SHORT COMMUNICATION

Inhibition of aflatoxin M₁ excretion in rat urine during dietary intervention with oltipraz

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4-Methyl-5-(2-pyrazinyl)-1,2-dithiole-3-thione (oltipraz) is an effective chemopreventive agent against several classes of carcinogens in many target organs. Induction of carcinogen detoxication enzymes, particularly glutathione S-transferases, appears to be an important component of the protective actions of oltipraz. It has recently been observed that addition of oltipraz to rat liver microsomes or to cultured human hepatocytes blocks the oxidative metabolism of aflatoxin B₁ (AFB₁) to its 8,9-oxide and the hydroxylated derivative aflatoxin M¹ (AFM¹). Oltipraz is a competitive and perhaps irreversible inhibitor of cytochromes P450 1A2 and 3A4. To determine whether oltipraz can affect cytochrome P450-dependent metabolism of AFB₁ in vivo we have assessed the effect of oltipraz on the urinary excretion of oxidative metabolites of AFB₁ before, during and after a transient intervention. Male F344 rats, housed individually in glass metabolism cages, were gavaged daily with 25 μg [³H]AFB₁ for 28 consecutive days. Starting on day 6 and extending to day 16 half of the rats were fed a diet supplemented with 0.075% oltipraz. Sequential 24 h urine samples were collected and a subset analyzed for AFB₁ metabolites. AFM¹ was the major metabolite detected in all urine samples, accounting for 2–6% of the administered dose. The excretion of AFM¹ was greatly reduced (77%) during the active phase of the intervention, when oltipraz was added to the diet, but rapidly returned to control levels after cessation of oltipraz administration. This inhibition of AFM¹ excretion was not seen in animals receiving oltipraz by gavage 24 h prior to dosing with AFB₁. Collectively these data are consistent with the view that oltipraz or a short-lived metabolite inhibits cytochrome P450 1A2 in vivo.

Chemoprevention against aflatoxin-induced carcinogenesis in experimental animals has been demonstrated using a variety of agents, such as butylated hydroxyanisole, butylated hydroxytoluene, ethoxyquin and 4-methyl-5-(2-pyrazinyl)-1,2-dithiole-3-thione (oltipraz*) (1–3). The chemopreventive actions of these agents have been associated with the induction of carcinogen detoxication enzymes (4–6). The ultimate carcino-
urinary excretion of oxidative metabolites of AFB\(_1\) before, during and after a transient intervention.

Aflatoxins B\(_1\), G\(_1\), P\(_1\) and M\(_1\) were obtained from Aldrich Chemical Co. (Milwaukee, WI). \([\text{H}]\text{AFB}_1\) was purchased from Moravek Biochemicals (Brea, CA) and was determined to be >98% radiochemically pure by HPLC. Oltipraz was obtained from the Chemoprevention Branch, National Cancer Institute. All chromatographic solvents were of HPLC grade quality and all other chemicals were of the highest grade commercially available.

Four male F344 rats were gavaged daily with 25 \(\mu\)g \([\text{H}]\text{AFB}_1\) (134 mCi/mmol) for 28 consecutive days. Starting on day 6 and extending to day 16 two of the rats were fed a diet supplemented with 0.075% oltipraz, as detailed in Bolton et al. (14). All rats were housed individually in glass metabolic cages throughout the study period. Sequential 24 h urine samples were collected on ice and then stored at \(-80^\circ\text{C}\) until analysis. A second group of male F344 rats were acclimated to the control AIN-76A diet for 1 week and then gavaged on two consecutive days with oltipraz (15 mg in 200 \(\mu\)l of a vehicle consisting of 1% Cremophor and 25% glycerol in distilled water), followed on the third day by dosing with AFB\(_1\). Groups of four rats were gavaged with graded doses of AFB\(_1\): 0.027, 0.160 and 0.800 mg AFB\(_1\)/kg body wt in 100 \(\mu\)l dimethyl sulfoxide. Urine was then collected daily over the following 3 days.

One milliliter aliquots of the 24 h urine samples were analyzed. Each sample was spiked with aflatoxin G\(_1\) (390 ng) as an internal standard, diluted with \(\text{H}_2\text{O}\) (3 ml), acidified with 5 M acetic acid (100 \(\mu\)l), loaded onto a pre-washed Sep-Pak and prepared for HPLC analysis as previously described (23). The HPLC column used was a Spherosorb C18 (II), 5 \(\mu\)m, 24 cm X 4.6 mm i.d. (Phenomenex), using 25 mM NH\(_4\)OAc, pH 5.0 as solvent A and 16% acetonitrile/EtOH as solvent B. A linear gradient of 12-35% B was generated over 45 min at a flow rate of 1.0 ml/min and a column temperature of 40°C. Diode array detection was part of a Millennium 2000 HPLC System (Waters-Millipore Corp.). Under these conditions the retention times of the standards for AFB\(_1\)-glutathione, AFB\(_1\)-N-acetylcysteine, AFB\(_1\)-N\(_7\)-guanine, AFM\(_1\), and aflatoxin P\(_1\) were 10.4, 14.4, 20.1, 28.2 and 31.1 min, respectively. For mass spectral analysis a urine sample was processed without addition of aflatoxin G\(_1\) for chromatography as described above. The liquid chromatography/mass spectrometry system used was a Finnegan TSQ 7000 equipped with a Hewlett Packard 1050 gradient HPLC. Shown in Figure 1 is the mass spectrum obtained with AFM\(_1\) isolated from the in vivo sample; it is identical to the spectrum of an authentic standard. Inasmuch as the HPLC conditions used for the liquid chromatography/mass spectrometry included acetonitrile, the AFM\(_1\) spectrum has a positive ion adduct (m/z 329), a monosodium adduct (m/z 351) and a monosodium-acetonitrile adduct (m/z 392).

To assess the effects of oltipraz on AFB\(_1\) metabolism in vivo urine samples were analyzed from every other day of the dosing protocol starting on day 2 and continuing to day 24. The major metabolite detected in all urine samples by HPLC was AFM\(_1\), accounting for 2-6% of the daily AFB\(_1\) dose. This finding is consistent with previous data from our laboratory (23). Figure 2 shows the excretion pattern for AFM\(_1\) following intervention with oltipraz. Introduction of oltipraz into the diet produced a rapid diminution in the levels of AFM\(_1\) excretion. Overall, AFM\(_1\) excretion throughout the 10 day intervention was decreased by 77\% (\(P < 0.01\), Student's t-test) as compared with the control. Excretion levels of AFM\(_1\) returned to control levels within 48 h of cessation of oltipraz administration. No enhancement of AFM\(_1\) excretion, which might be indicative of induction of cytochrome P450s by oltipraz, was seen during the post-intervention phase.

To further explore this finding the effects of oltipraz pre-treatment on urinary excretion of AFM\(_1\) after administration of AFB\(_1\), at three dose levels was determined. In this experiment oltipraz was administered by gavage and preceded exposure to AFB\(_1\) by 48 and 24 h. Over the range of doses (0.027-0.800 mg AFB\(_1\)/kg) ~3% of the AFB\(_1\) dose was excreted in urine within 24 h as AFM\(_1\). However, oral pre-treatment of rats with oltipraz had no effect on the total excretion of AFM\(_1\) during the 24 h period following AFB\(_1\) exposure at any dose of AFB\(_1\) (data not shown).

The failure to observe an effect of oltipraz on the excretion of AFM\(_1\) when AFB\(_1\) was administered 24 h after the last dose of oltipraz sharply contrasts with the pattern observed when oltipraz and AFB\(_1\) were administered simultaneously. The kinetic features of oltipraz disposition and cytochrome P450 turnover may account for these different effects of oltipraz on AFM\(_1\) metabolism. Pharmacokinetic studies in rats indicate that oltipraz is extensively metabolized and that the
plasma elimination half-life is ~2.5 h (24,25). Thus oltipraz appears to be rapidly eliminated. Consistent with this view, autoradiographs of mice following administration of [14]C)oltipraz indicate little residual drug in the liver and other tissues by 24 h. Enzyme kinetic studies on heterologously expressed CYP1A2 indicate that oltipraz is principally a competitive inhibitor, with a $K_i$ of 10 μM (16), a pharmacologically achievable concentration in rats and humans. CYP3A4 is also inhibited, but with an 8-fold higher $K_i$. On solely kinetic grounds concurrent administration of oltipraz and AFB₁ would be expected to inhibit aflatoxin metabolism in vivo. The dramatic reduction in the urinary elimination of AFB₁ during the feeding of oltipraz bears out this prediction. Moreover, the rapid clearance of oltipraz from rat tissues coupled with the rapid turnover of hepatic cytochrome P450s (26) explains the rapid restitution of AFB₁ metabolism to AFB₁ once oltipraz feeding was stopped. The failure of 24 h or earlier pre-treatment with oltipraz by gavage to alter AFB₁ metabolism to AFB₁ further exemplifies the short-term inhibition of cytochrome P450 activity in vivo.

Differences in patterns of AFB₁ excretion, which are dependent upon the relative timing of oltipraz and AFB₁ exposures, provide possible insights into the mechanisms of action of oltipraz in vivo. Primiano et al. (27) have recently reported that intermittent dosing with oltipraz (i.e. once weekly, per os) during continuous exposure to AFB₁ dramatically reduces the hepatic burden of tumors in rats. Such an outcome suggests that a protracted pharmacodynamic action contributes substantially to chemoprevention by oltipraz. The persistent induction of GST and other detoxification enzymes, such as aflatoxin-aldehyde reductase may be important components in the efficacy of intermittent dosing. Conversely, the transient inhibition of AFB₁ formation and elimination observed in the present study would suggest that alteration of cytochrome P450 activity need not be an essential component of oltipraz action in rats. Presumably aflatoxin oxidation (exo AFBO and AFB₁ formation) proceeded largely unabated during the intermittent chemoprevention regimen, where carcinogen was administered daily and oltipraz weekly. This point notwithstanding, daily administration of oltipraz is more effective than weekly. Perhaps reduction in AFB₁ production, and its contribution to the cytotoxic, autopromoting component of AFB₁ hepatocarcinogenesis, is an important element of the chemopreventive outcome.

These findings have practical implications for the use of oltipraz in clinical chemoprevention trials. The question has been posed as to whether enzyme inducers such as oltipraz, so effective in the rat, might demonstrate comparable protective activity against aflatoxin-associated hepatocarcinogenesis in humans (16). The potential for GST-dependent detoxification in human liver does not appear to be as great as in the rat, because human liver appears to have about one order of magnitude less GST activity towards exo AFBO (28). Thus induction of human GST may not be as effective in AFB₁ detoxication as it is in rats. If inhibition of cytochrome P450 activities were to be a major mode of action of oltipraz in humans, then only frequent administration of the drug would demonstrate chemopreventive activity. In that case, intervention drug costs and the likelihood of side-effects would be exaggerated, thereby counter-indicating its use. As an approach to discern the mechanisms relevant to humans, studies examining the effects of daily and weekly doses of oltipraz on aflatoxin metabolism and adduct formation are currently in progress in individuals exposed to aflatoxins and at high risk for liver cancer (29).

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