, 45, 46]. Recently, mutations in the human *FMO3* gene have been characterized in individuals with trimethylaminuria (i.e. fish-like odor disease, the inability to *N*-oxygenate TMA), confirming the observation that, *in vivo*, TMA *N*-oxygenation in humans is a bioindicator of human FMO3 function. Because consumption of Brussels sprouts decreases urinary TMA *N*-oxide formation, the conclusion is that potent selective inhibitors are present in or formed from the Brussels sprouts diet, and this treatment decreases human FMO3 activity. In addition, because no change in hepatic function was observed in the subjects examined throughout the course of the study, loss of human FMO3 activity was not a consequence of generalized hepatic cell death, but rather, a selective loss of FMO3 functional activity as a consequence of Brussels sprouts treatment. The K_i values observed (Table 1) for the inhibition of 10-([*N*,*N*-dimethylaminopentyl]-2-trifluoromethyl)phenothiazine *N*-oxygenation by I3C acid condensation products are in the region that is apparently physiologically relevant, and this is borne out by the observation that a diet of Brussels sprouts inhibited human FMO3 activity (i.e. TMA *N*-oxygenation) *in vivo* (Fig. 3). While the data herein point to a competitive inhibition

mechanism of action, it is also possible that the inhibitory effect of acid condensation products could stem from direct effects on human *FMO3* gene regulation, as appears to be the case for FMO1 in the rat [29]. Another possibility is that competitive inhibition is from sulfur-containing compounds in the Brussels sprouts. However, we do not favor this alternative mechanism involving competitive inhibition of FMO3 or substrate competitive inhibition by TMA, as the amounts of these types of compounds present in Brussels sprouts are too low to account for the results observed.

Although the level of indole-3-carbinol glucosinolate was not determined in this study, based on a previous report [10], the daily dose administered in this study was in the range of 0.002 to 0.014 mmol/kg. For comparison, administration of dietary I3C to rats at a daily dose of 0.88 mmol/kg for 28 days decreases hepatic FMO activity 3-fold and FMO1 protein levels 3.5-fold [29]. This dose results in a liver level of 3-6 μM I33′ and decreases hepatic FMO activity 3-fold and FMO1 protein levels 3.5-fold [29].

Porcine FMO1 is inhibited *in vitro* by ICZ (i.e. K_i value of 31 μM) and I33′ (i.e. K_i value of 42 μM) with kinetics indicative of competitive inhibition [47]. With regard to inhibition by I3C acid condensation products, porcine FMO1 differs from human FMO3 in not being sensitive to inhibition by LT or I3C. If one assumes that the K_i for I33′ inhibition of the rat FMO1 ortholog is similar, this implies that an apparent liver concentration of I3C acid condensation products an order of magnitude lower than the K_i still can affect FMO catalytic activity markedly. This observation, along with the low K_i value exhibited by I33′ for human FMO3, is consistent with the inhibition of catalytic activity observed *in vivo* in the present study.

It is possible that exchanging glucosinolate-rich vegetables for glucosinolate-free vegetables in the diet of the volunteers examined could introduce changes in the expression of human FMO3. For example, changes in caloric intake or a change in nutrient balance could alter human FMO3 activity. In the subjects examined, however, the caloric intake and macronutrient balance in both the treated and untreated groups were identical, and only the plasma thiocyanate concentration (a marker for glucosinolate exposure) was elevated [25]. However, we did not expressly examine effects of I3C acid condensation products on human *FMO3* gene regulation, and I3C acid condensation products have been observed previously to modulate gene expression [17, 19].

To elucidate the origin of the dietary indole effect as an inhibitor of human FMO3 activity, we examined the effect of the acid condensation products on human FMO3 function *in vitro*. Fusion proteins of human FMO3 (i.e. human FMO3-MBP) were examined because of the enhanced activity and stability of this highly purified enzyme [34] compared with its non-fusion protein. Generally, treatment of I3C with HCl under conditions that were anticipated to give products present after transit through the human stomach afforded a convenient source of these materials [48]. Direct incubation of neutralized I3C-acid condensation products in the presence of human FMO3-MBP Glu 158 or Lys 158 showed significant competitive 10-([*N*,*N*-dimethylaminopentyl]-2-trifluoromethyl)phenothiazine *N*-oxygenation inhibitory activity. To define more precisely the agent(s) in the I3C acid-condensation mixture responsible for the inhibitory activity, the 30 most prominent components from a preparative HPLC run were collected and tested individually as inhibitors of human FMO3-MBP 10-([*N*,*N*-dimethylaminopentyl]-2-trifluoromethyl)phenothiazine *N*-oxygenation. The major acid condensation products identified by GC–MS after derivatization as potent inhibitors of human FMO3-MBP *N*-oxygenation in the presence of human FMO3-MBP included I33′ and LT (Fig. 1). Although a few inhibitors of FMO have shown true competitive inhibition [49], most compounds that decrease FMO activity apparently do so as alternate substrate competitive inhibitors [41, 49]. Preliminary studies indicated that I3C was not a substrate for human FMO3. The lack of a nucleophilic heteroatom precludes I3C or any acid-condensation product from serving as an alternate substrate competitive inhibitor of human FMO3, and it is likely that the inhibition is due to direct competitive inhibition.

In conclusion, the data show that consumption of Brussels sprouts for 3 weeks resulted in decreased human FMO3 functional activity. Because little I3C is believed to survive the acid condensation chemical reactions upon transit through the stomach, it is likely that acid condensation products such as I33′ and LT are largely responsible for the human FMO3 inhibitory activity observed *in vivo*, although other gene regulation mechanisms also may be in operation as well. While the data point to I33′ and LT as responsible for the human FMO3-MBP inhibitory activity, it is notable that I3C itself possesses some inhibitory activity. However, after oral administration to humans, it is unlikely that sufficient I3C reaches the systemic circulation to exert a significant inhibitory effect on hepatic FMO3. While not specifically examined, it is likely that I3C and/or I3C acid condensation products also inhibit human FMO1. Based on the observations for inhibition of rat [29] and pig [47] FMO1 by I3C acid condensation products, inhibition of human FMO1 present in the intestine may have clinical consequences for the absorption and metabolism of chemicals.

Decreased human FMO3 activity could lessen the ability of an individual to detoxicate dietary nucleophiles and protect against potentially toxic xenobiotic exposure. However, because most reactive ultimate carcinogens are electrophilic, it is possible that decreased human FMO3 levels allow the hepatocyte to shift to up-regulation of enzymes that detoxicate electrophiles, including CYP and glutathione transferases. Induction of detoxication enzymes that inactivate electrophilic carcinogens potentially could increase the capacity of the cells to withstand cellular damage that could eventually lead to cancer. Previously, it has been proposed that FMO is induced constitutively [42], and

inhibition of human FMO may return the enzyme to a state of lower efficacy, although this level of enzyme activity apparently is more than sufficient to metabolize TMA, because in the subjects receiving a diet rich in Brussels sprouts, TMA was *N*-oxygenated to about 70% of normal.

In addition to the physiological importance of examining dietary modulation of FMO, it may be that monitoring the functional activity of FMO may serve as a convenient biomarker for studying the clinical utility of I3C as a chemoprotective agent in humans. Future studies may be directed toward this point.

The authors thank Dr. Greg Bruce for expressing the human FMO-MBP. The financial support of the National Institutes of Health (Grant GM36246) is acknowledged.

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