A Novel Mechanism Underlies the Hepatotoxicity of Pyrazinamide

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Relatively little is known about the hepatotoxicity of pyrazinamide (PZA). PZA requires activation by amidase to form pyrazinoic acid (PA). Xanthine oxidase then hydroxylates PA to form 5-hydroxypyrazinoic acid (5-OH-PA). PZA can also be directly oxidized to form 5-OH-PZA. Before this study, it was unclear which metabolic pathway or PZA metabolites led to hepatotoxicity. This study determines whether PZA metabolites are responsible for PZA-induced hepatotoxicity. PZA metabolites were identified and cytotoxicity in HepG2 cells was assessed. Potential PZA and PA hepatotoxicity was then tested in rats. Urine specimens were collected from 153 tuberculosis (TB) patients, and the results were evaluated to confirm whether a correlation existed between PZA metabolite concentrations and hepatotoxicity. This led to the hypothesis that coadministration of amidase inhibitor (bis-*p*-nitrophenyl phosphate [BNPP]) decreases or prevents PZA- and PZA metabolite-induced hepatotoxicity in rats. PA and 5-OH-PA are more toxic than PZA. Electron microscopy showed that PZA and PA treatment of rats significantly increases aspartate transaminase (AST) and alanine aminotransferase (ALT) activity and galactose single-point (GSP) levels ($P < 0.005$). PA and 5-OH-PA levels are also significantly correlated with hepatotoxicity in the urine of TB patients ($P < 0.005$). Amidase inhibitor, BNPP, decreases PZA-induced, but not PA-induced, hepatotoxicity. This is the first report of a cell line, animal, and clinical trial confirming that the metabolite 5-OH-PA is responsible for PZA-induced hepatotoxicity.

Tuberculosis (TB) is one of the most serious infectious diseases worldwide. Hepatotoxic effects attributable to anti-TB therapy are unique among drug-related liver problems, because almost all first-line anti-TB medications have these adverse effects (1). Adverse effects are an important clinical consideration for patients undergoing TB treatment because of the long therapy duration and concurrent use of several medications.

Hepatotoxicity is the most serious adverse effect of anti-TB therapy, because liver damage is associated with high morbidity and mortality. First-line anti-TB drugs, including isoniazid (INH), rifampin (RIF), and pyrazinamide (PZA), are associated with considerable hepatotoxic effects (2). Hepatotoxic effects negatively affect therapy adherence, decrease treatment success rates, and may increase treatment failure, relapse, or drug resistance (3). INH, RIF, and PZA are metabolized and detoxified in the liver, making the organ susceptible to liver injury. Toxic metabolites may play an important role in the development of anti-TB drug-induced hepatotoxicity (ATDH) (3, 4). RIF is a potent inducer of several cytochrome P450 isoenzymes (e.g., CYP2E1, 3A) (5). RIF increases INH toxicity, probably by increasing the formation of its toxic metabolite hydrazine (6). However, relatively little is known about PZA hepatotoxicity. Responsive enzymes for PZA metabolic pathways are amidase and xanthine oxidase; however, the proportions of these metabolic pathways in the human body are unclear.

PZA is a pyrazinoic acid (PA) prodrug that is an active inhibitor of *Mycobacterium tuberculosis* (7). PA is the main active metabolite of PZA, which is produced by liver microsomal amidase. Xanthine oxidase then hydroxylates PA to 5-hydroxypyrazinoic acid (5-OH-PA) (Fig. 1) (8, 9). After a single oral dose of 150 mg PZA/kg of body weight in humans, analysis of 0- to 6-h urine collections showed that PA, 5-OH-PA, 5-OH-PZA, and PZA accounted for $25.4 \pm 1.7\%$, $17.7 \pm 1.2\%$, $11.6 \pm 0.8\%$, and $2.7 \pm 0.2\%$, respectively, of the administered dose (8). The rate-limiting factor was liver microsomal amidase activity because of PA formation from PZA and 5-OH-PA formation from 5-OH-PZA. In con-

trast, xanthine oxidase oxidation occurred rapidly (5-OH-PZA formation and PA hydroxylated to 5-OH-PA) (10, 11). These PZA metabolites are excreted in urine. The other minor PZA metabolic pathway consists of PA and glycine-forming PA (12–16).

PZA is commonly used with INH and RIF in anti-TB chemotherapy. The introduction of PZA in the initial phase reduces the duration of RIF-containing anti-TB regimens from 9 to 6 months (7, 8). At the beginning of anti-TB treatment or during the follow-up maintenance phase, PZA must be taken for 6 months or longer (8). PZA has disadvantages, because it is associated with hepatotoxicity and hyperuricemia. The severity of this hepatotoxicity is no less than that associated with INH or RIF (3, 5, 6). A high incidence of hepatotoxicity was reported when a high dose of PZA (40 to 70 mg/kg) was used (9, 12). Studies have shown that of 114 TB patients treated with PZA for 2 months, 16% had increased liver enzymes, 7.9% had 5 times more liver enzymes than normal, and 5.3% had hepatitis symptoms (17, 18). These data show that PZA hepatotoxicity cannot be ignored.

General liver toxicity and liver histology markers and the U.S. Food and Drug Administration (FDA)-recommended galactose single-point (GSP) method were measured *in vivo*. The GSP method has been successfully used to predict prognoses and is correlated with the severity of liver disease, chronic hepatitis, cirrhosis, hepatocellular carcinoma, and nonalcoholic fatty liver disease (19–23). The FDA has recommended the GSP method in its industry pharmacokinetics guidelines for patients with impaired hepatic function.

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FIG 1 Metabolic pathway of PZA (8, 9).

Certain studies have demonstrated that amidase inhibitor pretreatment could prevent INH-induced hepatotoxicity in rabbits (24). However, the toxic mechanism of PZA remains unknown and hepatotoxicity remains unpreventable. This study determines whether PZA or PZA metabolites are responsible for PZA-induced hepatotoxicity. To clarify which PZA metabolites are responsible for toxicity, PZA and its metabolites were identified and cytotoxicity in HepG2 cells was assessed. Potential hepatotoxicity from PZA and PA was then tested in rats. Urine specimens were collected from 153 patients, and these were evaluated to determine whether a correlation exists between PZA metabolite concentration and hepatotoxicity. Based on these *in vitro* animal and patient findings, this study hypothesizes that coadministration of amidase inhibitor (bis-*p*-nitrophenyl phosphate [BNPP]) decreases or prevents PZA- and PZA metabolite-induced hepatotoxicity in rats.

MATERIALS AND METHODS

PZA and PZA metabolite preparation. Two PZA metabolites, 5-OH-PZA and 5-OH-PA, which are not commercially available, were synthesized using a method modified from Whitehouse et al. (8, 12). All organic solvents were high-performance liquid chromatography (HPLC) grade and were obtained from Tedia (Fairfield, OH). PZA, PA, and BNPP were purchased from Sigma (St. Louis, MO). Both 5-OH-PZA and 5-OH-PA were prepared by reacting PZA (0.5 mmol for 3 days at 37°C) or PA (0.35 mmol for 4 days at 37°C) dissolved in a phosphate buffer with xanthine oxidase (100 U/10 ml; Sigma, St. Louis, MO). The reaction was monitored by HPLC and then purified by a preparative HPLC system, producing 5-OH-PZA and 5-OH-PA.

Cytotoxicity of PZA and PZA metabolites in HepG2 cells. (i) **Cell lines and culture.** Human hepatoma cell line HepG2 was used to assess the cytotoxicity of PZA and PZA metabolites *in vitro*. M. J. Chou and C. S. Yang provided human hepatoma cell line HepG2. The cancer cells were maintained with an RPMI 1640 medium containing 10% fetal bovine serum (FBS) and 100 ng/ml penicillin and streptomycin at 37°C in a humidified atmosphere with 5% CO₂. *Mycoplasma* infection was excluded from the cultured cells by PCR screening methods before each experiment.

(ii) **Cytotoxicity analysis by MTT assay.** Viable cells treated with different concentrations of sinus extracts were evaluated before and after treatment using a modified MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay triplicate. The cells (5×10^3) were incubated in 96-well plates containing 200 μ l of the growth medium. Cells adhered for 8 to 12 h and were washed with phosphate-buffered saline and then treated with herbal extracts dissolved in a medium. After 24 h of

exposure, the drug-containing medium was replaced with fresh medium. Cells in each well were then incubated at 37°C in 50 μ l MTT (5 mg/ml) for 4 h. After the medium and MTT were removed, 200 μ l dimethyl sulfoxide (DMSO) and 25 μ l glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5) were added to each well. Absorbance was detected at 570 nm of the dissolved solutions by a microplate enzyme-linked immunosorbent assay (ELISA) reader (MRX II; DYNEX Technologies, Chantilly, VA). INH was used as a positive control. The absorbance of untreated cells was considered 100%. Cytotoxicity of PZA and PZA metabolites was tested on the HepG2 cell line.

Hepatotoxicity in animals. (i) **Animals.** Male Wistar rats between 300 and 325 g were obtained from the National Applied Research Laboratory and National Laboratory Animal Center (Taipei, Taiwan, Republic of China). All experiments adhered to the National Institutes of Health (NIH) guidelines for the treatment of animals. All rats were maintained in a room with air and humidity control, with a 12-h light-dark cycle. They were given *ad libitum* access to food and water throughout the experiment. All rats were anesthetized with ether, and galactose was intravenously injected. After 60 min, a tail-vein blood sample was used to measure the galactose blood concentration or GSP value.

(ii) **Hepatotoxicity study.** Rats were randomly divided into 5 treatment groups for 7 weeks. In the normal control group (NC; $n = 10$ rats), normal rats were orally administered 1 ml of saline/kg of body weight daily. In the PZA 500 group (C1; $n = 10$ rats), normal rats were orally administered 1 ml/kg of 500 mg/kg PZA in saline daily. In the PZA and BNPP group (S1; $n = 10$ rats), normal rats were orally administered 1 ml/kg of 50 mg/kg BNPP and 500 mg/kg PZA in saline. In the PA 500 group (C2; $n = 10$ rats), normal rats were orally administered 1 ml/kg of 500 mg/kg PA in saline daily. In the PA and BNPP group (S2; $n = 10$ rats), normal rats were orally administered 1 ml/kg of 50 mg/kg BNPP and 250 mg/kg PA in saline daily. The PZA dose corresponded to twice the human therapeutic dose, based on species sensitivity guidelines from the FDA. A GSP test was conducted 16 h after treatment at 0, 3, 5, and 7 weeks.

(iii) **Blood sampling.** At the end of treatment, the animals were sacrificed under diethyl ether anesthesia. Blood was collected in heparin tubes from rat hearts, and the plasma was separated by centrifugation at $13,000 \times g$ for 5 min at 4°C. Aliquots of the plasma were transferred to Eppendorf tubes and stored at -80°C until analysis.

(iv) **Hepatic AST and ALT determination.** Plasma enzyme activities were determined at 37°C (aspartate transaminase [AST] and alanine aminotransferase [ALT]) using a Synchron LXi 725 (Beckman Instruments, Palo Alto, CA) with manufacturer-provided kits.

(v) **Histological examinations.** Immediately after sacrifice, the rat livers were removed for histology analysis. For light microscopy, liver specimens ($n = 10$ from each group) were fixed in 10% phosphate-buffered formalin, dehydrated, and embedded in paraffin. Each paraffin-embedded block was cut into 5- μ m sections and then stained with hematoxylin, eosin, and periodic acid-Schiff for histology observation. A pathologist, who was blind to the treatment groups and the corresponding liver biochemistries, assessed liver histology. Histological assessment was graded using the histological activity index (HAI) according to the criteria of Knodell et al. (25). The HAI score is divided into 2 components: HAI-necroinflammation (HAI-NI) and fibrosis. The HAI-NI score includes 3 components: 0 to 10, piecemeal necrosis; 0 to 4, lobular necrosis and inflammation; and 0 to 4, portal inflammation.

(vi) **Quantitative testing of liver function.** Study animals received a rapid intravenous administration of 0.5 g/kg BW galactose solution (GSP, 0.4 g/ml). This injection must be completed in 30 s. Dried blood spots were taken from tail veins at 5, 10, 15, 30, 45, and 60 min after injection. GSP values were defined as the galactose blood concentration 60 min after injection (19).

PZA and PZA metabolite analysis of TB patient urine. (i) **Liquid chromatography.** This study recruited 153 Taiwanese TB patients (105 men and 48 women) diagnosed at Tri-Service General Hospital. The Tri-Service General Hospital Institutional Review Board approved the study,

TABLE 1 Cytotoxicity of pyrazinamide and its metabolites in HepG2 cells

Compound	IC ₅₀ (mg/ml) ^a
Pyrazinamide (PZA)	1,184.3 ± 120.2
Pyrazinoic acid (PA)	821.4 ± 83.8
5-Hydroxy-pyrazinamide (5-OH-PZA)	2,220.2 ± 562.0
5-Hydroxy-pyrazinoic acid (5-OH-PA)	509.0 ± 72.4
Isoniazid (INH)	2,440.0 ± 560.9

^a Data are shown as means ± SD. Each experiment was repeated in triplicate.

and patients signed an informed consent form before participating. Inclusion criteria included a sputum smear with acid-fast bacilli or a positive culture for *M. tuberculosis*, undergoing anti-TB PZA treatment or beginning treatment in the recruitment period, hepatitis serology recorded on a medical chart, and signed written consent. Patients were excluded if they were infected with hepatitis B virus (HBV) or HCV, had any other hepatic disease, or did not have PZA treatment in their medical records. Patient urine was collected and PZA and PZA metabolites were analyzed using HPLC.

HPLC was performed using a dual-piston reciprocating pump (LC-10ATVP), UV-visible dual-wavelength detector (SPD-10AVP), and autoinjector (SIL-10ADVP). Data were acquired using CLASS-VP software (Shimadzu Systems, Kyoto, Japan). PZA and PZA metabolites were separated on a 25-cm by 4.6-mm, 5-μm-particle, LiChroCART 250-4 LiChrospher 100RP-18 column. Separation was achieved using gradient elution. The gradient was prepared from mixtures of methanol and 0.01 M KH₂PO₄ (pH 5.2). The detection limits for PZA, PA, 5-OH-PZA, and 5-OH-PA were 10, 10, 25, and 25 ng/ml, respectively. A total of 100 μl of 2 M perchloric acid was placed in a 5-ml glass tube containing 0.5 ml sample, 0.3 ml distilled water, and 100 μl 10 mM 2,3-pyrazine-dicarboxamide as the internal standard. This was thoroughly mixed for 10 s. After centrifugation at 5,000 rpm for 10 min, 200 μl supernatant was diluted at a ratio of 1:100, and 20 μl was loaded onto the column.

Anti-TB efficacy assays. *M. tuberculosis* strain H37Ra was grown in 7H9 liquid medium (Difco) supplemented with 0.05% Tween 80 and 10% bovine serum albumin dextrose-catalase enrichment (Difco) at 37°C for 3 weeks with occasional shaking. *Mycobacterium smegmatis* mc26 (MC2) was similarly cultivated in 7H9 medium at 37°C for 4 days. To analyze the effect of PZA with and without BNPP on *Mycobacterium* survival, *M. tuberculosis* H37Ra or *M. smegmatis* cells were resuspended in the pH 6.5

7H9 liquid medium with 20 μg/ml PZA without or with 0.15, 1.5, and 15 mg/ml BNPP. When mycobacteria were incubated at a cell density of 0.3 at an optical density at 600 nm (OD₆₀₀) at 37°C, aliquots of the cell suspension were removed, washed, and diluted before plating on 7H11 plates. The plates were then incubated at 37°C for 4 weeks for *M. tuberculosis* and for 5 days for *M. smegmatis* to determine the number of surviving *Mycobacterium*.

RESULTS

Cytotoxicity of PZA and PZA metabolites in HepG2 cells. PZA metabolites (PA and 5-OH-PA) were more toxic than PZA (5-OH-PA [50% inhibitory concentration (IC₅₀) of 509 μg/ml] > PA [IC₅₀ of 821 μg/ml] > PZA [IC₅₀ of 1,184 μg/ml]) in HepG2 cells (Table 1).

Hepatotoxicity in animals. (i) Hepatotoxicity of PZA and PA in rats. The body and relative liver weights of the experimental animals were measured at the end of the study. No statistically significant differences existed between experimental and control animals.

Hepatotoxicity was successfully induced by oral administration of PZA or PA at a dose of 500 mg/kg/day over 7 weeks. Each group contained at least 7 rats. A relatively normal liver architecture was found in the NC group (Fig. 2A). Hepatocytes in the liver parenchyma were arranged into one-cell-thick anastomosing plates radiating from the central vein of the lobules. Hepatic sinusoids were found between 2 anastomosing plates of hepatocytes. Hepatocellular disintegration and vacuolation of the pericentral vein hepatocytes were observed in rats treated with PZA or PA (Fig. 2B and D). Plasma AST and ALT activity increased in rats treated with PZA, and ALT activity also increased in animals treated with PA (Table 2). GSP levels increased significantly in the PZA (776 ± 65 mg/liter; *P* < 0.005) and PA (588 ± 153 mg/liter; *P* < 0.005) groups compared to those in the NC group (260 ± 50 mg/liter), as shown in Table 2.

Liver histology was graded using the HAI, according to the criteria of Knodell et al. (25). A pathologist conducted the assessments. Table 2 shows that HAI-NI scores were significantly greater for the PZA (*P* < 0.005) and PA (*P* < 0.05) groups than for the NC group, with the PZA group showing the greatest differ-

FIG 2 Photomicrography of liver sections from rats after either no treatment (normal control [NC]) (A) or 7 weeks of treatment with 500 mg/kg PZA (B), 500 mg/kg PZA-BNPP (C), 500 mg/kg PA (D), or 500 mg/kg PA-BNPP (E). (A) Relatively normal liver tissue morphology in the NC group. (B and D) H&E stain; original magnification, ×200. CV, central vein; PA, portal vein.

TABLE 2 GSP levels, hepatic AST and ALT activities, and total HAI score in NC, PZA control, PZA-BNPP study, PA control, and PA-BNPP study groups after 7 weeks of treatment^a

Group	GSP (mg/liter) ^b	AST (IU/liter) ^c	ALT (IU/liter) ^c	Total HAI score ^d
Normal control (NC; <i>n</i> = 10)	248 ± 37	132 ± 33	50 ± 18	0.0 ± 0.0***
PZA 500 (C1; <i>n</i> = 7)	776 ± 65***	179 ± 21**	91 ± 11**	4.0 ± 1.2
PZA 500-BNPP (S1; <i>n</i> = 8)	293 ± 61**	124 ± 52	82 ± 26	1.2 ± 0.3**
PA 500 (C2; <i>n</i> = 8)	588 ± 153***	150 ± 29	82 ± 30*	2.3 ± 0.7*
PA 500-BNPP (S2; <i>n</i> = 8)	489 ± 65***	167 ± 31	88 ± 11*	2.6 ± 0.4

^a Data are shown as means ± SD. Each group contained at least seven rats (*n* = sample size). Statistical analysis: ANOVA and LSD test.

^b ***, *P* < 0.005 versus NC group, and S1 versus C1 group.

^c **, *P* < 0.01; *, *P* < 0.05 versus NC group.

^d ***, *P* < 0.005, each group versus NC group; *, *P* < 0.05; **, *P* < 0.01 versus C1 group.

ence. These results indicate that 5-OH-PA and PA could be the molecular species responsible for PZA-induced hepatotoxicity.

Analysis of PZA and PZA metabolites in TB patient urine.

Urine specimens were collected from 153 TB patients and evaluated to determine whether a correlation exists between PZA toxic metabolite concentration and hepatotoxicity. HPLC was used to analyze PZA and PZA metabolite concentration in 153 Taiwanese TB patient urine specimens (Table 3). Based on the exclusion and inclusion criteria, 39 patients were excluded: 30 patients had not undergone PZA treatment, 6 patients had hepatitis B or C, and the urine of 3 patients had not been examined for PZA levels. Of the remaining 114 patients, 35 presented with hepatotoxicity. PZA and PZA metabolite concentrations were adjusted by normalizing them to their respective risk factors (IC₅₀ of PZA/IC₅₀ of PZA metabolites). Of the patients treated with PZA, the urine of 6 patients (5.3%) exhibited 5 times more AST and ALT activity than the control group, indicating severe hepatotoxicity. The urine of 29 patients (25.4%) exhibited twice as much activity, indicating mild hepatotoxicity (Table 4).

In the urine of patients with severe hepatotoxicity, 5-OH-PA was at the highest relative concentration (527 ± 138), followed by PA (49 ± 19) and then 5-OH-PZA (14 ± 11). The concentration of PZA in the urine of TB patients with severe hepatotoxicity (6 ± 1 μg/ml) was significantly lower than in patients without hepato-

toxicity (18 ± 3 μg/ml; *P* < 0.005), indicating that PZA concentration is inversely correlated with hepatotoxicity. The relative ratio of 5-OH-PA to PZA was significantly higher in the urine of patients with severe hepatotoxicity (527 ± 138; *P* < 0.005) than in to patients with mild (157 ± 86) or no hepatotoxicity (49 ± 51). This suggests that increased hepatotoxicity may be caused by increased PZA metabolism (Table 4). Similarly, the ratio of PA to PZA in the urine of patients with severe and mild hepatotoxicity was significantly greater than in patients with no hepatotoxicity (severe, 49 ± 19; mild, 36 ± 28; and none, 11 ± 10; *P* < 0.005). However, no significant difference was observed in the PA-to-PZA ratio between patients with severe and mild hepatotoxicity. These results indicate that 5-OH-PA is the main metabolite responsible for PZA-induced hepatotoxicity in TB patients.

Effect of selected amidase inhibitors on PZA-induced hepatotoxicity. Based on these animal and patient *in vitro* findings, the coadministration of amidase inhibitor BNPP should decrease or prevent PZA- and PZA metabolite-induced hepatotoxicity in rats.

Oral administration of PZA or PA at a dose of 500 mg/kg/day over 7 weeks successfully induced hepatotoxicity (Table 2). Hepatoprotective treatment using amidase inhibitor BNPP decreased PZA-induced hepatotoxicity (GSP levels significantly decreased from 776 ± 65 to 293 ± 61 mg/liter) but did not prevent PA-induced hepatotoxicity. Table 2 shows that GSP levels in the PA group were 588 ± 153 mg/liter and 489 ± 65 mg/liter in the PA and BNPP group. Examination of liver histology by electron microscopy confirmed that BNPP treatment ameliorated PZA-induced, but not PA-induced, hepatotoxicity (Fig. 2). These results demonstrate that 5-OH-PA is the main toxic metabolite responsible for PZA-induced hepatotoxicity, which supports the original findings that 5-OH-PA is the main cause of PZA-induced hepatotoxicity.

TABLE 3 Characteristics of 153 TB patients with or without hepatotoxicity

Characteristic	Hepatotoxicity ^a		<i>P</i> ^b
	Present (<i>n</i> = 41 patients)	Absent (<i>n</i> = 112 patients)	
Age (yrs)	60.6 ± 19.5	57.8 ± 22.9	0.930
Gender (male/female)	25/16	80/32	0.729
Before treatment			
AST (U/liter)	23.9 ± 7.7	24.8 ± 6.2	0.990
ALT (U/liter)	23.0 ± 8.9	21.5 ± 8.4	0.891
Total bilirubin (mg/dl)	0.57 ± 0.34	0.49 ± 0.28	0.673
During treatment			
AST (U/liter)	181.8 ± 130.0	24.4 ± 7.2	0.000
ALT (U/liter)	198.8 ± 122.3	19.2 ± 9.0	0.000
Total bilirubin (mg/dl)	1.5 ± 0.6	0.5 ± 0.3	0.003

^a Subjects were TB patients. Data are shown as means ± SD. Normal interval of AST, 0 to 40 U/liter; ALT, 5 to 45 U/liter; total bilirubin, 0.2 to 1.6 mg/dl.

^b Statistical analysis, crosstabs chi-square test.

TABLE 4 Correlation between hepatotoxicity and urine PZA and its metabolites in 114 TB patients treated with fixed-dose antituberculosis combinations

Metabolite/PZA	Ratio ^a		
	No hepatotoxic (<i>n</i> = 79 patients)	2× hepatotoxic (<i>n</i> = 29 patients)	5× hepatotoxic (<i>n</i> = 6 patients)
5-OH-PA/PZA	49 ± 51	157 ± 86 B	527 ± 138 AB
PA/PZA	11 ± 10	36 ± 28 B	49 ± 19 B
5-OH-PZA/PZA	4 ± 3	16 ± 14 B	14 ± 11 C

^a PZA metabolites/PZA. Data are shown as means ± SD. Statistical analysis, ANOVA and LSD test. A, *P* < 0.005 relative to 2× hepatotoxic; B, *P* < 0.005 relative to no-hepatotoxic group; C, *P* < 0.01 relative to no-hepatotoxic group.

FIG 3 Effect of PZA treatment with or without BNPP on the survival of *M. tuberculosis* and *M. smegmatis*. Survival (%): colony numbers of different treatment groups/control group $\times 100$. Data are shown as means $\pm$ standard deviations (SD). Each treatment was repeated in triplicate. Statistical analysis: analysis of variance (ANOVA) and least significant difference (LSD) test.

Anti-TB efficacy assays. This study tested whether BNPP affects the efficacy of a combined PZA anti-TB treatment. *M. tuberculosis* and *M. smegmatis* were selected as anti-TB test strains. Adding various doses of BNPP caused no significant change in *Mycobacteria* survival (Fig. 3). These results indicate that combining 0.15 to 15 mg/ml BNPP with PZA does not affect the anti-TB activity of PZA. This suggests that BNPP may effectively protect rats against PZA-induced hepatotoxicity without interfering with PZA efficacy.

DISCUSSION

Before this study, no published work investigated whether PZA or its metabolites were responsible for PZA-induced hepatotoxicity. 5-OH-PZA and 5-OH-PA (PZA and PA metabolites) were purified by xanthine oxidase catalysis. PA and 5-OH-PA were more toxic than PZA in HepG2 cells (Table 1). A rat model was then used to confirm that PA had a hepatotoxic effect similar to that of PZA at a dose of 500 mg/kg (Table 2). These results indicate that 5-OH-PA and PA could be the molecular species responsible for PZA-induced hepatotoxicity.

Analysis of the urine of TB patients undergoing PZA treatment showed that 5-OH-PA/PZA and PA/PZA metabolic ratios increased with hepatotoxicity severity (Table 4). PZA decreased in patients with severe hepatotoxicity, probably because of increased conversion to 5-OH-PA and PA. These data support the first hypothesis that 5-OH-PA and PA are the main toxic metabolites responsible for PZA-induced hepatotoxicity.

Based on animal and patient *in vitro* findings, coadministration of amidase inhibitor BNPP should reduce PZA- and PZA metabolite-induced hepatotoxicity in rats. The rat hepatotoxic results show that an amidase inhibitor can reduce PZA-induced hepatotoxicity. Diagnostic indicators of liver damage (AST, ALT, and GSP) and histopathology show that oral administration of BNPP significantly decreased PZA-induced hepatotoxicity. In the rat model of hepatotoxicity, BNPP treatment prevented PZA-induced, but not PA-induced, hepatotoxicity (Table 2). This indicates that 5-OH-PA is the main toxic metabolite responsible for PZA-induced hepatotoxicity. Therefore, an amidase inhibitor such as BNPP may prevent PZA-induced hepatotoxicity. BNPP does not interfere with the anti-TB effects of PZA (Fig. 3).

Based on these results, coadministration of BNPP should decrease or prevent PZA-induced hepatotoxicity in TB patients. Se-

lected amidase inhibitors from 55 natural components in food and commonly used herbal medicines were used to explore whether they can prevent PZA-induced hepatotoxicity in rats. The results show that a natural compound, quercetin, is a potent amidase inhibitor that exhibits up to 75% inhibition. Because BNPP is a chemical dye that caused irritation or allergy through oral administration, dermal absorption, or inhalation, it is difficult to administer to patients. Quercetin is a flavonoid component found in vegetables and fruits, including apples, citrus fruit, broccoli, grapefruit, onions, tea, and *Ginkgo biloba* extract (26, 27). The FDA granted quercetin generally recognized as safe (GRAS) status on 22 November 2010 (FDA GRAS inventory number GRN 341). This demonstrates that quercetin has no safety concerns that could apply to patients. After confirming that quercetin significantly affects amidase inhibition in animals and humans, a clinical trial should be conducted to test the efficacy of amidase inhibitor in TB patients treated with PZA.

This is the first cell line, animal, and clinical study to confirm that metabolite 5-OH-PA causes PZA-induced hepatotoxicity. This study presents a novel and feasible strategy for preventing PZA-induced hepatotoxicity by inhibiting amidase. Because certain PZA metabolites contribute differently to PZA toxicity, the activity or expression level of metabolic enzymes, such as amidase and xanthine oxidase, may be correlated with PZA-related side effects, such as hepatitis and hyperuricemia. Future studies should explore single nucleotide polymorphism of amidase and xanthine oxidase, genotyping and phenotyping, and drug-induced hepatotoxicity correlation.

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The authors declare that they have no conflict of interests.

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