Monoterpenes reduced adducts formation in rats exposed to aflatoxin B₁

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Perillyl alcohol and d-limonene are naturally occurring plant compounds that exhibited anti-carcinogenic activities in mammary tumor models. The effects of these monoterpenes at the initiation stage of aflatoxin B₁-induced hepatocarcinogenesis were investigated. Male F₃₄₄ rats were fed control or treatment diets throughout the study and exposed to aflatoxin for 5 days. Three days after the last aflatoxin dose, blood and liver samples were obtained. Analysis of liver samples showed that both limonene and perillyl alcohol significantly inhibited (p<0.05) aflatoxin-DNA adducts formation in hepatocytes. The monoterpenes may have potential for use as chemopreventive agent against aflatoxin-induced liver cancer.

Keywords: Aflatoxin B₁, hepatocarcinogenesis, monoterpenes, chemoprevention.

INTRODUCTION

Liver cancer (primary hepatocellular carcinoma) is a major public health hazard in the developing countries of Africa and Asia. The etiology of this disease implicates both environmental (diet) and infectious (hepatitis B and C) factors. Aflatoxin B₁ (AFB₁) is a major contaminant of foods in the humid tropical regions of Africa and Asia and has been linked, with hepatitis B and C infections, to the high incidence of liver cancer in these regions (Montalto et al., 2002; Sarin et al., 2001; Wild et al., 2000). The poor prognosis and survival rates among liver cancer patients indicate that prevention may offer a useful approach to reducing the incidence of human liver cancer in high-risk areas. Economic considerations, however, dictate that such chemopreventive agent must be inexpensive and readily available to the general populace especially in the developing countries.

Monoterpenes (d-limonene, perillyl alcohol, etc) are naturally occurring, nontoxic and ubiquitous plant compounds. Dietary sources of monoterpenes include d-limonene in orange and citrus peel oils, caraway, dill and green leafy vegetables; perillyl alcohol in cherry and spearmint, other monoterpenes in lemongrass oil, and spearmint (Crowell, 1999). It was reported earlier that the monoterpenes, principally d-limonene and its analogs, elicited preventive (Elegbede et al., 1986; Haag et al., 1992) or therapeutic (Maltzman et al., 1989) effects against experimentally induced mammary tumors in rat cancer models. Mills et al. (1995) also reported that perillyl alcohol significantly inhibited the growth of diethylnitrosamine-induced liver tumors in rats by increasing the frequency of apoptosis. Although the mechanism of action of the monoterpenes is still not yet clearly understood, both limonene (Poon et al., 1996; Vigushin et al., 1998) and perillyl alcohol (Ripple et al., 1998, 2000) have been tested in Phase I human clinical trials.

In this study, the effects of the monoterpenes, d-limonene and perillyl alcohol (Figure 1), at the initiation stage of AFB₁-induced liver carcinogenesis in rats were investigated. Specifically, male F₃₄₄ rats were fed control or test diets [5% d-limonene, 2% perillyl alcohol, and 30mg% oltipraz] from two weeks before aflatoxin exposure to the end of the study. For aflatoxin exposure, each animal was anesthetized and intubated with [³H]-AFB₁ (260 µCi/µmol; 250 µg/kg body wt) in tricaprylin (Groopman et al., 1992). Three days after the last intubation with [³H]-AFB₁, animals were sacrificed and blood and liver were obtained. Twenty-four hour urine samples were also collected. Tritium-labeled...
aflatoxin B$_1$ (\(^{13}H\)-AFB$_1$) was used to facilitate tracking of aflatoxin metabolic products. A non-therapeutic dose of the known hepatic chemopreventive agent, oltipraz, was included as positive control.

**RESULTS AND DISCUSSION**

During the period of intubation with aflatoxin, rats in control, d-limonene and oltipraz groups lost 7, 8 and 4% of their body weights respectively while those on perillyl alcohol diets gained 5%. Livers of rats on the experimental diets appeared grossly normal. Average liver weights (g) for the respective groups were 5.6±1.29 for control, 8.0±1.76 for d-limonene, 9.8±0.78 for perillyl alcohol, and 9.6±0.94 for oltipraz. Animals on the treatment diets had significantly (p<0.05) larger livers than the Control animals.

The effect of dietary monoterpenes against AFB$_1$-induced initiation of liver cancer was assessed as the ability of the monoterpenes to inhibit the formation of \(^{13}H\) AFB$_1$-DNA adducts in liver cells. The levels of AFB$_1$-DNA adduct formed in the livers of animals fed different diets are shown in Table 1. Animals fed perillyl alcohol or d-limonene diets had significantly (p<0.01) lower reduction (52% and 46%, respectively) in the level of AFB$_1$-DNA adducts compared to the control group. Oltipraz, which was used at a non-therapeutic dose, elicited a 22% reduction in adduct formation compared to the control. This reduction in DNA adduct formation with oltipraz was consistent with earlier reports (Bolton et al., 1993; Groopman et al., 1994).

Urinary excretion of aflatoxin products has been used as a measure of exposure to the carcinogen. In this study we measured aflatoxin metabolic products excreted in the urine as \(^{13}H\) aflatoxin equivalents (nmoles/\(\mu\)g creatinine). Three days after the last AFB$_1$ intubation, the presence of labeled AFB$_1$ products in the blood and urine of exposed animals was measured. There were no significant differences in blood levels of aflatoxin products between the control and monoterpenes groups. Rats in the monoterpene-fed groups had lower urinary aflatoxin B$_1$ compared to the control group (Table 2).

### Table 1: Inhibition of hepatic AFB$_1$-DNA adduct formation in rats by dietary monoterpenes.

<table>
<thead>
<tr>
<th>Dietary Groups</th>
<th>AFB-DNA Adducts (dpm/mg DNA)</th>
<th>AFB-DNA Adducts (pmoles (^{13}H)AFB/mg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>424 ± 50.1</td>
<td>1654 ± 195.7</td>
</tr>
<tr>
<td>5% d-limonene</td>
<td>228 ± 38.9\textsuperscript{a}</td>
<td>890 ± 151.5\textsuperscript{a}</td>
</tr>
<tr>
<td>2% perillyl alcohol</td>
<td>205 ± 19.4\textsuperscript{a}</td>
<td>798 ± 75.4\textsuperscript{a}</td>
</tr>
<tr>
<td>30mg% oltipraz</td>
<td>333 ± 50.6\textsuperscript{b}</td>
<td>1298 ± 197.4\textsuperscript{b}</td>
</tr>
</tbody>
</table>

Liver DNA was extracted and quantitated by the method of Sambrook et al. (1989). Aliquots of DNA extracts were counted for \(^{13}H\) radioactivity in a Beckman LS 6000 IC Scintillation Counter. Values represent Mean ± SEM, (n=6).

\textsuperscript{a}Values are significantly different from the Control (p<0.01)

\textsuperscript{b}Values are significantly different from perillyl alcohol (p<0.05).

### Table 2: Distribution of \(^{13}H\) aflatoxin metabolites in blood and urine of rats intubated with aflatoxin B$_1$ and fed different monoterpenes diets.

<table>
<thead>
<tr>
<th>Dietary Groups</th>
<th>Aflatoxin-Albumin Adducts (nmoles (^{13}H)AFB/(\mu)g protein)</th>
<th>Urinary AFB Equivalents (nmoles (^{13}H)AFB/(\mu)g creatinine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.2 ± 2.06</td>
<td>5.5 ± 2.71</td>
</tr>
<tr>
<td>5% d-limonene</td>
<td>7.7 ± 2.45</td>
<td>3.8 ± 0.75</td>
</tr>
<tr>
<td>2% perillyl alcohol</td>
<td>7.5 ± 1.96</td>
<td>4.7 ± 0.20</td>
</tr>
<tr>
<td>30mg% oltipraz</td>
<td>8.7 ± 2.38\textsuperscript{a}</td>
<td>2.8 ± 1.43\textsuperscript{b}</td>
</tr>
</tbody>
</table>

Rats were fed respective diets throughout the study. Seventy-two hours after the last aflatoxin dose, 24-hr urine and blood samples were obtained. Serum albumin was extracted and quantified (Chapot and Wild, 1991) and urinary creatinine determined (Procedure No 555, Sigma Diagnostics, St Louis, MO). Aliquots of each sample were counted for total \(^{13}H\) activity. Values are Mean ± SD

\textsuperscript{a}Values are significantly different from the Control (p<0.05)

\textsuperscript{b}Values are significantly different from perillyl alcohol (p<0.05)
Incorporation of the monoterpenes in the diet of male F344 rats during the period of exposure to aflatoxin B₁ resulted in significant (p<0.01) reduction in the formation of aflatoxin-DNA adducts in the liver (Table I). The cumulative reduction in the formation of hepatic aflatoxin-DNA adducts were perillyl alcohol (52%), d-limonene (46%), and oltipraz (22%). Both d-limonene and perillyl alcohol significantly (p<0.01) lowered AFB-DNA adduct formation than in the Control group. In earlier studies, Bolton et al. (1993) and Groopman et al. (1994) showed that transient intervention with oltipraz caused about 25% cumulative reduction in levels of hepatic aflatoxin-DNA adducts. According to these authors, later analysis showed that oltipraz reduced the hepatic glutathione S-transferase placental form-positive foci by 54% and 72%, an indication that reduced cumulative adduct formation underestimated the potency of oltipraz (Bolton et al., 1993). By inference, the cumulative effects of d-limonene and perillyl alcohol against aflatoxin-induced liver carcinogenesis may be better than currently assessed.

The mechanism of action of many chemopreventive agents involves alteration of the metabolic fate of carcinogens by modulating the activities of either, or both, the phase I and/or phase II drug metabolizing enzymes. Induction of phase II detoxification enzymes enhances carcinogen inactivation by facilitating the clearance of activated metabolites through conjugation with glutathione (Coles et al., 1985; Kensler et al., 1987) and/or glucuronides (Elegbede et al., 1993). The mechanism of action of the synthetic anticarcinogen oltipraz is by the induction of glutathione S-transferases which facilitate the conjugation of glutathione to aflatoxin-8,9-oxide and its ultimate elimination in the urine (Sparfel et al., 2002). It was reported earlier that the monoterpenes, specifically limonene and a hydroxylated analog, induced phase II hepatic drug metabolizing enzymes (Elegbede et al., 1993) while limonene also decreased the formation of 7,12-dimethylbenz[a]anthracene (DMBA)-DNA adducts in the liver, lung, kidney and spleen of rats intubated with DMBA (Maltzman et al., 1991). DMBA is an inducer of carcinogenesis.

In a randomized, double-blind, placebo-controlled chemoprevention trial in China, Egner et al. (2001) reported that chlorophyllin, a mixture of semisynthetic, water-soluble derivatives of chlorophyll that is used as food colorant and over-the-counter medicine, inhibited aflatoxin adducts in urine samples collected 3 months into the study. It was therefore suggested that chlorophyllin may represent practical means to prevent the development of hepatocellular carcinoma (Egner et al., 2001). Limonene and perillyl alcohol are naturally occurring compounds that are abundant in the peel oil of oranges, lemons, limes, and other plant products that are readily available in the tropics.

In this study, we showed that perillyl alcohol and d-limonene significantly reduced the formation of aflatoxin-DNA adducts in the livers of F344 rats exposed to aflatoxin B₁ for 5 days. Mills et al. (1995) reported that perillyl alcohol inhibited the growth of diethylnitrosamine-induced liver tumors in rats by enhancing tumor cell loss through apoptosis. Our results suggested that perillyl alcohol and d-limonene offered protection against aflatoxin-induced liver tumor formation at initiation by reducing aflatoxin-DNA adducts formation. This finding, taken together with the low toxicity, ready availability and low cost suggests the importance of further studies to evaluate the efficacy of these monoterpenes as potential chemopreventive agents against aflatoxin-induced liver cancer in humans. The monoterpenes appear to have potential as chemopreventive agents against aflatoxin B₁-induced hepatocarcinogenesis in humans.

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