

Safety and Efficacy of Weekly Oral Oltipraz in Chronic Smokers

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Abstract

Cigarette smoking is thought to contribute to carcinogenesis by formation of DNA adducts of tobacco smoke constituents leading to genotoxic damage. The dithiolethione, oltipraz, is a putative cancer chemopreventive agent that induces phase II detoxifying enzymes in preclinical models and reduces aflatoxin adducts in humans living in areas with high dietary levels. To determine if oltipraz could reduce adduct levels of tobacco smoke constituents in the lungs and other target organs, chronic smokers were enrolled to one of three arms: 400 or 200 mg/wk oral oltipraz or placebo. Endobronchial tissue and bronchoalveolar lavage were done before and after 12 weeks of drug treatment; peripheral blood, urine, and oral saline rinse were also collected. Toxicity was assessed every 4 weeks. Fifty-nine of the 77 enrolled subjects completed the study. Of those receiving oltipraz, 15% experienced grade 2/3 toxicity, which was predominantly gastrointestinal. All

subject withdrawals occurred in the oltipraz groups. There was no significant difference between pre- and post-polycyclic aromatic hydrocarbon-DNA adduct levels in lung epithelial cells measured by immunoperoxidase staining between treatment and placebo groups. Likewise, no significant differences were found in polycyclic aromatic hydrocarbon or benzo(*a*)pyrene-7,8-diol-9,10-epoxide adducts measured in blood, oral lining cells, or bladder lining cells. There was also no increase in mRNA or enzymatic activity of phase II enzymes and no change in glutathione levels. Thus, despite moderate drug-related toxicity, there was no significant effect on pharmacodynamic or surrogate risk biomarkers. Other agents with lower toxicity and greater activity to induce phase II enzymes are needed to definitively test the detoxification-induction paradigm in smokers. (Cancer Epidemiol Biomarkers Prev 2005;14(4):892–9)

Introduction

Lung cancer is the leading cause of cancer-related mortality in the United States and is a major cause of death worldwide (1). Treatment strategies for established lung cancer are of limited efficacy (2). Although several early detection strategies have been tested, none has yet shown an ability to reduce lung cancer-specific mortality among those undergoing screening (3). Cigarette smoking, the primary cause of lung cancer, is highly addictive. The majority of people who smoke are unable to remain abstinent, with a typical median time to failure of only 7 days in smoking intervention studies (4). Thus, strategies are needed to modulate the carcinogenic effect of cigarette smoking in those who are unable to quit.

Tobacco smoke contains a large number of carcinogens, including polycyclic aromatic hydrocarbons (PAH). Benzo(*a*)pyrene is a prototypical PAH (5). Benzo(*a*)pyrene is activated by cytochrome *P*450 enzymes to a highly reactive species, benzo(*a*)pyrene-7,8-diol-9,10-epoxide (BPDE). BPDE can react with guanine residues in DNA, thereby forming a stable

DNA adduct. Alternatively, BPDE can be detoxified by conjugation to glutathione, a reaction catalyzed by glutathione *S*-transferases (GST). Several studies suggest that DNA adducts, including BPDE-DNA, are associated with the development of lung cancer (6). Therefore, one strategy to reduce lung cancer risk among smokers is to decrease DNA adduct levels (7).

Oltipraz [5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione; NSC 347901] is a dithiolethione originally developed as an antischistosomal agent. In a variety of preclinical models, oltipraz treatment was associated with induction of phase II detoxification enzymes as well as with chemopreventive activity. Specifically, oltipraz has been shown to induce hepatic GSTs, increase glutathione pools, and induce hepatic UDP-glucuronosyl transferases in rodent models. In a cell-free microsome-based system, oltipraz decreased benzo(*a*)pyrene-induced DNA adduct formation (8). In rats, oltipraz decreased DNA adduct formation after aflatoxin exposure (9) as well as after exposure to tobacco smoke (10). In addition, oltipraz decreased the transforming ability of human fetal lung explant DNA exposed to tobacco smoke condensate, suggesting that it protects against tobacco smoke carcinogen-induced mutagenesis (11). In chemical carcinogenesis-based models in rodents, oltipraz has been shown to protect against a variety of cancers, including lung, trachea, forestomach, small intestine, colon, breast, skin, liver, and urinary bladder (12). Taken together, these studies show that oltipraz can induce phase II metabolizing enzymes, which may therefore lead to decreased carcinogen-associated DNA adduct formation.

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Oltipraz has been administered to >1,000 individuals in clinical trials. Overall, toxicity was mild and primarily gastrointestinal. Several studies have evaluated pharmacodynamic end points relevant to cancer chemoprevention. In one such study, administration of single doses of 100 or 125 mg oltipraz to normal volunteers was associated with increases in glutathione and GST levels in lymphocytes within 24 hours (13). In a separate study involving patients at high risk for colon cancer, oltipraz was given in escalating single doses between 125 and 1,000 mg/m² to consecutive cohorts of subjects at high risk for development of colorectal cancer (14). Treatment-related increases in GST activity were observed in both peripheral blood mononuclear cells (PBMC) and colonic mucosa after a single 125 or 250 mg/m² dose. However, no blood or colon tissue phase II enzyme induction was seen in a subsequent multidose study by the same group (15). In a separate study, also in patients at risk for colon cancer, participants were given oltipraz 20 to 100 mg/d or 125 mg twice weekly for 6 months (16). In this study, oltipraz seemed to increase glutathione in lymphocytes but had no effect on glutathione in rectal mucosal tissue nor on GST in either lymphocytes or rectal tissues. Finally, in a clinical trial carried out in Qidong, Jiangsu Province, People's Republic of China, 500 mg/wk oltipraz were associated with decreased activation of aflatoxins by phase I metabolizing enzymes, whereas 125 mg/d oltipraz resulted in increased phase II conjugation of aflatoxin (17). However, in the same population, levels of urine mutagenic potency, which correlated with number of cigarettes smoked, were not altered by oltipraz treatment (18).

The human clinical trials completed to date show that oltipraz is generally well tolerated and that it may induce phase II metabolizing enzymes and decrease carcinogen adduct formation. In animal models, oltipraz induced similar effects and had cancer chemopreventive activity, including in a lung cancer model, suggesting that oltipraz may decrease adducts of cigarette smoke carcinogens in humans. We therefore initiated a study in chronic smokers to test this hypothesis and to examine related effects of oltipraz on phase II metabolizing enzymes and markers of oxidative state.

Materials and Methods

Subject Recruitment and Selection. Patients were entered onto institutional review board-approved protocols at Duke University Medical Center (Durham, NC) and Northwestern University (Chicago, IL) hospitals. Patients were eligible if they were currently smoking at least 20 cigarettes per day, had smoked for at least 10 years, and had failed at least one attempt to stop smoking within the past 3 years. The number of cigarettes smoked per day could not have varied by >10 in the 3 months before enrollment. Additional eligibility requirements were age \geq 18 years, Eastern Cooperative Oncology Group performance status of 0, willingness to undergo bronchoscopy twice, and ability to provide informed consent. Normal hemogram, blood chemistries (including renal and hepatic function tests), clotting variables, and electrocardiogram were required. Subjects must also have had a normal chest radiograph and forced expiratory volume in 1 second of >1.8 liters or 75% of predicted value. Subjects with an active malignancy were excluded as were those with a prior history of cancer, with the following exceptions: nonmelanomatous skin cancer, cervical dysplasia, stage I non-small cell lung cancer treated with surgical resection of at least a lobectomy, and stage I or II cancer of the head and neck treated with curative intent. Subjects who were pregnant, intending to become pregnant within the next 6 months, or breast feeding were excluded. Individuals with medical problems that in the opinion of the investigators would either compromise the subject or the data were excluded. Participants were not

allowed to simultaneously participate in any other treatment studies.

Drug Administration and Toxicity Assessment. Oltipraz was manufactured by Rhone Poulenc (Paris, France). Gelatin capsules containing either 100 mg oltipraz or no active drug were provided by the National Cancer Institute (Rockville, MD). Oltipraz and placebo capsules contained 560 and 660 mg lactose, respectively.

Participants were assigned in a double-blind fashion to one of three arms: oltipraz 400 mg/wk (four oltipraz capsules), oltipraz 200 mg/wk (two oltipraz + two placebo capsules), or placebo weekly (four placebo capsules). Treatment was for 12 consecutive weeks. Randomization was stratified by study site (Duke University and Northwestern University) using a permuted block randomization scheme at each site. At each monthly visit, sufficient study drug was dispensed to subjects for study compliance until the next visit. Compliance was encouraged through telephone contacts, which took place midway between monthly clinic visits. Compliance was monitored by the use of calendars (which were reviewed at monthly visits) and by monthly pill counts. All subjects with at least 80% compliance, as determined by both drug calendar review and pill count, were allowed to remain on study and were included in the analysis. Subjects who did not complete the planned 12 weeks of drug therapy and pre- and post-drug bronchoscopies were replaced.

Study subjects were monitored for toxicity every 4 weeks during the period of treatment and at a 4-week post-treatment time point by history and physical examination, complete blood count, and serum chemistries. Toxicity was graded using the National Cancer Institute Common Toxicity Criteria version 2.0. No dose modifications were made for grade 1 toxicity regardless of attribution. For grade 2 toxicity thought to be at least possibly due to study drug, a partial unblinding of the study arm assignment was done by the research pharmacist as follows. Subjects on the 200 mg arm were removed from study. For subjects on either 400 mg or placebo arm (each taking four capsules), blinding was maintained and subjects had their dose reduced by half to two capsules of oltipraz or placebo. If toxicity persisted, they were removed from study. Subjects experiencing grade \geq 3 toxicity were removed from study.

Sample Acquisition and Processing. Before therapy, the following baseline samples were collected: blood (~30 mL), oral mucosa cells (from saline oral rinse), bladder lining cells (from urine), two endobronchial biopsies, and bronchoalveolar lavage (BAL) fluid. An identical set of samples were collected between 2 and 5 days after completing the 12-week treatment course of study drug. Subjects were allowed 1-week flexibility in scheduling the second sample collection in which case an additional weekly dose of oltipraz was administered to allow the second sample collection to occur between 2 and 5 days after the last dose.

Bilateral bronchoscopy with BAL and endobronchial biopsy was done. Following a complete airway survey, bilateral BAL and bilateral endobronchial biopsy of a secondary carina in each lung (total of two biopsies) were done. For BAL, the bronchoscope was wedged into both right middle lobe and lingula, and serial lavage was done with 30 mL aliquots of 0.9% sterile saline solution (total of 150 mL lavage per lobe), instilled through the bronchoscope, and aspirated gently with a handheld syringe. The BAL fluid was filtered through sterile gauze, placed on ice, and processed immediately. Endobronchial biopsies were done at the carina between the medial and the lateral segments of the right middle lobe and at the carina of the medial basilar segment of the right lower lobe both before and after the interventions. Biopsies were fixed in formalin and paraffin embedded in a single block. BAL fluid was filtered through nylon gauze to remove debris and

centrifuged at 2,500 rpm, contaminating RBCs were removed from the resultant pellet by hypotonic lysis, and the remaining lung lavage cells were pelleted. BAL cells were counted by mixing equal aliquots of BAL fluid with 0.4% trypan blue and counting the number of viable and nonviable cells using a hemacytometer (Fisher Scientific, Pittsburgh, PA). Differential cell counts were obtained by first centrifuging 300 μ L BAL fluid at 600 rpm for 6 minutes in a Cytospin apparatus (Shandon, Pittsburgh, PA), air drying resultant slides, staining with Leukostat (Fisher Scientific), and differentially counting a total of 300 cells.

PBMCs were isolated by density gradient centrifugation and RBCs were removed by hypotonic lysis as described previously (19). DNA was isolated from PBMCs after treatment with proteinase K by phenol extraction. DNA concentration was measured by absorbance at 260 nm. RNA was isolated from PBMCs and BAL cells using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For measurement of hemoglobin adduct levels, blood samples were drawn into an EDTA-containing tube and centrifuged at 2,500 rpm for 20 minutes at 4°C and RBCs were washed thrice with 0.9% NaCl before freezing of the resultant RBC pellet. Samples were stored at -70°C and processed in a batch fashion. Oral mucosa and bladder lining cells were processed and cytospin preparations were made as described previously (20); slides were stored at -20°C until batch staining. Endobronchial biopsy specimens were placed immediately into formalin. The adequacy of biopsy samples was evaluated by demonstrating the presence of histologically intact epithelium by conventional histologic examination after H&E staining of tissue. For other samples, quality control measures varied as a function of the sample. In general, samples must have been processed in a timely fashion and in accordance to strict protocols, which were sample specific. For BAL cells, they must have had a viability of at least 80% and have yielded >100 μ g DNA. For PBMCs, resultant DNA yields must have been at least 100 μ g. For RNA, quality and quantity were evaluated by measurement of $A_{260/280}$ nm ratios as well as by agarose gel electrophoresis. Samples judged to be of poor quality were not evaluated. Finally, for oral and bladder derived cells, resultant cytospin preparations must have yielded at least 100 cells on a given slide, and individual cells must have been distinguishable from adjacent cells. Samples that were collected but did not meet these quality control measures were not analyzed further.

Sample Analysis. All samples were processed and analyzed without knowledge of the assigned treatment group. For all assays, samples were processed in a batch fashion, and both pretreatment and post-treatment specimens were processed within the same batch. The level of PAH-DNA in bronchial epithelium, oral lining cells, and bladder lining cells was measured by immunoperoxidase staining as described previously (21). Positive and negative controls were included with each batch, and all samples were scored for level of PAH-DNA adducts by the same individual. A minimum of 50 cells from each sample were counted. PAH-DNA adducts in peripheral blood DNA were measured by ELISA as described previously (22).

BPDE-hemoglobin adducts were measured by mass spectroscopic analysis using a modification of a method described previously (23). The detection limit was 0.004 fmol/mg globin using 500 mg globin. Blank samples subjected to the entire analytic procedure did not show detectable amounts of benzo(a)pyrene-tetraols. For analysis of BPDE-DNA adducts, essentially the same procedure was used, starting with acid treatment of DNA. The detection limit was 0.011 fmol/ μ g DNA using a 100 μ g sample. Positive control samples contained 10 fmol *trans*, anti-benzo(a)pyrene-tetraol added to 100 μ g calf thymus DNA. Analysis gave 11.3 ± 2.0 fmol ($n = 4$).

Reduced, oxidized, and protein-bound forms of glutathione were measured in both BAL and plasma as described previously using high-performance liquid chromatography separation and fluorescence detection of a conjugate with *o*-phthalaldehyde (24) with minor modifications. Authentic standards were used for calibration during each batch run. The level of reduced glutathione was calculated by subtracting oxidized and protein-bound glutathione from total glutathione.

Superoxide dismutase (SOD) activity in BAL fluid was measured using a Bioxytech SOD-525 kit (Oxis International, Portland, OR) according to the manufacturer's instructions. SOD activity in plasma was measured as described previously (25). Glutathione peroxidase activity in BAL fluid was determined by using the Bioxytech GPx-340 kit (Oxis International) according to the manufacturer's instructions with minor modifications. Plasma glutathione peroxidase activity was measured as described previously (26). GST activity in BAL fluid and plasma was measured using a Bioxytech GST-340 kit (Oxis International) according to the manufacturer's instructions.

Quantification of mRNA copy number for *GSTM1*, *GSTP1*, UDP-glucuronyltransferase (*UGT*), γ -glutamylcysteine synthetase (*GCLC*), and glutathione peroxidase 1 (*GPX1*) was done using a quantitative competitive template reverse transcription-PCR assay as described (27).

GSTM1 genotype was determined by multiplex PCR of genomic DNA based on a modification of procedures used by Comstock et al. (28). A 273-bp segment from exon 5 of *GSTM1* was amplified by PCR using oligonucleotide primers (GST-forward: CTGCCCTACTTGATTGATGGG and GST-reverse: CTGGATTGTAGCAGATCATGC) in the same reaction with amplification of a 360-bp genomic segment encompassing exon 8 of the *MYH9* gene on chromosome 22q (MYH9-8-forward: CCAACCTTTGAGGTCACAGC and MYH9-8-reverse: CATACTGAAGGCCTCCC). Total genomic DNA (0.1 μ g) was amplified in 10 μ L PCR reaction containing 50 ng of each oligonucleotide primer, 1.5 mmol/L MgCl₂, 200 μ mol/L of each deoxynucleotide triphosphate, 1 μ L of 10 \times reaction buffer, and 0.25 units Gold Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). Thermocycler settings were 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. PCR products were analyzed by agarose gel electrophoresis and detected by ethidium bromide staining under UV light. Samples known to be *GSTM1* null and *GSTM1* non-null, as well as a water control, were run with each assay batch. Samples were scored as either homozygous null or non-null.

Statistical Analysis. The primary end point used as the basis for power analyses was the change in PAH-DNA adduct formation in bronchial epithelium. Preliminary data showed a baseline mean and SD for levels of PAH-DNA adducts in oral mucosa cells, without any data transformation, of ~500 and 180, respectively (20). With the assumption that measurements collected at baseline and at week 12 are correlated with a correlation coefficient of 0.7, the SD of the mean change from baseline at week 12 for levels of DNA adduct (without any transformation) is 139. We also assumed that there would be a normal distribution of PAH-DNA adducts (29). With $\alpha = 0.05$ and a Bonferroni correction for two pairwise comparisons (high-dose oltipraz versus placebo and low-dose oltipraz versus placebo), a sample size of 60 individuals (i.e., 20 individuals per treatment arm) was determined to provide an 80% chance to detect a change of 77% of the SD in the baseline PAH-DNA adduct levels.

Except as noted above for the primary end point, a level of significance of 0.05 was used for other comparisons between groups without correction for multiple comparisons.

Results

Patient Characteristics. Between July 2000 and May 2002, 77 patients were accrued onto the study, and 59 (77%) patients completed it. Recruitment efforts resulted in a total of 667 initial telephone contacts by prospective participants, the scheduling of 301 screening clinic appointments, and 154 kept screening appointments. Of these 154 subjects, 53 (34%) were ineligible, 24 (26%) elected not to participate, and 77 (50%) were enrolled onto the study. All enrolled patients met the eligibility criteria. The fraction of initial contacts completing the study was only 9%.

The characteristics of enrolled subjects are presented in Table 1. Most subjects were middle aged, all had a performance status of 0, and there was a slight male predominance (58%). Of those accrued, 60% were White, 35% Black, and 5% other. The age and race/ethnicity of subjects in the three treatment groups were well balanced. The placebo group had more males (78%) than either of the oltipraz arms (48% and 52% for the 200 and 400 mg/wk groups, respectively). For all enrolled subjects, the mean duration of smoking was 28 years and the mean number of cigarettes per day at enrollment was 26. Both the duration and the amount of smoking were similar between the three arms.

Clinical Outcomes. Of 77 patients enrolled, 18 (23%) did not complete the study. Among those accrued, 6 subjects never received any drug; 4 because the bronchoscopy could not be safely done, 1 because the required endobronchial biopsy could not be done, and 1 because of a complication from the initial bronchoscopy. Of the 71 subjects who received drug, 12 (17%) either did not receive the planned 12 weeks of drug or did not have a second bronchoscopy. Of these 12, 7 were lost to follow-up, 3 had adverse drug events leading to removal from the study, and 2 refused the scheduled second bronchoscopy. The distribution of the subjects who did not complete the study was not balanced between the study arms (Table 2). No subjects in the placebo group failed to complete the study, whereas 10 and 8 subjects did not complete the study in the 400 and 200 mg/wk groups, respectively.

Of the 71 participants who received study drug, 26, 22, and 23 were in the 400 mg, 200 mg, and placebo groups, respectively. There were nine episodes of grade 2/3 toxicity that were possibly, probably, or definitely attributable to study drug among 7 subjects (Table 3). None of these toxicities were in the placebo group, whereas 5 (21%) and 2 (9%) subjects in the 400 and 200 mg/wk oltipraz groups, respectively, had these toxicities. Both episodes of transaminase elevation were seen in one individual in the 400 mg oltipraz group. The elevation began as grade 1 toxicity after 8 weeks of study drug and continued to increase to grade 2 and 3 toxicities after 12 weeks of study drug, at which time the subject was found to be

Table 2. Study compliance and completion by study arm

	400 mg	200 mg	Placebo	Total
Completed study (<i>n</i>)	19	17	23	59
Lost to follow-up	4	3	0	7
Withdrawn, drug toxicity	2	1	0	3
Withdrawn, procedure AE	1	0	0	1
Bronchoscopy failure	2	3	0	5
Refused second bronchoscopy	1	1	0	2

taking concomitant anti-HIV drugs. The transaminase elevation reverted to normal within 4 weeks of cessation of oltipraz.

For grade 2 toxicity, nausea, dyspnea, cough, hypertension, and photosensitivity were each experienced by one person. The individual with photosensitivity chose to discontinue the study after 6 weeks of drug. About 6 weeks later, he was diagnosed with pyoderma gangrenosum. Toxicity of any grade or attribution occurred in 54%, 32%, and 30% of people on the 400 mg, 200 mg, and placebo arms, respectively, and was primarily gastrointestinal (nausea, dyspepsia, bloating, flatulence, and diarrhea; data not shown).

Three individuals had procedure-related adverse events, one each with grade 2 pulmonary hemorrhage/pneumonitis, grade 1/2 culture-confirmed streptococcal pharyngitis, and grade 1 superficial thrombophlebitis. The subject with pulmonary hemorrhage/pneumonitis did not receive any study drug and recovered without sequelae after treatment with antibiotics and analgesics. The other two subjects also recovered after appropriate treatment and completed 12 weeks of study drug. Four subjects were found incidentally to have vocal cord polyps.

During the 12 weeks of study drug, most subjects continued to smoke the same number of cigarettes. One subject in the 200 mg oltipraz group quit smoking completely and one subject in the placebo group increased smoking by 15 cigarettes per day. No other subject changed smoking by >10 cigarettes per day. All subjects were again offered referral to a smoking cessation program at the end of drug treatment, but only one subject had quit smoking within 4 weeks after completion of the second bronchoscopy.

Tobacco Smoke-Related Adducts of Macromolecules. Macromolecule adducts of tobacco smoke constituents were measured before and after study drug. Measurements were taken only from subjects who had completed the planned 12-week treatment course, who had undergone both bronchoscopies, and in whom paired pre- and post-sample material existed that met the quality control standards described in Materials and Methods. Samples, of any type, not meeting the quality control standards were not evaluated further and not

Table 1. Subject characteristics

Study arm	400 mg	200 mg	Placebo	All
Age (y), median (range)	43 (26-63)	47 (22-67)	44 (26-65)	45 (22-67)
Race/ethnicity, <i>n</i> (%)				
White	16 (55)	17 (68)	13 (57)	46 (60)
African American	12 (41)	7 (28)	8 (35)	27 (35)
Hispanic	0 (0)	1 (4)	0 (0)	1 (1)
American Indian	1 (3)	0 (0)	1 (4)	2 (3)
Asian	0 (0)	0 (0)	1 (4)	1 (1)
Gender, <i>n</i> (%)				
Male	15 (52)	12 (48)	18 (78)	45 (58)
Female	14 (48)	13 (52)	5 (22)	32 (42)
Weight (kg), mean (range)	79 (51-108)	74 (43-101)	90 (67-130)	81 (43-130)
Smoking				
Duration (y), mean (range)	27 (10-41)	30 (10-50)	29 (10-50)	28 (10-50)
Amount (cigarettes/d), mean (range)	25 (20-40)	27 (20-50)	25 (20-55)	26 (20-55)

Table 3. Drug-related toxicities at least possibly related to study drug and at least grade 2 (National Cancer Institute Common Toxicity Criteria version 2)

Toxicity	400 mg		200 mg		Placebo	
	Grade 2	Grade 3	Grade 2	Grade 3	Grade 2	Grade 3
Elevated aspartate aminotransferase		1				
Elevated alanine aminotransferase	1					
Dyspnea	1					
Nausea	1					
Photosensitivity	1					
Hypertension	1					
Weight Loss	1					
Hyperbilirubinemia			1			
Cough			1			
Total	6	1	2	0	0	0

included in any of the data analyses. The primary study end point was change in PAH-DNA adducts in bronchial epithelial cells as measured immunohistochemically. The immunoperoxidase-based assay used to measure PAH-DNA adducts in epithelial cells uses a polyclonal antiserum raised against BPDE-DNA that has cross-reactivity with non-BPDE PAH diol epoxide-DNA adducts. The change in mean relative staining intensity for PAH-DNA adducts was not different between either of the oltipraz arms and the placebo arm ($P > 0.05$, unpaired t test, two sided; Table 4). The average of the PAH-DNA mean relative staining intensity in bronchial epithelial cells was not significantly different between pre- and post-drug samples in the 400 mg oltipraz group (0.076 ± 0.034 and 0.072 ± 0.038 , respectively), 200 mg oltipraz group (0.075 ± 0.036 and 0.076 ± 0.033 , respectively), or placebo group (0.081 ± 0.037 and 0.081 ± 0.037 , respectively; $P > 0.05$, paired t test, two sided). However, when analyzed across all three groups, mean relative staining intensity for PAH-DNA adducts in bronchial epithelium was significantly correlated between pre- and post-drug samples

Table 4. Mean change in tobacco smoke adduct levels by study arm

	400 mg	200 mg	Placebo
PAH-DNA in lung epithelial cells (endobronchial biopsies) by immunoperoxidase (mean relative staining intensity)			
Mean	-0.004	0.001	0.001
SD	0.042	0.031	0.045
n	17	17	21
BPDE-hemoglobin in blood by tandem mass spectrometry; benzo(a)pyrene-tetraol in sample (fmol/mg globin)*			
Mean	0.0020	-0.0004	0.0910
SD	0.0070	0.0040	0.3226
n	16	12	14
PAH-DNA in PBMCs by ELISA (adducts per 10^8 DNA bases)			
Mean	-0.467	-0.028	-0.125
SD	1.276	1.391	0.495
n	14	10	13
PAH-DNA in oral rinse cytopins by immunoperoxidase (mean relative staining intensity)			
Mean	-0.025	0.014	-0.022
SD	0.053	0.068	0.083
n	15	11	15
PAH-DNA in urine cytopins by immunoperoxidase (mean relative staining intensity)			
Mean	-0.023	-0.000	-0.026
SD	0.102	0.085	0.072
n	15	9	13

*Samples with adduct levels below the limit of detection were assigned values of 0. Excluding these values or assigning a value of half of the limit of detection also did not result in significant difference between the drug arms and placebo.

($r = 0.44$, $P < 0.001$, Spearman). A one-tailed statistical test conducted at the 0.05 level of significance with 80% power would have detected a difference in mean relative staining intensity for PAH-DNA adducts between the treatment group and the placebo group of 0.036 and 0.031 for the 400 and 200 mg oltipraz arms, respectively. The level of BPDE adducts in the blood DNA was below the limit of detection of the mass spectrometry assay. A planned analysis of PAH-DNA adducts in BAL fluid cells by ELISA could not be done due to lack of sufficient DNA isolated from these cells.

We also analyzed BPDE-hemoglobin adducts in peripheral blood by mass spectrometry, PAH-DNA adducts in PBMCs by ELISA, and PAH-DNA adducts by immunoperoxidase in both oral and bladder lining cells. For all sample and assay types, no significant differences in the change in adduct levels were found between either of the oltipraz arms and the placebo arm ($P > 0.05$, t test; Table 4). In addition, no significant differences between the mean adduct levels were found for any of these assays when comparing the pre- and post-values in the oltipraz or placebo arms (data not shown). Unlike PAH-DNA adduct levels measured in lung epithelium, there was no significant correlation between pre- and post-drug levels of PAH-DNA adducts in PBMCs. Interestingly, there was a modest inverse correlation between PAH-DNA adduct levels in lung and blood among the 38 subjects with paired pre-drug results ($r = -0.39$, $P = 0.016$, Spearman). This relationship was not observed in the post-drug samples. There was no correlation between PAH-DNA adduct levels in lung or blood cells and smoking duration, number of cigarettes smoked per day, or pack-years of smoking. Although there was imbalance of gender (and body weight) between the active drug and placebo arms (Table 1), there was no statistically significant interaction between study arm and either gender or body weight on any adduct level or pharmacodynamic end point (see below).

Gene Expression of Phase II Enzymes. To determine if weekly oltipraz induced phase II enzyme gene expression in the lungs of smokers, we measured mRNA levels of five genes or gene families (*GPX1*, *UGT*, *GCLC*, *GSTM*, and *GSTP*) by quantitative competitive PCR. No significant difference was found between pretreatment and post-treatment levels for *GPX1*, *GCLC*, *GSTM*, or *GSTP1* in any of the three arms (Fig. 1). For *UGT1*, there was a 52% decline in the mean mRNA copy number in the 200 mg arm ($P = 0.0353$, paired Wilcoxon). A 34% decline in *UGT1* mRNA in the placebo arm did not reach statistical significance and there was no change in the 400 mg arm. Analysis of mRNA levels of these five genes in the PBMCs of a subset of subjects also failed to show induction of expression of phase II enzymes (data not shown).

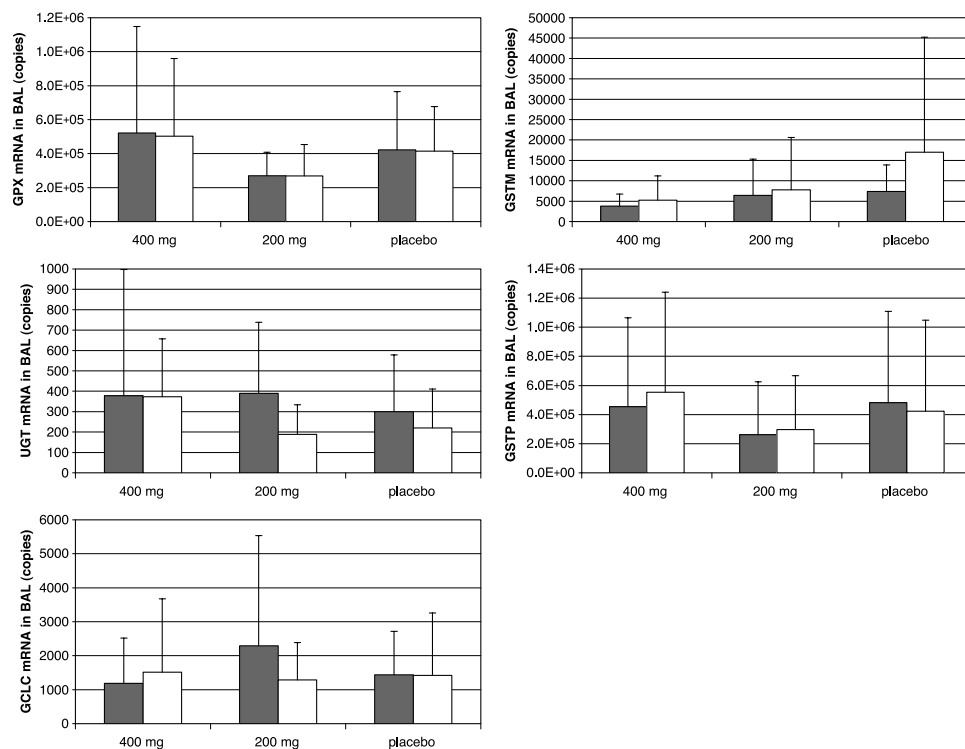


Figure 1. Effect of weekly oral oltipraz on phase II enzyme mRNA expression in BAL cells of smokers. mRNAs were measured by quantitative competitive reverse transcription-PCR in samples obtained by BAL before (*filled columns*) and after (*open columns*) study drug. *Columns*, mean; *bars*, SD. No statistically significant differences were noted within or between arms.

Measures of Oxidative State. Levels of total, reduced, oxidized, and protein-bound glutathione were measured in plasma and BAL fluid in both pretreatment and post-treatment samples. Overall, there was no significant difference in glutathione levels between or within treatment arms ($P > 0.05$ in all instances; data not shown). We also assayed plasma and BAL fluid for enzymatic activity levels of GST, glutathione peroxidase, and SOD. GST activity in plasma was undetectable in all samples. No differences between the groups were noted in the remaining assay values (Table 5).

Correlations with *GSTM1* Genotype. About half of the U.S. population is homozygous for the null allele of *GSTM1*, a condition that has been associated with an increased risk of development of lung cancer (30-32). Overall, 43% of subjects were biallelic null for *GSTM1* with similar proportions in each arm (40-48%). The levels of adducts, phase II enzymes, and oxidative state assays were not different between the *GSTM1* null subjects and those who were not biallelic null (data not shown).

Table 5. Change in enzymatic activities of SOD, glutathione peroxidase, and GST in BAL fluid and plasma

	BAL enzyme activities			Plasma enzyme activities		
	400 mg	200 mg	Placebo	400 mg	200 mg	Placebo
SOD (units/mL)						
Mean	-0.053	-0.079	0.045	0.100	-0.236	-0.040
SD	0.208	0.160	0.235	0.655	0.457	0.676
<i>n</i>	19	17	19	14	11	15
Glutathione peroxidase (mU/mL)						
Mean	-0.016	-0.065	-0.011	9.726	-16.882	24.211
SD	0.433	0.343	0.205	43.979	38.427	64.891
<i>n</i>	20	17	20	14	11	15
GST (mU/mL)						
Mean	0.113	-1.295	0.066			
SD	3.910	3.634	1.323			
<i>n</i>	19	17	19			

Discussion

Lung cancer is a major public health problem. Although cigarette smoking is a major causal factor, smoking cessation is not attained for the vast majority of chronic smokers, even those motivated to stop smoking. A significant amount of preliminary data support the concepts that reduction in the level of cigarette-derived DNA adduct formation will reduce the rate of lung cancer, that changes in the cellular redox state and activity of phase I and II enzymes can alter DNA-adduct levels, and that oral oltipraz has the potential to induce such effects in humans. The current study was designed to test this hypothesis.

A high rate of toxicity was identified in the current study, thus raising concerns about the future of oltipraz as a chemopreventive agent for broad consumption. Exact quantitation of toxicity may have been confounded by the high dropout rate of 23% observed in the current study. Although we were concerned that the requirement for two bronchoscopic procedures may have decreased compliance with the study plan, we did not have any subjects from the placebo group who dropped out of the study, suggesting that those in the oltipraz groups who were lost to follow-up may have been experiencing drug-related toxicity. Despite this limitation, grade 2/3 toxicity was observed in 7 of 46 (15%) people who completed 12 weeks of oltipraz. Although the placebo group had a higher proportion of males and an associated greater mean weight of subjects than either treatment group, the apparent dose dependency of the toxicity in the two drug arms cannot be explained by a drug-independent effect of gender or weight on toxicity.

Oltipraz treatment was not associated with any significant change in BPDE or PAH adduct levels despite sampling multiple sites of epithelial cells in the lung, oral cavity, and bladder. The lack of induction of phase II enzymes, however, leaves the underlying hypothesis untested. Concerning phase II enzyme induction, our results contrast with the demonstration of an ~4- to 6-fold induction of *GCLC* and DT-diaphorase mRNA in colonic epithelium following single doses of oltipraz (14). Given the large number of assays examined in the current study (including *GCLC*), we believe it unlikely we have simply

chosen the wrong marker to examine. The peak elevation in the colonic epithelium was noted at 2 to 4 days following drug administration and returned to baseline over the ensuing 7 to 10 days (14), so it is possible that there was transient induction of phase II enzyme transcription in our study that was not detected. The lack of phase II enzyme induction in multidose studies of oltipraz (the current study and refs. 15, 16) but not in a single dose study (14) suggests possible tachyphylaxis. Alternatively, chronic smokers may either have maximally induced phase II enzymes or be otherwise unable to respond to induction in response to dithiolethiones.

We believe the design of the current study was extremely rigorous and its implementation is arduous. Specifically, to complete the trial, multiple recruitment efforts were employed, and from the 667 initial encounters with prospective participants, only 9% went on to complete the study. Despite the rigors of implementing this type of study design, the strength of the ultimate conclusions supports the rationale underlying the design. Future study designs must account for the large overhead in accrual to studies of similar design.

Completed phase III clinical trials testing retinol (vitamin A), β -carotene, *N*-acetylcysteine, and selenium for primary and tertiary chemoprevention of lung cancer have not shown a reduction in lung cancer incidence or, in the case of β -carotene, have been associated with increased lung cancer incidence and mortality (33). Subsequent investigations have focused on smaller phase IIa and IIb trials using putative markers of lung cancer risk as the primary end point to test an increasing array of potential agents. The lack of a confirmed biomarker for prediction of lung cancer risk has hampered the design of such trials. One study of *N*-acetylcysteine in smokers measured adducts of tobacco smoke constituents as biomarkers (34). In this study, lipophilic DNA and 8-OH-dG adducts in BAL cells were decreased but lipophilic DNA in peripheral blood cells was not. Likewise, micronuclei in cells from the floor of the mouth were decreased but not in cells from the soft palate. The strength of using carcinogen-macromolecule adducts as an intermediate biomarker for lung cancer risk is the association of adduct levels with risk in case-control studies (35) and the implication of tobacco smoke carcinogens, such as benzo(*a*)pyrene, in the development of lung cancer-associated molecular genetic alterations. However, in the current study, there was no association of adduct levels with cigarette smoking in contrast to previous studies (36, 37). However, there are other studies that do not show a correlation with smoking (38-40). Therefore, further validation of these adduct levels will be required to strengthen the dependence of future trials on similar end points (41).

In conclusion, the current study found that oral weekly oltipraz is associated with relatively high rates of toxicity in a cohort of chronic smokers yet did not result in measurable effects on pharmacodynamic end points in the lung. Furthermore, oltipraz treatment did not modulate PAH or BPDE adduct levels. Although the current study does not support further development of oltipraz as a chemopreventive agent, additional study of alternative inducers of detoxification pathways as a chemopreventive strategy for smoking-related malignancies is warranted.

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References

- Jemal A, Tiwari RC, Murray T, et al. Cancer statistics, 2004. *CA Cancer J Clin* 2004;54:8-29.
- Breathnach OS, Freidlin B, Conley B, et al. Twenty-two years of phase III trials for patients with advanced non-small-cell lung cancer: sobering results. *J Clin Oncol* 2001;19:1734-42.
- Patz EF Jr, Goodman PC, Bepler G. Screening for lung cancer. *N Engl J Med* 2000;343:1627-33.
- Westman EC, Behm FM, Simel DL, Rose JE. Smoking behavior on the first day of a quit attempt predicts long-term abstinence. *Arch Intern Med* 1997;157:335-40.
- Hecht SS. Tobacco smoke carcinogens and lung cancer. *J Natl Cancer Inst* 1999;91:1194-210.
- Cuzick J, Routhledge MN, Jenkins D, Garner RC. DNA adducts in different tissues of smokers and non-smokers. *Int J Cancer* 1990;45:673-8.
- Kensler TW. Chemoprevention by inducers of carcinogen detoxication enzymes. *Environ Health Perspect* 1997;105 Suppl 4:965-70.
- Smith WA, Gupta RC. Determining efficacy of cancer chemopreventive agents using a cell-free system concomitant with DNA adduction. *Mutat Res* 1999;425:143-52.
- Kensler TW, Egner PA, Dolan PM, Groopman JD, Roebuck BD. Mechanism of protection against aflatoxin tumorigenicity in rats fed 5-(2-pyrazinyl)-4-methyl-1,2-dithiol-3-thione (oltipraz) and related 1,2-dithiol-3-thiones and 1,2-dithiol-3-ones. *Cancer Res* 1987;47:4271-7.
- Arif JM, Gairola CG, Glauret HP, Kelloff GJ, Lubet RA, Gupta RC. Inhibition of cigarette smoke-related lipophilic DNA adducts in rat tissues by dietary oltipraz. *Carcinogenesis* 1998;19:1515-7.
- Li ZQ, Luo XM, Wei HJ, Feng YM, Zhang C. Inhibition of the transforming ability of cigarette smoking condensate-treated human fetal lung DNA induced by oltipraz. *J Environ Pathol Toxicol Oncol* 1994;13:133-5.
- Kensler TW, Helzlsouer KJ. Oltipraz: clinical opportunities for cancer chemoprevention. *J Cell Biochem Suppl* 1995;22:101-7.
- Gupta E, Olopade OI, Ratain MJ, et al. Pharmacokinetics and pharmacodynamics of oltipraz as a chemopreventive agent. *Clin Cancer Res* 1995;1:1133-8.
- O'Dwyer PJ, Szarka CE, Yao KS, et al. Modulation of gene expression in subjects at risk for colorectal cancer by the chemopreventive dithiolethione oltipraz. *J Clin Invest* 1996;98:1210-7.
- Szarka CE, Yao KS, Pfeiffer GR, et al. Chronic dosing of oltipraz in people at increased risk for colorectal cancer. *Cancer Detect Prev* 2001;25:352-61.
- Benson AB III, Olopade OI, Ratain MJ, et al. Chronic daily low dose of 4-methyl-5-(2-pyrazinyl)-1,2-dithiole-3-thione (oltipraz) in patients with previously resected colon polyps and first degree female relatives of breast cancer patients. *Clin Cancer Res* 2000;6:3870-7.
- Wang JS, Shen X, He X, et al. Protective alterations in phase 1 and 2 metabolism of aflatoxin B1 by oltipraz in residents of Qidong, People's Republic of China. *J Natl Cancer Inst* 1999;91:347-54.
- Camoirano A, Bagnasco M, Bencicelli C, et al. Oltipraz chemoprevention trial in Qidong, People's Republic of China: results of urine genotoxicity assays as related to smoking habits. *Cancer Epidemiol Biomarkers Prev* 2001;10:775-83.
- Schwartz GN, Liu YQ, Tisdale J, et al. Growth inhibition of chronic myelogenous leukemia cells by ODN-1, an aptamer inhibitor of p210bcra-tyrosine kinase activity. *Antisense Nucleic Acid Drug Dev* 1998;8:329-39.
- Romano G, Sgambato A, Boninsegna A, et al. Evaluation of polycyclic aromatic hydrocarbon-DNA adducts in exfoliated oral cells by an immunohistochemical assay. *Cancer Epidemiol Biomarkers Prev* 1999;8:91-6.
- Zhang YJ, Weksler BB, Wang L, Schwartz J, Santella RM. Immunohistochemical detection of polycyclic aromatic hydrocarbon-DNA damage in human blood vessels of smokers and non-smokers. *Atherosclerosis* 1998;140:325-31.
- Dickey C, Santella RM, Hattis D, et al. Variability in PAH-DNA adduct measurements in peripheral mononuclear cells: implications for quantitative cancer risk assessment. *Risk Anal* 1997;17:649-56.
- Melikian AA, Goldin BF, Prahalad AK, Hecht SS. Modulation of benzo(*a*)pyrene-DNA adducts in hamster cheek pouch by chronic ethanol consumption. *Chem Res Toxicol* 1990;3:139-43.
- Paroni R, De Vecchi E, Cighetti G, et al. HPLC with *o*-phthalaldehyde pre-column derivatization to measure total, oxidized, and protein-bound glutathione in blood, plasma, and tissue. *Clin Chem* 1995;41:448-54.
- Crapo JD, McCord JM, Fridovich I. Preparation and assay of superoxide dismutases. *Methods Enzymol* 1978;53:382-93.
- Abushamaa AM, Sporn TA, Folz RJ. Oxidative stress and inflammation contribute to lung toxicity after a common breast cancer chemotherapy regimen. *Am J Physiol Lung Cell Mol Physiol* 2002;283:L336-45.
- Willey JC, Crawford EL, Knight CR, et al. Standardized RT-PCR and the standardized expression measurement center. *Methods Mol Biol* 2004;258:13-41.
- Comstock KE, Sanderson BJ, Claffin G, Henner WD. GST1 gene deletion determined by polymerase chain reaction. *Nucleic Acids Res* 1990;18:3670.
- Wiencke JK, Thurston SW, Kelsey KT, et al. Early age at smoking initiation and tobacco carcinogen DNA damage in the lung. *J Natl Cancer Inst* 1999;91:614-9.
- McWilliams JE, Sanderson BJ, Harris EL, Richert-Boe KE, Henner WD. Glutathione S-transferase M1 (GSTM1) deficiency and lung cancer risk. *Cancer Epidemiol Biomarkers Prev* 1995;4:589-94.
- Houlston RS. Glutathione S-transferase M1 status and lung cancer risk: a meta-analysis. *Cancer Epidemiol Biomarkers Prev* 1999;8:675-82.
- Benhamou S, Lee WJ, Alexandrie AK, et al. Meta- and pooled analyses of the effects of glutathione S-transferase M1 polymorphisms and smoking on lung cancer risk. *Carcinogenesis* 2002;23:1343-50.

33. Kelley MJ, McCrory DC. Prevention of lung cancer: summary of published evidence. *Chest* 2003;123:50–95.
34. Van Schooten FJ, Nij AB, De Flora S, et al. Effects of oral administration of *N*-acetyl-L-cysteine: a multi-biomarker study in smokers. *Cancer Epidemiol Biomarkers Prev* 2002;11:167–75.
35. Tang D, Santella RM, Blackwood AM, et al. A molecular epidemiological case-control study of lung cancer. *Cancer Epidemiol Biomarkers Prev* 1995;4:341–6.
36. Phillips DH, Schoket B, Hewer A, Bailey E, Kostic S, Vincze I. Influence of cigarette smoking on the levels of DNA adducts in human bronchial epithelium and white blood cells. *Int J Cancer* 1990;46:569–75.
37. Garner RC, Cuzick J, Jenkins D, et al. Linear relationship between DNA adducts in human lung and cigarette smoking. *IARC Sci Publ* 1990;104:421–6.
38. Gammon MD, Santella RM, Neugut AI, et al. Environmental toxins and breast cancer on Long Island. I. Polycyclic aromatic hydrocarbon DNA adducts. *Cancer Epidemiol Biomarkers Prev* 2002;11:677–85.
39. Phillips DH. Smoking-related DNA and protein adducts in human tissues. *Carcinogenesis* 2002;23:1979–2004.
40. Kriek E, Rojas M, Alexandrov K, Bartsch H. Polycyclic aromatic hydrocarbon-DNA adducts in humans: relevance as biomarkers for exposure and cancer risk. *Mutat Res* 1998;400:215–31.
41. Hecht SS. Carcinogen biomarkers for lung or oral cancer chemoprevention trials. *IARC Sci Publ* 2001;154:245–55.