Inhibition of Isoniazid-Induced Hepatotoxicity in Rabbits by Pretreatment with an Amidase Inhibitor1,2

TROY C. SARICH,3 STEPHEN P. ADAMS, GIORGIO PETRICCA, and JAMES M. WRIGHT

Departments of Pharmacology and Therapeutics (T.C.S., S.P.A., G.P., J.M.W.) and Medicine (J.M.W.), Faculty of Medicine, University of British Columbia, Vancouver British Columbia, Canada

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ABSTRACT

Isoniazid (INH), a widely used drug in the prophylaxis and treatment of tuberculosis, is associated with a 1 to 2% risk of severe and potentially fatal hepatotoxicity. There is evidence that the INH metabolite hydrazine plays an important role in the mechanism of this toxicity. Metabolism of INH leads to the production of hydrazine via both direct and indirect pathways. In both cases, the activity of an INH amidase is required to hydrolyze an amide bond. In the present study, using a model of INH-induced hepatotoxicity in rabbits, pretreatment of rabbits with the amidase inhibitor bis-p-nitrophenyl phosphate 30 min before injection of INH inhibited the formation of INH-derived hydrazine and decreased measures of hepatocellular damage, hepatic triglyceride accumulation, and hypertriglyceridemia. Bis-p-nitrophenyl phosphate also potently inhibited the production of hydrazine from INH in vitro microsomal incubations (IC50 2 μM). Although hepatic glutathione stores are decreased, they are not depleted in animals with INH-induced hepatotoxicity. Significant effects on hepatic microsomal cytochrome P-450 1A1/2 and cytochrome P-450 2E1 activities suggest that these isozymes may be involved in the mechanism of the toxicity. In conclusion, this study demonstrates the importance of amidase activity in this rabbit model of hepatotoxicity and provides additional evidence in support of the role of hydrazine in the mechanism of INH-induced hepatotoxicity.

Daily administration of isoniazid (INH), a highly effective drug in the chemoprophylaxis and treatment of tuberculosis, is associated with mild elevations of liver enzyme activities in plasma in up to 20% of patients (Mitchell et al., 1975) and severe hepatotoxicity (predominantly hepatocellular damage) in approximately 1 to 2% of patients (Barlow et al., 1974). If this hepatotoxicity is not recognized early, it can be fatal. Over 25 years after the toxicity was detected in INH-treated patients, the mechanism remains unknown and the hepatotoxicity remains neither preventable nor treatable. Our laboratory has developed a model of INH-induced hepatotoxicity in rabbits that closely resembles the toxicity in humans (Sarich et al., 1995). Histopathological changes in INH-induced hepatotoxicity in humans range in severity from focal and diffuse necrosis to multilobular, bridging, and massive necrosis (Maddrey and Boitnott, 1973). Histopathological evaluation of INH-induced hepatotoxicity in rabbits reveals focal and centrilobular inflammatory infiltration and necrosis (Sarich et al., 1995).

Hydrazine is a known hepatotoxin (Yard and McKennis, 1955; Patrick and Back, 1965). We have previously reported a positive correlation between plasma hydrazine levels and severity of INH-induced hepatic cellular damage in rabbits (Sarich et al., 1996). This evidence suggesting that hydrazine plays a role in INH-induced hepatotoxicity is supported by previous reports that suggest a role for hydrazine in INH-induced hepatotoxicity in animals and humans (Noda et al., 1983; Peretti et al., 1987; Woo et al., 1992; Gent et al., 1992). The role of acetylhydrazine has also been studied and it has been proposed to be the INH-derived hepatotoxic metabolite in INH-induced hepatic cellular damage (Mitchell et al., 1976).

During metabolism of INH, hydrazine can be produced both directly (from INH) and indirectly (from acetylhydrazine; Fig. 1). The direct pathway involves hydrolysis of the amide bond of INH to produce isonicotinic acid and hydrazine. The indirect pathway involves acetylation of INH to acetyl-INH by N-acetyltransferase, hydrolysis of acetyl-INH to isonicotinic acid and acetylhydrazine, and hydrolysis/deacetylation of acetylhydrazine to hydrazine. Both hydrolysis reactions involve an amidase enzyme.

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ABBREVIATIONS: ALT, alanine aminotransferase; ASAL, argininosuccinic acid lyase; BNPP, bis-p-nitrophenyl phosphate; CYP, cytochrome P-450; EROD, ethoxyresorufin-O-deethylase; INH, isoniazid; PROD, pentoxyresorufin-O-dealkylase; VEH, vehicle.
A previous study in rats demonstrated that bis-p-nitrophenyl phosphate (BNPP), an amidohydrolase (amidase) inhibitor, prevented acetyl-INH-induced hepatocellular damage (Mitchell et al., 1976). The investigators suggested this to be due to prevention of hydrolysis of acetyl-INH to acetylhydrazine, the suspected INH-derived hepatotoxic agent at that time. However, Mitchell and colleagues (1976) did not propose the possibility that inhibition of acetyl-INH-induced hepatocellular damage by BNPP could be due to inhibition of production of hydrazine from acetylhydrazine. In 1984, Sendo et al. reported that BNPP acts as a potent amidase inhibitor in vitro, inhibiting the conversion of INH to hydrazine in hepatic microsomal incubations. Because there is evidence that hydrazine is the INH-derived hepatotoxin in our model of INH-induced hepatotoxicity, we have included BNPP in our INH/amidase investigations. BNPP was investigated in vitro. The activity of several CYP isozymes were measured during an active phase of INH-induced hepatotoxicity to evaluate their potential role. Hepatic glutathione levels were also measured to determine whether it plays a role in this model of INH-induced hepatotoxicity in rabbits.

**Experimental Procedures**

**Animals.** Seventy male New Zealand White rabbits weighing 2 to 3 kg were used in this study. Throughout the experiment, the rabbits were housed in pairs in stainless steel cages with a 12-h light/dark cycle and free access to food and water. This project was reviewed and approved by the University of British Columbia Committee on Animal Care.

**Materials.** INH, BNPP, β-NADPH (for the reductase and CYP2E1 assays) and p-nitrophenol (for the CYP2E1 assay) were obtained from Sigma Chemical Co. (St. Louis, MO). Reagents for the argininosuccinic acid lyase (ASAL) assay were obtained as follows: barium argininosuccinate (which was converted to the sodium salt by admixture with sodium sulfate and centrifugation) and 2,4-dichloro-l-naphthol from Sigma; the other reagents required for the ASAL assay were obtained from local chemical suppliers and were all of reagent grade.

**INH Injection Protocol.** The dosing schedule for INH was as described previously (Sarich et al., 1996, 1998).

**Treatment Groups.** Animals were randomized into one of five groups, each involving two treatments. The first treatment involved either a BNPP injection of 25 mg/kg (0.074 mmol/kg) or a BNPP-vehicle (VEH) injection (saline). BNPP was dissolved in saline (0.9% NaCl) at 7.65 mg/ml after heating to approximately 60°C (Heymann and Krisch, 1969) and, after cooling, injected i.p. at a volume of 3.3 ml/kg.

The second treatment involved s.c. injections of either INH or INH-VEH (0.9% NaCl; saline). The first treatment (BNPP or BNPP-VEH) was administered 30 min before each of the INH or INH-VEH injections.

In total there were five treatment groups: BNPP-VEH and INH-VEH (VEH-VEH); BNPP-VEH and INH (VEH-INH); BNPP and INH (BNPP-INH); BNPP and INH-VEH (BNPP-VEH); and BNPP-VEH and INH-VEH (VEH-VEH*, a food and water control group; Table 1). The VEH-VEH* group had treatments delayed one day to match their food and water intake to the VEH-INH animals.

TABLE 1

<table>
<thead>
<tr>
<th>Treatment groups</th>
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<tbody>
<tr>
<td>Group 1</td>
</tr>
<tr>
<td>Number Name: VEH-VEH</td>
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<tr>
<td>Group 2</td>
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<tr>
<td>Number Name: VEH-VEH</td>
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<tr>
<td>Group 3</td>
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<tr>
<td>Number Name: BNPP-INH</td>
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<tr>
<td>Group 4</td>
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<tr>
<td>Number Name: BNPP-VEH</td>
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<tr>
<td>Group 5*</td>
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<tr>
<td>Number Name: VEH-VEH*</td>
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</table>

*This group was delayed by 1 day and allowed only the food and water consumed by group 3, the INH-only group. The animals in group 5 were matched by weight to the animals in group 3.
During the INH-injection protocol, food intake in the VEH-INH group was decreased to 20 to 40 g/day and water intake ranged from 50 to 100 ml/day. The VEH-VEH animals were paired based on weight and were only allowed the amount of water and food which was consumed by its weight-matched counterpart in the VEHN-INH group. The purpose of this group was to control for any pathological or biochemical changes that may occur as a result of decreased food and water intake.

The rabbits were housed and treated in groups of ten. The first six groups of 10 included two randomly selected animals for each treatment group. The final group of 10 (numbers 61–70) included only randomly selected animals placed into VEHN-INH or BNP-PP-VEHN groups (five of each).

Blood Sampling. Blood samples (1 ml) were taken from the lateral ear vein using a heparinized syringe at 0, 12, 24, 32, and 48 h after the first dose of INH, as described previously (Sarich et al., 1996). The animals were sacrificed at 48 h by cervical dislocation and exsanguination.

Biochemical Analysis. Hepatocellular damage was quantitated by measuring peak plasma ASAL activity and peak plasma alanine aminotransferase (ALT) activity. ASAL activity in the plasma has previously been used as a sensitive marker of liver damage (Campanini et al., 1970; Sims and Rautanen, 1975). The quantitation of plasma ASAL activity (expressed as μmol/100 ml/h; Takahara units) was done according to Campanini et al. (1970) with modifications as described in Sarich et al. (1995). ALT activity (U/l), a commonly used marker of liver toxicity, was determined using a kit from Sigma Diagnostics (kit no. 59–20).

Hepatic triglyceride accumulation (hepatic steatosis) was determined as previously described (Sarich et al., 1996). Plasma triglyceride levels (mM triolein equivalent) were quantitated in 32-h plasma samples using a triglyceride kit from Sigma Diagnostics (kit no. 336–10).

Tissue Handling. Immediately after sacrifice of the rabbits, the livers were removed, weighed, and homogenized (using a glass tube and Teflon pestle) with a homogenizing buffer (1.15% KCL, 10 mM KH2PO4 buffer, pH 7.0; 3 mM initial concentration) was incubated with 150 μl hepatic microsomes (3–10 mg protein/ml) and 50 μl 67 mM KH2PO4 buffer (pH 7.0) at 37°C for 30 min (300 μl total volume). The reaction was stopped by the addition of 0.3 ml acetonitrile, followed by mixing, standing for 6 min, addition of 0.3 ml 0.6 N perchloric acid and centrifugation at 12,700g for 6 min. The rest of the procedure was the same as for determination of plasma hydrazine levels. In control microsomes, the time course of the reaction was monitored over 120 min and the reaction was found to be linear up to 30 min. A standard curve was prepared (in triplicate) using microsomes spiked with hydrazine. The standard curve had a correlation coefficient (r) of 0.998 and a slope of 0.235 (95% confidence intervals 0.222–0.248) M ν s/μM azine and a y-intercept of −0.64 (95% confidence intervals −1.41 to 0.13) M ν s. The peak areas of the azine (each sample in duplicate) were converted into micromoles per liter (μM). Hydrazine concentration in the plasma samples ranged from below 4 μM (undetectable) to 74 μM. Recovery of hydrazine was approximately 20%, but could be improved with the addition of 100 μl of a saturated K2CO3 as described above.

Amidase Activity Determination. Hepatic amidase activity was determined by incubation of INH with microsomes (Whitehouse et al., 1983; Sendo et al., 1984) followed by measurement of hydrazine using HPLC as described above. Incubation of INH with microsomes was done as follows: One hundred microliters of INH in 67 mM KH2PO4 buffer (pH 7.0; 3 mM initial concentration) was incubated with 150 μl hepatic microsomes (3–10 mg protein/ml) and 50 μl 67 mM KH2PO4 buffer (pH 7.0) at 37°C for 30 min (300 μl total volume). The reaction was stopped by the addition of 0.3 ml acetonitrile, followed by mixing, standing for 6 min, addition of 0.3 ml 0.6 N perchloric acid and centrifugation at 12,700g for 6 min. The rest of the procedure was the same as for determination of plasma hydrazine levels. In control microsomes, the time course of the reaction was monitored over 120 min and the reaction was found to be linear up to 30 min. A standard curve was prepared (in triplicate) using microsomes spiked with hydrazine. The standard curve had a correlation coefficient (r) of 0.998, a slope of 0.152 (95% confidence intervals 0.140–0.165) M ν s/μM azine and a y-intercept of −1.19 (95% confidence intervals −2.56 to 1.86) M ν s. Hydrazine production was calculated as nmol hydrazine produced/h/mg protein.

Plasma amidase activity was determined by incubation of INH with an aliquot of plasma followed by measurement of the production of hydrazine using HPLC. One hundred microliters of INH in 67 mM KH2PO4 buffer (pH 7.0; 3 mM initial concentration) was incubated with 150 μl plasma and 50 μl 67 mM KH2PO4 buffer (pH 7.0) at 37°C for 30 min (300 μl total volume). The rest of the procedure was identical with that described above for microsomal hydrazine production from INH. A standard curve was prepared (in triplicate) using blank rabbit plasma spiked with hydrazine. The standard curve gave a correlation coefficient (r) of 0.999 and slope of 0.166 (95% confidence intervals 0.163–0.170) M ν s/μM azine and a y-intercept of 0.040 (95% confidence intervals −0.062 to 0.142) M ν s. Hydrazine production was calculated as nmol hydrazine produced/h/mg protein [plasma protein (mg/ml) was determined].

Microsomal CYP Enzymes. Hepatic NADPH CYP reductase activity (nmol/min/mg protein) was determined using procedures outlined in Phillips and Langdon (1962), using an extinction coefficient of 19.6 cm−1 mM−1 and a final reaction volume of 1.575 ml. p-Nitrophenol hydroxylase activity (nmol/min/mg protein; used as a marker of CYP2E1 activity) was assayed using an initial substrate concentration (p-nitrophenol) of 100 μM, a peak absorbance of 510 nm and an extinction coefficient of 9.53 cm−1 mM−1 (Reinke and Moyer, 1985; Koop, 1986; Jenner and Timbrell, 1994).

Microsomal ethoxyresorufin-O-deethylase (EROD) activity, benzoxyresorufin-O-dealkylase activity, and pentoxyresorufin-O-dealky-
lase (PROD) activity were determined based on previous methods (Burke et al., 1985; Grimm et al., 1994). The specificity of benzoyloxyresorufin-O-dealkylase activity (used as a marker for CYP2B4) and PROD activity (used as a marker for CYP2B4/5) have been tested in rabbits; however, the specificity of EROD activity (CYP1A1/2) in rabbits has not. The analyses were performed on a Shimadzu recording spectrofluorophotometer (model RF-540) with a DR-3 recorder. Scans were performed on resorufin standard curve solutions to identify the peak excitation and emission wavelengths of resorufin. A peak excitation wavelength of 575 nm and a peak emission wavelength of 585 nm were identified. The scans confirm no interference from the light-scattering peak that appears from approximately 580 to 600 nm. Using 2-nm slit widths (for both the excitation and emission beams) allowed for resolution of the peak at 575 nm. Samples were analyzed in duplicate. Activity units were expressed as nmol resorufin produced/min/mg protein.

Protein quantitation of liver homogenate and microsomal samples was carried out using methods outlined by Bradford (1976).

INH, Acetylhydrazine, and Hydrazine Comparison. In a study comparing the toxicity of s.c. administered INH, acetylhydrazine, or hydrazine, these compounds were administered at molar equivalent doses using our standard dosing protocol (0.37 mM/kg followed by 3 doses of 0.26 mM/kg at 3-h intervals; Sarich et al., 1996). INH (n = 5), acetylhydrazine (n = 9), and hydrazine (n = 3) were each administered at this equimolar dose. Hydrazine was also administered (n = 8) using the same dosing protocol but at one-half the molar dose (0.19 mM/kg followed by three doses of 0.13 mM/kg at 3-h intervals).

Statistics. Plasma ASAL and ALT activities were logarthimically-transformed to give normally distributed data suitable for statistical analysis. For the purpose of clarity, plasma ASAL and ALT activities are presented in the untransformed form (mean ± S.E.; calculated from raw data) in text, tables, and figure legends, but as the log-transformed form in figures. Student’s t tests assuming either equal or unequal variances (as determined by F-tests) were used when comparing two groups (Zar, 1984). ANOVA and the Newman-Keul’s multiple comparison test were done for comparison of more than two groups (Zar, 1984). All data are presented as mean ± S.E. unless indicated otherwise.

Results

In this study, four of seventy animals died before 48 h, the planned time of sacrifice. Three were from the INH-only (VEH-INH) group and one was from the BNPP-INH group. Plasma samples from all four of these animals before death showed highly elevated plasma liver enzymes, indicating hepatocellular damage, and were included in the toxicity analyses. The liver of one of the VEH-INH animals that died between 12 and 24 h was recovered at the time of death and immediately frozen in liquid nitrogen and included in the microsomal preparation and analyses. The livers from the other three were not available for microsomal analyses.

Peak plasma ASAL and ALT activities, as well as hepatic and plasma triglycerides, were significantly increased above control levels only in the VEH-INH group (Table 2).

Plasma hydrazine concentrations at 24 h were not significantly different between the VEH-INH and BNPP-INH groups (Table 3). However, at 32 h, plasma hydrazine concentrations were significantly different (approximately 4 times higher) in the VEH-INH group as compared to the BNPP-INH group (Table 3).

Hepatic amidase activity, determined by measurement of the amount of hydrazine produced after incubation of INH with microsomes for 30 min, was significantly decreased in the VEH-INH group (by 38%) versus the VEH-VEH control group. However, the greatest decrease was found in the BNPP-VEH and the BNPP-INH groups (both decreased to approximately 10% of control levels; Table 4).

In vitro incubation of BNPP with INH in control microsomes (from VEH-VEH control rabbits) showed that BNPP is a potent inhibitor of hydrolysis of INH to hydrazine with an KI of approximately 2.0 μM (Fig. 2). Microsomes were also incubated with various concentrations of INH (0.3, 1, 3, 6, 10, and 15 mM) in the presence of BNPP at concentrations of 0, 1, or 3 μM. A Lineweaver-Burk plot of the results (not shown) indicated that at 0 μM BNPP, Vmax was 226 nmol/min/mg protein and Km was 9.3 mM; at 1 μM BNPP, Vmax was 167 nmol/min/mg protein and Km was 8.3 mM; and at 3 μM BNPP, Vmax was 99.4 nmol/min/mg protein and Km was 12.1 mM. An Eadie-Hofsteet plot of the results (not shown) indicated that at 0 μM BNPP, Km was 167 nmol/min/mg protein and Km was 7.8 mM; at 1 μM BNPP, Vmax was 116 nmol/min/mg protein and Km was 5.3 mM; and at 3 μM BNPP, Vmax was 85.5 nmol/min/mg protein and Km was 10.0 mM. In general, these data suggest that increasing concentrations of BNPP decreases the Vmax of INH-amidase but does not significantly change its Km.

Hepatic glutathione content was significantly decreased in the VEH-INH group versus the other four groups (Table 2). There were no significant correlations between hepatic glutathione and log peak plasma ASAL activity, log peak plasma

### Table 2

<table>
<thead>
<tr>
<th>Toxicological markers by group</th>
<th>VEH-VEH</th>
<th>VEH-INH</th>
<th>BNPP-INH</th>
<th>BNPP-VEH</th>
<th>VEH-VEH*</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak plasma ASAL activity</td>
<td>3.9 ± 0.4</td>
<td>239 ± 83*</td>
<td>57.6 ± 47</td>
<td>3.8 ± 0.4</td>
<td>4.6 ± 0.8</td>
<td>p &lt; .0001</td>
</tr>
<tr>
<td>(Takehara units)</td>
<td>n = 12</td>
<td>n = 17</td>
<td>n = 17</td>
<td>n = 12</td>
<td>n = 12</td>
<td></td>
</tr>
<tr>
<td>Peak plasma ALT activity</td>
<td>24.9 ± 2.3</td>
<td>240 ± 81*</td>
<td>62.8 ± 24</td>
<td>25.2 ± 2.8</td>
<td>31.3 ± 3.9</td>
<td>p = .003</td>
</tr>
<tr>
<td>(U/l)</td>
<td>n = 12</td>
<td>n = 17</td>
<td>n = 17</td>
<td>n = 12</td>
<td>n = 12</td>
<td></td>
</tr>
<tr>
<td>Hepatic Triglycerides (mg TG/g liver)</td>
<td>7.6 ± 1.7</td>
<td>29.4 ± 3.6*</td>
<td>11.9 ± 2.3</td>
<td>8.1 ± 2.2</td>
<td>10.5 ± 1.6</td>
<td>p &lt; .0001</td>
</tr>
<tr>
<td>(mM)</td>
<td>n = 12</td>
<td>n = 15</td>
<td>n = 16</td>
<td>n = 12</td>
<td>n = 12</td>
<td></td>
</tr>
<tr>
<td>32-h Plasma Triglycerides (mM)</td>
<td>1.7 ± 0.7</td>
<td>6.5 ± 1.6*</td>
<td>2.5 ± 1.0</td>
<td>1.3 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td>p = .001</td>
</tr>
<tr>
<td>(μmol/g liver)</td>
<td>n = 12</td>
<td>n = 14</td>
<td>n = 16</td>
<td>n = 12</td>
<td>n = 12</td>
<td></td>
</tr>
<tr>
<td>Hepatic Glutathione (μmol/g liver)</td>
<td>7.6 ± 0.4</td>
<td>4.3 ± 0.3*</td>
<td>6.3 ± 0.3*</td>
<td>7.5 ± 0.4</td>
<td>6.0 ± 0.3*</td>
<td>p &lt; .0001</td>
</tr>
</tbody>
</table>

* Food- and water-restricted control group.
* Significantly different from the VEH-VEH group (p < .05) using Newman-Keuls’s multiple comparison test.
ALT activity, or hepatic triglyceride content in animals receiving INH (BNPP-INH and VEH-INH groups).

The activities of hepatic reductase, EROD, benzoyloxyresorufin-O-dealkylase, PROD, and p-nitrophenol hydroxylase are presented in Table 5.

A negative correlation of EROD activity in the VEH-INH group with hepatocellular damage (peak plasma ASAL, \( r^2 = 0.66, n = 15, p < .01 \); peak plasma ALT activity, \( r^2 = 0.70, n = 15, p < .005 \)) was observed. In addition, p-nitrophenol hydroxylase activity correlated (negatively) with hepatic steatosis (liver triglycerides; \( r^2 = 0.71, n = 22, p < .001 \)). None of the other P-450 enzyme activities correlated with measures of toxicity.

In the toxicity comparison of s.c. administered INH, acetylhydrazine, or hydrazine, peak plasma ASAL activities in animals administered INH (292 ± 616 Takahara units; \( n = 5 \)) or acetylhydrazine (142 ± 48 Takahara units; \( n = 9 \)) resulted in statistically significant increases from baseline (\( p < .0001 \)). However, in animals administered hydrazine at an equimolar dose, death resulted in the animals (\( n = 3 \)) within 48 h. Plasma ASAL activity was only obtained in one animal, and the activity was extremely elevated (976 Takahara units). Reducing the dose of hydrazine by one-half did not result in the death of any animals and did cause a statistically significant increase in plasma ASAL activity versus baseline (101 ± 33 Takahara units; \( n = 8; p < .0001 \)). There was no statistically significant difference in peak plasma ASAL activities between animals treated with INH and acetylhydrazine at full dose or hydrazine at half dose (\( p = 0.82; \text{ANOVA} \)).

**Discussion**

Treatment with BNPP before INH prevented INH-induced hepatocellular damage, hepatic steatosis, and hypertriglyceridemia. The VEH-INH group was the only group to expe-
experience significant INH-induced hepatotoxicity. This is an important and novel observation because it has not been previously demonstrated in an in vivo animal model that INH-induced hepatotoxicity could be prevented using an inhibitor of INH-amidase.

The present study shows that BNPP inhibits the production of hydrazine from INH in vivo and in vitro. The significantly decreased plasma hydrazine levels at 32 h in the BNPP-INH group versus the VEH-INH group shows that BNPP inhibited in vivo formation of hydrazine from INH. BNPP was found to be a long-acting or irreversible inhibitor of INH-amidase because inhibition by BNPP persisted in vitro in microsomes from BNPP-treated animals. This observation is consistent with the phosphorylating mechanism of action of BNPP (Heymann and Krisch, 1967). The present data also suggest that BNPP inhibits amidase via noncompetitive inhibition. The direct conversion of INH to hydrazine in in vitro incubations of control microsomes can be completely inhibited by the addition of BNPP at low μM concentrations. Based upon these observations, a possible mechanism of action of the inhibitory activity of BNPP involves phosphorylation of a hydroxyl group at an allostERIC site on the amidase, which results in long-acting/irreversible and noncompetitive enzyme inhibition.

In addition to the inhibition of production of hydrazine, BNPP likely decreased the production of acetylhydrazine from acetyl-INH. Thus, it is possible that the decreased production of acetylhydrazine played a role in the decreased severity of INH-induced hepatotoxicity. However, previous evidence (Sarich et al., 1996) that hydrazine, and not acetylhydrazine, correlates with INH-induced hepatocellular damage suggests that decreased production of hydrazine most likely explains the prevention of INH-induced-hepatocellular damage in this rabbit model.

Additional data from a toxicity comparison of INH, acetylhydrazine, and hydrazine were also described in the present study. Administration of hydrazine to rabbits, at the same molar dose as INH in this animal model, resulted in severe toxicity and early death (after severe convulsions). In addition, plasma ASAL activity was extremely elevated (976 Takahara units) in the one animal in which ASAL could be determined. The occurrence of convulsions in this model of INH-induced hepatotoxicity, and the suspected causative role of hydrazine, has been reported previously (Sarich et al., 1995, 1998). When the dose of hydrazine was lowered to one-half the molar dose, the severity of hepatocellular damage was not different from that caused by a full molar dose of INH or acetylhydrazine. On a molar basis, therefore, hydrazine appears to be more toxic than INH or acetylhydrazine. One might have expected an even greater degree of hepatotoxicity from this dose of hydrazine; however, the proportion of hydrazine, administered s.c., that gets to the liver could be quite small, and may not necessarily be a good model for hepatotoxicity of hydrazine directly formed from INH in the liver.

Of course, additional studies are required to fully understand the roles of hydrazine and acetylhydrazine in the mechanism of INH-induced hepatotoxicity in rabbits. This study does, however, confirm that a hydrolysis product(s) of INH is, or becomes, the hepatotoxic species in this model of INH-induced hepatotoxicity, and that hydrolysis product is most likely hydrazine.

The observed decrease in glutathione levels in the INH-treated animals may be due to increased scavenging of reactive substances that are produced as a result of the necrotic and/or steatotic state of the hepatocytes and/or possibly decreased hepatic production of glutathione. Nevertheless, hepatic glutathione levels are not depleted. The 21% decrease in hepatic glutathione stores observed in the food- and water-deprived group suggests that at least some of the decrease in glutathione levels in the VEH-INH group was due to the decreased intake of food and water. This is consistent with previous findings that fasting of rats decreases hepatic glutathione levels possibly via protein deprivation (Pessayre et al., 1979; reviewed in Mandl et al., 1995).

Hepatic EROD activity, a measure of CYP1A1/2 activity (Aix et al., 1994; Rey-Grobellet et al., 1996), was significantly decreased in the VEH-INH, BNPP-INH, and food- and water-deprived VEH-VEH* groups as compared with controls. Although BNPP itself had no effect, it appears that INH and food and water deprivation both have effects on CYP1A1/2 activity. About 42% of the decrease in the VEH-INH and BNPP-INH groups is due to decreased food and water intake. Although there are no reports of decreased CYP1A1/2 activity due to decreased food and water intake in the literature, there are reports of decreased CYP1A1/2 activity in acetaminophen-induced hepatotoxicity in mice (Snawder et al., 1994) and aflatoxin B1-induced hepatotoxicity in rabbits (Guerre et al., 1996).

In addition to the effect of food and water deprivation on CYP1A1/2 activity, the activity in the VEH-INH group was significantly decreased as compared with the BNPP-INH group and this group was significantly decreased compared to the VEH-VEH* group. A significant negative correlation of CYP1A1/2 activity with INH-induced hepatocellular damage (versus ASAL and ALT activity) in the two groups receiving

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### TABLE 5

<table>
<thead>
<tr>
<th>Microsomal enzyme activities by group</th>
<th>VEH-VEH</th>
<th>VEH-INH</th>
<th>BNPP-INH</th>
<th>BNPP-VEH</th>
<th>VEH-VEH*</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reductase activity (nmol/min/mg protein)</td>
<td>361 ± 15</td>
<td>320 ± 17</td>
<td>343 ± 14</td>
<td>360 ± 10</td>
<td>362 ± 19</td>
<td>p = .22</td>
</tr>
<tr>
<td>EROD activity (pmol/min/mg protein)</td>
<td>804 ± 42</td>
<td>180 ± 20</td>
<td>354 ± 36</td>
<td>804 ± 68</td>
<td>490 ± 35</td>
<td>p &lt; .00001</td>
</tr>
<tr>
<td>BROD Activity (nmol/min/mg protein)</td>
<td>1254 ± 142</td>
<td>988 ± 131</td>
<td>1103 ± 136</td>
<td>1209 ± 138</td>
<td>780 ± 113</td>
<td>p = .13</td>
</tr>
<tr>
<td>PROD Activity (nmol/min/mg protein)</td>
<td>105 ± 14</td>
<td>121 ± 15</td>
<td>112 ± 12</td>
<td>112 ± 8</td>
<td>82 ± 6</td>
<td>p = .18</td>
</tr>
<tr>
<td>CYP2E1 Activity (nmol/min/mg protein)</td>
<td>1.8 ± 0.1</td>
<td>0.25 ± 0.1</td>
<td>3.0 ± 0.4</td>
<td>1.9 ± 0.2</td>
<td>1.4 ± 0.1</td>
<td>p &lt; .00001</td>
</tr>
</tbody>
</table>

a Food- and water-restricted control group.

b Significantly different from the VEH-VEH group (p < .05) using Newman-Keuls’s multiple comparison test.
INH (VEH-INH and BNPP-INH) shows that more enzyme activity is inhibited as the severity of hepatocellular damage increases. It is still possible, however, given the 48-h time course of this study that a regulatory effect, for example, decreased RNA, or protein synthesis, caused the decreased CYP1A1/2 activity.

As previously reported, p-nitrophenol hydroxylase (a measure of CYP2E1) activity is significantly decreased after treatment with INH (VEH-INH group; Sarich et al., 1998). Inhibition of CYP2E1 activity has been previously found to occur after acetaminophen-induced hepatotoxicity in mice (Snawder et al., 1994). Aniline hydroxylase activity (another measure of CYP2E1 activity) was also decreased in aflatoxin B1-induced hepatotoxicity in rabbits (Guerre et al., 1996). Consistent with the findings in the present study, activation of alkylhydrazines into free radical intermediates by CYP2E1 has also been suggested as a mechanism of toxicity of hydrazines (Albano et al., 1995). In acetylaminophen-induced hepatotoxicity, CYP1A1/2 and CYP2E1 are involved in the production of reactive and toxic intermediates (Raucy et al., 1989; Patten et al., 1993; Thummel et al., 1993) and have decreased activity after the development of toxicity in mice (Snawder et al., 1994). Interestingly, CYP2E1 activity was increased in the BNPP-INH group (67% greater than the controls) but not in the BNPP-VEH group. BNPP, therefore, resulted in an increase in CYP2E1 activity in the presence of INH, rather than the expected inhibition of CYP2E1 activity in the presence of INH alone. This is possibly due to the inhibitory actions of BNPP on INH hydrolysis.

An interesting finding in this study is that hepatic amida- dase, EROD, and p-nitrophenyl hydroxylase activities are all significantly decreased in animals treated with INH. Could a common link between these enzymes be that they all come into contact with hydrazine? INH-amidase does because it cleaves hydrazine from INH and it appears that some amidase becomes inactivated in the process. Thus, like INH-amidase, the decreased activities of EROD and p-nitrophenyl hydroxylase may be due to their affinity for hydrazine. It is possible that these P-450s activate hydrazine to a damaging intermediate(s), which results in damage to the producing enzyme and surrounding intracellular components. As has been proposed for acetaminophen and its more reactive anal- log N-acetyl-m-aminophenol, if a metabolite intermediate is too reactive, as soon as it is produced it reacts with the producing enzyme as a suicide inactivator and the production of reactive metabolites and toxicity becomes self-limiting (Pumford and Halmes, 1997). In this situation, no toxicity would occur to the cell other than to the producing enzyme because the reactive metabolites would immediately bind to the enzyme before causing damage away from the producing enzyme. This reaction, and its inherent potential for causing toxicity, is thus self-limiting. Therefore, if hydrazine is converted to a reactive intermediate by these isozymes, it appears the reactive intermediate is reactive enough to damage and decrease the activity of these isozymes, but not so reactive that some does not get away from the producing isozyme(s) to cause damage to vital intercellular components, causing cell death.

In conclusion, administration of BNPP 30 min before INH administration in this model of INH-induced hepatocellular damage in rabbits prevents elevations of plasma ASA and ALT activities and hepatic and plasma triglyceride levels, decreases plasma hydrazine levels, and prevents a decrease in the activity of markers for CYP1A1/2 and CYP2E1. These findings suggest that BNPP inhibits a key step in INH me- tabolism that prevents production of hepatotoxic intermedi- ates. It was found in this study that BNPP is a potent and irreversible inhibitor of hydrolysis of INH to hydrazine with an IC50 of approximately 2 μM. Based on a key previous study implicating hydrazine in the mechanism of INH-in- duced hepatocellular damage (Sarich et al., 1996), the mech- anism by which BNPP prevents INH-induced hepatocellular damage is most likely through the inhibition of the produc- tion of hydrazine from INH.

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References

Send reprint requests to: Dr. James M. Wright, M.D., Ph.D., F.R.C.P.(C), Department of Pharmacology and Therapeutics, University of British Columbia, 2176 Health Sciences Mall, Vancouver British Columbia, Canada V6T 1Z3. E-mail: jmwright@unixg.ubc.ca.