

Oltipraz Chemoprevention Trial in Qidong, People's Republic of China: Results of Urine Genotoxicity Assays as Related to Smoking Habits¹

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Abstract

A Phase II chemoprevention trial was carried out in Qidong, Jiangsu Province, People's Republic of China. The recruited subjects, all of whom were positive for serum aflatoxin-albumin adducts, were divided into three treatment arms: placebo; oltipraz ([5-(2-pyrazinyl)-4-methyl-1,2-dithiol-3-thione]) given daily at 125 mg p.o.; and oltipraz given once per week at 500 mg p.o. Besides biomarkers related to aflatoxin B₁ exposure, the genotoxicity of blind-coded urine XAD-2 concentrates was evaluated in 201 subjects on the fifth and seventh week of intervention. Genotoxicity was assessed both in the Ames reversion test in strain YG1024 of *Salmonella typhimurium*, in the presence of an exogenous metabolic system (S9 mix), with or without β -glucuronidase, and in a DNA repair test in *Escherichia coli*. Heating of concentrated urine samples or of cigarette smoke condensates was discovered to result in a significant enhancement of their mutagenicity. It was also found that the mutagenicity of condensates from the most extensively used brands of cigarettes in Qidong was much lower than that of Western cigarette brands. Urine mutagenicity was unrelated to treatment with oltipraz, intervention time, gender, and supplement of S9 mix with β -glucuronidase. Mutagenicity was significantly but variably higher in cigarette smokers than in nonsmokers, which suggests that the urinary excretion of mutagens in the examined

population was not exclusively attributable to smoking. Nevertheless, within smokers (28% of the recruited subjects; 67% of all males), the mutagenic potency was significantly correlated with the self-reported number of cigarettes smoked per day and, even more sharply, with the cotinine concentrations in urines. In conclusion, this study demonstrated the validity of urine mutagenicity assays as a biomarker of tobacco smoke exposure that can be investigated on a relatively large scale in chemoprevention trials and provided evidence that oltipraz treatment had no influence on this parameter in the examined population.

Introduction

Dithiolethiones such as oltipraz³ offer considerable promise as cancer chemopreventive agents. Oltipraz is an effective anti-carcinogen in rodent models of experimental carcinogenesis in target organs such as the liver, forestomach, colon, pancreas, trachea, lung, mammary gland, and skin (see Ref. 1 and references therein). This drug has now advanced through Phase I clinical trials to determine its pharmacokinetics and dose-limiting side effects during chronic administration to humans (2, 3). We have conducted a Phase IIa clinical intervention trial in Qidong, Jiangsu Province, People's Republic of China with a primary objective to define a dose and schedule of oltipraz for reducing levels of validated biomarkers of exposure to the human hepatocarcinogen AFB₁ (4–6). Risk for development of hepatocellular carcinoma is high in this region and appears to be related to infection with hepatitis B virus and dietary exposure to aflatoxins (6, 7).

One of the end points of this trial was the assessment of urine genotoxicity, which provides a biomarker of internal dose evaluating the amounts of mutagenic and DNA-damaging agents excreted with this biological fluid. To this purpose, we used both the classical Ames reversion test in *Salmonella typhimurium his⁻* strains and a differential lethality test in *Escherichia coli* strains having distinctive repair capacities. It was of interest to assess the load of exposure to genotoxic agents in the examined population and to evaluate its possible modulation in subjects treated with oltipraz. Tobacco smoking is a major source of urinary genotoxins, and a positive response can also be elicited by other exposures, either dietary, occupational, pathological, or therapeutical (*e.g.*, see Refs. 8 and 9 for review).

Urinary genotoxicity assays have several advantages, including the circumstances that collection of samples is noninvasive, genotoxicity assays can be adapted to yield quantitative data, and the costs are reasonable. Because the renal elimination of genotoxins is rapid and continuous, their detection in urine reflects acute exposures with high sensitivity. Therefore, the timing of sample collection is important in case of short-lived markers. For instance, a mutagenicity peak could be detected in the urine of a volunteer just 2 h after smoking a single cigarette

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³ The abbreviations used are: oltipraz, [5-(2-pyrazinyl)-4-methyl-1,2-dithiol-3-thione]; AFB₁, aflatoxin B₁; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; CSC, cigarette smoke condensate; NR, nitroreductase; OAT, O-acetyltransferase; β G, β -glucuronidase; MI, mutagenicity index.

(10). Urine genotoxicity reacts very rapidly to variations either in exposure to mutagens or in their bioavailability and excretion. Hence, urinary genotoxicity assays are expected to provide a particularly sensitive end point for selecting cohorts of individuals at risk as well as for evaluating modulation of this parameter after administration of chemopreventive agents (10).

On the other hand, urine genotoxicity assays have some limitations. In fact, although xenobiotics circulating in the blood are physiologically concentrated by the kidney, their low amounts in urine require preliminary concentration steps, which are time consuming and may not be equally effective for all classes of mutagens. Another disadvantage is that these assays are not suitable for detecting cumulative exposures. Finally, the biological significance and interpretation of data are sometimes uncertain.

We report herein the results of preliminary assays, aimed at optimizing the experimental conditions of the test systems, followed by a large-scale analysis of urine samples collected from subjects recruited into the trial. The main finding was that treatment with oltipraz did not modulate the investigated biomarker. The internal validity of the study was documented, however, after unblinding the sample codes, by the high correlations between urinary mutagenic potency among cigarette smokers, number of smoked cigarettes, and concentrations of cotinine in urine. Ancillary experiments provided evidence that both urines and CSCs have a greater mutagenicity after heating and that the mutagenicity of some Chinese cigarette brands is substantially lower than that of Western cigarette brands.

Materials and Methods

Recruitment of Participants and Study Design. Study participants were recruited from Daxin Township, Qidong, Jiangsu Province, People's Republic of China. All participants signed an informed consent in accordance with institutional and federal guidelines in the People's Republic of China and the United States. Full methodological details of this chemoprevention trial are described elsewhere (4), as well as assessments of compliance and adverse effects (11). The study included three intervention arms: (a) placebo, (b) 125-mg oltipraz administered once daily, and (c) 500-mg oltipraz administered once per week for 8 weeks. Of the 234 subjects randomized in the three arms, 201 (79 males and 122 females) provided urine samples for genotoxicity assays. A group of 192 of these participants provided a second series of urine samples 2 weeks later. A group of 56 of the recruited subjects were cigarette smokers (53 males and 3 females). Ten subjects were HBV carriers, as assessed by positivity for serum HBsAg.

Sampling of Urine Specimens. For preliminary assays, aimed at optimizing the conditions for genotoxicity assays, urine samples were collected from 20 individuals, living in Qidong, not recruited into the trial. Each sample was the pool of overnight urines collected on 3 consecutive days. For the same purposes, concentrates of 24-h urine samples from Italian and Dutch smokers were also available.

Because of the logistics of screening >1000 individuals for participation in the trial, it was not possible to obtain preintervention samples from the study subjects. Further, because of the climate and the rural setting for the trial, it was not practicable to have 24-h samples from the enrolled subjects. However, the representativeness of the samples was established by the fact that each of them was the pool of overnight urine voids collected on 3 consecutive days. Moreover, almost all of the 201 subjects under study, divided into the three treatment arms, were monitored both on the 5th and 7th week of inter-

vention (July–August 1995), accounting for a total of 394 urine samples.

Concentration of Urine Samples. Urine samples were collected at the participant's homes by village doctors and delivered by motorbike courier to the Qidong Liver Cancer Institute by mid-morning of each collection day, at which time they were logged in and frozen at -20°C . The urine samples were then thawed 24 h after the third collection, centrifuged at $800 \times g$, and acidified to pH 5 if necessary. Urine (500 ml) was pooled and concentrated through 10-g Amberlite XAD-2 resin (Bio-Rad) columns, as described by Yamasaki and Ames (12). Aliquots were removed from the pooled urine before XAD-2 chromatography for creatinine and cotinine determinations. The samples, taken to dryness, were shipped to Genoa as blind-coded material. Immediately after arrival in Genoa, the residue of each sample was collected, taken up to 5-ml DMSO, divided into 10 aliquots, and stored at -70°C until use.

Determination of Urinary Creatinine and Cotinine. To normalize mutagenicity data from samples collected as overnight voids, creatinine was measured spectrophotometrically with the Sigma Chemical Co. (St. Louis, MO) creatinine kit in Baltimore using aliquots prepared in Qidong of the pooled, unconcentrated urine samples from all subjects. Cotinine was measured by RIA at the American Health Foundation, Valhalla, NY in replicate aliquots of the pooled urine samples from smokers as described in Langone *et al.* (13).

Preparation of CSCs. CSCs were prepared from three brands of Chinese cigarettes and four brands of Western cigarettes, which were manufactured in the United States, United Kingdom, France, and Switzerland, respectively. The cigarettes were available on the market, and their choice was totally casual, although we tried to select the three brands of cigarettes that were more extensively used in Qidong. Each CSC sample was obtained by aspirating the mainstream smoke generated by two cigarettes through a disposable cigarette holder, using a water pump at a rate of 5 min/cigarette. The tar deposited inside the cigarette holder was eluted by rinsing with 2-ml DMSO and stored at -70°C until use.

Bacterial Strains. Eight *S. typhimurium his⁻* strains were used in the Ames reversion test. They included TA98 (*hisD3052*, *rfa*, Δ *uvrB*, pKM101), TA100 (*hisG46*, *rfa*, Δ *uvrB*, pKM101), TA97a (*hisD6610*, *rfa*, Δ *uvrB*, pKM101), and TA102 (*hisG428*, *rfa*, pKM101; gifts of Dr. B. N. Ames, University of California, Berkeley, CA) and derivatives of TA98 or TA100 overproducing either NR or acetyl-CoA:*N*-hydroxyarylamine OAT, *i.e.*, YG1021 (as TA98 but overproducing NR), YG1024 (as TA98 but overproducing OAT), YG1026 (as TA100 but overproducing NR), and YG1029 (as TA100 but overproducing OAT; gifts of Dr. T. Nohmi, National Institute of Hygienic Sciences, Tokyo, Japan).

Two *E. coli trp⁻* strains were used in the differential lethality test, *i.e.*, WP2 (wild type) and its DNA repair-deficient derivative CM871 (*uvrA⁻*, *recA⁻*, *lexA⁻*; gifts of Dr. B. A. Bridges, Medical Research Council, University of Sussex, Falmer, Brighton, United Kingdom).

Metabolic Conditions for Genotoxicity Assays. The results of the pilot study with samples of urines from 20 individuals living in Qidong and preliminary assays with samples of subjects enrolled in the study showed that no mutagenic response was detectable in the Ames test in the absence of S9 mix. Therefore, all mutagenicity assays in *S. typhimurium* were carried out in the presence of S9 mix incorporating the 10% of liver S12 fraction from Aroclor-pretreated Sprague Dawley rats, with or without the addition of β G type IX-A, extracted

from *E. coli* (1000 units/plate; Sigma Chemical Co.). In contrast, a number of preliminary experiments showed that the presence of S9 mix and/or β G did not affect the results in the differential lethality test. Therefore, all final experiments in *E. coli* were run in the absence of an exogenous metabolic system.

Mutagenicity Assays in *S. Typhimurium*. Assays were carried out with samples of CSCs and urine concentrates and kept for varying times at various temperatures, as described in "Results." Basically, all samples were assayed according to the standard plate incorporation test (14).

In particular, CSC samples were assayed in triplicate at eight doses, ranging between 0.008 and 0.1 cigarette equivalent/plate. Urine XAD-2 concentrates were tested in triplicate at three doses (100, 50, and 25 μ l/plate). Samples which were toxic at the above doses were retested at 25.0, 12.5, and 6.25 μ l/plate and, in case of further toxicity, at 6.25, 3.12, and 1.56 μ l/plate, at 1.56, 1.78, and 0.39 μ l/plate, and even at 0.39, 0.20, and 0.10 μ l/plate.

In each experiment, DMSO was assayed as a negative control (spontaneous revertants). AFB₁ (0.1 μ g/plate; Sigma Chemical Co.) and a CSC stock, kept in small aliquots at -70°C (0.05 cigarette equivalents/plate), were assayed as positive controls.

In the case of CSC samples, the results were expressed as induced revertants (total less spontaneous revertants) per plate. In the case of urine concentrates, an MI was calculated by dividing the induced revertants by spontaneous revertants. By this way, a sample yielding the same revertants as DMSO has an MI of 0, a sample yielding a number of revertants which is the double of those recorded in the presence of DMSO has an MI of 1, a sample yielding a number of revertants which is the triple of those recorded in the presence of DMSO has an MI of 2, etc. The MI was calculated at each tested dose, and the MI corresponding to 100- μ l concentrated urine, equivalent to 10 ml of unconcentrated urine, was inferred from the equation of the regression line. This was calculated on three doses (*e.g.*, 100, 50, and 25 μ l or 25.0, 12.5, and 6.25 μ l, etc.), except when either (a) the MI at the top dose within a triplet of doses (*e.g.*, 100 μ l) was lower than the MI at the lower dose (*e.g.*, 50 μ l), indicating the occurrence of toxic effects, or (b) when the MI recorded at the lowest tested dose (*e.g.*, 25 μ l) was close to 0 and to the MI yielded by the upper dose (*e.g.*, 50 μ l), which was therefore considered to represent the starting point of the dose-response curve. In these cases, the regression line was based on two doses only.

For each sample, the MI/10-ml unconcentrated urine was related to the creatinine content (g/10 ml), thereby obtaining the MI/g creatinine value.

Differential Lethality Assays in *E. Coli*. A DNA repair test was carried out in *E. coli* strains evaluating the differential lethality in the DNA repair-proficient strain WP2 and its DNA repair-deficient counterpart CM871 (*uvrA*⁻, *recA*⁻, *lexA*⁻). As validated with a number of compounds belonging to different chemical classes (15), a significantly higher lethality of a test substance in CM871 is assumed as an indicator of genotoxicity.

Spot tests were performed by assaying 15 μ l of 100-fold concentrated urines, diluted with DMSO to contain 1- μ g creatinine, imbued in paper discs, and deposited onto the surface of agar plates incorporating broth cultures of strains WP2 or CM871. As previously reported, these assays were performed in the absence of exogenous metabolic system. A CSC (0.015 cigarette equivalents/disc) and a pool of concentrated urines, previously found to be positive in this test, were used as

Table 1 Preliminary assays with 20 urine samples, CSC and AFB₁ tested for mutagenicity with various *S. typhimurium his*⁻ strains under various metabolic conditions

<i>S. typhimurium</i> strain	Metabolic conditions		MI		
	S9	β G	Urine samples ^a	CSC ^b	AFB ₁ ^c
TA97a	+	-	0.09 \pm 0.07	2.38	4.95
TA98	+	-	0.27 \pm 0.18	10.34	27.12
TA100	-	-	0.02 \pm 0.02	1.03	1.15
	+	-	0.10 \pm 0.08	0.84	8.69
	+	+	0.03 \pm 0.04	1.06	9.19
TA102	+	-	0.07 \pm 0.06	0.18	0.27
YG1021	+	-	0.44 \pm 0.23	18.69	30.73
YG1024	-	-	0.02 \pm 0.03	0.97	1.34
	+	-	0.53 \pm 0.38	28.68	32.33
	+	+	0.56 \pm 0.51	30.11	29.86
YG1026	+	-	0.04 \pm 0.05	2.53	7.96
YG1029	+	-	0.09 \pm 0.11	5.67	7.73

^a Mean \pm SD of 20 samples from individuals living in Qidong, tested at 100 μ l/plate.

^b Tested at 0.05 cigarette equivalent/plate.

^c Tested at 0.1 μ g/plate.

positive controls in each experiment. All samples were tested in triplicate.

The results were expressed in terms of area of bacterial killing, after having subtracted the disc area (28.3 mm²). A significant difference between the net areas of bacterial killing produced by a given sample in the wild-type strain and in the triple mutant was assumed as an indicator of positivity in this test system.

Statistical Analyses. The effect of heating on the mutagenicity of urine samples was evaluated by Student's *t* test for paired samples. Frequencies of positive results in the differential lethality test were compared by χ^2 analysis and Fisher's exact test. Correlations between dose of samples and mutagenicity and between mutagenicity data, smoking habits, and cotinine levels were evaluated by using Spearman's and simple regression tests. Comparisons of mean mutagenicity data as related to variables, such as intervention arm, collection time, use of β G in the metabolic system, gender, and smoking habits were made by nonparametric analyses (Wilcoxon test and Kruskal-Wallis test) after having checked the normality of data distribution. Comparisons of mean mutagenicity data in small groups were made by Student's *t* test for unpaired data.

Results

Choice of the *S. Typhimurium* Strain for Mutagenicity Assays. Eight *S. typhimurium his*⁻ strains were comparatively assayed in preliminary experiments, under various metabolic conditions (Table 1). Of the positive controls, AFB₁ was positive (at least a doubling of spontaneous revertants) after metabolic activation, with the following rank of sensitivity: YG1024 \geq YG1021 \geq TA98 > TA100 \geq YG1026 \geq YG1029 > TA97a. TA102 was negative. Metabolically activated CSC was positive with the following rank of sensitivity: YG1024 > YG1021 > TA98 > YG1029 > YG1026 > TA97a > TA100. TA102 was negative. The mutagenicities of AFB₁ and CSCs were not appreciably affected by supplementing the exogenous metabolic system with β G.

Table 1 also summarizes the results of the mutagenicity assays performed with urine concentrates from 20 male individuals living in Qidong County, aged 32–58 years, not re-

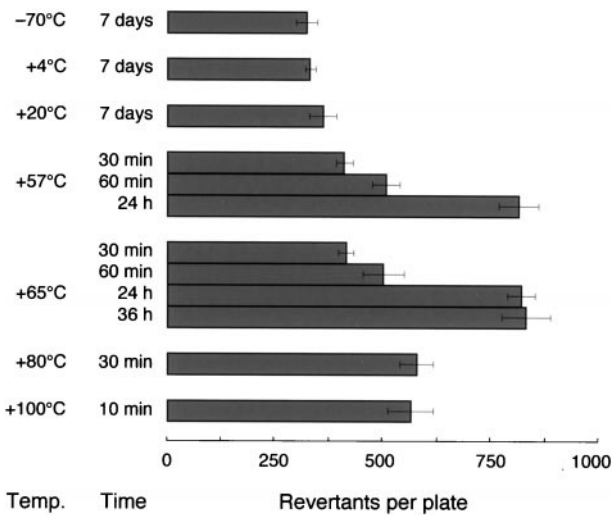


Fig. 1. Mutagenicity of CSCs (amount equivalent to 0.05 cigarettes/plate) in *S. typhimurium* YG1024, in the presence of S9 mix, after storage at varying temperatures for varying times. Values are means \pm SD of triplicates.

cruited into the trial. All samples were negative in the absence of S9 mix. Assuming an arbitrary biological criterion of positivity when the MI ≥ 1 , which means at least a doubling of spontaneous revertants at 100- μ l concentrated urine, and of borderline positivity when the MI is in the 0.5–1.0 range, two samples (10%) were positive, and eight samples (40%) were borderline positive in strain YG1024 in the presence of S9 mix. The addition of β G had variable effects on mutagenicity, but on the whole, it did not change the mean MI values (Table 1). In strain YG1021, four samples (20%) were borderline positive, and one (5%) was positive, whereas in TA98, three samples (15%) were positive. All samples were negative in TA97a, TA102, TA100 (irrespective of the addition of β G), and its derivatives YG1026 and YG1029. Thus, as compared with TA98, sensitivity to both CSCs and urine condensates was higher in the NR-overproducing derivative (YG1021) and even more in the OAT-overproducing derivative (YG1024), whereas sensitivity to AFB₁ mutagenicity was of the same order of magnitude in these three strains. On the basis of these data and on our previous experience with cigarette smoke, CSCs, and smokers' urines (16), we decided to use YG1024 for the mutagenicity assay of all urine samples from the subjects enrolled into the trial.

Effect of Heating on the Mutagenicity of CSCs and Urine Concentrates. With the goal of evaluating the influence of storage conditions on the stability of CSC mutagenicity, we assayed the mutagenic activity of a CSC sample kept at varying temperature for varying time intervals (Fig. 1). The results were somewhat surprising, in that storage at high temperatures produced an enhancement rather than a decrease of mutagenicity. In particular, mutagenicity was very similar by storing CSC aliquots for 7 days at either -70°C , 4°C , or 20°C . At either 57°C or 65°C , there was an increase of mutagenicity which reached its maximum after 24 h. Heating at 65°C for 36 h did not further increase the mutagenic response. Even heating at high temperature (80°C for 30 min or 100°C for 10 min) enhanced CSC mutagenicity.

We then carried out additional experiments to evaluate the effect of heating at high temperature on the mutagenicity of XAD-2-concentrated urine samples. The comparative analysis

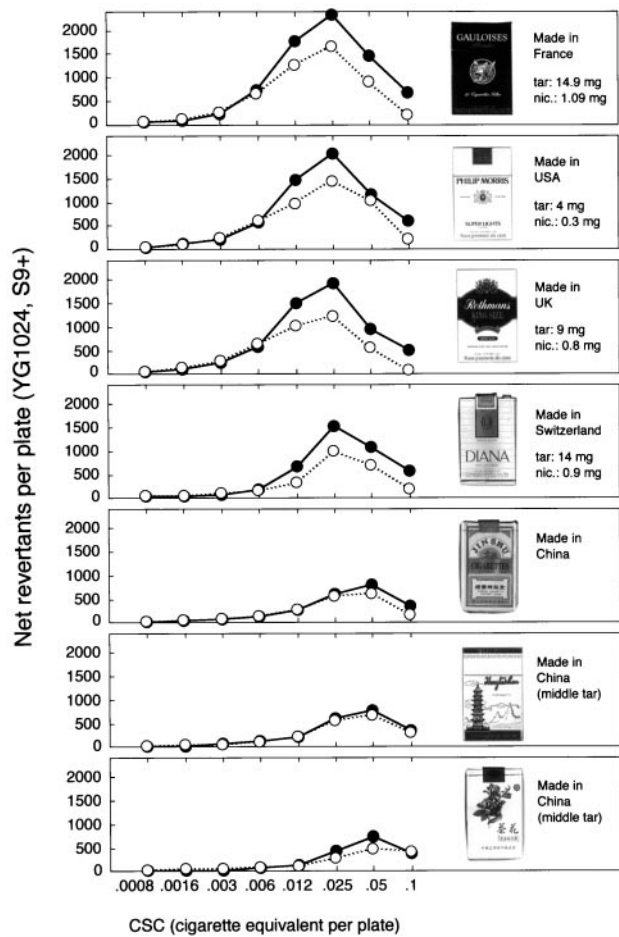


Fig. 2. Dose-dependent mutagenicity of CSCs from seven brands of cigarettes (\circ — \circ , untreated CSCs; \bullet — \bullet , CSCs heated for 24 h at 65°C). SD values are not indicated because, in almost all cases, they fell within the size of symbols.

of 27 samples from smokers (3 Italian, 5 Dutch, and 19 Chinese) provided evidence that the mutagenic response was almost invariably higher in samples heated at 65°C for 24 h than in the same samples kept at -70°C . The mean (\pm SD) values were 2.1 ± 1.9 versus 2.8 ± 2.7 , respectively. As assessed by Student's *t* test for paired samples, this difference was statistically significant ($P < 0.001$).

On the basis of these data, all urine concentrated samples from the subjects recruited into the trial were heated at 65°C for 24 h immediately before testing.

Mutagenicity Assay of Condensates of Chinese and Western Brands of Cigarettes. Because the mutagenicity of urine concentrates from the 20 subjects examined in the pilot study was rather low, even in smokers, we decided to comparatively assess the mutagenicity of three brands of Chinese cigarettes and four brands of Western cigarettes, all of them with filters. Dose-response curves with CSC samples prepared under standardized conditions from these brands of cigarettes led to the results shown in Fig. 2, which can be summarized as follows: (a) the CSC samples prepared from the Chinese brands were considerably less mutagenic than the CSC samples prepared from the Western brands of cigarettes; (b) with all brands, there was a dose-dependent increase of mutagenicity up to 0.025–0.05 cigarette equivalents/plate, followed by a drop of the

curve, in the absence of evident toxic effects; and (c) after heating, CSC mutagenicity of Western brands of cigarettes became significantly higher, and the drop of mutagenicity at 0.05–0.1 cigarette equivalent/plate was less pronounced. This effect was less evident with Chinese cigarettes.

Spontaneous Revertants and Positive Controls. The mutagenicity assessment in strain YG1024, with or without β G, of the 394 urine samples collected during the trial required 49 separate experiments. In each experiment, DMSO was tested as a negative control, and CSCs and AFB₁ were tested as positive controls.

Spontaneous revertants ranged between a minimum of 38.7 ± 3.8 and a maximum of 48.7 ± 3.5 in the absence of β G and between 41.3 ± 4.0 and 53.7 ± 4.2 in the presence of β G (means \pm SD of triplicate plates). The overall means (\pm SD) of spontaneous revertants recorded in the 49 experiments were 43.7 ± 2.7 in the absence of β G and 47.1 ± 5.2 in the presence of β G.

The overall mean (\pm SD) of MI produced by AFB₁ (0.1 μ g/plate) in the 49 experiments was 32.1 ± 2.9 in the absence of β G (range, 25.1–36.7) and 31.7 ± 3.1 in the presence of β G (range, 25.4–38.1). The values for CSCs (0.05 cigarette equivalents/plate) were 21.9 ± 3.1 (range, 16.2–26.7) and 23.3 ± 2.6 (17.2–26.8), respectively.

Mutagenicity of Urine Concentrates from All Subjects Recruited into the Trial. For internal use, the results of mutagenicity assays performed with all urine samples from subjects recruited into the trial, which were analyzed as blind-coded samples, were reported in 889 standardized forms, which are available in both Genoa and Baltimore laboratories.

Before analyzing the results, we decided to clean the data by eliminating all samples having either an anomalous creatinine (≤ 0.1 mg/ml) or an excessive toxicity in the Ames test (maximum nontoxic dose ≤ 12.5 μ l/plate). In fact, it was apparent that even very small changes of MI at low doses (e.g., MI values of 0.25, 0.11, and 0.02 versus 0.25, 0.06, and 0 at 1.56, 0.78, and 0.39 μ l/plate, respectively) dramatically affected the slope of the regression line, thereby rendering the MI value (at 100- μ l unconcentrated urine) unreliable. After this criterion, 10 samples were discarded because of too low creatinine, and 22 samples were discarded because of the excessive toxicity in the Ames test. Of these, 18 were toxic both in the presence and in the absence of β G, 3 were toxic only in the presence of β G, and 1 was toxic only in the absence of β G.

Before normalization according to creatinine concentrations, in the absence of β G, MI values ranged between 0.11 and 11.08; 31 (9.0%) had an MI value < 0.5 , 137 (39.7%) had an MI value of 0.5–1.0, and 177 (51.3%) had an MI value > 1.0 , meaning at least a doubling of spontaneous revertants at 100 μ l/plate. In the presence of β G, MI values ranged between 0.13 and 9.94; 36 (10.5%) had an MI value < 0.5 , 163 (47.5%) had an MI value of 0.5–1.0, and 144 (42.0%) had an MI value > 1.0 .

The identity of samples was disclosed by the study coordinator (T. W. Kensler) after having sent all data from Genoa to Baltimore. Table 2 summarizes the results of mutagenicity assays (MI/g creatinine), expressed either as median or mean \pm SD, as related to the intervention arm (either placebo or oltipraz 125 mg daily or 500 mg once per week), collection time (either 5th or 7th week), and supplement of the metabolic system with β G. No statistically significant variation of mutagenicity could be ascribed to these variables. In particular, after log transformation, because of the skewness of the data, the MI/g creatinine values overlapped among the three treatment groups. Irrespective of log transformation, no significant difference was ob-

Table 2 Synthesis of the results of urine mutagenicity assays (all subjects) performed in the framework of the oltipraz chemoprevention trial

Intervention arm	Intervention time (wk)	β G in the assay	No. of samples	Mutagenicity (MI/g creatinine)	
				Median	Mean \pm SD
Placebo	5	–	64	140.1	190.9 \pm 283.3
		+	63	128.9	180.2 \pm 260.2
	7	–	68	164.5	208.6 \pm 190.5
		+	68	144.6	186.2 \pm 148.8
Oltipraz (125 mg daily)	5	–	53	132.7	213.9 \pm 304.0
		+	54	127.5	191.7 \pm 158.9
	7	–	49	146.8	212.4 \pm 224.1
		+	48	139.8	186.2 \pm 153.4
Oltipraz (500 mg weekly)	5	–	53	153.7	198.2 \pm 153.0
		+	52	147.3	171.8 \pm 172.5
	7	–	58	154.5	199.4 \pm 163.4
		+	58	154.1	207.4 \pm 170.6

served for either oltipraz arm as compared with placebo, as assessed by nonparametric analyses (Wilcoxon test and Kruskal-Wallis test).

The comparison of smokers and nonsmokers by nonparametric analyses yielded inconsistent results. In fact, urine mutagenicity was significantly higher in smokers ($P < 0.05$) but only on the 5th intervention week and when β G was added to S9 mix. Additionally, after having excluded smokers, no statistically significant difference was observed as related either to the intervention arm, collection time, or gender.

Twenty of the urine samples were from 10 subjects positive for HBsAg. In this small subset of subjects, the overall means (\pm SD) of MI/g creatinine were 200.9 ± 92.0 when the samples were assayed in the absence of β G and 194.7 ± 108.3 when the samples were assayed in the presence of β G. There was no statistically significant difference between HBsAg-positive subjects and the remaining subjects recruited into the trial. However, within HBsAg-positive subjects, there was a general trend to a decrease of mutagenicity in those treated with oltipraz as compared with the placebo. Despite the small number of samples, the observed decrease was statistically significant on the 5th intervention week in the subjects treated with the drug at the weekly dose of 500 mg, when the samples were assayed in the absence of β G (176.4 ± 49.0 versus 317.6 ± 75.1 ; $P < 0.05$ as assessed by Student's *t* test), and was borderline to significance when the samples were assayed in the presence of β G (175.9 ± 45.8 versus 347.1 ± 152.8 ; $P = 0.08$).

Mutagenicity of Urine Concentrates from the Cigarette Smokers Recruited into the Trial. Of the 345 urine samples available after cleaning the data, 101 (29.3%) were from smokers, smoking 2–30 cigarettes/day (14 ± 7 , mean \pm SD). The results of mutagenicity assays performed in this subset of subjects are shown in Table 3. Again, there was no statistically significant difference as related either to the presence of β G in the metabolic system, to the intervention arm, and to the collection time.

The ratio of urinary cotinine:creatinine ranged between 5 and 23,861 ($2,228 \pm 3,381$, mean \pm SD) and was correlated with the self-reported number of cigarettes smoked per day both on the 5th and 7th intervention week (Table 4). Irrespective of the supplement of S9 mix with β G, the urinary mutagenicity (MI/g creatinine) was significantly correlated with the number of cigarettes smoked per day, which was especially evident

Table 3 Synthesis of the results of urine mutagenicity assays (smokers only) performed in the framework of the oltipraz chemoprevention trial

Intervention arm	Intervention time (wk)	β G in the assay	No. of samples	Mutagenicity (MI/g creatinine)	
				Median	Mean \pm SD
Placebo	5	-	19	165.3	183.3 \pm 104.5
		+	18	145.7	175.5 \pm 111.0
	7	-	21	128.4	185.1 \pm 140.0
		+	21	115.0	183.0 \pm 147.7
Oltipraz (125 mg daily)	5	-	19	154.9	202.5 \pm 119.6
		+	20	197.6	258.8 \pm 188.0
	7	-	17	128.6	161.3 \pm 69.0
		+	17	167.9	184.4 \pm 111.9
Oltipraz (500 mg weekly)	5	-	12	154.4	263.3 \pm 247.4
		+	12	158.7	261.9 \pm 324.7
	7	-	13	177.9	205.2 \pm 108.7
		+	13	155.7	183.3 \pm 95.8

when pooling the data of the samples collected on the 5th and 7th intervention week (Table 4, Fig. 3). Even higher was the correlation between urinary mutagenicity and cotinine/creatinine, which was statistically significant not only by pooling the data but also by evaluating the samples collected on each intervention time (Table 4, Fig. 3).

Genotoxicity of Urine Concentrates in *E. Coli*. A total of 36 of the 394 urine samples tested (9.1%) displayed a significantly increased lethality in the DNA repair-deficient strain CM871 (*uvrA*⁻, *recA*⁻, *lexA*⁻) as compared with the wild-type WP2 (Table 5). There was no significant difference amenable to the intervention arm and time both in the frequency of positive results and in the genotoxic potency evaluated in this test system. Among 107 samples from smokers, 5 were genotoxic (4.7%), whereas among 287 samples from nonsmokers, 31 were genotoxic (10.8%). This difference is close to statistical significance when analyzed either by χ^2 ($P = 0.06$) or Fisher's exact test ($P = 0.08$).

Discussion

The present study provides an example of extensive application of urinary genotoxicity assays as a tool for evaluating possible protective effects in Phase II chemoprevention trials. On a more limited scale, some modulation of urinary mutagenicity could be detected in previous studies in humans, *e.g.*, in individuals eating cooked beef after treatment with *Lactobacillus acidophilus*-fermented milk (17), individuals eating fried ground beef after treatment with *Lactobacillus casei* (18), individuals smoking beedies or cigarettes after treatment with turmeric (19), cigarette smokers after treatment with *N*-acetylcysteine (10), and individuals eating raw fish and cooked beef after administration of Tochu tea (20).

From a technical point of view, of the eight *S. typhimurium* strains tested, the OAT-overproducing strain YG1024 was found in preliminary assays to be the most sensitive to the mutagenic actions of CSCs and urine concentrates from individuals living in Qidong County. At the same time, sensitivity to AFB₁ was of the same order of magnitude in YG1024, its parental strain TA98, and the NR-overproducing strain YG1021. These findings confirm the particularly high sensitivity of these derivatives to a number of mutagenic compounds (21), as well as to complex mixtures, particularly those which are rich in aromatic amines and heterocyclic amines, such as

Table 4 Correlations between self-reported number of cigarettes smoked per day, urinary cotinine/creatinine, and urinary mutagenicity (MI/g creatinine), with (β G+) or without (β G-) supplement of β G to S9 mix

Correlations	Intervention time		
	5th wk	7th wk	5th + 7th wk
No. of cigarettes vs cotinine/creatinine	$r = 0.463$	$r = 0.425$	$r = 0.394$
	$P = 0.0006$	$P = 0.002$	$P < 0.0001$
MI/g creatinine (S9 + β G-) vs number of cigarettes	$r = 0.243$	$r = 0.151$	$r = 0.200^a$
	$P = 0.10$	$P = 0.30$	$P = 0.05$
MI/g creatinine (S9 + β G+) vs number of cigarettes	$r = 0.219$	$r = 0.319$	$r = 0.269^a$
	$P = 0.14$	$P = 0.025$	$P = 0.008$
MI/g creatinine (S9 + β G-) vs cotinine/creatinine	$r = 0.513$	$r = 0.488$	$r = 0.413^a$
	$P = 0.0002$	$P = 0.0005$	$P < 0.0001$
MI/g creatinine (S9 + β G+) vs cotinine/creatinine	$r = 0.399$	$r = 0.402$	$r = 0.336^a$
	$P = 0.007$	$P = 0.005$	$P = 0.001$

^a See Fig. 3.

cooked proteinaceous food (22), cigarette smoke, CSCs, and smokers' urines (16, 23).

In all cases, mutagenicity of urines required the presence of an exogenous metabolic system. Supplementation with β G, aimed at hydrolyzing mutagens that are excreted in urine primarily as β G conjugates, had variable effects on the mutagenicity of urine concentrates. On the whole, however, the mean values of mutagenicity obtained by testing the urine samples collected both from the 20 subjects examined in preliminary experiments and the 201 subjects recruited into the trial were not significantly affected by β G. The variable effects of β G on urinary mutagenicity have already been reported and discussed (*e.g.*, see Ref. 24 and the references therein reported).

An unexpected result was that heating of both CSCs and concentrated urine samples resulted in some increase of their mutagenicity, although this was not a general rule and, as also confirmed by more recent analyses,⁴ the effect may vary depending on the type of CSCs and from experiment to experiment. This finding is reassuring as to the stability of urine mutagenicity under nonoptimal storage conditions. A tentative interpretation is that urine contains inhibitors of mutagenicity, which can at least partially be removed upon heating. Irrespective of smoking habits, human urine has already been shown to contain substances which strongly inhibit the bacterial mutagenicity of aromatic and heterocyclic amines, presumably by binding the parent compounds before their metabolic activation (25). An alternative hypothesis is that heating might favor oxidation of promutagenic components to mutagenic derivatives. Both hypotheses warrant further studies.

In addition to the reversion test in *his*⁻ *S. typhimurium*, we assayed all urine concentrates in a DNA repair test in *trp*⁻ *E. coli*, which is particularly sensitive to direct-acting genotoxins (15). Irrespective of the intervention time and arm, only the 9.1% of tested samples was genotoxic in this test system. Although CSCs consistently induced a greater lethality in the DNA repair-deficient strain (CM871) than in the wild type

⁴ A. Camoirano and S. De Flora, unpublished data.

Fig. 3. Correlation between mutagenicity (MI/g creatinine) of smokers' urines in *S. typhimurium* YG1024 in the presence of S9 mix, without (S9+ β G-) or with β G (S9+ β G+), and either the number of cigarettes smoked daily or the concentrations of urinary cotinine/creatinine. The data recorded on the 5th and 7th intervention weeks were pooled.

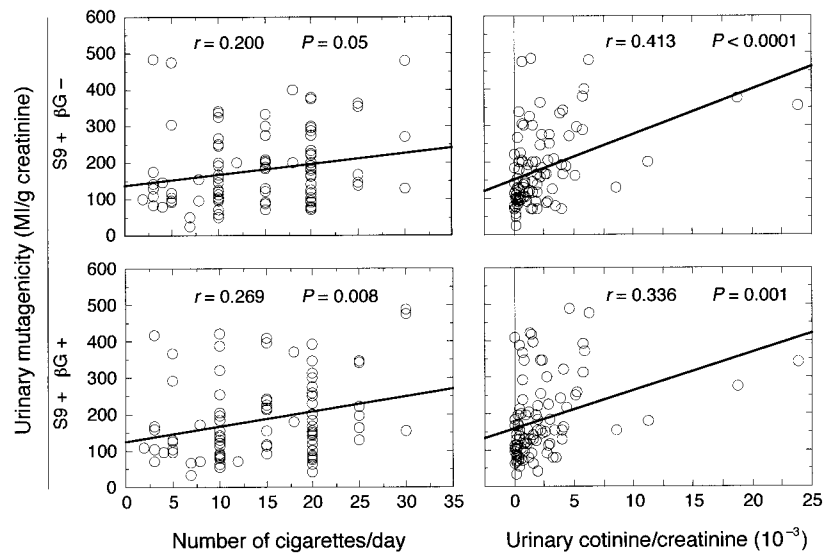


Table 5 Differential lethality of urine samples in *E. coli*

Intervention arm	Intervention time (wk)	No. of positive/total samples (%)	Genotoxic potency ^a (mean \pm SD)
Placebo	5	7/73 (9.6%)	74.6 \pm 81.5
	7	6/74 (8.1%)	79.6 \pm 50.7
Oltipraz (125 mg daily)	5	5/61 (8.2%)	61.4 \pm 54.8
	7	7/57 (12.3%)	110.2 \pm 21.6
Oltipraz (500 mg weekly)	5	6/65 (9.2%)	229.2 \pm 207.5
	7	5/64 (7.8%)	100.8 \pm 45.1

^a The genotoxic potency is expressed as the mean (\pm SD) difference in the areas of bacterial killing (in mm²) produced in strains CM871 and WP2, calculated among those samples in which the difference between these two strains was statistically significant.

(WP2), without any need for an exogenous metabolic system, urine samples from nonsmokers were more frequently positive than samples from smokers. This suggests that the direct activity of a minority of urine samples in this system is mainly attributable to genotoxic agents other than cigarette smoke.

The reliability of the methodology used for assessing the mutagenicity of urines in the chemoprevention trial is supported both by the satisfactory intraindividual reproducibility and by the observed relationships to the smoking dose. In fact, mutagenicity did not vary >2-fold in the majority of paired samples collected on the 5th and 7th intervention week and, irrespective of treatment, the mean mutagenicity values of urines collected on the two intervention times were almost overlapping. As to smoking habits, urine mutagenicity was significantly higher in smokers than in nonsmokers but only at one collection time and in the presence of β G. This indicates that mutagenicity of the urines in the examined population is not only attributable to cigarette smoking but also to other exposures. In this context, it is noteworthy that the mutagenicity of condensates from the brands of cigarettes which are the most commonly used in Qidong was rather low when compared with brands of Western cigarettes. This outcome provides limited consolation, however, as it has been estimated that in the People's Republic of China, there are >300 million smokers, which is roughly the same number as in all industrialized countries combined (26, 27).

In any case, the mutagenicity of urine samples from smokers was significantly correlated with the self-reported number of cigarettes smoked per day, as well as with the cotinine levels measured in the same urine samples. Indeed, after having analyzed almost 400 blind-coded samples in a biological test system involving the use of more than 10,000 agar plates, this finding speaks in favor of the appropriate design and conduction of the study and of the internal validity of the results.

Accordingly, the consistent lack of modifying effects of oltipraz not only in the entire cohort but also in the subset of smokers should be interpreted as a true negative result. In fact, this chemopreventive agent failed to modulate the urinary excretion of promutagens resulting either from cigarette smoking or other unidentified sources. Focusing on smoke-related pulmonary carcinogenesis, oral oltipraz displayed conflicting results in animal models. It inhibited the formation of lung tumors induced by benzo[a]pyrene in ICR/Ha mice (28) but was ineffective in A/J mice (29). It was less effective toward lung tumors induced in ICR/Ha mice by uracil mustard or diethylnitrosamine (28) and failed to modulate the formation of lung tumors after treatment with the tobacco-specific nitrosamine 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone in A/J mice (30). In Sprague Dawley rats exposed whole-body to cigarette smoke, the oral administration of oltipraz inhibited the formation of lipophilic DNA adducts in the lung, as assessed by ³²P postlabeling (31). However, it was ineffective when measuring by means of a suitable chromatographic procedure the total levels of ³²P postlabeled DNA adducts, including massive smoke-related diagonal radioactive zones (32). Collectively, the failure to observe any protective action of oltipraz against urinary mutagenesis in smokers coupled with the limited effects of oltipraz on tobacco-related carcinogenesis in experimental models suggests that this agent will not be effective in the prevention of lung cancer in humans.

The primary goal of the oltipraz trial was to evaluate biomarkers related to dietary exposures to AFB₁. Sequential immunoaffinity and liquid chromatography coupled with mass spectrometry and fluorescence detection was used to identify and quantify the Phase 1 metabolite, AFM₁, and the Phase 2 metabolite, aflatoxin-mercapturic acid, in the collected urine samples. One month of weekly administration of 500-mg oltipraz led to a significant (51%) decrease in median levels of

AFM₁ excreted in urine compared with placebo, whereas daily intervention 125 mg of oltipraz led to a significant, 2.6-fold increase in the median levels of aflatoxin-mercaptopuric acid but did not appreciably affect formation of AFM₁. Thus, sustained low dose oltipraz increased Phase 2 conjugation of aflatoxin, yielding higher levels of mercaptopuric acid, whereas intermittent, high-dose oltipraz inhibited the Phase 1 activation of aflatoxin.

Although AFM₁ is usually considered to be a detoxification product of AFB₁, it still maintains a weak carcinogenicity compared with its parent compound (33) and is about 10 times less potent than AFB₁ in the Ames test (34). After completion of the present study, we evaluated the influence of excreted levels of AFM₁ measured on the samples collected during the 5th intervention week (35) on urine genotoxicity. Irrespective of treatment with oltipraz, we did not find any positive correlation between urinary AFM₁ and mutagenicity (data not shown). Moreover, because AFM₁ has been reported to be highly toxic to human cell lines, in the absence of metabolic activation (36), we checked whether urinary AFM₁ levels on the 5th intervention week were correlated with the direct DNA damaging activity of concentrated urines in *E. coli*. Again, we did not find any relationships (data not shown). Therefore, it appears that our urine genotoxicity assays did not provide information concerning the ability of oltipraz to modulate the internal dose to AFB₁.

The simultaneous exposure to AFB₁ and infection with HBV deserves particular interest, also because infection with hepadnaviruses has been shown to enhance the metabolism of chemical hepatocarcinogens and formation of DNA adducts in the liver (37, 38). In the present study, there was no significant difference in the urinary mutagenicity evaluated in 10 HBV carriers and in the remaining 191 subjects recruited into the trial. No effect of the HBsAg status had previously been observed on the levels of serum aflatoxin-albumin adducts in people living in the same geographic area where this trial was carried out (6). Interestingly, we observed a trend to a decrease of urinary mutagenicity in HBV carriers treated with oltipraz as compared with those receiving the placebo, which was statistically significant on the 5th intervention week in HBV carriers receiving the drug at a dose of 500 mg once per week. Because of the small number of HBV carriers, we cannot draw any firm conclusion, but this result warrants attention, also in the light of the recent finding that oltipraz inhibits HBV transcription through elevation of p53 protein (39) and knowledge that HBV elevates the expression of Phase 2 genes (40).

On the basis of our data, we cannot speculate on the identity of other factors, presumably resulting from the diet, which contributed to the observed genotoxicity of urines in the examined population. Like other end points that are influenced by metabolic mechanisms, a considerable interindividual variability in the urinary excretion of genotoxins can be ascribed to genetic polymorphisms related to the metabolism of carcinogens (22, 41, 42). We raise the hypothesis that genetic polymorphisms not only may account for part of the interindividual variability in susceptibility to carcinogens, and in the expression of biomarkers of exposure and effect, but also may play a role in the individual responsiveness to certain chemopreventive agents.

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