Biochemical and Biophysical Research Communications 497 (2018) 485–491

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

First-line anti-tuberculosis drugs induce hepatotoxicity: A novel mechanism based on a urinary metabolomics platform

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ARTICLE INFO

Article history:
Received 28 January 2018
Accepted 5 February 2018
Available online 15 February 2018

Keywords:
Metabolomics
Tuberculosis
Pyrazinamide
Purine metabolism
First-line anti-tuberculosis drugs
Prediction

ABSTRACT

Tuberculosis (TB) has become a global public health and social threat. As clinical first-line drugs, rifampicin and isoniazid used in combination with pyrazinamide and ethambutol (the HRZE regimen) usually induce hepatotoxicity. However, the mechanisms underlying this phenomenon remain unclear, and studying the metabolic impact of co-treating TB patients with the HRZE regimen can provide new hepatotoxicity evidence. In this study, urine metabolites from TB patients were profiled using a high-resolution ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) platform. The tricarboxylic acid circulation, arginine and proline metabolism and purine metabolic pathways were found to be affected by anti-TB drugs. The levels of pyroglutamate, isocitrate, citrate, and xanthine were significantly decreased after the administration of HRZE. The above mentioned pathways were also different between drug-induced liver injury (DILI) and non-DILI patients. Urate and *cis*-4-octenedioic acid levels in the DILI group were significantly increased compared to those in the non-DILI group, while the *cis*-aconitate and hypoxanthine levels were significantly decreased. These results highlight that superoxide generation can aggravate the hepatotoxic effects of the HRZE regimen. In addition, our metabolomic approach had the ability to predict hepatotoxicity for clinical applications.

1. Introduction

Tuberculosis (TB), an infectious disease caused by the bacillus *Mycobacterium*, has gradually become a major global health and social threat. Despite the successful control and prevention of TB for the past 20 years, China now has the third highest TB incidence rate worldwide due to recent outbreaks [1]. Anti-TB drugs play vital roles in TB control and patient rehabilitation, and while individual variances usually affect treatment options and forms, "early, regular, full, joint, and appropriate" treatment protocols must be followed. Since the 1950s, first-line anti-TB drugs, including isoniazid (INH, H), rifampicin (RMP, R), ethambutol (EMB, E), pyrazinamide (PZA, Z) and streptomycin (SM, S), have been used gradually in standard treatment regimens for 6–9 months [2].

DILI is one of the most important and serious treatment-related adverse effects that leads to reduced therapy effectiveness [3,4]. Unlike in the USA and Europe, tuberculostatics was the most common DILI aetiology in China in 2013 [5]. Hepatotoxicity caused by anti-TB drugs due to the combined use of several medications and long therapy duration has been a major concern of clinical treatment. The risk of DILI is considerably higher for patients receiving the HRZE regimen than for those receiving single treatment [6]. Most studies have focused on the adverse reactions of INH [7,8], a drug metabolized by hepatic *N*-acetyltransferase and cytochrome P450 2E1 (CYP2E1) to form hepatotoxins [9] and reactive oxygen species (ROS) [10]. As an inducer of CYP2E1, RMP could increase INH-induced hepatotoxicity by aggravating the production of toxic metabolites [11], and human pregnane X receptor (PXR) modulates hepatotoxicity associated with RMP and INH co-therapy [7]. PZA and its metabolites are responsible for PZA-induced

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https://doi.org/10.1016/j.bbrc.2018.02.030
0006-291X/

hepatotoxicity [12], and PPARα plays a critical role in PZA-induced hepatotoxicity [13]. Until now, the toxic mechanisms underlying HRZE co-therapy have remained partially characterized, and hepatotoxicity has remained unpreventable.

In recent years, new analytical techniques, such as metabolomics, genomics, proteomics, and transcriptomics, have provided a new approach and platform for exploring the toxicity, mechanism of action and safety evaluation of certain compounds [14–16]. As a downstream effector of the genome, proteome and transcriptome, the metabolome not only reflects changes in various omics but also displays environmental and lifestyle differences, thus promising to be applicable in individual clinical administration. Numerous studies have shown that metabolomics techniques could detect biomarker differences between TB patients and healthy volunteers [17]. This study aimed to investigate the related mechanisms of liver injuries in TB patients induced by tuberculostatics co-therapy based on the urine metabolomics platform.

2. Material and methods

2.1. Participants

We targeted male and female adults who were diagnosed with TB and subjected to treatment with INH, RMP, EMB, PZA (the HRZE regimen) in this study, which was approved by the Ethics Committee of The First Affiliated Hospital of Soochow University. Participating patients without other serious disease fully understood the risks and benefits and provided informed consent.

2.2. Sample collection and preservation

As shown in Fig. S1, morning urine was collected before dosing from newly diagnosed TB patients who were administered HRZE for at least one month. Total urine volume was recorded, and 1.5 mL of each urine sample was frozen at −80 °C in a medical freezer until analysis. Simultaneously, the participants' serum biochemical indices were used to evaluate their liver states, and urine samples and serum biochemical indices were collected once a week.

2.3. Sample extraction protocol

For analysis, 400 μL of a mixed precipitator (methanol: acetonitrile = 5:3, v:v) was added to 100 μL of each thawed urine sample. After being vortexed for 3 min, the sample was centrifuged at 20,000 rpm at 4 °C for 10 min. Then, 400 μL of the supernatant was dried and redissolved in 120 μL of the mix precipitator as the analytical sample. For liquid chromatography-mass spectrometry (LC-MS) performance quality control (QC), a 20-μL aliquot of each redissolved supernatant was mixed, and the LC-MS experiment was performed together with samples and replicates from the same LC-MS condition. The QC sample was applied before and after the injection of every 10 samples, and all samples, including the QCs, were shown to be highly reproducibility in principal component analysis (PCA) (Fig. S2).

2.4. Metabolomics data acquisition

Ultra-performance liquid chromatography (UPLC) separation was performed on a Waters ACQUITY UPLC® HSS T3 column (2.1 × 150 mm, 1.8 μm) at 40 °C at a flow rate of 0.35 mL/min. The auto-sampler was conditioned at 4 °C, and the injection volume was 3 μL. The two mobile phases consisted of 0.1% formic acid (solvent A) and 0.1% formic acid acetonitrile (solvent B), and separation was carried out in 17 min under the following conditions: 0–1 min, 2% B; 1–8 min, 2%–50% B; 8–14 min, 50%–98% B; 14–15 min, 98% B; 15–15.5 min, 98%–2% B; and 15.5–17 min, 2% B.

An LTQ Orbitrap XL mass spectrometer equipped with an electron spray ionization (ESI) source was used to acquire mass spectra profiles (Thermo Fisher Scientific, Bremen, Germany). The optimized operating parameters were as follows: source voltage, 4.80 kV (positive mode), 4.50 kV (negative mode); sheath gas, 40 arbitrary units; auxiliary gas, 15 arbitrary units; capillary temperature 325 °C; capillary voltage: 35 V/-15 V; and tube lens voltage: 50 V/-50 V. MS acquisition was performed using both negative and positive ionization with a mass resolution of 70,000. The m/z range for all full scan analyses was 50–1200. The collision energy for collision-induced dissociation (CID) was adjusted to 40% of the maximum.

2.5. Metabolomics data processing

The acquired LC-MS data was deconvoluted using XCMS software package with R Gui as previously reported. The matrix was normalized followed by urine volume and QC samples. Multivariate data analysis was performed with SIMCA-P software (version 13.0, Umetrics, Sweden) based on the data set. PCA and orthogonal projection to latent structures-discriminant analysis (OPLS-DA) were employed for data analysis. Three quantitative parameters were used to evaluate the fit of a model: R2X was the explained variation in X; R2Y was the explained variation in Y; and Q2Y was the predicted variation in Y. Major metabolites were screened and identified using Thermo Xcalibur software based on accurate mass measurements (mass errors < 5 p.p.m.). Statistical analysis was performed using analysis of variance (ANOVA) tests ($P < 0.05$) for all samples in both groups, and correlation analysis was performed using SPSS 17.0 software.

2.6. Functional interpretation of metabolomics data

Metabolites were characterized by comparisons with reference standards or MS/MS fragment information obtained from the METLIN (http://metlin.scripps.edu) database, the Human Metabolome Database (HMDB) (http://www.hmdb.ca/), the MassBank database (http://www.massbank.jp/), the LipidMaps database (http://www.lipidmaps.org) or the mzCloud (https://www.mzcloud.org) database. Perturbation was interrogated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (http://www.kegg.jp/kegg/pathway.html), which serves as a valuable tool in metabolomics pathway analysis.

3. Results

3.1. Participant characteristics

In this study, 77 enrolled participants (Table 1) were divided into three groups: a preliminary diagnosis group (TB, without therapeutics, n = 25), a non-DILI group (HRZE regimen, ALT<40, n = 49), and a DILI group (HRZE regimen, ALT>40, n = 11). The distributions of sex, age and total bilirubin levels among the three groups were not significantly different, and the median ALT and AST values were significantly higher in TB patients with DILI than in newly diagnosed TB patients or non-DILI patients after administration ($p < 0.05$). Serum uric acid levels in post-dose patients were also higher than those in pre-dose TB patients, which was consistent with previous reports [18].

3.2. Metabolic shifts between pre- and post-dose patients

After raw LC-MS data pre-processing, 1282 total ion signals (606 in positive mode and 676 in negative mode) were detected and