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Research Article
Curcumin Enhances the Systemic Exposure of Isoniazid in Rats: Role of NAT2 in the Liver and Intestine

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Abstract

Background and Objective: N-acetyltransferase 2 (NAT2) is the main enzyme that responsible for isoniazid (INH) metabolism, the expression and function of NAT2 play important roles in the pathogenesis of INH-induced hepatotoxicity. Concerning the potential inhibitory effect of curcumin on NAT2, it is of great importance to know the effect of curcumin on INH metabolism and evaluate enzyme-mediated drug-drug interaction. **Materials and Methods:** Male Wistar rats were orally administered with INH (60 mg kg^{-1}) alone or in combination with curcumin (100 mg kg^{-1}). The serum concentration and tissue distribution of INH and its metabolite acetylisoniazid (AcINH) were determined using HPLC-MS/MS, the protein expression and function of NAT2 in the liver and small intestine were evaluated further and molecular docking technique was also applied to explore the interaction between curcumin and NAT2. **Results:** Curcumin pretreatment significantly elevated the serum concentration and tissue distribution of INH accompanied with marked reduction of AcINH, the parameter AUC_{0-t}, $t_{1/2}$ and C_{max} in curcumin pretreated rats were all significantly increased, while CL/F was markedly reduced. NAT2 activity in the liver and small intestine were significantly inhibited by curcumin pretreatment. Molecular docking study showed that curcumin could locate at the hydrophobic pocket and form a strong binding to NAT2. **Conclusion:** Results from this study indicate that curcumin could inhibit NAT2 activity and further affect the pharmacokinetics of INH. This study may provide a cue for reducing INH-induced hepatotoxicity and improving INH potency by curcumin co-administration.

Key words: Curcumin, isoniazid, N-acetyltransferase 2, pharmacokinetics, drug-drug interaction

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Nowadays, tuberculosis continues to be the main threat to global health, it is estimated that over 2 billion people are carrying latent tuberculosis infection globally[1]. Isoniazid (INH), the commonly used first-line chemotherapeutic drug for tuberculosis, is usually prescribed alone or in combination with other anti-tuberculosis drugs, such as rifampicin, pyrazinamide or ethambutol[2]. Despite the highly beneficial effect of INH for tuberculosis treatment, hepatotoxicity, the most frequently reported side effect of INH, has hampered its clinical application. Although the exact mechanism of INH-induced hepatotoxicity is still not fully identified, the metabolites of INH have been thought to be involved in INH related liver injury[3].

The major metabolic pathways of INH are enzymatic-dependent reactions involving acetylation and hydrolysis reactions. N-acetyltransferase 2 (NAT2), one of the phase II drug-metabolizing enzymes, is the dominant enzyme that catalyzes the acetylation of INH, which expression is limited to the liver and gastrointestinal tract[4].Upon the action of NAT2, INH can be firstly metabolized to acetylisoniazid (AcINH) and AcINH is further hydrolyzed to generate acetylhydrazine (AcHz) through amidase[5]. It has been proposed that AcINH as well as AcHz, the 2 metabolites of INH, were responsible for INH-induced liver injury, for these metabolites were found to cause hepatic necrosis and cell death[6]. Nowadays, the prediction model has been studied using NAT2 gene in order to assess INH-induced liver injury[7]. Evidence from clinical studies showed that INH-induced hepatotoxicity had close correlation with NAT2 gene polymorphisms[8,9], indicating the important role of NAT2 in the pathogenesis of INH related hepatotoxicity[10].

Curcumin, a phenolic compound from *Curcuma longa*, showed multiple bioactivities, such as anti-inflammatory, anti-microbial, anti-carcinogenic and anti-aging activities[11]. Accumulated evidence showed that curcumin had hepatoprotective effect on anti-tubercular drug-induced hepatotoxicity in cells model[12] and also to prevent isoniazid/rifampicin-induced liver injury in mice[13], indicating the protective role of curcumin against INH-induced liver injury. Results from previous studies showed that NAT2 activity could be suppressed by curcumin in a dose-dependent manner[14,15], we speculate that the hepatoprotective effect of curcumin may have correlation with its inhibitory effect on NAT2 activity. Recently, curcumin and its derivatives have been identified to have anti-bacterial activity against drug-resistant *Mycobacterium abscessus* and *Mycobacterium tuberculosis*[16,17], indicating the potential therapeutic role of curcumin on tuberculosis.

In view of the protective effect of curcumin on INH-related hepatotoxicity and the potential therapeutic effect of curcumin on tuberculosis, it is of great importance to know the effect of curcumin on INH metabolism as well as on NTA2 function *in vivo*. In this present study, we investigated the effect of curcumin on serum concentration and tissue distribution of INH and AcINH in rats. To investigate the enzyme-mediated drug interaction, the expression as well as function of NAT2 in the liver and intestine was evaluated after curcumin pretreatment and molecular docking study was also applied to explore the interaction between curcumin and NAT2. The aim of this study was to study the effect of curcumin on INH metabolism and pharmacokinetic profile, the finding of this study may provide important information for enzyme-mediated drug-drug interaction, especially for the co-administration of curcumin and INH.

MATERIALS AND METHODS

Study area: This study was carried out in the Lab of Department of Pharmacy in First Hospital of Lanzhou University from May to December in 2018.

Animals: Male Wistar rats (9-month-old, weighing 260-280 g) were obtained from the Experimental Animal Center of Gansu University of Chinese Medicine (Lanzhou, China). The rats were housed in cages on a 12-12 h light-dark cycle with free access to food and water. The study was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the committee on use of Animal Subjects in Teaching and Research of Lanzhou University (LDYYLL2017-37).

Pharmacokinetic experiment: To investigate the effects of curcumin on the pharmacokinetics of INH, rats were randomly divided into 3 groups (n = 6/group). Rats in INH group were orally administered INH (60 mg kg^{-1}, Sigma-Aldrich, Missouri, USA), while rats in co-administration group were orally treated with curcumin (100 mg kg^{-1}, HaoXuan Pharmaceutical Co. Ltd., Xi' an, China) at 30 min prior to INH (60 mg kg^{-1}) administration. After single drugs administration, blood samples (0.25 mL) were collected through the right side of the femoral artery at 0.5, 1, 2, 4, 6, 8, 12 and 24 h after INH administration, respectively[18]. Serum concentration of INH and AcINH were determined using HPLC-MS/MS, the pharmacokinetic parameters were calculated using DAS2.0 software (Medical College of Wannan, China). The pharmacokinetic parameters included area under the plasma

concentration-time curve (AUC), time-averaged total body clearance (CL/F), terminal half-life ($t_{1/2}$), apparent volume of distribution at steady state (V/F) and peak plasma concentration (C_{max}).

Tissue distribution experiment: To investigate the effect of curcumin on tissue distribution of INH, rats were randomly divided into 2 groups (n = 5). The group setting and drug treatment were as same as that of pharmacokinetic study[19]. At 1 or 3 h post INH administration, the tissues of brain, lung, liver, small intestine and kidney were collected. After rinsing with saline, the tissues were homogenized in water (200 mg mL^{-1}), centrifuged at 18000 g for 10 min. The supernatants were collected, prepared as that of serum samples and then the concentration of INH as well as AcINH was determined using LC-MS/MS.

Western-blot analysis: Liver and small intestine were collected and homogenized in lysis buffer (Beyotime Biotechnology, Nantong, China) for protein extraction. The samples were denatured at 95 for 5 min, separated by 10% SDS-PAGE gel and transferred to PVDF membrane (GE healthcare, Princeton, USA). The membrane was first incubated with 5% non fat milk for 1 h in room temperature and further incubated with primary antibody (anti-NAT2, 1:500, Proteintech, Wuhan, China) overnight at 4 . After rinsing in TBST solution, the immunoreaction was detected using ECL Western blotting kit (Thermo Fisher Scientific, USA), the optical density of each band was semi-quantified by Image J. software (NIH, USA) [14].

NAT2 activity assay: Approximately 300 mg of liver tissues or small intestine were homogenized in deionized water to prepare homogenate and then centrifuged at 13,000 g for 5 min. The supernatant was collected for NAT2 activity assay and the protein concentration was also determined using Protein Assay Kit (Thermo Fisher, USA). NAT activity was evaluated by Ng *et al.*[20]. Briefly, the reaction vials contained cell lysate and INH (1.25-10 mM), curcumin was added and pre-incubated for 10 min before the reaction. Reactions were started by the addition of acetyl-CoA (100 μM), incubated at 37 for 30 min. After centrifugation at 16,000 g for 3 min, AcINH in the supernatant was quantified by LC-MS/MS.

HPLC-MS/MS detection: The amount of INH and AcINH was determined by LC-MS/MS by Ruan *et al.*[21]. To each 20 μL of sample, 60 μL of methanol were added to precipitate the proteins. The mixture was vortexed and centrifuged at 16,000 g for 10 min, the supernatant was collected for HPLC-MS/MS analysis. The detection were performed on an Agilent 1260 infinity HPLC coupled to an Agilent 6460 mass spectrometer equipped with an electrospray ionization interface (Agilent Technologies, USA). Agilent HC-C18 column (4.6×250 mm, 5 μm particles) and Agilent Eclipse XDB-C18 4.6×12.5 mm analytical guard column (Agilent Technologies, USA) were used in this study. The transition was 138→121 with positive ESI for INH and 180→121 with positive ESI for AcINH.

Molecular docking: Molecular docking study was performed to investigate the binding mode between curcumin and NAT2 using[22] AutoDock Vina 1.1.2. The three-dimensional (3D) structure of theNAT2 (PDB ID: 2PFR) was downloaded from RCSB Protein Data Bank (http://www.rcsb.org/). The 2D structure of curcumin was drawn by ChemBioDraw Ultra 14.0 and converted to 3D structure. The AutoDock Tools 1.5.6 package was employed to generate the docking input files[23,24]. The ligand was prepared for docking by merging non-polar hydrogen atoms and defining rotatable bonds. The search grid of the NAT2 site was identified as center x: 9.229, center y: 40.208 and center z: 59.163 with dimensions size x: 15, size y: 15 and size z: 15. For Vina docking, the default parameters were used if it was not mentioned. The best-scoring pose as judged by the Vina docking score was chosen and visually analyzed using PyMoL 1.7.6 software (http://www.pymol.org/).

Statistical analysis: The data are expressed as Mean±SD and the differences between 2 groups were analyzed by Student's t-test. In all statistical analysis, $p<0.05$ was considered to indicate statistical significance.

RESULTS

Effects of curcumin on the pharmacokinetic profile of INH and AcINH: The serum concentration-time curves of INH and AcINH after single dose INH administration were shown in Fig. 1. Compared with INH administration group, the serum concentration of INH were significantly elevated at each time point ($p<0.05$), while the serum concentration of AcINH were all markedly decreased in curcumin and INH co-administration group ($p<0.05$). As shown in Table 1, compared with INH administration group, the pharmacokinetic parameters of INH, such as $AUC_{0\to t}$, $t_{1/2}$ and C_{max}, were all significantly increased in curcumin and INH co-administration group ($p<0.05$), while CL/F was markedly reduced ($p<0.05$). No statistical difference was found in the value of V/F between INH group and curcumin and INH co-administration group ($p<0.05$).

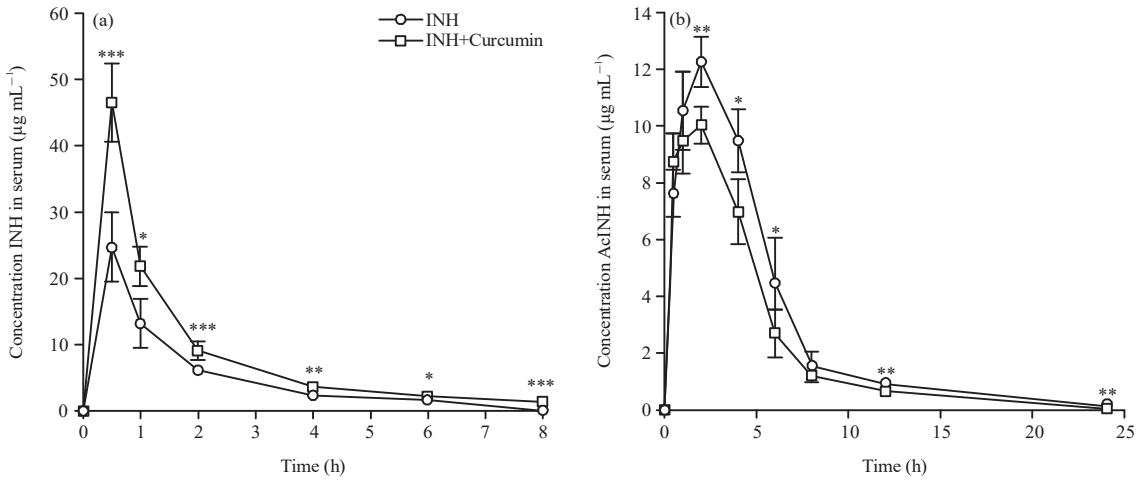

Fig. 1(a-b): Effects of curcumin pretreatment on serum concentration-time curves of (a) Isoniazid (INH) and (b) Acetylisoniazid

Concentration-time curves of INH and AcINH after single dose INH (60 mg kg^{-1}) or curcumin (100 mg kg^{-1})+INH (60 mg kg^{-1}) oral administration respectively, data are expressed as Mean±SD, *p<0.05, **p<0.01, ***p<0.001compared to the INH group (n = 6)

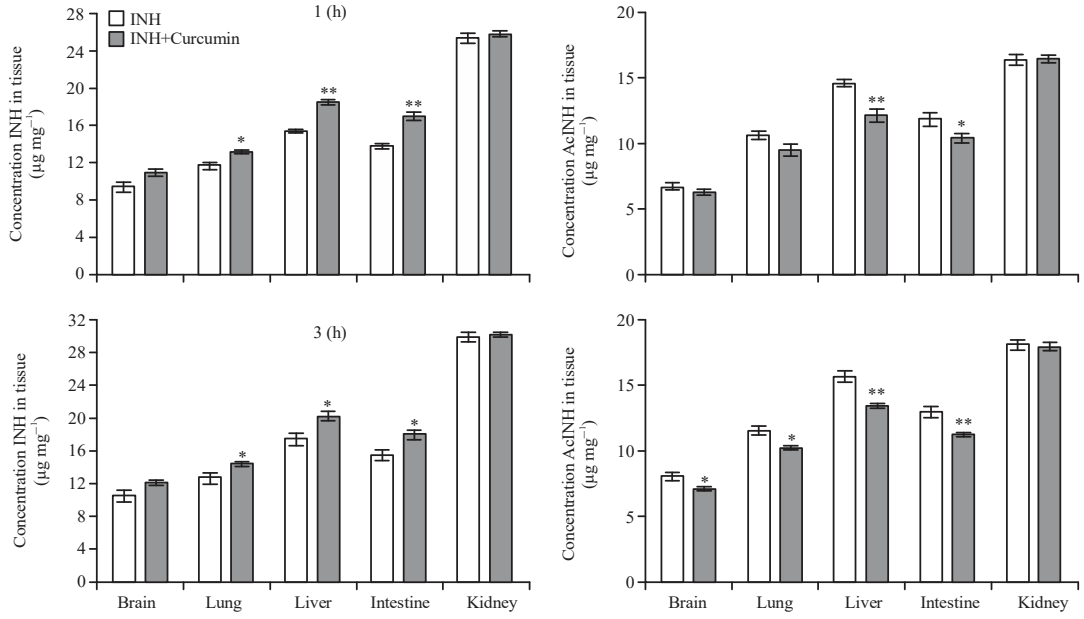

Fig. 2: Effects of curcumin on tissue distribution of isoniazid (INH) and acetylisoniazid (AcINH)

Tissues were collected at 1 h or 3 h after single dose INH (60 mg kg^{-1}) or curcumin (100 mg kg^{-1})+INH (60 mg kg^{-1}) oral administration in rats, data are expressed as Mean±SD, *p<0.05, **p<0.01 compared to the INH group (n = 5)

Table 1: Effect of curcumin on the pharmacokinetic parameters of isoniazid

Parameters	Isoniazid	Isoniazid+Curcumin
AUC_{0-t} (mg h L^{-1})	42.17±4.33	65.71±5.67***
$AUC_{0-\infty}$ (mg h L^{-1})	42.44±4.33	69.25±5.89***
$t_{1/2}$ (h)	1.10±0.16	2.13±0.41***
CL/F (L h^{-1} kg^{-1})	1.43±0.16	0.90±0.09***
V/F (L kg^{-1})	2.28±0.50	2.75±0.50
C_{max} (mg L^{-1})	25.52±4.49	47.07±8.85***

***p<0.001 compared to the isoniazid group, Mean±SD, n = 6

Effects of curcumin on tissue distribution of INH and AcINH: As shown in Fig. 2, when compared to INH treated group, at 1 h after INH administration, INH concentration of lung, liver and small intestine were markedly increased in curcumin and INH co-administration group (p<0.05), at 3 h after INH, INH concentration of lung, liver and small intestine were also markedly elevated in curcumin and INH co-administration

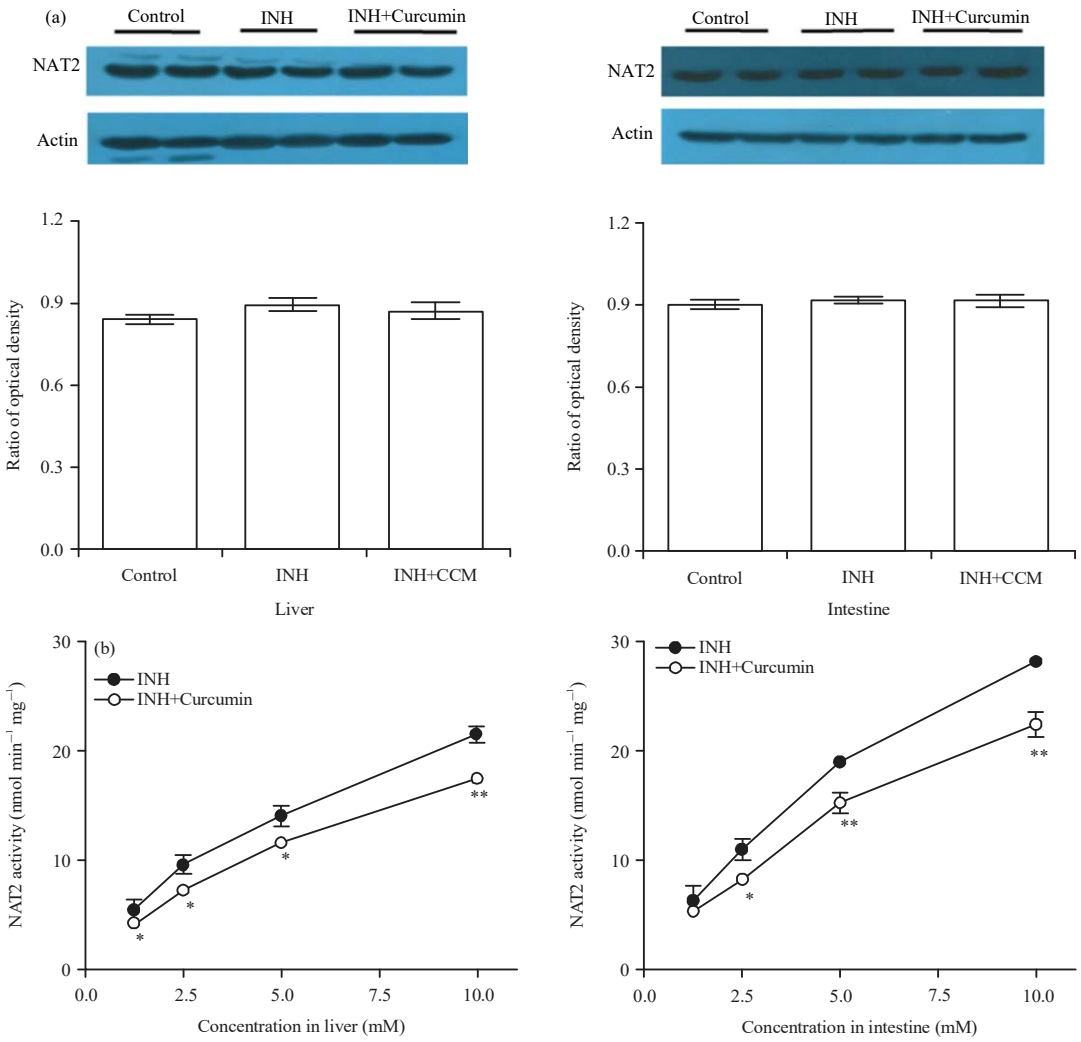

Fig. 3(a-b): Effects of curcumin on the protein expression and function of NAT2 in the liver and small intestine, (a) Western blot results of hepatic and intestinal protein expression of NAT2 and (b) NAT2 activity in the liver and intestine

Tissue were collected after single dose INH (60 mg kg^{-1}) or curcumin (100 mg kg^{-1})+INH (60 mg kg^{-1}) oral administration in rats, data are expressed as Mean±SD, *p<0.05, **p<0.01 compared to the INH group (n = 5)

group (p<0.05), no difference was found in INH concentration in the brain and kidney (p<0.05). In addition, when compared to INH treated group, at 1 h after INH administration, AcINH concentration of liver and small intestine were both significantly reduced in curcumin and INH co-administration group (p<0.05), at 3 h after INH administration, AcINH concentration of brain, lung, liver and small intestine were also markedly decreased, no difference was found in AcINH concentration in the kidney.

Effects of curcumin on NAT2 expression and activity of the liver and intestine: As shown in Fig. 3, no difference was found in NAT2 expression between INH group and curcumin plus INH co-administration group both in the liver and small intestine of rats (p>0.05, Fig. 3a). Compared with INH administration group, the NAT2 activity in the liver and intestine were all markedly decreased in curcumin and INH co-administration group (p<0.05, Fig. 3b). These results indicated that curcumin pretreatment can reduce intestinal and hepatic NAT2 activity but not NAT2 protein expression.

Binding mode of curcumin and NAT2: Curcumin was docked into the binding site of the NAT2 and the results were shown in Fig. 4. The compound curcumin adopted a compact conformation to bind at the site of the NAT2 (Fig. 4a). The

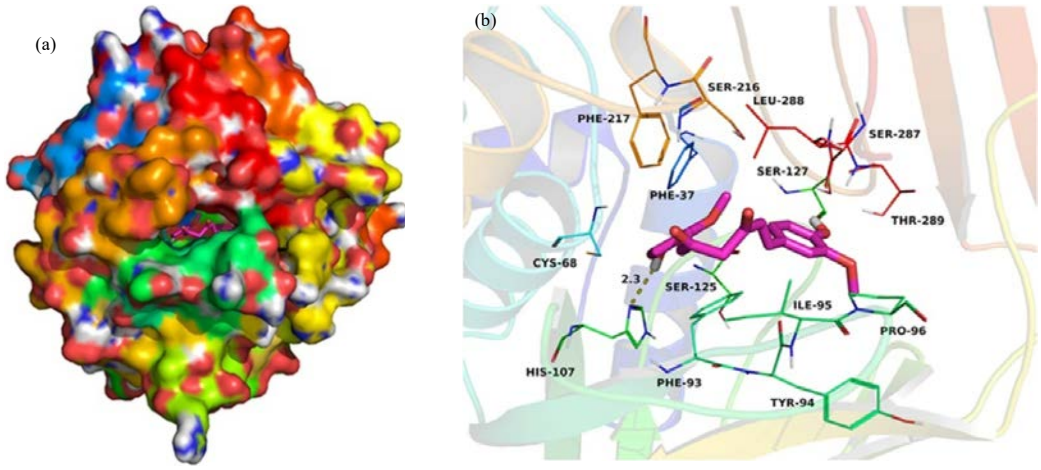

Fig. 4(a-b): Binding mode of curcumin and NAT2 (a) Compound curcumin binding at the site of NAT2 and (b) Binding mode of curcumin in the hydrophobic pocket of NAT2

key hydrogen bond interaction was located between curcumin and the residue HIS-107, which was the main interaction between curcumin and NAT2

compound curcumin located at the hydrophobic pocket, surrounded by the residues PHE-37, CYS-68, PHE-93, ILE-95, PRO-96, PHE-217 and LEU-288, forming a strong hydrophobic binding (Fig. 4b). Detailed analysis showed that one of the phenyl group of the curcumin formed π-π stacking interaction and CH-π interaction with the residues PHE-93 and PHE-217, respectively. Importantly, one key hydrogen bond interaction was observed between the curcumin and the residue HIS-107 (bond length: 2.3 Å), which was the main interaction between the curcumin and the NAT2. All these interactions helped curcumin to anchor in the binding site of NAT2.

DISCUSSION

From the data of this study, we found that curcumin pretreatment elevated the serum exposure and tissue distribution of INH in rats via inhibiting NAT2 activity. INH is an effective therapeutic and preventive agent against tuberculosis but the high rate of hepatotoxicity occurred during drug treatment largely hindered its application[25]. NAT2 is the primary enzyme that has close correlation with INH-induced hepatotoxicity[26]. Based on our previous findings[14], this study confirmed the effect of curcumin on NAT2 expression and function in vivo and further identified the enzyme-mediated drug-drug interaction on pharmacokinetics of INH.

It is well known that NAT2 is the primary enzyme that responsible for INH metabolism, the production of AcINH can reflect the function[27] of NAT2. This study firstly determined the concentration of INH and AcINH after single dose of INH. The result showed that curcumin pretreatment significantly increased the serum concentration of INH accompanied with reduced content of AcINH and the alterations from pharmacokinetic parameters also indicated that INH metabolism was significantly reduced by curcumin pretreatment. Consistent well with pharmacokinetic findings, data from tissue distribution showed that curcumin pretreatment markedly increased the tissue distribution of INH accompanied with decreased content of AcINH. Furthermore, we found that curcumin pretreatment could suppress NAT2 activity both in the liver and intestine but had no effect on NAT2 protein expression. All these data indicated that curcumin pretreatment could reduce the metabolism of INH via inhibiting NAT2 activity and thus enhanced the exposure of INH both in the serum and tissue.

In our study, we have studied the binding mode of curcumin and NAT2 using molecular docking technique, the result showed that curcumin could locate at the hydrophobic pocket and form a strong binding to NAT2. Based on the finding of this study, it seems that curcumin may act as an inhibitor of NAT2. The concentration-dependent inhibitory effect of curcumin was firstly reported in human colon tumor cells[28] and this effect was further confirmed in studies using human liver tissue, cholangiocarcinoma cell line and hepatoma cell line[14,15]. It is reported that curcumin could suppress NAT2 activity in a noncompetitive manner, suggesting that curcumin is a noncompetitive inhibitor of NAT2. It is reported that there was a poor absorption of curcumin in the gastrointestinal tract after oral intake, because

the major part of the administered curcumin was found in the small intestine[29]. It is also found that the small absorbed portion of curcumin can also be eliminated by biliary excretion, no accumulation occurs in organ tissues[30]. Considering the low oral bioavailability and poor tissue distribution of curcumin, it seems that the intestinal curcumin, instead of the hepatic curcumin, contributed much to the increased serum level and tissue distribution of INH by inhibiting NAT2 in the gut.

The multiple bioactivities of curcumin have been reported extensively, which also included the hepatoprotective effect on antitubercular drug-induced hepatotoxicity[12,13]. Results from our study showed that curcumin pretreatment markedly reduced the concentration of AcINH both in the serum and in organ tissues, which may contribute to the protective role of curcumin against INH-induced hepatotoxicity. Currently, the metabolites of INH have been considered to be the key factors in the pathogenesis of INH induced liver injury[31]. Knowing that AcINH as well as AcHz are the 2 important metabolites of INH[32], we tried to detect the content of these 2 metabolites in this study. We found that AcINH, the main metabolite of INH, could be successfully detected in the serum and tissue, while AcHz was no detectable because the low concentration. Even though, this study also provides clues for the effective role of curcumin in reducing INH metabolism, for AcINH production was the first step of INH metabolism[5]. Furthermore, in view of the newly reported anti-bacterial activity of curcumin on drug-resistant *Mycobacterium tuberculosis*[16,17], the elevated concentration of INH achieved by curcumin pretreatment may also enhance the efficiency of INH on tuberculosis. The limitation of this study is that we did not observe the systematic exposure of INH after multiple dose of curcumin or long-term INH administration.

CONCLUSION

Results of this study showed that curcumin pretreatment could elevate serum exposure and tissue distribution of INH in rats via inhibiting NAT2 activity in the liver and intestine and curcumin also markedly reduced the systemic exposure of AcINH, the harmful metabolite of INH that related to INH-induced hepatotoxicity. These findings provide important clues showing that curcumin may protect against INH-induced liver injury and enhance the therapeutic effect of INH though inhibiting NAT2 activity in the liver and intestine.

SIGNIFICANCE STATEMENT

This study discover the possible inhibitory effect of curcumin on N-acetyltransferase 2 that can be beneficial for elevating the serum exposure and tissue distribution of INH, which is accompanied with reduced systemic exposure of its harmful metabolite. This study will help the researcher to uncover the critical areas of N-acetyltransferase 2 and INH metabolism that many researchers were not able to explore. Thus a new theory on enhancing the therapeutic effect of INH though inhibiting N-acetyltransferase 2 activity may be arrived at.

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