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Dysregulation of protein degradation pathways may mediate the liver injury and phospholipidosis associated with a cationic amphiphilic antibiotic drug

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ABSTRACT

A large number of antibiotics are known to cause drug-induced liver injury in the clinic; however, interpreting clinical risk is not straightforward owing to a lack of predictivity of the toxicity by standard preclinical species and a poor understanding of the mechanisms of toxicity. An example is PF-04287881, a novel ketolide antibiotic that caused elevations in liver function tests in Phase I clinical studies. In this study, a mouse diversity panel (MDP), comprised of 34 genetically diverse, inbred mouse strains, was utilized to model the toxicity observed with PF-04287881 treatment and investigate potential mechanisms that may mediate the liver response. Significant elevations in serum alanine aminotransferase (ALT) levels in PF-04287881-treated animals relative to vehicle-treated controls were observed in the majority (88%) of strains tested following a seven day exposure. The average fold elevation in ALT varied by genetic background and correlated with microscopic findings of hepatocellular hypertrophy, hepatocellular single cell necrosis, and Kupffer cell vacuolation (confirmed as phospholipidosis) in the liver. Global liver mRNA expression was evaluated in a subset of four strains to identify transcript and pathway differences that distinguish susceptible mice from resistant mice in the context of PF-04287881 treatment. The protein ubiquitination pathway was highly enriched among genes associated with PF-04287881-induced hepatocellular necrosis. Expression changes associated with PF-04287881-induced phospholipidosis included genes involved in drug transport, phospholipid metabolism, and lysosomal function. The findings suggest that perturbations in genes involved in protein degradation leading to accumulation of oxidized proteins may mediate the liver injury induced by this drug.

Introduction

Drug-induced liver injury (DILI) is the major adverse drug event that leads to regulatory actions on drugs (Abboud and Kaplowitz, 2007), and antibiotics are among the most commonly implicated culprits (Hussaini and Farrington, 2007; Robles et al., 2010). While both the widespread prescription of antibiotics and the large doses required for therapeutic effect contribute to the prevalence of adverse reactions, exposure alone does not fully explain the hepatotoxicity associated with these compounds. The mechanisms responsible for these adverse reactions are not well understood owing in part to a lack of experimental models to study the toxicity. As a result, liver injury remains one of the major liabilities in the development of new antibiotic therapies.

PF-04287881 is a ketolide antibiotic that was in development at Pfizer, Inc. for the treatment of acute bacterial respiratory infections. Preclinical toxicology studies to evaluate the safety of this compound were performed in both Sprague–Dawley rats and beagle dogs, and the liver was identified as one of the target organs of toxicity based on increased serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and microscopic findings in the liver consistent with phospholipidosis. The microscopic findings were not surprising given the propensity of other cationic amphiphilic antibiotics (*e.g.* azithromycin and erythromycin) to cause phospholipidosis in animal and *in vitro* models, despite positive clinical utility (Baronas et al., 2007; Matsumori et al., 2006). Overall, PF-04287881 was well tolerated in both animal species tested at doses where systemic exposure was approximately 30 times the anticipated clinical efficacious exposure. However, mild elevations in liver function tests (LFTs) were observed in study participants enrolled in the Phase I clinical study and the

Abbreviations: MDP, mouse diversity panel; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LFT, liver function test; DILI, drug-induced liver injury.

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development of this compound was discontinued. Because there was inter-individual variation in the liver response profile across study subjects, it was hypothesized that genetic variation may, in part, underlie sensitivity to the adverse liver effects of PF-04287881.

A mouse diversity panel (MDP) has been previously shown to provide a population-based approach to modeling and predicting inter-individual variability in adverse drug reactions. An MDP is a commercially available panel of inbred strains that are genetically different from one another and selected to maximize genetic variation across strains (Bogue and Grubb, 2004; McClurg et al., 2007). This resource can be utilized to model human genetic diversity, and was recently shown to both predict and provide mechanistic insight into the kidney injury induced by DB289, a promising drug therapy for sleeping sickness that was suspended after kidney toxicity was observed in clinical studies (Harrill et al., 2012). The genetic diversity of an MDP has also been shown to enable the identification of genomic, transcriptomic, and metabolomic biomarkers associated with acetaminophen toxicity that inform human sensitivity to acetaminophen-induced liver injury (Court et al., 2013; Harrill et al., 2009a,b; Liu et al., 2010).

To better understand inter-individual sensitivity to PF-04287881, an MDP was utilized to identify mouse strains that showed sensitivity or resistance to PF-04287881-induced liver injury. Select strains demonstrating differential pathologic findings in the liver following treatment were then utilized to identify transcripts and pathways that distinguish susceptible and resistant strains in the context of PF-04287881 treatment. It was hypothesized that these transcriptional changes are important for mediating PF-04287881-induced liver injury, and would inform mechanisms associated with the hepatotoxicity induced by antibiotic drugs.

Materials and methods

Animals. Female mice aged 6–8 weeks were obtained from The Jackson Laboratory (129S1/SvlmJ, A/J, AKR/J, BALB/cJ, BTBR T + tf/J, BUB/BnJ, C3H/HeJ, C57BL/6J, C57BLKS/J, C57BR/cdJ, C58/J, CBA/J, CE/J, DBA/2J, FVB/NJ, I/LnJ, KK/HIJ, LG/J, LP/J, MA/MyJ, MRL/MpJ, NOD/ShiLtJ, NON/ShiLtJ, NOR/LtJ, NZW/LacJ, P/J, PL/J, PWK/PhJ, RIIIS/J, SEA/GnJ, SJL/J, SM/J, SWR/J, WSB/EiJ) or Charles River (CD-1) and housed in the Laboratory Animal Resources and Technical Support Facility at The Hamner Institutes. Mice were allowed to acclimate to the facility for two weeks prior to dosing; during this time, animals were randomized to treatment groups by body weight using the Instem Provantis system. Animals were given water *ad libitum* throughout the study and were fed NIH-07 wafer feed except for the time period 18 h prior to necropsy. Animals were maintained on a 12 h light–dark cycle. Care of mice followed institutional guidelines under a protocol approved by the Institutional Animal Care and Use Committee.

Experimental design. PF-04287881 was obtained from Pfizer, Inc. Dose selection was based on a pilot study in which CD-1 female mice were administered 0, 150, 300, or 600 mg/kg ($N = 5$ per group) of PF-04287881 intragastrically (*i.g.*) for 7 consecutive days (data not shown). All doses were well tolerated with no adverse clinical reactions and, thus, the 600 mg/kg dose was selected for the MDP study. Female mice from 34 inbred strains were randomized by body weight into treatment groups ($N = 4$ per strain) and dosed *i.g.* with 600 mg/kg PF-04287881 or vehicle (sterile water) once daily for 7 days in a dosing volume of 10 ml/kg. The first day of dosing was designated as Day 1. Dosing was performed at the same time of day throughout the study to avoid diurnal variability. Animals were fasted 18 h prior to necropsy to reduce hepatic glycogen levels and improve histopathological assessment. A subsequent study was conducted with the same experimental design to collect additional blood and tissues from four strains (MA/MyJ, NZW/LacJ, SM/J, and WSB/EiJ) selected for transcriptomic analysis.

At 2 h post dosing on Day 7, blood (25–50 μl) from each mouse was collected in a heparinized microcapillary tube *via* puncture of the lateral tail vein. Blood samples were immediately blotted onto Whatman FTA DMPK-C dried blood spot filters (GE Healthcare) in duplicate. Dried blood spot filters were sent to Pfizer Global Research and Development, Groton, CT for analysis of drug concentration by LC-MS/MS as previously described (Li and Tse, 2010). On Day 8, animals were euthanized by CO_2 asphyxia and blood was collected *via* cardiac puncture. A blood sample was taken for analysis of drug concentration as previously described. The livers were quickly excised and weighed. Sections of the left liver lobe were placed in 10% phosphate buffered formalin for histological analyses. The remaining tissue was snap-frozen in liquid nitrogen and stored at −80 °C. In the follow-up study with four mouse strains, samples of lung, spleen, and liver tissue were collected for histological analyses, and an additional sample from each of these tissues was fixed for electron microscopy (EM) analysis in McDowell's and Trump's 4F:1G containing 4% formaldehyde and 1% glutaraldehyde in phosphate buffer.

Of the 272 animals included in the initial study, 11 animals died prematurely due to non-compound related events and were therefore excluded from data analysis. Animals that were excluded came from the following strains (number and treatment): C57BR/cdJ (one, PF-04287881), BUB/BnJ (one, vehicle), SWR/J (one, PF-04287881), MA/MyJ (one, PF-04287881), CE/J (one, PF-04287881), RIIIS/J (one, PF-04287881), WSB/EiJ (one, vehicle), KK/HIJ (one, vehicle and one, PF-04787881), and FVB/NJ (two, PF-04287881). Four animals (one NOR/LtJ and three A/J) also died due to morbidity associated with PF-04287881 administration, and these animals were excluded from subsequent data analysis. Upon repeating the experiments to collect tissue for transcriptomics analysis, a single non-compound-related death occurred in a PF-04287881-treated animal (WSB/EiJ), and thus it was excluded from subsequent analyses.

Clinical chemistry. Blood was collected in serum separation tubes with a gel clotting activator (Starstedt) and serum was separated by centrifugation (1300 ×*g* for 15 min). Serum was transferred to a clean tube and standard enzymatic procedures were used to assay for ALT, AST, and alkaline phosphatase (ALP) on an Advia 1800/2400 analyzer (Siemens). In the follow-up study with four mouse strains, blood was collected in EDTA-containing microcentrifuge tubes (Starstedt) and plasma was separated by centrifugation (2000 ×*g* for 15 min). ALT was assayed using the Infinity ALT Liquid Stable Reagent (Thermo Scientific) on a SpectraMax microtiter plate reader (Molecular Devices).

microRNA-122 (miR-122) measurements. Total RNA was isolated from 25 μl of plasma using the miRNeasy Serum/Plasma isolation kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Synthetic *Caenorhabditis elegans* miR-39 was added as a spike-in control. Plasma levels of miR-122 were calculated as previously described (Kroh et al., 2010). Briefly, reverse transcription reactions were performed using the TaqMan miRNA Reverse Transcription Kit and miRNA-specific stem-loop primers for miR-122 and miR-39 (Applied Biosystems). Standard curves with known concentrations of miR-122 and miR-39 were generated in parallel using miScript miRNA mimics (Qiagen). qPCR was performed on both samples and standards using the TaqMan Universal PCR Master Mix II with no UNG and miRNA-specific TaqMan primer/probe mixes (Applied Biosystems) on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Cycle threshold (C_T) values for miR-122 were normalized using C_T values for the spiked-in miR-39 as previously described (Kroh et al., 2010). Absolute values of miR-122 copy number were determined using the synthetic miR-122 standard curve.

Histology. Formalin-fixed, paraffin embedded liver sections (5 μm) were stained with hematoxylin and eosin (H&E). Liver tissues were assessed by light microscopy by a veterinary pathologist (Pfizer).