Research Article

Cancer Prevention Research

2-Phenethyl Isothiocyanate, *Glutathione S-transferase M1* and *T1* Polymorphisms, and Detoxification of Volatile Organic Carcinogens and Toxicants in Tobacco Smoke

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Abstract

Cigarette smoke contains relatively large quantities of volatile organic toxicants or carcinogens such as benzene, acrolein, and crotonaldehyde. Among their detoxification products are mercapturic acids formed from glutathione conjugation, catalyzed in part by glutathione *S*-transferases (GST). A randomized phase II clinical trial with a crossover design was conducted to evaluate the effect of 2-phenethyl isothiocyanate (PEITC), a natural product formed from gluconasturtiin in certain cruciferous vegetables, on the detoxification of benzene, acrolein, and crotonaldehyde in 82 cigarette smokers. Urinary mercapturic acids of benzene, acrolein, and crotonaldehyde at baseline and during treatment were quantified. Overall, oral PEITC supplementation increased the mercapturic acid formed from benzene by 24.6% ($P = 0.002$) and acrolein by 15.1% ($P = 0.005$), but had no effect on crotonaldehyde. A remarkably stronger effect was observed among subjects with the null genotype of both *GSTM1* and *GSTT1*: in these individuals, PEITC increased the detoxification metabolite of benzene by 95.4% ($P < 0.001$), of acrolein by 32.7% ($P = 0.034$), and of crotonaldehyde by 29.8% ($P = 0.006$). In contrast, PEITC had no effect on these mercapturic acids in smokers possessing both genes. PEITC had no effect on the urinary oxidative stress biomarker 8-*iso*-prostaglandin $F_{2\alpha}$ or the inflammation biomarker prostaglandin E_2 metabolite. This trial demonstrates an important role of PEITC in detoxification of environmental carcinogens and toxicants which also occur in cigarette smoke. The selective effect of PEITC on detoxification in subjects lacking both *GSTM1* and *GSTT1* genes supports the epidemiologic findings of stronger protection by dietary isothiocyanates against the development of lung cancer in such individuals. *Cancer Prev Res; 9(7); 598–606. ©2016 AACR.*

Introduction

Naturally occurring isothiocyanates such as 2-phenethyl isothiocyanate (PEITC) and sulforaphane, which occur abundantly as glucosinolates in certain cruciferous vegetables including watercress and broccoli sprouts, respectively, have multiple effects on xenobiotic metabolizing enzymes and molecular pathways related to cancer (1, 2). Some of these effects, such as inhibition of certain cytochrome P450s involved in the metabolic activation of carcinogens or induction of protective enzymes such as glutathione-*S*-transferases (GST), are associated with protection against carcinogenesis. Indeed, PEITC is a powerful inhibitor of lung carcinogenesis in rats and mice induced by the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) while sulforaphane inhibits mammary and skin carcinogenesis induced by 7,12-dimethylbenz[*a*]anthracene 3–1)). These naturally occurring compounds provide a potentially inexpensive and widely available approach to cancer prevention, and recent clinical studies have generated some encouraging results (4, 5).

We have focused on the potential effects of PEITC as an inhibitor of lung carcinogenesis in cigarette smokers (6). While the best method of lung cancer prevention is clearly prevention of cigarette smoking, there are still 39 million smokers in the United States and 1 billion worldwide (7, 8). Smoking cessation is difficult due to the addictive power of nicotine. Inhibiting the carcinogenic and toxic effects of cigarette smoke is an alternate approach to decrease the enormous death toll from diseases caused by smoking. With this goal in mind, we recently completed a clinical trial of PEITC as an inhibitor of the metabolic activation of NNK in cigarette smokers. The results of that trial did demonstrate a modest but significant reduction of NNK metabolic activation and provided some important insights for further clinical trials of PEITC (5). As there are multiple toxic and carcinogenic agents in cigarette smoke, we have explored the effects of PEITC on several other agents and pathways that could plausibly be modified by this compound.

Thus, in the study presented here, we investigated the effects of PEITC on the metabolism of benzene, acrolein, and

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crotonaldehyde and on oxidative damage and inflammation caused by cigarette smoking. Benzene, acrolein, and crotonaldehyde are three important compounds in cigarette smoke because of their high concentrations in smoke and their established carcinogenic or toxic effects. Air pollution is also a significant, but lesser source of exposure to these three volatile organic compounds (9). Benzene, with a concentration of 6–59 μg per cigarette in mainstream smoke, causes acute myeloid leukemia and acute nonlymphocytic leukemia in humans and is the likely cause of this disease in smokers (10, 11). Acrolein, which is present in smoke at concentrations ranging from 5 to 60 μg per cigarette (12), is a highly toxic compound widely considered as one of the most dangerous in cigarette smoke, and possibly involved in lung cancer etiology in smokers (13, 14). Crotonaldehyde is also highly toxic although weakly carcinogenic (15). Many studies demonstrate that cigarette smoking causes oxidative damage and inflammation, both of which are believed to play a role in the etiology of diseases caused by smoking (16, 17). The 8-*iso*-prostaglandine $F_{2\alpha}$ (8-*iso*-$PGF_{2\alpha}$) and the prostaglandin E2 metabolite (PGEM) are biomarkers of oxidative damage and inflammation, respectively (18, 19).

Glutathione conjugation, non-enzymatically mediated or glutathione *S*-transferases (GSTs) catalyzed, plays an important role in the metabolism of benzene, acrolein, crotonaldehyde, and PEITC. The results of this study showed a remarkable enhancement of benzene detoxification by PEITC in smokers who were null for both *GSTM1* and *GSTT1*; PEITC had more modest effects on the detoxification of acrolein and crotonaldehyde.

Materials and Methods

This study was approved by the Institutional Review Boards of the University of Minnesota (0712M22651) and the University of Pittsburgh (PRO11110669). The study was a randomized, placebo-controlled, double-blind, phase II clinical trial with a crossover study design. Details of the study are described in a separate publication (5). In brief, current smokers of 10–45 cigarettes/day who were 21 years or older and in good health were enrolled in the study. Upon the subject's consent to participate in the trial, he or she was asked to complete a questionnaire that asked about history of tobacco use, medical conditions, and medication use at the initial clinic visit (week 1). A blood sample was collected from each consented participant for hemogram and liver function test as part of the eligibility screening test. All eligible subjects were asked to smoke commercial cigarettes with added [pyridine-D_4]NNK (to allow measurement of NNK metabolic activation) for the entire study course (5). After two weeks of adaptation to smoking these cigarettes, smokers were randomly assigned to either the PEITC then placebo arm (PEITC-placebo group), or the placebo then PEITC arm (placebo-PEITC group) of the trial. During the treatment period, each subject was asked to take PEITC (10 mg in 1 mL olive oil, 4 times/day, once every 4 hours, for five days, week 3 or 5) or the placebo agent (olive oil), on the same schedule (week 3 or 5). There was a one-week washout period between the PEITC and placebo treatments (week 4).

Twenty-four-hour urine samples were collected at the end of the smoking adaptation period (week 2), on three days (3rd, 4th, and 5th day) of each of the two treatment periods (weeks 3 and 5), and at the end of the washout period (week 4). Spot urine samples were collected on the second day of each treatment period. Blood and buccal cell samples were collected at baseline, and the end of the smoking adaptation period, each of the two treatment periods, and the washout period. A diagram of the study design is presented in Supplementary Fig. S1.

Laboratory assays

Genomic DNA was isolated from circulating blood lymphocytes collected at the initial clinic visit (week 1). *GSTM1* and *GSTT1* were genotyped using standard methods (20). Briefly, two separate PCR reactions were used to determine *GSTM1* and *GSTT1* homozygous deletion. Each amplification included an internal PCA control (*CYP1A1* fragment) to ensure null results were attributable to GST gene deletion but not due to PCR failure. PCR products were analyzed by electrophoresis (1.5% agarose) and ethidium bromide staining. The presence or absence of *GSTM1* and *GSTT1* genes was detected by the presence or absence of a band at 480 base-pairs (corresponding to *GSTT1*) and a band at 215 base-pairs (corresponding to *GSTM1*).

We quantified *S*-phenyl mercapturic acid (SPMA), a metabolite of benzene; 3-hydroxypropyl mercapturic acid (HPMA), a metabolite of acrolein; and 3-hydroxy-1-methylpropyl mercapturic acid (HMPMA), a metabolite of crotonaldehyde in all 8 individually collected 24-hour urine samples over the study period per subject. The analyses for the mercapturic acids were carried out essentially as described previously (21, 22). The limits of quantitation were 0.15 pmol/mL for SPMA, 20 pmol/mL for HPMA, and 12 pmol/mL for HMPMA. The inter-day precisions of the assays were 14% relative standard deviation (RSD) for SPMA, 9% RSD for HPMA, and 11% RSD for HMPMA.

8-*iso*-$PGF_{2\alpha}$ and PGEM (as its dehydration product PGAM) were quantified in all 8 individually collected 24-hour urine samples per subject using liquid chromatography/tandem mass spectrometry (LC/MS-MS) as described previously (23, 24). The limits of quantitation were 0.1 pmol/mL for 8-*iso*-$PGF_{2\alpha}$ and PGEM. The inter-day precision figures were 5.5% RSD for 8-*iso*-$PGF_{2\alpha}$ and 9.9% for PGEM. Total nicotine equivalents and total NNAL were quantified using high-throughput liquid chromatography-MS/MS assays described previously (25, 26).

Statistical analysis

The mean values of all biomarkers measured separately in three 24-hour urine samples for each of the two treatment periods, respectively, were calculated and used for statistical analysis. All urinary biomarkers were expressed per milligram of urinary creatinine to account for varying water content of individual urine samples. Given the markedly skewed distributions of the urinary biomarkers, formal statistical testing was performed on logarithmically (natural log) transformed values, and geometric means are presented.

One study participant did not provide a 24-hour urine at baseline (week 2). In addition, we were unable to determine baseline urinary concentrations of acrolein metabolite HPMA on 8 subjects, crotonaldehyde metabolite HMPMA on 6 subjects, and oxidative biomarker 8-*iso*-$PGF_{2\alpha}$ on 3 subjects due to poor chromatography. To maximize the sample size, we retained these subjects in the analysis for baseline measurements by using a statistical model to estimate these missing values. For baseline HPMA and HMPMA, the model included age, sex, race, body mass index, all individual metabolites of nicotine, total NNAL, SPMA, PGEM and 8-*iso*-$PGF_{2\alpha}$ at baseline and during the placebo period as well as HPMA and HMPMA during the placebo period only. We used the same model with 8-*iso*-$PGF_{2\alpha}$ replaced by HPMA and

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HMPMA at baseline to estimate baseline 8-*iso*-$PGF_{2\alpha}$. Both models performed very well. The correlation coefficient between the predicted and measured pairs of values was 0.90 for HPMA, 0.94 for HMPMA, and 0.89 for 8-*iso*-$PGF_{2\alpha}$.

The two-group *t* test (for continuous variables) or χ^2 statistics (for discrete or nominal variables) were used to compare the differences in the distributions of demographic characteristics and urinary metabolites of nicotine and NNK between two treatment sequence assignments. The ANOVA method was used to examine the differences in urinary biomarkers at baseline (week 2) between different groups defined by demographic characteristics of study participants such as age, sex, race, level of education, body mass index, number of cigarettes smoked per day, alcohol intake, and genotypes of *GSTM1* and *GSTT1*.

This study was a randomized crossover trial, a type of longitudinal study in which participants were randomly assigned to receive a sequence of treatment with PEITC or placebo. We used the linear mixed model with random effect to simultaneously examine the effect of treatment (PEITC vs. placebo), study period (period 1 vs. period 2), treatment sequence (the carryover effect), and their interaction with the urinary biomarkers in all subjects as well as in subgroup analyses stratified by *GSTM1* and/or *GSTT1* genotypes. An interaction term between PEITC treatment and *GSTM1* and/or *GSTT1* genotypes was included in the linear mixed models to evaluate the potential modifying role of the *GST* genotypes on PEITC's effect on the urinary biomarkers. Because the statistical analyses for all urinary biomarkers were done on the log-transformed variables, the difference of log-transformed means after back-transformation is presented as the percentage change, the equivalents of the ratio of the least-squared means on the original scale.

Statistical analyses were carried out using SAS software version 9.3 (SAS Institute). All *P* values reported are two-sided, and those that were less than 0.05 were considered to be statistically significant.

Results

Characteristics of the study subjects are summarized in Table 1. There were no statistically significant differences in distributions of age, gender, race, level of education, smoking history (smoking intensity, duration, and age when beginning to smoke), and alcohol consumption between the two groups of randomly assigned individuals, that is, the PEITC-Placebo and Placebo-PEITC groups. Overall, the mean age (SD) was 41.0 (10.0) years. Among the 82 study participants, 46% were women, 67% were

Table 1. Distributions of demographic and lifestyle factors, and urinary biomarkers at baseline by the treatment sequence assignment, The PEITC Intervention Study 2008–2013

	Treatment sequence assignment		
Characteristics or biomarkers	**PEITC-Placebo**	**Placebo-PEITC**	***P***
Number of subjects	41	41	
Age (years), mean (SD)	40.9 (10.6)	41.1 (9.6)	0.939
Body mass index (kg/m^2)[a], mean (SD)	28.0 (4.8)	28.0 (6.3)	0.957
Gender, *n* (%)			0.376
Male	24 (59)	20 (49)	
Female	17 (41)	21 (51)	
Race, *n* (%)			0.839
African American	8 (20)	10 (24)	
Caucasian American	28 (68)	27 (66)	
Others	5 (12)	4 (10)	
Level of education, *n* (%)			0.179
High school or lower	14 (34)	20 (49)	
College or higher	27 (66)	21 (51)	
Cigarette smoking, mean (SD)			
Cigarettes/day	19.3 (6.6)	19.0 (6.7)	0.843
Years of smoking	14.7 (10.3)	17.9 (9.4)	0.157
Age at starting smoking (year)	15.2 (4.9)	15.1 (4.6)	0.963
Age when became regular smokers (year)	18.6 (7.1)	17.7 (5.2)	0.514
Alcohol drinking, *n* (%)[a]			0.784
Never	14 (35)	17 (42)	
Monthly or less	14 (35)	12 (30)	
Weekly	12 (30)	11 (28)	
GSTM1 and *GSTT1* genotypes, *n* (%)			0.585
Present and present	20 (49)	18 (44)	
Present and null	3 (7)	4 (10)	
Null and Present	14 (34)	11 (27)	
Null and null	4 (10)	8 (19)	
Urinary biomarkers, geometric mean (95% CI)[b]			
Total nicotine, ng/mg Cr	2,114 (1,670–2,676)	3,015 (2,389–3,806)	0.039
Total cotinine, ng/mg Cr	2,839 (2,368–3,402)	3,371 (2,819–4,031)	0.190
Total 3′-hydroxycotinine, ng/mg Cr	6,638 (5,355–8227)	6,993 (5,657–8,646)	0.736
Total nicotine equivalents, nmol/mg Cr	45.1 (37.6–54.0)	58.1 (48.6–69.5)	0.054
Benzene SPMA (pmol/mg creatinine)	2.36 (1.68–3.28)	2.38 (1.72–3.30)	0.955
Acrolein HPMA (nmol/mg creatinine)	6.16 (5.14–7.38)	7.04 (5.90–8.42)	0.300
Crotonaldehyde HMPMA (nmol/mg creatinine)	4.22 (3.46–5.12)	5.04 (4.14–6.12)	0.207
PGEM (pmol/mg creatinine)	46.66 (38.68–56.26)	42.68 (35.48–51.34)	0.509
8-*iso*-$PGF_{2\alpha}$ (pmol/mg creatinine)	0.90 (0.76–1.04)	0.78 (0.68–0.90)	0.233

[a]Two subjects were excluded from this analysis due to missing data.

[b]One subject who did not provide a urine sample at baseline was excluded from the analyses.

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whites, and 22% were African Americans. On average, study subjects smoked 19.1 (6.6) cigarettes per day and smoked cigarettes for 16.3 (9.8) years. There were 45.1% of study participants with the *GSTM1*-null genotype, 23.2% with the *GSTT1*-null genotype, and 14.6% with null genotype of both those genes. The baseline urinary level of total nicotine was slightly higher in smokers who were randomly assigned to the Placebo-PEITC group than those assigned to the PEITC-Placebo group. All other measures in urine at baseline were comparable between the two treatment assignment groups (Table 1).

Among biomarkers measured, total NNAL and metabolites of nicotine were highly correlated with metabolites of acrolein (HPMA) and crotonaldehyde (HMPMA), and moderately correlated with the benzene metabolite (SPMA) and with 8-*iso*-$PGF_{2\alpha}$. PGEM was not correlated with any measured urinary biomarker except for nicotine, and this was moderate ($r = 0.25$; $P = 0.023$; Supplementary Table S1).

Women had significantly higher baseline urinary mercapturic acid metabolites of acrolein and crotonaldehyde, and oxidative stress biomarker 8-*iso*-$PGF_{2\alpha}$, but lower level of the inflammatory biomarker PGEM than men (Table 2). Urinary levels of 8-*iso*-$PGF_{2\alpha}$ and metabolites of acrolein and crotonaldehyde were significantly higher in older ($\geq$ 40 years) than younger subjects. There was no statistically significant difference in urinary levels of all measured biomarkers between subjects of different race/ethnicity, body mass index, alcohol consumption, and number of cigarettes per day, except for lower PGEM in smokers of 20 or more cigarettes per day than in those who smoked less than 20 cigarettes per day.

GSTT1 and *GSTM1* genotype significantly affected the concentration of SPMA in smokers' urine (Table 3). Smokers who carry both copies of both *GSTTM1* and *GSTT1* gene excreted the highest levels of SPMA (3.56 pmol/mg creatinine), whereas those who were null for both genes excreted 8-fold lower levels (0.44 pmol/mg creatinine). Overall, the urinary levels of SPMA were about 80% and 315% higher in individuals possessing the *GSTM1* or the *GSTT1* gene, respectively, compared with their counterparts lacking the respective gene. The relation between urinary SPMA and the number of genes present was dose-dependent ($P_{trend} < 0.001$). Furthermore, among individuals lacking the *GSTT1* gene, the presence of the *GSTM1* gene was associated with more than 4-fold higher SPMA than the absence of *GSTM1* (1.94 vs. 0.44 pmol/mg creatinine; $P < 0.001$). However, among individuals possessing the *GSTT1* gene, the urinary levels of SPMA in smokers possessing and lacking the *GSTM1* gene were comparable ($P = 0.571$). The *GST* genotypes had no impact on urinary levels of acrolein and crotonaldehyde metabolites, PGEM and 8-*iso*-$PGF_{2\alpha}$ at baseline.

Intake of PEITC significantly increased urinary excretion of the benzene metabolite SPMA by 24.6% [95% confidence interval (CI), 9.3%–42.1%; $P = 0.002$] and the acrolein metabolite HPMA by 15.1% (95% CI, 4.5%–26.7%; $P = 0.005$), but did not have a statistically significant effect on urinary levels of the crotonaldehyde metabolite HMPMA, the inflammation biomarker PGEM, or

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Table 2. Geometric means (95% CIs) of baseline urinary benzene, acrolein, and crotonaldehyde mercapturic acids, prostaglandin E_2 metabolite (PGEM), and 8-*iso*-prostaglandin $F_{2\alpha}$ (8-*iso*-$PGF_{2\alpha}$) in smokers stratified by demographic and lifestyle factors, The PEITC Intervention Study 2008–2013

	N^a	Benzene SPMA (pmol/mg Cr)	Acrolein HPMA (nmol/mg Cr)	Crotonaldehye HMPMA (nmol/mg Cr)	PGEM (pmol/mg Cr)	8-*iso*-$PGF_{2\alpha}$ (pmol/mg Cr)
All subjects	81	2.36 (1.88–2.98)	6.60 (5.80–7.48)	4.62 (4.02–5.30)	44.60 (39.12–50.84)	0.84 (0.74–0.92)
Gender						
Male	43	2.18 (1.58–3.00)	5.78 (4.88–6.84)	3.94 (3.28–4.74)	54.62 (46.14–64.66)	0.72 (0.62–0.84)
Female	38	2.60 (1.86–3.64)	7.66 (6.40–9.18)	5.52 (4.52–6.70)	35.46 (29.62–42.42)	0.98 (0.84–1.14)
P		0.462	0.028	0.018	<0.001	0.006
Race						
Caucasians	54	2.48 (1.86–3.30)	6.94 (5.94–8.10)	5.02 (4.24–5.94)	41.24 (35.18–48.32)	0.90 (0.78–1.02)
Non-Caucasians	27	2.16 (1.44–3.24)	5.96 (4.78–7.40)	3.90 (3.08–4.94)	52.16 (41.68–65.30)	0.72 (0.60–0.86)
P		0.590	0.266	0.091	0.098	0.063
Age (years)						
<40	33	2.22 (1.54–3.20)	5.10 (4.24–6.14)	3.66 (2.98–4.50)	41.78 (34.00–51.32)	0.72 (0.62–0.86)
≥40	48	2.46 (1.82–3.34)	7.86 (6.74–9.18)	5.40 (4.54–6.42)	46.64 (39.32–55.32)	0.92 (0.80–1.06)
P		0.673	0.001	0.006	0.422	0.039
Level of education						
High school or lower	33	2.92 (2.04–4.18)	6.92 (5.68–8.44)	4.62 (3.72–5.74)	46.66 (37.96–57.34)	0.84 (0.70–1.00)
College or higher	48	2.04 (1.52–2.76)	6.38 (5.40–7.52)	4.60 (3.84–5.52)	43.24 (36.44–51.30)	0.84 (0.72–0.96)
P		0.144	0.533	0.985	0.579	0.973
Body mass index (kg/m^2)[b]						
<25	26	2.26 (1.48–3.42)	7.24 (5.46–9.08)	5.22 (4.08–6.68)	43.38 (34.30–54.88)	0.78 (0.64–0.94)
25–≤30	26	2.62 (1.72–3.98)	6.22 (4.96–7.82)	3.94 (3.08–5.04)	48.72 (38.52–61.62)	0.80 (0.66–0.96)
≥30	27	2.36 (1.56–3.56)	6.40 (5.12–8.00)	4.80 (3.76–6.12)	41.48 (32.94–52.26)	0.96 (0.80–1.16)
P_{trend}		0.880	0.451	0.644	0.783	0.120
Number of cigarettes per day						
<20	36	2.20 (1.56–3.12)	6.20 (5.12–7.50)	4.28 (3.48–5.26)	51.76 (42.68–62.76)	0.80 (0.68–0.94)
≥20	45	2.50 (1.84–3.42)	6.92 (5.84–8.22)	4.90 (4.06–5.90)	39.58 (33.32–47.04)	0.86 (0.74–1.00)
P		0.585	0.396	0.342	0.046	0.444
Alcohol intake[b]						
Never	30	2.46 (1.68–3.60)	6.74 (5.48–8.30)	4.94 (3.92–6.20)	47.12 (38.06–58.36)	0.78 (0.66–0.92)
Monthly or less	26	2.34 (1.56–3.54)	7.38 (5.90–9.22)	4.68 (3.66–6.00)	42.28 (33.60–53.20)	0.92 (0.76–1.12)
Weekly	23	2.26 (1.46–3.48)	5.94 (4.70–7.54)	4.36 (3.36–5.66)	46.90 (36.74–59.86)	0.82 (0.66–1.00)
P_{trend}		0.763	0.491	0.482	0.932	0.674

[a]One subject who did not provide a urine sample at baseline was excluded from all analyses.
[b]Two additional subjects were excluded from this analysis due to their missing data on body mass index.

Table 3. Geometric means (95% confidence intervals) of baseline urinary benzene, acrolein and crotonaldehyde mercapturic acids, prostaglandin E_2 metabolite (PGEM), and 8-*iso*-prostaglandin $F_{2\alpha}$ (8-*iso*-$PGF_{2\alpha}$) in smokers separated by *glutathione S-transferase* (*GST*) *M1* and *T1* genotypes, The PEITC Intervention Study 2008–2013

GST genotype	N^a	Benzene SPMA (pmol/mg Cr)[b]	Acrolein HPMA (nmol/mg Cr)[b]	Crotonaldehye HMPMA (nmol/mg Cr)[b]	PGEM (pmol/mg Cr)[b]	8-*iso*-$PGF_{2\alpha}$ (pmol/mg Cr)[b]
GSTM1						
Null	36	1.72 (1.22–2.40)	7.12 (5.98–8.48)	5.16 (4.22–6.28)	41.18 (34.22–49.58)	0.88 (0.76–1.02)
Present	45	3.10 (2.28–4.20)	6.28 (5.36–7.36)	4.28 (3.58–5.12)	46.42 (39.26–54.88)	0.82 (0.70–0.94)
P		0.014	0.309	0.186	0.357	0.442
GSTT1						
Null	19	0.80 (0.52–1.18)	7.22 (5.64–9.22)	5.26 (3.98–6.94)	45.32 (34.90–58.86)	0.78 (0.64–0.98)
Present	62	3.34 (2.68–4.18)	6.48 (5.66–7.40)	4.48 (3.86–5.22)	43.58 (37.78–50.26)	0.86 (0.76–0.96)
P		<0.001	0.459	0.338	0.799	0.495
GSTM1 and *GSTT1*						
Both null	12	0.44 (0.28–0.74)	8.58 (6.26–11.74)	5.76 (4.02–8.26)	43.18 (30.76–60.62)	0.86 (0.64–1.14)
One present	31	2.84 (2.12–3.82)	6.28 (5.22–7.58)	4.82 (3.88–5.96)	42.06 (34.38–51.48)	0.84 (0.72–1.00)
Both present	38	3.56 (2.74–4.66)	6.40 (5.40–7.58)	4.22 (3.48–5.12)	45.88 (38.20–55.12)	0.84 (0.72–0.98)
P_{trend}		<0.001	0.244	0.130	0.617	0.891

[a]One subject who did not provide urine sample at baseline was excluded from all analyses.
[b]All geometric means were adjusted for age and gender.

the oxidative biomarker 8-*iso*-$PGF_{2\alpha}$ (Table 4). The *GST* genotypes significantly modified the effect of PEITC on the detoxification of benzene (Table 5). Among smokers lacking the *GSTT1* gene, PEITC increased the urinary benzene metabolite SPMA by 74.2% (95% CI, 33.9%–126.6%; $P < 0.001$) compared with the placebo. Similarly among smokers lacking the *GSTM1* gene, intake of PEITC increased SPMA by 43.1% (95% CI, 18.1%–73.4%; $P < 0.001$) compared with the placebo. When subjects were separated by the presence or absence of both *GSTM1* and *GSTT1* genes, compared with the placebo controls, intake of PEITC increased SPMA by 95.4% (95% CI, 40.7%–171.5%; $P < 0.001$) in smokers lacking both the *GSTM1* and *GSTT1* genes, and by 27.4% (95% CI, 4.3%–55.6%; $P = 0.020$) in smokers who carried either *GSTM1* or *GSTT1* gene, but had no effect in those subjects possessing both genes ($P_{interaction} = 0.009$; Table 5). The level of SPMA excreted after PEITC consumption by individuals who were null for both *GSTT1* and *GSTM1* (1.45 pmol/mg creatinine) was still significantly lower than the level in smokers who carried both genes without PEITC treatment (3.84 pmol/mg creatinine) or with PEITC treatment (4.07 pmol/mg creatinine; Table 5).

Although PEITC did not have a statistically significant effect on the urinary level of the crotonaldehyde metabolite HMPMA in all subjects (Table 4), when the subjects were separated by *GST* genotypes, intake of PEITC significantly increased urinary HMPMA by 15.4% ($P = 0.009$) in subjects lacking the *GSTM1* gene alone, by 22.0% ($P = 0.010$) in those lacking the *GSTT1* gene alone, and by 29.8% ($P = 0.006$) in those subjects lacking both these genes, but had no such effect in subjects possessing one or both genes (all $P_{interaction} < 0.05$; Table 5). PEITC intake had a slightly greater effect on the increase in the urinary acrolein metabolite HPMA for individuals lacking one or both *GST* genes than those possessing the gene. However, the difference was not statistically significant (Table 5). The deletion polymorphism of *GSTM1* and *GSTT1* genes had no effect on PEITC's modulation of urinary levels of PGEM and 8-*iso*-$PGF_{2\alpha}$ (data not shown).

We previously examined the effects of *GSTM1* and *GSTT1* status on urinary PEITC-NAC and total isothiocyanates in this study and the results are summarized in Supplementary Table S2, which has been published previously (5) and is included here because of its relevance to the results. There were modest but significant increases in levels of total isothiocyanates in the *GSTM1*-null groups compared with the non-null, but no significant effects on PEITC-NAC.

Discussion

The mercapturic acid metabolites SPMA, HPMA, and HMPMA measured here are detoxification products of benzene, acrolein, and crotonaldehyde, respectively. Glutathione conjugation, whether non-enzymatically mediated or GSTs catalyzed, plays an important role in the metabolism of benzene, acrolein, and crotonaldehyde. The results of this clinical trial clearly demonstrate that intake of PEITC, up to 40 mg per day by cigarette smokers, enhances the detoxification of benzene and acrolein by increasing formation of their glutathione conjugates, as measured by the corresponding urinary mercapturic acid metabolites. The increase in the mean urinary concentration of the mercapturic acids was driven by the significant increases in smokers who were null for *GSTM1* or *GSTT1*, or both genes. Compared with controls, excretion of SPMA was nearly doubled in the double null individuals who consumed PEITC, while levels of HPMA from acrolein and HMPMA from crotonaldehyde increased by 32.7% and 29.8%, respectively. These results support the further

Table 4. The changes of urinary concentrations of benzene, acrolein and crotonaldehyde mercapturic acids, prostaglandin E_2 metabolite (PGEM), and 8-*iso*-prostaglandin $F_{2\alpha}$ (8-*iso*-$PGF_{2\alpha}$) in smokers before and after intake of PEITC, The PEITC Intervention Study 2008–2013

	Geometric means			
Urinary biomarkers	Placebo ($n = 82$)	PEITC ($n = 82$)	% Difference (95% CI)	P^a
Benzene SPMA (pmol/mg creatinine)	2.60	3.25	24.6 (9.3–42.1)	0.002
Acrolein HPMA (nmol/mg creatinine)	7.34	8.45	15.1 (4.5–26.7)	0.005
Crotonaldehyde HMPMA (nmol/mg creatinine)	5.16	5.44	5.5 (−1.8–13.4)	0.148
PGEM (pmol/mg creatinine)	49.3	50.9	3.3 (−4.9–12.3)	0.444
8-*iso*-$PGF_{2\alpha}$ (pmol/mg creatinine)	0.86	0.87	1.3 (−4.9–8.0)	0.684

[a]Two-sided *P* values were derived from the mixed models that test the PEITC treatment effect.

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Table 5. The effect of PEITC on changes of urinary concentrations of merpcaturic acid metabolites of benzene, acrolein and crotonaldehyde in smokers stratified by the *glutathione S-transferase (GST) M1* and *T1* genotype, The PEITC Intervention Study 2008–2013

GST genotype	*N*	Geometric mean Placebo	Geometric mean PEITC	% difference (95% CI)	P^a	$P_{interaction}$
GSTM1			Benzene SPMA (pmol/mg creatinine)			
Null	37	2.09	2.99	43.1 (18.1–73.4)	<0.001	0.060
Present	45	3.12	3.47	11.2 (−6.5–32.4)	0.234	
GSTT1						
Null	19	0.83	1.45	74.2 (33.9–126.6)	<0.001	0.006
Present	63	3.67	4.14	12.7 (−2.4–30.4)	0.108	
GSTM1 and *GSTT1*						
Both null	12	0.74	1.45	95.4 (40.7–171.5)	<0.001	0.009
One present	32	2.64	3.36	27.4 (4.3–55.6)	0.020	
Both present	38	3.84	4.07	6.1 (−11.6–27.5)	0.526	
GSTM1			Acrolein HPMA (nmol/mg creatinine)			
Null	37	8.07	10.05	24.6 (7.9–43.8)	0.005	0.179
Present	45	6.79	7.37	8.6 (−4.3–23.2)	0.210	
GSTT1						
Null	19	8.00	10.12	26.6 (5.4–52.0)	0.022	0.320
Present	63	7.15	8.00	12.0 (−0.1–25.6)	0.057	
GSTM1 and *GSTT1*						
Both null	12	9.84	13.06	32.7 (5.9–66.2)	0.034	0.315
One present	32	6.90	8.28	20.0 (1.8–41.3)	0.038	
Both present	38	7.04	7.53	7.0 (−6.9–22.9)	0.347	
GSTM1			Crotonaldehyde HMPMA (nmol/mg creatinine)			
Null	37	5.89	6.80	15.4 (3.9–28.1)	0.009	0.027
Present	45	4.62	4.53	−1.9 (−10.8–7.8)	0.686	
GSTT1						
Null	19	5.34	6.52	22.0 (5.3–41.4)	0.010	0.031
Present	63	5.10	5.15	1.0 (−6.8–9.5)	0.808	
GSTM1 and GSTT1						
Both null	12	6.19	8.04	29.8 (8.2–55.8)	0.006	0.017
One present	32	5.36	5.85	9.2 (−2.3–22.0)	0.124	
Both present	38	4.71	4.53	−4.0 (−13.3–6.3)	0.438	

[a]Two-sided *P* values were derived from the mixed models that test PEITC treatment effect on the change of urinary levels of biomarkers within each specific *GST* genotypes before and after PEITC intake.
[b]Two-sided *P* values were derived from the mixed models that test the interaction term between PEITC intake and *GST* genotype on the levels of urinary biomarkers.

development of PEITC as a chemopreventive agent to counteract some of the carcinogenic and toxic effects of cigarette smoking.

We were able to observe the marked increase in SPMA in the *GSTM1* and *GSTT1*-null individuals as a consequence of the low basal levels of SPMA in these subjects. We and others have previously shown that *GSTT1*-null status has a strong effect on levels of SPMA (22, 27), and that was also observed in this study, where levels of SPMA were significantly lower in *GSTT1*-null individuals than in non-nulls (Table 3). The 95.4% increase in SPMA in these individuals may be due to PEITC effects on GSTP1, which is a good catalyst of SPMA formation from benzene oxide (28). GSTP1 is also an excellent catalyst of acrolein and crotonaldehyde conjugation with glutathione (29) and induction of this enzyme may contribute to the effects of PEITC on the urinary levels of HPMA and HMPMA. However, a study carried out in human hepatocytes did not find induction of GSTP1 by PEITC, but did note a strong induction of NAD(P)H:quinone oxidoreductase (NQO; ref. 30). NQO1 participates in the detoxification of benzoquinones (31), and may have an indirect effect on the pathways leading to the production of SPMA. Thus, the mechanism of PEITC on modulation of benzene metabolism whether through the regulation of NQO1 and/or GSTP1 may be complex (32). Furthermore, future studies may be warranted to examine the effect of PEITC on the formation of DNA adducts by benzene and other volatile organic compounds.

While the deletion polymorphism of *GSTT1* is known to diminish SPMA urinary excretion in individuals with occupational or environmental exposures, the effect of *GSTM1* on urinary excretion of SPMA is far less certain. In the current study, we found that *GSTM1* genotype may play a role in the metabolism and excretion of benzene conditional on the presence or absence of the *GSTT1* gene. Among subjects null for the *GSTT1* gene, the levels of urinary SPMA were more than four-fold greater in subjects possessing *GSTM1* than in subjects lacking *GSTM1*. However, the presence of the *GSTM1* gene did not have a significant impact on the urinary level of SPMA among subjects possessing *GSTT1*. Overall there was an 8-fold difference in urinary SPMA at baseline between subjects who had both or neither *GSTM1* and *GSTT1* genes. The deletion polymorphism of *GSTM1* and *GSTT1* genes is associated with increased risk of many malignancies including lung cancer (33), particularly in smokers (34). The current study demonstrates that smokers lacking both *GSTM1* and *GSTT1* genes may derive the most benefit from intake of PEITC due to their increased detoxification of carcinogens and toxicants.

The findings of our clinical trial are consistent with those of a recent randomized clinical trial, which demonstrated that intake of a broccoli sprout beverage containing high levels of sulforaphane (40 μmol/day) and its precursor glucoraphanin (600 μmol/day) induced a rapid and sustained increase in the urinary excretion of the mercapturic acids of benzene and acrolein. The subjects of the broccoli sprout trial were mostly nonsmokers with exposures to substantial levels of airborne pollutants in China (4). Although the two clinical trials used

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different dietary isothiocyanates (PEITC vs. sulforaphane) for treatment, they produced very similar results, which generalize the detoxification effect of dietary isothiocyanates on environmental carcinogens and toxicants present in both tobacco smoke and the general environment. However, the clinical trial in China did not find a statistically significant modifying effect of *GSTT1* or *GSTM1* genotype on the effect of sulforaphane on urinary excretion of SPMA. This may be due to the much lower benzene exposure experienced by this 89% nonsmoking population, whereas all of our study participants were current smokers with high exposures to benzene and other volatile compounds (2- to 3-fold higher levels of all mercapturic acid metabolites). Limited data showed that exposure to cigarette smoke significantly altered hepatic and pulmonary GST activities in male rats that were differentially influenced by the age of rats and the tar contents of cigarette smoke (35, 36). Compared with nonsmokers, gene expression of *GSTP1* and *GSTA2* was significantly elevated in the lungs of smokers (37). However the impact of cigarette smoking on the metabolism of benzene, acrolein, and crotonaldehyde is unknown. Alternatively, the lack of an effect of *GSTT1* or *GSTM1* genotype on SPMA levels in the Chinese study may be due to the different isothiocyanates administered, the study populations or the study design. The China study was a randomized study design with two parallel groups of study participants—one receiving a broccoli sprout beverage and the other a placebo beverage as controls. The current study was a crossover study design, and therefore used the same study participants as controls for determining the effects of PEITC treatment, eliminating potential confounding effects due to interindividual differences such as genetic background, lifestyle, and other unmeasured characteristics.

Benzene is considered a cause of various types of leukemia in humans (10, 11). In mice, benzene also causes tumors at multiple sites including the lung when administered by gavage, and in some cases, by inhalation (38, 39). Occupational studies found increased risks of mortality from hematopoietic malignancies and sometimes lung cancer in workers who were exposed to benzene in the workplace (40, 41). Acrolein is toxic to the cilia of the lung and is an intense irritant for the skin, eyes, and nasal passages (42). Acrolein–DNA adducts are present in the human lung (43). In addition, acrolein reacts with the *p53* gene at hot spots associated with lung cancer, leading some to propose that it is important in lung cancer etiology in smokers (13). Acrolein and crotonaldehyde are products of lipid peroxidation and may be involved in inflammation, and both are recognized as extremely hazardous substances (42, 44). The current study demonstrates that intake of PEITC enhances the urinary excretion of benzene, acrolein, and crotonaldehyde detoxification metabolites. Thus, supplementation of PEITC could be an efficient and cost-effective way to reduce the harmful effects of exposure to these toxicants and carcinogens.

Epidemiologic studies from different parts of the world provide strong evidence for reduced risk of several cancers including cancers of the lung, stomach, and colon associated with high intake of cruciferous vegetables or isothiocyanates (45). A meta-analysis including more than 8,000 lung cancer cases and 684,000 noncancer subjects demonstrated an overall 20% lower risk of lung cancer associated with high intake of cruciferous vegetables; the risk reduction was stronger in subjects with null genotype of *GSTM1* or *GSTT1*, particularly in those with null genotype of both genes (OR = 0.41; 95% CI, 0.20–0.68; ref. 46). Within the Shanghai Cohort Study, we previously found that the urinary concentration of total isothiocyanates was associated with a significant 35% reduced risk of developing lung cancer, the protective effect was greater in subjects lacking either *GSTM1* or *GSTT1* gene, and greatest in those lacking both genes (OR = 0.28; 95% CI, 0.13–0.57; ref. 47). In contrast, urinary total isothiocyanates levels were not associated with the risk of lung cancer in subjects who possessed both *GSTM1* and *GSTT1* genes (OR = 1.04; 95% CI, 0.60–1.67). The difference in the isothiocyanate–lung cancer risk association between the two genotype groups was statistically significant. Similar results were found for colorectal cancer (48).

In our initial report (47), we speculated that the stronger chemopreventive effect of isothiocyanates in *GSTM1* and *GSTT1*-null individuals was the result of decreased formation of isothiocyanate glutathione conjugates in these individuals and hence increased levels of free PEITC available to exert a protective effect. Our hypothesis was supported by observational studies reporting that among subjects who consumed similar amounts of cruciferous vegetables, urinary levels of total isothiocyanates were higher in those possessing the *GSTM1* and/or *GSTT1* gene compared with those who carried neither of these genes (48, 49). This was not confirmed by previous feeding studies (50, 51) or the current study, in which we found little effect of *GSTT1* or *GSTM1* status on the urinary levels of PEITC-NAC in the individuals treated with PEITC (Supplementary Table S2). Therefore, *GSTM1* and *GSTT1* status may have little or no effect on circulating levels of PEITC; the level of which in double null individuals are likely from both the nonenzymatic reaction of PEITC with glutathione as well as catalysis of this reaction by *GSTP1*, which has similar catalytic efficiency to *GSTM1* (52). The significant effect of PEITC on the formation of mercapturic acids derived from benzene, acrolein, and crotonaldehyde in the *GSTM1* and *GSTT1*-null individuals but not in non-nulls reported here suggest a different mechanism for chemoprevention by isothiocyanates: that isothiocyanate consumption results in a significant increase in toxicant detoxification in the *GSTM1* and *GSTT1*-null individuals, but not in individuals who carry two functional alleles for both of these genes. PEITC, like sulforaphane, is expected to induce detoxification via the Nrf2 pathway (53, 54). However, previous studies have shown that chemopreventive agents such as ethoxyquin and butylated hydroxyanisole can actually attenuate the Nrf2 response; therefore, other pathways of induction may be involved (32).

In experimental studies in animals, isothiocyanates derived from a variety of cruciferous vegetables have been identified as inducers of GSTs (55). In feeding studies in humans, intake of the cruciferous plant Brussels sprouts significantly increased the plasma GST-α (56). Furthermore, intake of a diet consisting of high quantities of mixed cruciferous vegetables elevated serum GST-α only in subjects lacking the *GSTM1* gene (this study was unable to examine the *GSTT1* gene separately due to the limited number of study subjects; ref. 57). These findings of an inducing effect of cruciferous vegetables on GSTs support the observation of the current study with a relatively stronger effect of PEITC on the detoxification of carcinogens and toxicants in subjects lacking the *GSTT1* or *GSTM1* gene, and suggest that future studies of watercress consumption may be warranted.

In summary, intake of PEITC has a significant effect on the detoxification of the environmental carcinogens and toxicants benzene, acrolein, and crotonaldehyde. This effect is strikingly large in subjects lacking both the *GSTM1* and *GSTT1* genes, in fact would not have been observed in the absence of genotyping.

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These data support the epidemiologic findings of stronger protection of dietary isothiocyanates against the development of several cancers in this subset of individuals. Intake of cruciferous vegetables with high concentrations of isothiocyanate precursors (glucosinolates) or supplementation with specific isothiocyanates such as PEITC and sulforaphane may be an efficient and cost-effective strategy for cancer prevention, particularly for individuals with exposure to high levels of environmental and tobacco carcinogens and toxicants who lack the *GSTM1* and *GSTT1* genes. The findings of the current study provide supporting evidence for the development of a personalized strategy for cancer chemoprevention related to cruciferous vegetables and their bioactive constituents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed by the authors.

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