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Effects of bicyclol on aflatoxin B_1 metabolism and hepatotoxicity in rats[1]

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KEY WORDS bicyclol; aflatoxin B_1; cytochrome P450; metabolism; hepatotoxicity

ABSTRACT

AIM: To study the effect of new antihepatitis drug, bicyclol, on the metabolism and hepatotoxicity of aflatoxin B_1 (AFB_1) in rats. **METHODS:** Rats were given bicyclol 300 mg· kg^{-1}· d^{-1} ig for 3 d and then injected ip with AFB_1 1.5 mg· kg^{-1}. Liver damages were examined 16 h after ip AFB_1. The *in vitro* metabolism of AFB_1 by bicyclol-pretreated liver microsomes was investigated by HPLC assay. **RESULTS:** Bicyclol (300 mg· kg^{-1}· d^{-1} for 3 d) pretreatment provided protection against AFB_1 hepatotoxicity as evidenced by the decrease of AFB_1-elevated serum aminotransferase and hepatic malondialdehyde in rats. Bicyclol pretreatment slightly increased the production of the less toxic metabolite aflatoxin Q_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_1 hepatotoxicity by increasing the detoxifying metabolism of AFB_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[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_1 (AFB_1), a potent hepatotoxin and hepatocarcinogen, is closely related to the incidence of liver cancer[3]. Liver cytochrome P450 1A, 2B, 3A, and other minor isozymes catalyze the oxidative metabolism of AFB_1[4]. The formation of the metabolite, AFB_1-8,9-epoxide, and its subsequent covalent binding to DNA to form AFB_1-DNA adducts, are critical steps leading to hepatocarcinogenesis[5]. Aflatoxin Q_1 (AFQ_1), another oxidative metabolite of AFB_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 hepatoxins[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 AFB_1 in rats.

MATERIALS AND METHODS

Chemicals Bicyclol was provided by the Beijing Union Pharmaceutical Plant (purity >99 %). AFB_1, aflatoxin M_1 (AFM_1), AFQ_1, aflatoxin G_1 (AFG_1), 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-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 $mg\cdot kg^{-1}\cdot d^{-1}$ 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 were received bicyclol 300 $mg\cdot kg^{-1}\cdot d^{-1}$ ig once a day for 3 d and the groups of control animals were given an equivalent volume of PEG-400. AFB_1 at 1.5 $mg\cdot kg^{-1}$ was intraperitoneally injected to rats in AFB_1 treated groups 8 h after last dose of bicyclol. Rats were killed by decapitation 24 h after injection of AFB_1 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[7]. Liver protein and cytochrome P450 content were determined by the methods of Lowry *et al*[8] and Omura *et al*[9], respectively. The hepatic glutathione (GSH) level was determined as described by Ellman[10]. The spectrophotometric determination of hepatic cytosol glutathione *S*-transferase (GST) activity was carried out in the presence of potassium phosphate 100 $mmol\cdot L^{-1}$ buffer (pH 6.5) with GSH 1 $mmol\cdot L^{-1}$ and CDNB 1 $mmol\cdot L^{-1}$ or with GSH 5 $mmol\cdot L^{-1}$ and DCNB 1 $mmol\cdot L^{-1}$ [11]. 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[12]. The assay of microsomal cytochrome P450 isozyme 3A activity was carried out with erythromycin as a substrate[13]. Serum levels of ALT and AST were assayed with the test kits.

***In vitro* biotransformation of AFB_1 by hepatic microsomes** This assay was performed according to the method described by Monroe *et al* with modification[14]. The determination was carried out in a final volume of 0.25 mL containing a final concentration ($mmol\cdot L^{-1}$, pH 7.6) of GSH 5, NADPH 1, sucrose 190, potassium phosphate 60, Tris-HCl 80, NaCl 15, KCl 5, and $MgCl_2$ 4 and maintained at 37 ℃. Rat liver microsomes equivalent to 750 μg protein were measured in the presence of excess mouse cytosol (750 μg) to scavenge the AFB_1-epoxide as the 8,9-dihydro-8-(*S*-glutathionyl)-9-hydroxy-aflatoxin B_1 (AFB-GSH) conjugate. The reaction was initiated by the addition of 5 μL AFB_1 (0.25 g/L in Me_2SO) and terminated after 30 min by the addition of ice-cold 50 μL acetic acid 2 $mol\cdot L^{-1}$ and 10 μL methanol containing AFG_1 0.03 g/L as an internal standard. After overnight storage at -20 ℃ to enhance precipitation of macromolecules, the samples were centrifuged to remove the precipitate. The resulting supernatants were analyzed by HPLC method to determine AFB_1 and its metabolites.

HPLC analysis of AFB_1 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 $mL\cdot min^{-1}$. Peaks were detected by absorbency at 363 nm with Waters 996 photodiode array detector (Milford, MA). Authentic standards of AFM_1, AFQ_1, AFB_1, and AFG_1 were used to determine the retention time (4.5, 5.8, 8.9, and 7.2 min, respectively). AFB_1-GSH was identified by an excellent accordance of the spectrum of 2.8-min fraction with the spectrum of AFB_1. Peak areas were integrated with a Millennium 2010 chromatography manager and converted to nanomolar AFB_1 equivalents with AFG_1 as an internal standard and an AFB_1 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 ℃ 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 AFB_1-induced liver damage in rats Intraperitoneal injection of AFB_1 (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 AFB_1 hepatotoxicity as shown by the decreased serum levels of ALT and AST. The decrease of MDA in the liver of AFB_1-treated rats indicated that bicyclol had an anti-oxidative effect. Bicyclol itself had no effect on these serum and liver markers in rats.

Tab 1. Effect of bicyclol on liver damage in AFB_1-treated rats. n=5. Mean±SD. ^aP>0.05, ^cP<0.01 *vs* control. ^fP<0.01 *vs* AFB_1 group. U=Karman's unit.

Treatment	ALT/U	AST/U	MDA/ nmol· g^{-1} protein
Control	35.6±1.2	85±4	14±3
Bicyclol	38±3[a]	97±10[a]	10.4±1.1[a]
AFB_1	122±15[c]	160±10[c]	22±4[c]
AFB_1+Bicyclol	47±18[f]	133±11[f]	13±3[f]

Effect of bicyclol on metabolic rate and ratio of AFB_1 metabolites in rats Bicyclol pretreatment at 300 mg· kg^{-1}· d^{-1} for 3 d slightly accelerated the *in vitro* metabolism of AFB_1 by rat microsomes (Tab 2). Bicyclol pretreatment increased the level of the less toxic metabolite, AFQ_1.

Tab 2. Effect of bicyclol on the metabolism of aflatoxin B_1 (AFB_1) in rats. n=4. Mean±SD. ^aP>0.05, ^cP<0.01 *vs* control.

Treatment	Metabolized AFB_1/nmol· g^{-1}· min^{-1} protein	AFB_1-8,9-epoxide (% of total AFB_1 metabolites)	AFM_1	AFQ_1
Control	11.12±0.21	66±5	20±4	14.5±2.1
Bicyclol	17.3±2.4	61.5±2.8[a]	18.6±2.7[a]	19.9±1.0[c]

Effect of bicyclol on liver drug metabolizing enzymes in rats Bicyclol-treated rats had higher levels of microsomal cytochrome P450 content and isoenzyme 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-

Tab 3. Effect of bicyclol on liver drug metabolizing enzymes in rats. n=4. Mean±SD. ^bP<0.05, ^cP<0.01 *vs* control.

Enzyme	Unit	Control	Bicyclol
P450	μmol· g^{-1} (protein)	0.59±0.14	0.92±0.14[c]
EROD	μmol· min^{-1}· g^{-1} (protein)	0.69±0.19	1.45±0.24[c]
PROD	nmol· min^{-1}· g^{-1} (protein)	31±9	1302±281[c]
Erythromycin-demethylase	μmol· min^{-1}· g^{-1} (protein)	0.47±0.06	1.18±0.29[c]
GSH	μmol· g^{-1} (protein)	36.8±2.0	70±18[c]
GST (CDNB)	μmol· min^{-1}· g^{-1} (protein)	795±139	1168±225[b]
(DCNB)	μmol· min^{-1}· g^{-1} (protein)	32±4	62±9[c]

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 AFB_1-induced liver injury by increasing the detoxifying metabolism of AFB_1. 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 AFB_1, is the determining factor in species susceptibility to the toxic and carcinogenic effects of AFB_1[15,16]. 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 AFB_1-8,9-epoxide to GSH, and consequently are relatively susceptible to AFB_1 hepatotoxicity[17]. In view of its antihepatitis and detoxifying effects, bicyclol may contribute to the chemoprevention of liver cancer.

REFERENCES

1 Gonzalez FJ, Gelboin HV. Role of human cytochromes P450 in the metabolic activation of chemical carcinogens and toxins. Drug Metab Rev 1994; 26: 165-83.

2 Lu H, Li Y. Cytochrome P450s and cancer. Prog Physiol Sci 1997; 28: 178-80.

3 Wogan GN. Aflatoxins as risk factors for hepatocellular carcinoma in humans. Cancer Res 1992; 52 (7 Suppl): 2114s-2118s.

4 Pong RS, Wogan GN. Toxicity and biochemical and fine structure effects of synthetic aflatoxins M1 and B1 in rat liver. J Natl Cancer Inst 1971; 47: 585-92.

5 Gallagher EP, Wienkers LC, Stapleton PL, Kunze KL, Eaton DL. Role of human microsomal and human complementary DNA-expressed cytochromes P450 1A2 and P450 3A4 in the bioactivation of aflatoxin B_1. Cancer Res 1994; 54: 101-8.

6 Li Y, Dai GW, Liu GT, Li Y. Effect of bicyclol on acetaminophen-induced hepatotoxicity: energetic metabolism and mitochondrial injury in acetaminophen-intoxicated mice. Acta Pharm Sin 2001; 36: 723-6.

7 Lesca P, Lecointe P, Paoletti C, Mansuy D. Induction des monoxygenases hepatiques par l' ellipticine chez le rat: formation de cytochrome P448. Activite hydroxylante. C R Acad Sci 1976; 282: 1457-60.

8 Lowry OH, Rosebrough NG, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265-75.

9 Omura T, Sato SR. The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification, and properties. J Biol Chem 1964; 239: 2379-85.

10 Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959; 82: 70-7.

11 Habing WH, Pabst MJ, Jakoby WB. Glutathione *S*-transferases. The first enzymatic step in mecaptaric acid formation. J Biol Chem 1974; 249: 7130-9.

12 Lubet RA, Mayer RT, Cameron JW, Nims RW, Burke MD, Wolff T, *et al*. Dealkylation of pentoxyresorufin: a rapid and sensitive assay for measuring induction of cytochrome(s) P450 by phenobarbital and other xenobiotics in the rat. Arch Biochem Biophys 1985; 238: 43-8.

13 Chang T, Levine M, Bellward GD. Influence of sex and inducer treatment on the high- and low-affinity forms of hepatic microsomal erythromycin *N*-demethylase in rats. Can J Physiol Pharmacol 1990; 68: 1510-3.

14 Monroe DH, Eaton DL. Comparative effects of butylated hydroxyanisole on hepatic *in vivo* DNA binding and *in vitro* biotransformation of aflatoxin B_1 in the rat and mouse. Toxicol Appl Pharmacol 1987; 90: 401-9.

15 Raney KD, Meyer DJ, Ketterer B, Harris TM, Guengerich FP. Glutathione conjugation of aflatoxin B_1 exo- and endo-epoxides by rat and human glutathione *S*-transferases. Chem Res Toxicol 1992; 5: 470-8.

16 Hayes JD, Judan DJ, McLellan LI, Neal GE. Contribution of the glutathione *S*-transferases to the mechanisms of resistance to aflatoxin B_1. Pharmacol Ther 1991; 50: 443-72.

17 Kim ND, Kim SG. Chemopreventive effects of 2-(allylthio) pyrazine. Arch Pharm Res 1999; 22: 99-107.