Effects of Glucosinolate-Rich Broccoli Sprouts on Urinary Levels of Aflatoxin-DNA Adducts and Phenanthrene Tetraols in a Randomized Clinical Trial in He Zuo Township, Qidong, People's Republic of China

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Abstract

Residents of Qidong, People's Republic of China, are at high risk for development of hepatocellular carcinoma, in part due to consumption of aflatoxin-contaminated foods, and are exposed to high levels of phenanthrene, a sentinel of hydrocarbon air toxics. Cruciferous vegetables, such as broccoli, contain anticarcinogens. Glucoraphanin, the principal glucosinolate in broccoli sprouts, can be hydrolyzed by gut microflora to sulforaphane, a potent inducer of carcinogen detoxication enzymes. In a randomized, placebo-controlled chemoprevention trial, we tested whether drinking hot water infusions of 3-day-old broccoli sprouts, containing defined concentrations of glucosinolates, could alter the disposition of aflatoxin and phenanthrene. Two hundred healthy adults drank infusions containing either 400 or <3 μmol glucoraphanin nightly for 2 weeks. Adherence to the study protocol was outstanding; no problems with safety or tolerance were noted. Urinary levels of aflatoxin-N^7-guanine were not different between the two intervention arms (P = 0.68). However, measurement of urinary levels of dithiocarbamates (sulforaphane metabolites) indicated striking interindividual differences in bioavailability. An inverse association was observed for excretion of dithiocarbamates and aflatoxin-DNA adducts (P = 0.002; R = 0.31) in individuals receiving broccoli sprout glucosinolates. Moreover, *trans, anti*-phenanthrene tetraol, a metabolite of the combustion product phenanthrene, was detected in urine of all participants and showed a robust inverse association with dithiocarbamate levels (P = 0.0001; R = 0.39), although again no overall difference between intervention arms was observed (P = 0.29). Understanding factors influencing glucosinolate hydrolysis and bioavailability will be required for optimal use of broccoli sprouts in human interventions. (Cancer Epidemiol Biomarkers Prev 2005;14(11):2605–13)

Introduction

Environmental exposures to foodborne and airborne carcinogens contribute to overall cancer burden. Hepatocellular carcinoma is one of the most common cancers worldwide and results in >300,000 deaths annually in the People's Republic of China, principally in the coastal and southern regions of the country (1). Hepatocellular carcinoma can account for up to 10% of adult deaths in some rural townships, such as He Zuo, in the Qidong region of eastern Jiangsu Province (2). Chronic infection with hepatitis B virus, coupled with exposure to aflatoxins in the diet, likely contributes to the high risk of hepatocellular carcinoma in People's Republic of China and other regions of the world (3, 4). From a public health perspective, hepatitis virus vaccination programs and efforts to reduce aflatoxin exposures could have major effects on the incidence of this disease. It is too early to ascertain the extent to which vaccination programs are altering the burden of hepatocellular carcinoma on the mainland of the People's Republic of China; however, a universal vaccination program against hepatitis B virus started over a decade ago on Taiwan has shown lower rates of hepatocellular carcinoma in children (5). The extent of aflatoxin contamination in foods is a function of the ecology of molds and is not completely preventable, such that chemoprevention may be especially useful in the setting of ongoing aflatoxin exposure and prior infection with hepatitis B virus. We have previously evaluated two chemopreventive strategies through randomized clinical trials in the Qidong region that sought to attenuate the toxic manifestations of aflatoxins by (*a*) blocking carcinogen bioavailability and (*b*) altering the metabolic phenotype of exposed individuals, thereby enhancing the detoxication and elimination of aflatoxin. Both approaches, using chlorophyllin (6) and oltipraz (7), respectively, led to significant modulation of aflatoxin biomarker levels consistent with reductions in risk of hepatocellular carcinoma.

The intervention with chlorophyllin, which acted in part through formation of molecular complexes with aflatoxin in the gastrointestinal tract, used thrice daily administration of tablets. Although inexpensive and safe, such an intensive intervention is unlikely to be sustainable over a long period of time in large numbers of at-risk individuals. Oltipraz, by

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contrast, was administered once daily and lead to enhanced metabolic detoxication of aflatoxin, presumably through induction of glutathione *S*-transferases (GST). As modeled in animal studies, in practice, use of small molecules that selectively induce protective phase 2 enzyme pathways may only require weekly rather than daily administration and is, therefore, considerably more attractive as a chemoprevention strategy (8). However, oltipraz itself has significant deficiencies, including availability, cost, and side effects (7, 9). Thus, we sought to examine more feasible means to induce aflatoxin detoxication in a clinical trial setting.

Numerous epidemiologic studies indicate that consumption of large quantities of fruits and vegetables, particularly cruciferous vegetables (e.g., broccoli, cabbage, kale, and Brussels sprouts), is associated with a reduced incidence of cancers of the gastrointestinal tract and other sites (10-14). Although not broadly studied, several cohort and case-control studies have shown inverse associations between vegetable consumption and liver cancer (14). In a series of laboratory studies, Talalay and colleagues (15-17) have observed that broccoli is rich in phytochemicals that induce phase 2 detoxication enzymes and bolster antioxidant activities in mammalian cells. Nearly all of this inducer activity is attributable to sulforaphane, which is an isothiocyanate. Many edible plants belonging to Cruciferae and other families contain glucosinolates (β-thioglucoside *N*-hydroxysulfates), which are the precursors of isothiocyanates (18). Young broccoli plants are an especially good source of chemopreventive glucosinolates, with levels 20 to 50 times those found in mature market-stage plants (19). The most abundant glucosinolate found in broccoli seeds and 3-day-old broccoli sprouts is glucoraphanin, the precursor to sulforaphane [(−)1-isothiocyanato-4-(methylsulfinyl)butane]. Broccoli seeds contain glucoraphanin levels ranging from ~5 to 100 μmol/g (20) and the sprouts that are grown from them contain glucoraphanin in quantities commensurate with seed levels. Dietary broccoli sprouts and their component glucosinolates and isothiocyanates induce phase 2 enzymes and afford protection against chemically induced tumors in rodents (16, 19). Their glucosinolates account almost exclusively for the phase 2 response induced from these plant extracts in cultured cells (19).

Glucosinolates, such as glucoraphanin, which are stable, water-soluble molecules, are converted enzymatically to their cognate isothiocyanate by the coexisting but normally segregated plant enzyme myrosinase (which is released when food is prepared or chewed) and by the flora of the human intestinal tract (Fig. 1). Thus, this clinical trial used broccoli sprouts as a rich and consistent source of glucoraphanin to determine whether an infusion (a hot-water extract) could serve as a simple, easily distributed medium for use in interventions that attempt to modulate levels of carcinogen biomarkers. The primary goal was to determine whether daily consumption of a broccoli sprout infusion could diminish the level of aflatoxin-N^7-guanine in urine samples collected from the study participants. As depicted in Fig. 2, this aflatoxin-DNA adduct excretion product serves as a biomarker of the biologically effective dose of aflatoxin and elevated levels are associated with increased risk of liver cancer (21). In animals, induction of GSTs is associated with reduced aflatoxin-N^7-guanine excretion and chemoprevention of hepatocellular carcinoma (22-24). A secondary end point, *r*-1,*t*-2,3,*c*-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene (*trans, anti*-PheT), reflecting exposure to and subsequent biotransformation of polycyclic aromatic hydrocarbons (PAH), was also evaluated. PAHs are well-established environmental carcinogens that are also likely to play a significant role in the etiology of human cancer through both foodborne and airborne exposures (25, 26). Like the prototypical PAH, benzo(*a*)pyrene, the simplest PAH containing a bay region, phenanthrene, undergoes sequential metabolism to a diol epoxide. Thus, it is a useful marker for carcinogen metabolite phenotyping (27) and is applied here for the first time for the assessment of pharmacodynamic action in an intervention.

Materials and Methods

Study Design and Participants. Adults in good general health without a history of major chronic illnesses were randomized into two intervention arms: placebo and broccoli sprout infusions ingested shortly before dinner. The trial included men and women and hepatitis B surface antigen–positive individuals with normal liver function. Study participants were recruited from the rural farming community of He Zuo Township, Qidong, Jiangsu Province, People's Republic of China. Seven hundred individuals were screened at the He Zuo Township Hospital over 3 days in mid January 2003. Written informed consent was obtained from all participants. The protocol was approved by the Institutional Review Boards of the Johns Hopkins Medical Institutions and the Qidong Liver Cancer Institute. A medical history, physical examination, and routine hepatic and renal function tests were used to screen the individuals, ages 25 to 65 years, using methods identical to those described for previous interventions in this region (28). Three hundred thirty-three individuals from the screened group were eligible, of which 200 were randomized using a fixed randomization scheme with a block size of 10. In the few instances when multiple family members were participants, they were randomized to the same intervention arm. Participants returned to the He Zuo Township Hospital on the first day of the study (February 20, 2003) where they were given their identification number.

All participants were asked to refrain from eating any green vegetables that could possibly contain glucosinolates and they were thus provided a comprehensive list of food items to avoid. Participants were assigned to local doctors in groups of roughly 20 based on area of residence. Local doctors were responsible for monitoring diets through daily completion of a questionnaire as well as distributing the intervention beverages and recording compliance. In practice, study investigators from the Qidong Liver Cancer Institute and Johns Hopkins visited the participants at their homes at dinnertime to observe composition of meals during a 3-day run-in period. Thereafter, participants, local doctors, and study investigators would meet at the homes of the local doctors between 5 and 6 p.m. for distribution of the intervention beverages and monitoring of compliance. Compliance was determined by visual observation and measures of urinary excretion of isothiocyanates in the form of their dithiocarbamate metabolites. Placebo and broccoli sprout infusions were stored at −20°C and brought to He Zuo daily for distribution. Overnight (roughly 12 hours) urine samples were collected each morning, volumes were measured, and aliquots were prepared and transported to Qidong for initial storage at −20°C. Semiquantitative measures of dithiocarbamate levels were conducted on urine samples obtained during the run-in and intervention phases to monitor for abstinence from green vegetables and to verify consumption of the infusions. In the few circumstances where unexpectedly high levels of dithiocarbamates were detected in urine samples from the run-in, study investigators visited participants to discuss possible dietary sources. Blood samples were collected on the first and last day of the study. Serum alanine aminotransferase activities were determined at the Qidong Liver Cancer Institute on all collected samples. Aliquots of urine and serum from each sample were shipped frozen to Baltimore at the end of the study, where serum samples were transferred immediately to a clinical laboratory (Hagerstown Medical Laboratory, Hagerstown, MD) for comprehensive blood chemistry analyses.

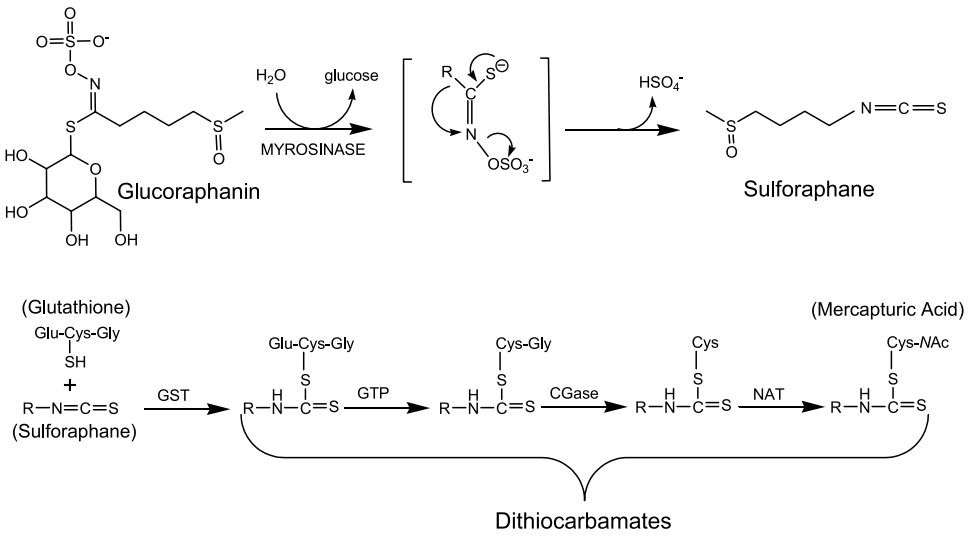

Figure 1. Glucoraphanin is converted to sulforaphane by the enzymatic action of myrosinase. Sulforaphane is conjugated to glutathione by GSTs, then metabolized sequentially by γ-glutamyltranspeptidase (*GTP*), cysteinylglycinase (*CGase*), and *N*-acetyltransferase (*NAT*) ultimately to form mercapturic acids. Mercapturic acids are the predominant form of sulforaphane metabolites found in urine; however, all glutathione-derived conjugates (collectively called dithiocarbamates) are measured by the cyclocondensation assay using 1,2-benzenedithiol.

Preparation of the Broccoli Sprout Infusion. Following extensive screening for glucoraphanin content, broccoli seeds (*Brassica oleracea* L., Italica Group; lot BR0302 of a cultivar Marathon-derived F2 hybrid; not treated with pesticides or dyes) were purchased from Caudill Seed Co. (Louisville, KY) and delivered to the study site. Forty-five kilograms of seeds were surface-disinfested according to standard practices for commercial green sprout growers (29). Briefly, seeds were contacted with 2% (v/v) sodium hypochlorite prepared by appropriate dilution of commercial household bleach into tap water. Seeds and bleach were agitated periodically for 15 minutes, bleach was poured off, and seeds were thoroughly rinsed with running tap water for 2 hours. Seeds (~40 kg remaining after rinsing loss) were then spread in thin layers over at total of 56 especially designed 14 × 22.5 × 1.5 in. sprouting trays (Green Valley Food Corp., Dallas, TX). Trays were stacked on carts at a slight inclination to allow water runoff and were maintained at ~22 ± 2°C, illuminated with low-level ambient indoor filtered sunlight, and irrigated with tap water delivered from a spray nozzle at 1- to 2-hour intervals.

After 3 days of growth, broccoli sprouts were deep green in color and were estimated to have increased in weight (fresh weight basis) by ~5-fold; thus, 40 kg of seed resulted in ~200 kg of sprouts. Sprouts were harvested batch-wise, by sequentially plunging seven full trays (each containing ~3.5 kg of sprouts) into 50 L of rapidly boiling water, such that the water returned to a full boil between each tray. Following the addition of the final tray, boiling was continued for 30 minutes, after which sprouts were separated from the hot water by filtration through a 1 × 1 mm nylon mesh screen. Preparations were pooled and the final filtered volume of hot water extract from 200 kg of sprouts was ~400 L.

Preparation of Placebo Infusion. Following the first boiling water extraction of sprouts to produce the broccoli sprout infusion, sprouts were rinsed thoroughly in cold tap water and reextracted thrice in boiling water as described above. The second and third extractions were discarded and the fourth overall hot water extract was used as the placebo. Both broccoli sprouts and placebo infusions were cooled overnight at 5°C, bottled in 125 mL aliquots in sterile 596 mL commercial bottled water bottles, and immediately frozen.

Reagents. All reagents used were analytic or high-performance liquid chromatography (HPLC) grade. Glucosinolate standards used for HPLC were isolated and identified according to published methods (30-32).

Glucosinolate Composition of Doses. The hot aqueous extracts were subsequently analyzed by HPLC according to methods developed for separation of intact glucosinolates (30). Confirmation of glucosinolate identities was done by comparison of retention times with previously obtained glucosinolate standards and by mass spectrometry (31, 32). Infusions were also analyzed following digestion with added myrosinase and ascorbic acid as described by Shapiro et al. (33). Total isothiocyanates were assayed both before and after enzymatic hydrolysis by the cyclocondensation assay (15, 34, 35).

Bioassay of Phase 2 Enzyme Inducer Activity. Aliquots of the broccoli sprouts and placebo infusions were diluted 200-fold into microtiter plates for bioassay of quinone reductase

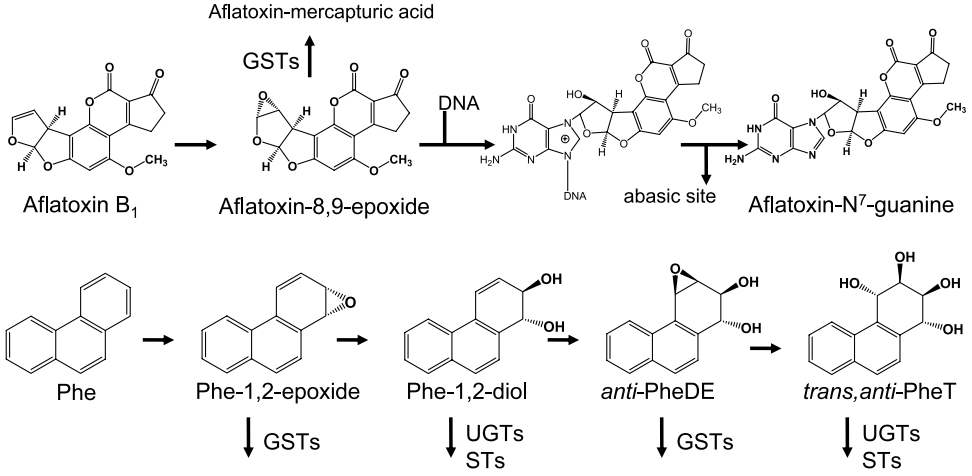

Figure 2. Pathways for the formation of aflatoxin-N^7-guanine and *trans, anti*-PheT after environmental exposures to aflatoxin and phenanthrene (*PheT*), respectively. *Anti-PheDE,anti*-1,2-dihydroxy-3,4-epoxy-1,2,3,4-tetrahydrophenanthrene; *EH,* epoxide hydrolase; *P450,* cytochrome P450; *UGT,* UDP-glucuronosyl transferase; *ST,* sulfotransferase.

(NQO1), a representative phase 2 enzyme, using Hepa 1c1c7 cells as described originally by Prochaska et al. (36) and modified by Fahey et al. (19). Excess, highly purified myrosinase (0.0003 units/mL of cell culture medium) and 500 μmol/L ascorbate were added at the same time infusions were added to microtiter plates to achieve complete hydrolysis of glucosinolates during a 48-hour incubation at 37°C. With this method, conversion of extracted glucosinolates to their cognate isothiocyanates is essentially quantitative.

Dithiocarbamates in Urine. Measurement of isothiocyanates as their dithiocarbamate metabolites in urine was done on 0.5 mL aliquots by the cyclocondensation reaction with1,2-benzenedithiol (Fig. 1) as described by Ye et al. (35).

Aflatoxin-N^7-Guanine in Urine. Twenty-milliliter aliquots of urine were acidified, added to 1.0 ng of aflatoxin B2 as an internal standard, and loaded onto preconditioned Varian Bond-Elut SPE columns. Aflatoxins were then eluted with acidified methanol and concentrated under nitrogen in silanized vials to ~300 μL. Eluants were subsequently diluted with water before application to aflatoxin-specific monoclonal antibody immunoaffinity resin columns. Aflatoxins were then eluted with 12 mL of 70% DMSO/water, subsequently diluted to <10% DMSO with water, and reapplied sequentially to three SPE columns to remove the solvent. Finally, aflatoxins were eluted with acidified methanol and concentrated under an argon stream to a final volume of 25 μL. This concentrate was then injected onto a Thermo LCQ Deca liquid chromatography/electrospray ionization mass spectrometer operated in the positive ionization mode. The limit for detection of the nucleic acid adduct (signal-to-noise <3) was ~0.5 pg (~1 fmol). Aflatoxin-N^7-guanine and aflatoxin B2 were quantified by measuring the mass area of specific tandem mass spectrometry daughter fragments MH^+ 152.1 and 259.1 derived from the parent ions of m/z 480.1 and 315.1, respectively. All values were normalized to creatinine levels measured in the original urinary aliquots by Hagerstown Medical Laboratories.

Phenanthrene Tetraol in Urine. Urinary levels of *trans, anti*-PheT were determined by gas chromatography-mass spectrometry as described by Hecht et al. (25).

Statistical Analysis. Comparisons of characteristics between randomized treatment groups were made using Wilcoxon rank-sum tests (continuous variables) and χ^2 tests of association (categorical variables). Dithiocarbamate levels in the run-in and intervention periods were compared using parametric (ANOVA and t tests) and nonparametric (Kruskal-Wallis and Wilcoxon tests) methods, resulting in identical inferences. The intraclass correlation between repeated measurements of dithiocarbamate of individuals randomized to the group receiving the broccoli sprout glucosinolates during the intervention period were calculated as the ratio of within-individual to total variability; 95% confidence intervals were computed using bootstrap methods. To compare the primary and secondary end points, Student's t tests were used in an intention-to-treat analysis on $\log_{10}$-transformed data. Associations between aflatoxin-N^7-guanine concentrations or *trans, anti*-PheT and urinary dithiocarbamate were evaluated using linear regression. All analyses were conducted using SAS (version 9, SAS Institute, Cary, NC) and Splus (version 6.2, Mathsoft, Seattle, WA) software.

Results

Enrollment and Comparability of Intervention Arms. As indicated in Fig. 3, 200 individuals, representing 100% of the recruitment goal, were randomized into the two intervention arms. Table 1 shows that the intervention groups did not differ significantly ($P > 0.05$) by age, gender, hepatitis B surface antigen status, smoking status, or alcohol consumption. For most variables, these distributions represented the screened population (data not shown).

Compliance and Data Collection Completeness. Adherence to the study protocol was outstanding. Only one person, who was randomized to the placebo arm, dropped out of the study. With the exception of one dropout, overall, 100% of the placebo and broccoli sprout beverages were consumed during the study, as assessed by daily monitoring of intake and periodic urinary measures of isothiocyanate derivatives. All blood and 98% of requested urine samples were collected from each participant over the duration of the study. No adverse events were reported in the study group nor were there any abnormal clinical chemistry values for blood samples collected on the last day of the study.

Characterization of the Glucosinolate Composition of the Broccoli Sprout Infusion. Both broccoli sprout and placebo infusions were analyzed by multiple complementary methods to confirm that the broccoli sprout preparation contained the expected glucoraphanin levels and that the placebo contained at least 100-fold lower levels of this glucosinolate. Infusions were chromatographed directly and were also subjected to hydrolysis with purified myrosinase to convert glucosinolates to isothiocyanates and other minor metabolites. Disappearance of glucosinolates, and appearance of isothiocyanates, and their induction of a marker mammalian phase 2 enzyme NQO1 in cell culture could thus be followed. Glucoraphanin was the primary glucosinolate in the broccoli sprout infusion, accounting for 61% of the total glucosinolates, followed by glucoerucin (18%), glucoiberin (14%), glucosinalbin (3.9%), glucoiberverin (1.7%), and 4-hydroxyglucobrassicin (1.7%; Fig. 4). Total glucosinolates measured by direct HPLC amounted to 5.3 μmol/mL, of which 3.2 μmol/mL were glucoraphanin. Thus, a 125 mL dose contained 400 μmol glucoraphanin. Hydrolysis of the dose with myrosinase resulted in no detectable remaining glucosinolates by direct HPLC, but yielded 5.2 μmol/mL of isothiocyanates measured as their cyclocondensation reaction

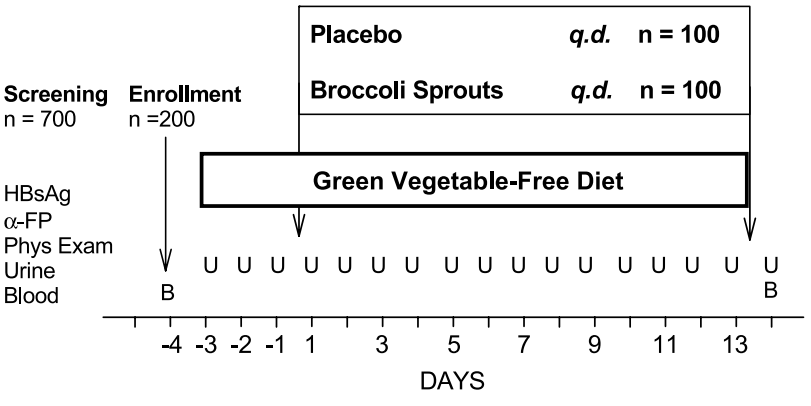

Figure 3. Outline of the intervention protocol, schedule, and timeline. Broccoli sprouts or placebo beverages were administered daily, shortly before dinner, for 14 consecutive days. Overnight voids were collected each morning whereas blood samples were obtained at the beginning and end of the study. *HBsAg,* hepatitis B surface antigen; *α-FP,* α-fetoprotein.

Table 1. Demographic characteristics of enrolled participants by treatment

	Placebo (n = 100)	Broccoli sprouts (n = 100)	P
Gender			1.00
Female	69 (69%)	69 (69%)	
Male	31 (31%)	31 (31%)	
Median age (min, max)	48 (25, 65)	48 (27, 65)	0.37
Hepatitis B surface antigen positive	5 (5%)	10 (10%)	0.28
Smokers	12 (12%)	16 (16%)	0.42
Median alcoholic drinks/wk (min, max)	0 (0, 96)	0 (0, 84)	0.92

product (the 0.1 μmol/mL of 4-hydroxyglucobrassicin present in the dose is not converted to an isothiocyanate by myrosinase). Only 0.024 μmol/mL of glucoraphanin could be detected in the placebo (no other glucosinolates were detectable) and upon hydrolysis by myrosinase, 0.041 μmol/mL of isothiocyanate, over 100-fold less than in the broccoli sprout infusion, were detected by cyclocondensation.

Bioassay of the infusions for phase 2 enzyme induction activity showed no detectable activity in either placebo or myrosinase-hydrolyzed placebo. The NQO1 induction potential of the myrosinase-hydrolyzed broccoli sprout infusion was 71,400 units/mL, thus confirming the presence of high-potency inducers (e.g., glucoraphanin/sulforaphane) in this glucosinolate preparation.[8]

Effect of the Broccoli Sprout Glucosinolates on Urinary Levels of Dithiocarbamates and Carcinogen Biomarkers. Isothiocyanates are metabolized in mammals principally by the mercapturic acid pathway. As shown in Fig. 1, an initial conjugation with glutathione promoted by GSTs gives rise to the corresponding isothiocyanate-glutathione conjugates. These undergo further enzymatic modifications to give rise sequentially to the cysteinylglycine-, cysteinyl-, and *N*-acetylcysteinyl-isothiocyanate conjugates, all of which are dithiocarbamates (dithiocarbamates). Inasmuch as nearly all isothiocyanates and dithiocarbamates react quantitatively with the vicinal sulfhydryl groups of 1,2-benzenedithiol to form a cyclic condensation product (1,3-benzenedithiole-2-thione), measurement of this product by HPLC provides a facile assay for determining total dithiocarbamate excretion, and hence isothiocyanate bioavailability, following dosing with glucosinolates, such as glucoraphanin (34, 35, 37). Table 2 shows the amounts of dithiocarbamates excreted in overnight voids at the conclusion of the run-in period (day −1) and at several time points (days 5, 9, 10, and 12) during the intervention with the broccoli sprout and placebo preparations. Because isothiocyanates have short whole-body half-lives (33, 38, 39), 12-hour urine collections provide a reasonable estimate of the fraction of administered glucoraphanin hydrolyzed by the enteric flora, absorbed by the study participants and subsequently excreted. Very low levels (0.1-0.2 μmol/12 hours) of dithiocarbamates were detected in most all urine samples during the run-in phase, highlighting the restraint of the study group from consuming green vegetables. The amount of excreted dithiocarbamate did not significantly increase in individuals assigned to drink the placebo, reflecting the low glucosinolate content of this beverage prepared from a fourth hot water extraction of the 3-day-old sprouts used for preparation of the broccoli sprout infusion. Excreted dithiocarbamate amounts increased dramatically ($P < 0.001$ compared with run-in period) for those individuals randomized to drink the broccoli sprout beverage and the dithiocarbamate levels in the intervention group exceeded those found in urine of individuals receiving placebo in all cases at all times. The average amount of dithiocarbamate excreted in individuals randomized to receive the broccoli sprout infusion was 49 μmol/12 hours during days 9, 10, and 12. This output represents ~12% of the administered dose. Less than half of this steady-state excretion level was observed at day 5 of the intervention (21.2 μmol/12 hour), suggesting that an adaptive response of increased enteric hydrolysis of glucoraphanin or enhanced glutathione conjugation of sulforaphane occurs over time with repeated dosing. There was unexpectedly high interindividual variability in the excretion rates, ranging from 4.1 to 180.4 μmol (1-45% of the administered dose). Interestingly, repeated measures of dithiocarbamate elimination on days 5, 9, 10, and 12 in individuals receiving the broccoli sprout infusion indicated a moderately strong intraclass correlation with a value of 0.29 (95% confidence interval, 0.27-0.45), indicating that dithiocarbamate ''tracks'' within individuals over time. Overall then, although sulforaphane bioavailability was reasonably consistent between doses within an individual, there was 3-fold greater variability in dithiocarbamate excretion rates between participants.

The study was designed to have the statistical power to evaluate biomarker modulation in a single, cross-sectional analysis. Such an approach is necessary because of the short biological half-life of urinary aflatoxin and phenanthrene metabolites. Unlike glucosinolate dosing with the broccoli sprout infusion, daily environmental exposures to aflatoxins and PAHs were likely to be highly variable and were

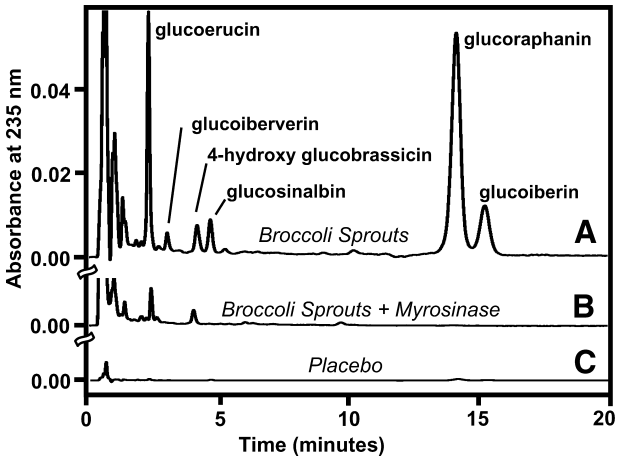

Figure 4. HPLC analysis of the glucosinolate composition of the broccoli sprouts (**A**) and placebo (**C**) beverages prepared from the first and fourth aqueous extracts of 3-day-old broccoli sprouts, respectively. **B,** HPLC profile of the broccoli sprouts beverage pretreated with myrosinase to hydrolyze glucosinolates to their cognate isothiocyantes. Absorbance was measured at 235 nm.

[8] The cognate isothiocyanates of glucoerucin, glucoiberin, and glucoiberverin (erucin, iberin, and iberverin, respectively) have been shown to be over 10-fold less potent as inducers of NQO1 than sulforaphane (the cognate isothiocyanate of glucoraphanin; ref. 15). The isothiocyanate metabolites of glucosinalbin and 4-hydroxyglucobrassicin are even less potent inducers. Thus, ≥94% of the measured induction of NQO1 was probably due to the isothiocyanate (sulforaphane) derived from myrosinase hydrolysis of glucoraphanin.

Table 2. Urinary levels of excreted dithiocarbamates during the run-in and intervention

Day	Urinary dithiocarbamate (μmol excreted/12 h)	
	Placebo	Broccoli sprouts
Run-in		
−1	0.2 (0, 4.9)*	0.1 (0, 3.5)
Intervention		
5	ND	21.2 (4.1, 81.3)†
9	0.1 (0, 2.3)	52.0 (7.4, 180.4)†,‡
10	0.1 (0, 4.6)	46.5 (10.1, 144.1)†,‡
12	ND	48.7 (5.0, 119.0)†,‡

Abbreviation: ND, not determined.
*Mean (min, max).
†Differs from run-in, $P < 0.001$.
‡Differs from day 5 broccoli sprouts, $P < 0.001$.

uncontrolled. Day 10 urine samples were selected for aflatoxin biomarker analysis for two reasons: (*a*) steady-state excretion rates for dithiocarbamate were achieved (Table 2) and (*b*) a presumption that any pharmacodynamic action of glucoraphanin would be maximal by this time. Aflatoxin-N^7-guanine could be detected in 188 (94%) of the 199 available urine samples from the day 10 collection. Eleven samples could not be evaluated because of interfering peaks during chromatography. As shown in Table 3, the mean level of aflatoxin-N^7-guanine in the urine of study participants receiving the placebo was 2.70 fmol/mg creatinine. Administration of the broccoli sprout glucosinolate preparation daily for nine evenings led to a statistically nonsignificant 7% reduction in urinary excretion of aflatoxin-N^7-guanine (mean = 2.51). *Trans, anti*-PheT, a secondary end point biomarker for this study, was detectable in all 199 urine samples collected on day 9. Urinary concentrations of *trans, anti*-PheT averaged 4,000-fold higher than those observed for aflatoxin-N^7-guanine on the following day. The mean level of *trans, anti*-PheT in the urine of participants receiving the placebo was 12.50 pmol/mg creatinine. Administration of broccoli sprout infusions daily for eight evenings led to a statistically nonsignificant 28% reduction in urinary excretion of *trans, anti*-PheT (mean = 8.99).

Effect of Sulforaphane Bioavailability on Carcinogen Biomarker Modulation. An effect of the intervention with broccoli sprouts was observed with both carcinogen biomarkers after controlling for the bioavailability of sulforaphane by monitoring for dithiocarbamate excretion in each individual. Such an evaluation was feasible because of the absolute visual verification of compliance for all study participants. Presented in Fig. 5 is a scatterplot of log[aflatoxin-N^7-guanine] versus log[dithiocarbamate] for each individual receiving the broccoli sprout infusion. There was no association between dithiocarbamate concentration and aflatoxin-N^7-guanine >concentration in the placebo group ($P = 0.39$). However, a significant inverse association between dithiocarbamate and aflatoxin-N^7-guanine concentrations was observed in the group receiving the broccoli sprout beverage ($P = 0.002$; $R = 0.31$). A similar, slightly more robust inverse association was observed when a 1 day lag was introduced between dithiocarbamate and aflatoxin-N^7-guanine measurements ($P = 0.001$; $R = 0.33$). Such an outcome, which is supported by mechanistic studies (8) and is consistent with the tracking of urinary dithiocarbamate levels among individuals, suggests that yesterday's pharmacodynamic action of sprouts principally affects today's carcinogen biomarker excretion. Measures of *trans, anti*-PheT levels also provide evidence of pharmacodynamic action by the broccoli sprout glucosinolates. There was a highly significant inverse association between log[*trans, anti*-PheT] and log[dithiocarbamate] in urine samples collected on day 9 from participants in the broccoli sprout infusion group ($P = 0.0001$; $R = 0.39$). Thus, two independent biomarkers of carcinogen metabolism were modulated following ingestion of the broccoli sprout beverage.

Discussion

The biomarkers used in this study provide measures of carcinogen exposure in the residents of Qidong, People's Republic of China, an area of high risk for development of hepatocellular carcinoma (6, 7). Urinary levels of aflatoxin-N^7-guanine adducts (median = 1.90 fmol/mg creatinine) measured from the placebo arm of this winter 2003 study were comparable with those found in the placebo arm during a summer 1997 intervention trial with chlorophyllin in a nearby township, Daxin (median = 0.96 fmol/mg creatinine; ref. 6). The modest difference may reflect the influence of seasonality on aflatoxin exposure: A 2-fold winter versus summer difference was seen with aflatoxin biomarkers in an ecological study in Daxin in 1993 (40). Similar analytic approaches of immunoaffinity chromatography followed by liquid chromatrography-mass spectrometry were used in the two intervention studies. Thus, aflatoxin exposures have not changed appreciably over the past decade in the rural areas of Qidong despite urbanization of the center of this county. Incipient industrialization in the Qidong region and expansive urbanization and industrialization in nearby Shanghai have affected air quality in eastern People's Republic of China. PAHs, such as phenanthrene, are ubiquitous in the general environment and are released into the ambient air by tobacco smoke, vehicle exhausts, and other incomplete combustion sources such as cooking stoves. Analysis of the organic extract of indoor air particles from homes during cooking in Yunnan, People's Republic of China, indicated phenanthrene to be the most abundant PAH (41). Hecht et al. (25) previously reported mean levels of 1.5 pmol *trans, anti*-PheT/mg creatinine in nonsmokers in the United States, whereas smokers had levels of 4.6 pmol/mg. In the current study, we observed a modest increase in *trans, anti*-PheT levels in the 28 smokers compared with the 171 nonsmokers (11.9 versus 10.6; $P = 0.09$). Although the magnitude of the difference between smokers and nonsmokers is lower in Qidong (perhaps reflecting a modest average consumption of 10 cigarettes per day) than in the U.S. study, the baseline values of the PAH biomarker are substantially higher in the nonsmoking residents of Qidong randomized to placebo compared with the U.S. nonsmokers (12.2 versus 1.5 pmol/mg creatinine). Thus, nontobacco-derived sources, such as industrial, automotive, and cooking emissions, seem to account for the bulk of PAH exposures in this rural area adjacent to and downwind of Shanghai. Continued monitoring of PAH biomarkers in this region may provide objective measures of changing exposure patterns, which in turn suggests further needs for simple chemopreventive modalities in this population.

Table 3. Urinary levels of study end points by treatment

Biomarker	Intervention arm		*P*
	Placebo	Broccoli sprouts	
Aflatoxin-N^7-guanine (fmol/mg creatinine)	2.70 (0.13, 11.27)*	2.51 (0.10, 7.75)	0.68
Trans, anti-PheT (pmol/mg creatinine)	12.50 (0.45, 68.92)	8.99 (1.62, 28.53)	0.29

*Mean (min, max).

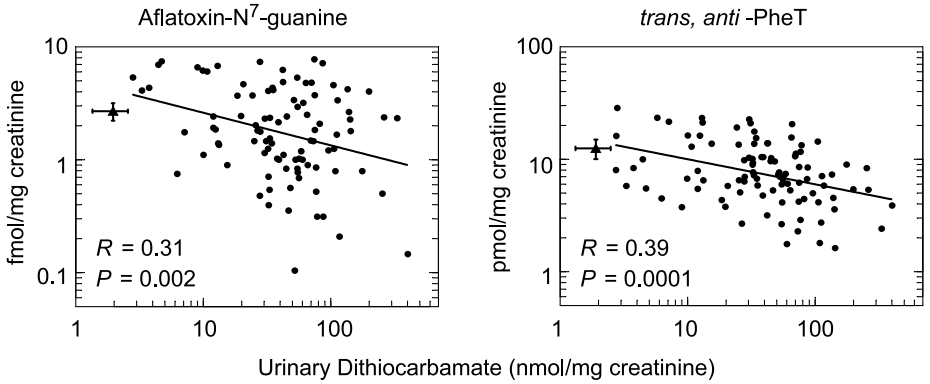

Figure 5. Scatterplots of urinary levels of aflatoxin-N^7-guanine (*left*) and *trans, anti*-PheT (*right*) versus dithiocarbamate after 10 or 9 days of intervention, respectively, for each of the individuals randomized to receive the broccoli sprouts beverage (●). ▲, mean of biomarker levels for the 100 participants receiving the placebo; —, 95% confidence interval.

In previous clinical trials, we evaluated the efficacy of pharmacologic interventions to modulate aflatoxin bioavailability and disposition in residents of Qidong. These randomized, placebo-controlled interventions with chlorophyllin and oltipraz led to significant reductions in urinary levels of aflatoxin-N^7-guanine and increases in the phase 2 metabolite aflatoxin-mercapturic acid, respectively. In both instances, the directionality and magnitude of biomarker modulation was consistent with protection against hepatocellular carcinoma development as had been observed in animal models (22, 42). However, issues of availability, cost, frequency of administration, and safety render the long-term use of these agents in prevention trials problematic. Thus, we sought to evaluate a potentially inexpensive, simple, safe, food-based intervention targeting similar molecular mechanisms of action as with the oltipraz and chlorophyllin interventions. Oltipraz, chlorophyllin, and sulforaphane exert chemopreventive actions at least in part by inducing phase 2 gene expression through the Keap1-Nrf2 signaling pathway (43-45).

The selection of broccoli sprouts was predicated upon extensive preclinical and clinical studies (15, 16, 19, 33, 35, 38). Broccoli sprouts are an exceptionally rich source of sulforaphane that differ from mature, market-stage broccoli in two important aspects: (*a*) on a gram-fresh-weight basis, they contain up to 50 times more glucoraphanin, the glucosinolate precursor of sulforaphane and (*b*) they contain substantially less or no detectable amounts of the indole and β-hydroxyalkenyl glucosinolates that are associated with potential toxicity (Fig. 3). Hot water extracts were used as the vehicle for administration of the glucoraphanin/sulforaphane for additional pragmatic reasons. First, residents of this region dislike consuming raw vegetables. Second, immersion of broccoli sprouts into boiling water provides a near quantitative extraction of glucoraphanin into the aqueous phase. Thus, with proper selection of seeds, preparation of a beverage with a defined content of glucoraphanin is readily achievable. Moreover, glucoraphanin is stable to freezing, allowing for large-batch preparation of an infusion for consistency of content as well as ease of storage and distribution. By contrast, provision of a fresh, consistent source of young sprouts for a long-term intervention in rural, underdeveloped regions provides many logistical and hygiene challenges. A major disadvantage of using hot water extracts of broccoli sprouts as opposed to intact broccoli sprouts lies in the capacity for hydrolysis of glucoraphanin to sulforaphane. The plant enzyme catalyzing this hydrolysis, myrosinase, becomes inactivated by boiling. Glucosinolates per se are inert as phase 2 enzyme inducers and must be hydrolyzed to generate the active isothiocyanates (19, 33, 38). Earlier studies established striking differences in the excretion of total dithiocarbamates following ingestion of either uncooked sprouts (~39% with chewing and ~26% without chewing), a glucosinolate preparation containing heat-inactivated myrosinase (~15%) or an isothiocyanate preparation derived from a myrosinase-treated glucosinolate preparation (~90%) prepared from broccoli sprouts (33). Getahun and Chung (37) have similarly observed a limited bioavailability of isothiocyanates from their precursor glucosinolates when watercress is cooked and myrosinase is destroyed. Thus, the bioavailability of sulforaphane following dosing with a hot water extract is entirely dependent on enteric flora for hydrolysis, which can be limiting (37, 38). Small clinical studies on healthy volunteers in inpatient facilities indicated that ~15% of administered glucosinolate could be recovered in urine as dithiocarbamates over 72 hours; 80% of this amount could be recovered within 16 hours after administration (33).

In complete accord with these initial clinical studies, we observed on average that 12% of the administered dose of glucoraphanin was excreted into urine as dithiocarbamates over the subsequent 12 hours in the 100 participants on the broccoli sprout arm of the clinical trial in Qidong. However, unexpected was the large interindividual variability in glucoraphanin pharmacokinetics, such that overnight dithiocarbamate excretion reflected between 1% and 45% of the administered dose. The reason underlying this interindividual variation is unknown, but may reflect difference in the composition of enteric microflora that influence the extent of glucoraphanin hydrolysis, polymorphisms in GSTs affecting dithiocarbamate formation from sulforaphane, or other factors that affect bioavailability, biotransformation, and excretion. Repeated measures of dithiocarbamate excretion rates over the course of the intervention indicate a strong level of tracking, with an intraclass correlation coefficient of 0.29. This degree of stability in dithiocarbamate excretion is identical to the intra-animal variance of weekly measures of aflatoxin-albumin adduct levels in a chronic dosing bioassay of 120 animals (46). Thus, factors influencing sulforaphane uptake and elimination following consumption of the broccoli sprout infusion are reasonably constant within an individual. Such tracking has been observed in earlier clinical trials with feeding of broccoli sprout preparations (33). It should be noted, however, that overall excretion rates doubled from early in the intervention (day 5) to later in the intervention (days 9, 10, and 12). This result suggests that some dynamic process, such as elevation in GST activities or in glutathione levels, may have occurred over the course of the intervention. Such outcomes are consistent with the potent actions of sulforaphane as an inducer of GSTs involved in conjugation with glutathione and γ-glutamylcysteine ligase, which controls the rate-limiting step for biosynthesis of glutathione (47). *GSTM1* or *GSTT1* genotype, however, had no effect on urinary excretion of dithiocarbamates (data not shown).

The randomized, placebo-controlled intervention with the broccoli sprout infusion was, *strictu sensu*, negative with respect to modulating levels of the primary (aflatoxin-N^7-guanine) and secondary (*trans, anti*-PheT) urinary biomarkers as only small, nonsignificant diminutions were observed with a simple comparison by treatment arm. This outcome is likely

a consequence of the highly variable bioavailability of sulforaphane from the broccoli sprout preparation. Although the dose of 400 μmol was selected with an expectation of 15% bioavailability, to yield an absorbed and excreted dose of 50 to 60 μmol/d, the unexpected variability compromised the overall power of the study to detect differences between treatment arms. However, the strong, highly significant inverse associations ($P < 0.002$) observed when biomarker levels were compared with actual levels of dithiocarbamate excretion, rather than comparing by treatment assignment, suggest that sulforaphane was exerting a pharmacodynamic action in at least a subset of the participants. Reductions in aflatoxin-N^7-guanine levels likely reflect induction of GSTs by sulforaphane, thereby shunting the reactive epoxide intermediate away from nucleophiles in DNA and toward glutathione (Fig. 2). Elevations in urinary excretion of aflatoxin-mercapturic acid, a glutathione-derived conjugate, were observed in the clinical trial with oltipraz, a phase 2 enzyme inducer with lower potency than sulforaphane *in vitro*. The observation that a similar reduction in excretion of *trans, anti*-PheT was observed as a function of dithiocarbamate elimination adds strong credence to the likelihood of an action of sulforaphane on phase 2 enzymes in study participants. Phenanthrene is a combustion-derived PAH that undergoes metabolism in a manner similar to the prototypic PAH, benzo(*a*)pyrene. GSTs, and UDP-glucuronosyl transferases likely divert phenanthrene metabolism at several steps before tetraol formation, although this hypothesis has not as yet been directly tested. Benzo(*a*) pyrene metabolism to tetraols is affected by conjugating enzymes (48, 49). Thus, excretion patterns of both foodborne and airborne toxicants can be modulated by consumption of an infusion derived from broccoli sprouts. Future studies will be required to define approaches for providing consistency and optimization of the yield and bioavailability of sulforaphane from glucoraphanin-containing beverages. Nonetheless, these results provide an expectation that food-derived chemopreventive agents can be administered in defined, rational, and practical ways to favorably modulate the disposition of unavoidable exposures to environmental carcinogens.

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