Toxicology and Applied Pharmacology 268 (2013) 79–89

Contents lists available at SciVerse ScienceDirect

Toxicology and Applied Pharmacology

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

Perturbation of bile acid homeostasis is an early pathogenesis event of drug induced liver injury in rats

Makoto Yamazaki [a], Manami Miyake [a], Hiroko Sato [a], Naoya Masutomi [a], Naohisa Tsutsui [a], Klaus-Peter Adam [b], Danny C. Alexander [b], Kay A. Lawton [b], Michael V. Milburn [b], John A. Ryals [b], Jacob E. Wulff [b], Lining Guo [b,*]

[a] Mitsubishi Tanabe Pharma Corporation, Kisarazu, Chiba 292-0818, Japan
[b] Metabolon Inc., 617 Davis Drive, Suite 400, Durham, NC 27713, USA

ARTICLE INFO

Article history:
Received 4 September 2012
Revised 3 January 2013
Accepted 18 January 2013
Available online 27 January 2013

Keywords:
Metabolomics
Biomarker
Hepatotoxicity
Oxidative stress
Mechanism of action

ABSTRACT

Drug-induced liver injury (DILI) is a significant consideration for drug development. Current preclinical DILI assessment relying on histopathology and clinical chemistry has limitations in sensitivity and discordance with human. To gain insights on DILI pathogenesis and identify potential biomarkers for improved DILI detection, we performed untargeted metabolomic analyses on rats treated with thirteen known hepatotoxins causing various types of DILI: necrosis (acetaminophen, bendazac, cyclosporine A, carbon tetrachloride, ethionine), cholestasis (methapyrilene and naphthylisothiocyanate), steatosis (tetracycline and ticlopidine), and idiosyncratic (carbamazepine, chlorzoxasone, flutamide, and nimesulide) at two doses and two time points. Statistical analysis and pathway mapping of the nearly 1900 metabolites profiled in the plasma, urine, and liver revealed diverse time and dose dependent metabolic cascades leading to DILI by the hepatotoxins. The most consistent change induced by the hepatotoxins, detectable even at the early time point/low dose, was the significant elevations of a panel of bile acids in the plasma and urine, suggesting that DILI impaired hepatic bile acid uptake from the circulation. Furthermore, bile acid amidation in the hepatocytes was altered depending on the severity of the hepatotoxin-induced oxidative stress. The alteration of the bile acids was most evident by the necrosis and cholestasis hepatotoxins, with more subtle effects by the steatosis and idiosyncratic hepatotoxins. Taking together, our data suggest that the perturbation of bile acid homeostasis is an early event of DILI. Upon further validation, selected bile acids in the circulation could be potentially used as sensitive and early DILI preclinical biomarkers.

Introduction

Drug induced liver injury (DILI) is a major challenge for both clinicians and pharmaceutical researchers. DILI has been associated with a wide variety of drugs and accounts for a major portion of acute liver failure (Ramachandran and Kakar, 2009). It is the leading cause of drug withdraws from the market (Temple and Himmel, 2002) and is also among the main reasons for drug candidate attrition (Schuster et al., 2005). Because of the significant impact of DILI, there is clearly a need to improve DILI detection during the drug developmental process.

DILI can be generally classified as hepatocellular, cholestatic, or mixed. Its histological outcomes consist of a wide spectrum of conditions, ranging from steatosis, hepatitis, to acute liver failure (Ramachandran and Kakar, 2009). The current pre-clinical assessment for DILI relies primarily on histopathology and clinical chemistry based on a panel of serum biomarkers, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), sorbitol dehydrogenase (SDH), glutamate dehydrogenase (GLDH) (for hepatocellular damage), alkaline phosphatase (ALP), γ-glutamyltransferase (GGT), and total bilirubin (TBil) (for hepatobiliary dysfunction) (Boone et al., 2005). However, there are considerable limitations associated with these standard tests. Histopathology is typically examined at a single end point and may lack sufficient sensitivity. Changes with the liver clinical markers may be contributed by factors other than DILI (Ennulat et al., 2010). Additionally, the current preclinical DILI assessment only correlates with approximately 50% of the cases of human liver toxicity; thus leading to false positives or false negatives (Olson et al., 2000). These limitations in sensitivity and specificity have prompted the discovery of new DILI biomarkers that can complement the current methodology.

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANIT, 1-naphthylisothiocyanate; APAP, acetaminophen; AST, aspartate aminotransferase; BDZ, bendazac; CBZ, carbamazepine; CCL4, carbon tetrachloride; CSA, cyclosporine A; CZS, chlorzoxasone; DILI, drug-induced liver injury; ETN, DL-ethionine; FTM, flutamide; GGT, gamma-glutamyl transferase; GLDH, glutamate dehydrogenase; MTP, methapyrilene; NMS, nimesulide; SDH, sorbitol dehydrogenase; TBil, total bilirubin; TCP, ticlopidine; TTC, tetracycline.

* Corresponding author. Fax: +1 919 572 1721.
E-mail address: lguo@metabolon.com (L. Guo).

0041-008X/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.taap.2013.01.018

The key to predicting the progression and outcome of hepatic injury is understanding the underlying mechanisms. Various studies have been conducted to discover biomarkers for improved DILI detection using technologies such as genomics, proteomics, and metabolomics (Andrade et al., 2009; Beger et al., 2010; Collins et al., 2010). However, many of these studies analyzed only a single or a very limited number of hepatotoxins at a single dose/time point, thereby restricting the scope of the findings. More extensive efforts toward developing DILI biomarkers carried out by various consortia, such as InnoMed PredTox, Critical Path Liver Toxicity Biomarker Study (LTBS), and the Consortium for Metabonomic Toxicology (COMET), have yet to come to full fruition.

Here we report the use of untargeted metabolomics to gain insights into DILI induced pathogenesis and explore the feasibility of identifying early and sensitive DILI biomarkers. Rats were dosed with diverse types of hepatotoxins that are known to cause hepatic necrosis, cholestasis, or steatosis in rats, as well as with hepatotoxins that do not cause observable injury in rats but are known to be associated with liver injury in humans (idiosyncratic hepatotoxins). We were able to identify perturbation of bile acid homeostasis as a common and early event of DILI by these diverse hepatotoxins. Negative controls such as testicular and muscular toxins did not significantly alter the levels of major plasma bile acids. This understanding led to the identification of specific bile acid species as potential early and more sensitive DILI biomarkers in rats.

Materials and methods

Drugs, dosing regimen, and animal studies. Thirteen compounds that are known to cause liver toxicity were selected for this study. For each compound, a low dose and a high dose were administrated. The list of these hepatotoxins, doses, administration method, and vehicles were summarized in Table 1. Crl:CD (SD) male rats of six weeks of age used in this study were from Charles River Japan, Inc. (Atsugi Breeding Center, Tokyo, Japan). All the experimental animals were housed in stainless-steel cages in a room that was lit for 12 h (7:00–19:00) daily, ventilated with an air-exchange rate of 10–20 times per hour, and maintained at 20–26 °C with a relative humidity of 30–70%. All animals were allowed free access to water and food (CRF-1, sterilized by radiation, Oriental Yeast Co., Tokyo, Japan) except for a 4-hour fasting period prior to plasma collection. After a 5-day acclimatization period, the rats were randomly assigned to experimental groups ($n = 5$ for each group). For each compound, two groups of animals received the low dose, two groups of animals received the high dose, and the other two groups of animals received a corresponding vehicle control. The plasma (anti-coagulated with sodium EDTA), urine, and liver samples were collected on day 2 and day 5. Urine was collected over 24 h with the collection vessels surrounded by dry ice. After urine collection, rats were maintained under fasting conditions for 4 h after which plasma samples were collected. The animals were then euthanized to collect liver for pathological examination. All procedures of animal studies were performed in accordance with the rule of the Institutional Animal Care and Use Committee at the study facility.

Table 1
Hepatotoxins and their doses used in this study. All hepatotoxins were administrated with oral gavage. CMC-Na: sodium carboxymethyl cellulose.

Hepatotoxin	Abbreviation	Doses (mg/kg/day)		Vehicle
1-naphthyl isothiocyanate	ANIT	15	50	Corn oil
Acetaminophen	APAP	500	1000	0.5% CMC-Na
Bendazac	BDZ	300	1000	Corn oil
Carbamazepine	CBZ	150	300	0.5% CMC-Na
Carbon tetrachloride	CCL4	100	300	Corn oil
Chlorzoxasone	CZS	500	1000	0.5% CMC-Na
Cyclosporine A	CSA	50	100	Corn oil
DL-ethionine	ETN	125	250	0.5% CMC-Na
Flutamide	FTM	50	150	0.5% CMC-Na
Methapyrilene	MTP	50	150	0.5% CMC-Na
Nimesulide	NMS	100	200	0.5% CMC-Na
Tetracycline	TTC	1000	2000	0.5% CMC-Na
Ticlopidine	TCP	150	300	0.5% CMC-Na

Metabolomic profiling. The metabolomic platform consisted of three independent methods: ultrahigh performance liquid chromatography/tandem mass spectrometry (UHLC/MS/MS^2) optimized for basic species, UHLC/MS/MS^2 optimized for acidic species, and gas chromatography/mass spectrometry (GC/MS). The detailed descriptions of the platform, including sample processing, instrument configuration, data acquisition, and metabolite identification and quantitation, were published previously (Evans et al., 2009; Ohta et al., 2009). Essentially, the samples were extracted and split into three equal aliquots for analysis by the three methods. For the two LC methods, chromatographic separation followed by full scan mass spectra was carried out to record retention time, molecular weight (m/z) and MS/MS^2 of all detectable ions presented in the samples. For GC, the samples were derivatized using bistrimethyl-silyl-triflouroacetamide. The retention time and molecular weight (m/z) for all detectable ions were measured. The metabolites were identified by comparison of the ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular weight (m/z), preferred adducts, and in-source fragments as well as their associated MS/MS^2 spectra.

Data imputation and statistical analysis. For statistical analysis, the missing values for a given metabolite were imputed with the observed minimum detection value based on the assumption that they were below the limits of instrument detection sensitivity. Statistical analysis of the data was performed using JMP (SAS, http://www.jmp.com) and "R" (http://cran.r-project.org/). Welch's two-sample t-tests were performed on the log-transformed data to compare the treatment and control groups for each drug dose at each time point. Multiple comparisons were accounted for with the false discovery (FDR) rate method, and each FDR was estimated using q-values. For the convenience of data visualization, the raw area counts for each biochemical were re-scaled by dividing the value for a specific biochemical in each sample by the median value observed for that specific biochemical.

Quantitative determination of glycocholate and taurocholate in the plasma and cholate in the urine. For absolute quantitation, the metabolites were analyzed by isotope dilution UHPLC-MS/MS. The range of quantitation was 0.0250 to 5.00 μg/mL for cholate, 0.0400 to 16.0 μg/mL for glycocholate, and 0.0500 to 20.0 μg/mL for taurocholate. Samples with higher levels were appropriately diluted with PBS to be covered by the calibration range. The calibration standard and the internal standard solutions were prepared in methanol/water (4:1). For the plasma sample preparation, 50.0 μL/mL of rat plasma was spiked with 20.0 μL internal standard solution (4.00 μg/mL glycocholate-D_4, 5.00 μg/mL taurocholate-D_5,) and subjected to protein precipitation by vigorously mixing with 200 μL methanol. Following centrifugation, 1.0 μL of the supernatant was injected onto a Agilent 1290/Sciex 5500 QTrap LC-MS/MS system. For the urine sample preparation, 50.0 μL/mL of rat urine was spiked with 20.0 μL internal standard solution (1.00 μg/mL cholate-D_4) and vigorously mixed with 50.0 μL methanol. Following centrifugation, 1.0 μL of the supernatant was injected onto a Agilent 1290/Sciex 5500 QTrap LC-MS/MS system.

The chromatographic conditions were identical for plasma and urine analyses and included the following: Acquity C18 BEH column, 1.7 μm, 2.1 × 50 mm (Waters); mobile phase A: 6.5 mM ammonium bicarbonate in water; mobile phase B: 6.5 mM ammonium bicarbonate in methanol/water (95:5); flow rate: 0.550 mL/min; gradient: initial, 30% mobile phase A; 0.7 min, 10% phase mobile A, linear; 1.00 min, 10% phase mobile A; linear; 1.05 min 30% phase mobile A, linear; retention times