Effects of bicyclol on aflatoxin B\textsubscript{1} metabolism and hepatotoxicity in rats\textsuperscript{1}

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KEY WORDS bicyclol; aflatoxin B\textsubscript{1}; cytochrome P450; metabolism; hepatotoxicity

ABSTRACT

AIM: To study the effect of new antihepatitis drug, bicyclol, on the metabolism and hepatotoxicity of aflatoxin B\textsubscript{1} (AFB\textsubscript{1}) in rats. METHODS: Rats were given bicyclol 300 mg·kg\textsuperscript{-1}·d\textsuperscript{-1} ig for 3 d and then injected ip with AFB\textsubscript{1} 1.5 mg·kg\textsuperscript{-1}. Liver damages were examined 16 h after ip AFB\textsubscript{1}. The \textit{in vitro} metabolism of AFB\textsubscript{1} by bicyclol-pretreated liver microsomes was investigated by HPLC assay. RESULTS: Bicyclol (300 mg·kg\textsuperscript{-1}·d\textsuperscript{-1} for 3 d) pretreatment provided protection against AFB\textsubscript{1} hepatotoxicity as evidenced by the decrease of AFB\textsubscript{1}-elevated serum aminotransferase and hepatic malondialdehyde in rats. Bicyclol pretreatment slightly increased the production of the less toxic metabolite aflatoxin Q\textsubscript{1}. Bicyclol increased liver cytochrome P450 content, CYP 2B1-mediated 7- pentoxyresorufin O-dealkylase (PROD) activity, cytosolic glutathione (GSH) level, and GSH S-transferase (GST) activities. Moreover, bicyclol increased CYP 3A-mediated erythromycin-demethylase and CYP 1A-mediated 7-ethoxyresorufin O-deethylase (EROD) activities. CONCLUSION: Bicyclol protected rats against AFB\textsubscript{1} hepatotoxicity by increasing the detoxifying metabolism of AFB\textsubscript{1} in the liver.

INTRODUCTION

Liver drug metabolizing enzymes are important in the conversion of exogenous drugs, toxins, carcinogens, endogenous steroid hormones, vitamins, and fatty acids\textsuperscript{[1,2]}. A decrease in the activity of these enzymes has been noted in patients with chronic hepatitis or cirrhosis. Furthermore, these patients are highly susceptible to develop liver cancer. Aflatoxin B\textsubscript{1} (AFB\textsubscript{1}), a potent hepatotoxin and hepatocarcinogen, is closely related to the incidence of liver cancer\textsuperscript{[3]}. Liver cytochrome P450 1A, 2B, 3A, and other minor isozymes catalyze the oxidative metabolism of AFB\textsubscript{1}\textsuperscript{[4]}. The formation of the metabolite, AFB\textsubscript{1}-8,9-epoxide, and its subsequent covalent binding to DNA to form AFB\textsubscript{1}-DNA adducts, are critical steps leading to hepatocarcinogenesis\textsuperscript{[5]}. Aflatoxin Q\textsubscript{1}, another oxidative metabolite of AFB\textsubscript{1}, is of low toxicity and carcinogenicity. Bicyclol, 6-methoxycarbonyl-6'-hydroxymethyl-2,3,2',3'-bis (methylenedioxy)-4,4'-dimethoxybiphenyl is a new drug for the treatment of chronic hepatitis B to improve the hepatic function and the rate of HBV-DNA and HBeAg seroconversion in China. Previous studies have shown that bicyclol is very effective in the detoxication of certain hepatotoxins\textsuperscript{[6]}. The protective effect of bicyclol was associated with the modulation of liver microsomal cytochrome P450s. The purpose of our investigation was to study the effect of bicyclol on liver drug metabolizing enzymes and on the metabolism and hepatotoxicity

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of AFB₁ in rats.

MATERIALS AND METHODS

Chemicals  Bicyclol was provided by the Beijing Union Pharmaceutical Plant (purity >99%). AFB₁, aflatoxin M₁ (AFM₁), AFQ₁, aflatoxin G₁ (AFG₁), ethoxyresorufin, 7-pentoxyresorufin, 1-chloro-2,4-dinitrobenzene (CDNB), 5′;5-dithio-bis(2-nitrobenzoic) acid (DTNB), NADPH, NADP, glutathione (GSH), glucose-6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase (Type XV), and erythromycin base were purchased from Sigma Chemical Co (St Louis, MO). 1,2-Dichloroquinone (DCNB), 5′,5-dithio-resorufin, 7-pentoxyresorufin, 1-chloro-2,4-dinitrobenzene (CDNB), 5′;5-dithio-bis(2-nitrobenzoic) acid (DTNB), NADPH, NADP, glutathione (GSH), glucose-6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase (Type XV), and erythromycin base were purchased from Sigma Chemical Co (St Louis, MO). 1,2-Dichloro-4-nitrobenzene (DCNB) was obtained from Fluka AG (Swiss). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) assay kits were purchased from Beijing Chemical Plant (Beijing, China). Other chemicals were of analytical grade and were obtained from the local market.

Treatment of animals  All animals received food and water ad libitum and were maintained under identical conditions. Male Wistar rats weighing (200±20) g were obtained from the Animal Center at the Chinese Academy of Medical Sciences. Four rats in bicyclol group received bicyclol, ig (dissolved in polyethylene glycol-400, PEG-400) 300 mg·kg⁻¹·d⁻¹ once a day for 3 d. The group not given bicyclol received an equivalent volume of PEG-400 ig as vehicle control. Animals were killed by decapitation 24 h after the last dose of bicyclol to obtain tissues for microsome preparation and enzyme assays. For hepatoprotection study, twenty rats were divided into four groups, five rats in each group. Rats in bicyclol group received bicyclol 300 mg·kg⁻¹·d⁻¹ ig once a day for 3 d and the groups of control animals were given an equivalent volume of PEG-400. AFB₁ at 1.5 mg·kg⁻¹ was intraperitoneally injected to rats in AFB₁ treated groups 8 h after last dose of bicyclol. Rats were killed by decapitation 24 h after injection of AFB₁ and then blood and liver samples were promptly collected for assay of serum levels of ALT, AST, and hepatic level of MDA.

Enzyme assays  We prepared hepatic microsomes and cytosols as described by Lesca[27]. Liver protein and cytochrome P450 content were determined by the methods of Lowry et al[89] and Omura et al[91], respectively. The hepatic glutathione (GSH) level was determined as described by Ellman[109]. The spectrophotometric determination of hepatic cytosol glutathione S-transferase (GST) activity was carried out in the presence of potassium phosphate 100 mmol·L⁻¹ buffer (pH 6.5) with GSH 1 mmol·L⁻¹ and CDNB 1 mmol·L⁻¹ or with GSH 5 mmol·L⁻¹ and DCNB 1 mmol·L⁻¹[111]. We determined the activities of microsomal 7-ethoxyresorufin O-deethylase (EROD) and 7-pentoxyresorufin O-dealkylase (PROD) in duplicate, using the dynamic fluorimetric method of Lubet et al with excitation and emission wavelengths of 522 and 586 nm[112]. The assay of microsomal cytochrome P450 isozyme 3A activity was carried out with erythromycin as a substrate[113]. Serum levels of ALT and AST were assayed with the test kits.

In vitro biotransformation of AFB₁ by hepatic microsomes  This assay was performed according to the method described by Monroe et al with modification[114]. The determination was carried out in a final volume of 0.25 mL containing a final concentration (mmol·L⁻¹, pH 7.6) of GSH 5, NADPH 1, sucrose 190, potassium phosphate 60, Tris-HCl 80, NaCl 15, KCl 5, and MgCl₂ 4 and maintained at 37 °C. Rat liver microsomes equivalent to 750 μg protein were measured in the presence of excess mouse cytosol (750 μg) to scavenge the AFB₁-epoxide as the 8,9-dihydro-8-(S-glutathionyl)-9-hydroxy-aflatoxin B₁ (AFB₁-GSH) conjugate. The reaction was initiated by the addition of 5 μL AFB₁ (0.25 g/L in Me₂SO) and terminated after 30 min by the addition of ice-cold 50 μL acetic acid 2 mol·L⁻¹ and 10 μL methanol containing AFG₁, 0.03 g/L as an internal standard. After overnight storage at -20 °C to enhance precipitation of macromolecules, the samples were centrifuged to remove the precipitate. The resulting supernatants were analyzed by HPLC method to determine AFB₁ and its metabolites.

HPLC analysis of AFB₁ metabolites  We analyzed 50 μL samples of supernatant by HPLC with a Waters C18 Nova-Pak column (3.9 mm×300 mm) and a mobile phase consisting of acetonitrile:ethyl acetate:water, 18:6:76 (v:v:v), pumped at 1.0 mL·min⁻¹. Peaks were detected by absorbency at 363 nm with Waters 996 photodiode array detector (Milford, MA). Authentic standards of AFM₁, AFQ₁, AFB₁, and AFG₁ were used to determine the retention time (4.5, 5.8, 8.9, and 7.2 min, respectively). AFB₁-GSH was identified by an excellent accordance of the spectrum of 2.8-min fraction with the spectrum of AFB₁. Peak areas were integrated with a Millennium 2010 chromatography manager and converted to nanomolar AFB₁ equivalents with AFG₁ as an internal standard and an AFB₁ standard curve.

Malondialdehyde (MDA) assay  We incubated a
reaction mixture containing 0.05-0.2 mL of liver samples, 8.1 % sodium lauryl sulfate 0.2 mL, 20 % acetic acid-NaOH buffer (pH 3.5) 1.5 mL, 0.8 % thiobarbituric acid 1.5 mL, and 0.75 mL distilled water at 100 °C for 60 min. After cooling, the mixture was centrifuged at 10,500× g for 10 min. The UV absorbance of the supernatant was detected at 532 nm. MDA was quantified by an external standard method with MDA as standard.

Statistical analysis All results expressed as mean±SD were analyzed with a two-tailed unpaired Student’s t-test. P value of less than 0.05 was considered statistically significant.

RESULTS

Protection of bicyclol against AFB1-induced liver damage in rats Intraperitoneal injection of AFB1 (1.5 mg· kg⁻¹) induced acute liver damage in rats as evidenced by increase in serum ALT and AST and hepatic MDA levels (Tab 1). Bicyclol (300 mg· kg⁻¹· d⁻¹ for 3 d) pretreatment markedly reduced AFB1 hepatotoxicity as shown by the decreased serum levels of ALT and AST. The decrease of MDA in the liver of AFB1-treated rats indicated that bicyclol had an anti-oxidative effect. Bicyclol itself had no effect on these serum and liver markers in rats.

Effect of bicyclol on metabolic rate and ratio of AFB1 metabolites in rats Bicyclol pretreatment at 300 mg· kg⁻¹· d⁻¹ for 3 d slightly accelerated the in vitro metabolism of AFB1 by rat microsomes (Tab 2). Bicyclol pretreatment increased the level of the less toxic metabolite, AFQ1.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Metabolized AFB1 (μmol· g⁻¹· min⁻¹ protein)</th>
<th>AFB1-8,9-epoxide (% of total AFB1 metabolites)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.12±0.21</td>
<td>66±5</td>
</tr>
<tr>
<td>Bicyclol</td>
<td>17.3±2.4</td>
<td>61.5±2.8</td>
</tr>
</tbody>
</table>

Effect of bicyclol on liver drug metabolizing enzymes in rats Bicyclol-treated rats had higher levels of microsomal cytochrome P450 content and isozyme CYP 2B1-mediated PROD activity than did control rats (Tab 3). The induction of CYP3A-mediated erythromycin-demethylase and CYP1A-mediated EROD activities were found in bicyclol-treated rats (Tab 3). Bicyclol treatment also increased the activities of liver cytosolic GST with both CDNB and DCNB as substrates. The level of GSH in the livers of bicyclol-treated rats was twice than that of control rats.

DISCUSSION

Treatment of rats with bicyclol increased the activity of CYP 2B1 and GST (which catalyze detoxifica-
tion of chemicals) in the liver. Furthermore, bicyclol increased the production of AFQ1, which is of low toxicity. Therefore, we believe that bicyclol prevents rats from AFB1-induced liver injury by increasing the detoxifying metabolism of AFB1. Our results suggest that bicyclol may be helpful in detoxifying carcinogens and preventing liver cancer.

Many previous studies have indicated that the activity of hepatic GST, rather than the activation of AFB1, is the determining factor in species susceptibility to the toxic and carcinogenic effects of AFB1. GST and GSH play a key role in detoxifying toxins and carcinogens. In our study, bicyclol increased levels of hepatic GST and GSH, which helped to eliminate the toxic metabolite. Humans and rats possess extremely limited capacity of catalyzing the binding of AFB1-8,9-epoxide to GSH, and consequently are relatively susceptible to AFB1 hepatotoxicity. In view of its anthepatitis and detoxifying effects, bicyclol may contribute to the chemoprevention of liver cancer.

REFERENCES

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