Nitrosative Stress and Lipid Homeostasis as a Mechanism for Zileuton Hepatotoxicity and Resistance in Genetically Sensitive Mice

Dahea You,^{*} Lascelles E. Lyn-Cook Jr,[†] Daniel M. Gatti,[‡] Natalie Bell,^{*,§} Philip R. Mayeux,[¶] Laura P. James,^{||} William B. Mattes,^{|||} Gary J. Larson,^{||||} and Alison H. Harrill ^{*,#,1}

^{*}Division of the National Toxicology Program, The National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709; [†]Graduate Program in Interdisciplinary Biomedical Sciences, The University of Arkansas for Medical Sciences and Arkansas Children's Research Institute, Little Rock, Arkansas 72205; [‡]The Jackson Laboratory, Bar Harbor, Maine 04609; [§]East Carolina University, Greenville, North Carolina 27858; [¶]Department of Pharmacology and Toxicology and ^{||}Department of Pediatrics, The University of Arkansas for Medical Sciences and Arkansas Children's Research Institute, Little Rock, Arkansas 27705; ^{|||}Division of Systems Biology, The National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, Arkansas 72079; ^{||||}Social & Scientific Systems, Inc., Durham, North Carolina 27703; and [#]Department of Environmental and Occupational Health, The University of Arkansas for Medical Sciences and Arkansas Children's Research Institute, Little Rock, Arkansas 72205

Dahea You and Lascelles E. Lyn-Cook Jr contributed equally to this study.

¹To whom correspondence should be addressed at Division of the National Toxicology Program, National Institute of Environmental Health Sciences, National Institutes of Health, 111 TW Alexander Drive, MSC K2-17, Research Triangle Park, NC 27709. Fax: 301-451-5759. E-mail: alison.harrill@nih.gov.

ABSTRACT

Zileuton is an orally active inhibitor of leukotriene synthesis for maintenance treatment of asthma, for which clinical usage has been associated with idiosyncratic liver injury. Mechanistic understanding of zileuton toxicity is hampered by the rarity of the cases and lack of an animal model. A promising model for mechanistic study of rare liver injury is the Diversity Outbred (DO) mouse population, with genetic variation similar to that found in humans. In this study, female DO mice were administered zileuton or vehicle daily for 7 days (i.g.). Serum liver enzymes were elevated in the zileuton group, with marked interindividual variability in response. Zileuton exposure-induced findings in susceptible DO mice included microvesicular fatty change, hepatocellular mitosis, and hepatocellular necrosis. Inducible nitric oxide synthase and nitrotyrosine abundance were increased in livers of animals with necrosis and those with fatty change, implicating nitrosative stress as a possible injury mechanism. Conversely, DO mice lacking adverse liver pathology following zileuton exposure experienced decreased hepatic concentrations of resistin and increased concentrations of insulin and leptin, providing potential clues into mechanisms of toxicity resistance. Transcriptome pathway analysis highlighted mitochondrial dysfunction and altered fatty acid oxidation as key molecular perturbations associated with zileuton exposure, and suggested that interindividual differences in cytochrome P450 metabolism, glutathione-mediated detoxification, and farnesoid X receptor signaling may contribute to zileuton-induced liver injury (ZILI). Taken together, DO mice provided a platform for investigating mechanisms of toxicity and resistance in context of ZILI which may lead to targeted therapeutic interventions.

Key words: zileuton; idiosyncratic hepatotoxicity; Diversity Outbred; genetic variability; drug-induced liver injury.

Highlights

- Use of a genetically diverse mouse population, the Diversity Outbred, enabled mechanistic study of liver injury caused by zileuton in sensitive patients.
- Transcriptomic, histopathological, and hormone analysis indicated that disruption in lipid homeostasis and pathways governing nitrosative stress preferentially occurred in sensitive individuals, informing the overall susceptibility mechanism.

Zileuton, a medication that is commonly used for maintenance treatment of asthma, is an orally active inhibitor of 5-lipoxygenase which inhibits leukotriene synthesis (Bailie et al., 1995). The drug has been used to treat related conditions, including chronic obstructive pulmonary disease, conditions associated with upper airway inflammation, and dermatological conditions such as acne and atopic dermatitis (Sorkness, 1997). While also a promising candidate for additional inflammatory diseases involving leukotrienes, such as rheumatoid arthritis and inflammatory bowel disease, enthusiasm for expanding its indications is tempered by instances of associated drug-induced liver injury (DILI) (Lewis et al., 1990). Liver injury due to zileuton is relatively rare (up to 5% of patients) (Lazarus et al., 1998; Pohl et al., 2017; Wang et al., 2016). The typical clinical presentation is elevation of serum enzymes suggestive of hepatocellular injury, along with jaundice, often resolving rapidly within 1–2 months following cessation (Watkins et al., 2007). Studies in rat models have suggested that zileuton-induced liver injury (ZILI) may be initiated by formation of reactive metabolite intermediates of the drug (Joshi et al., 2004). However, mechanistic understanding of ZILI has been hampered by the rarity of clinical cases and a lack of animal models for ZILI; thus, the underpinnings of interindividual variability in susceptibility to adverse liver responses to zileuton have remained elusive.

Variation in gene sequences has recently become appreciated as a significant source of interpatient variability in drug responses including adverse events (Somogyi and Phillips, 2017). A potential opportunity for the mechanistic study of rare (or idiosyncratic) toxicities is to utilize animal models that represent genetic diversity analogous to human populations (Harrill, 2016). If genetic variation largely underlies interindividual variation in the clinical response, then animal models that contain broad genetic variation may provide insight into the underlying mechanisms of toxicity. One such model is the Diversity Outbred (DO) mouse population, a genetically heterogeneous mouse population that may be used as a surrogate model for differential toxicity that occurs among human patients. DO mice are an outbred stock with genetic variability that is analogous to, and greater than, the human population (Churchill et al., 2012). DO mice have been used successfully as a translational model for rare hepatotoxicity induced by green-tea extract containing herbal supplements in sensitive consumers (Church et al., 2015). Likewise, DO mice have been used to elucidate genetic risk factors for interindividual susceptibility to neutropenia induced by a number of chemotherapy drugs (Gatti et al., 2017).

In the course of our investigations, DO mice were utilized with the hypothesis that zileuton hepatotoxicity is a result of as yet unidentified genetic predispositions. Upon short-term exposure to zileuton, unique hepatic manifestations of pathology occurred in sensitive DO mice. The focus of this study was to investigate contributing molecular mechanisms that underlie differences in response pathways between susceptible and

nonsusceptible DO mice exposed to zileuton. As little is known with respect to ZILI's mode of action in sensitive individuals, common DILI modalities were considered, including generation of reactive oxygen species and reactive nitrogen species, glutathione depletion, generation of protein adducts that may propagate cellular injury responses, excess lipid accumulation, inflammatory signaling process, and metabolic signaling processes. Here, we link observations relating to these injury modalities with liver histopathology findings and differentially expressed genes (DEGs) as identified via transcriptomic analysis of the liver tissue to infer potential mechanisms that underlie sensitivity to ZILI in the clinic.

MATERIALS AND METHODS

Animals. Four hundred and fifty female Diversity Outbred (J:DO) mice were received from The Jackson Laboratory at 6–8 weeks of age. Females were chosen for investigation due to the clinical observation of a female preponderance in DILI clinical cases, in general, and a lack of zileuton pharmacokinetic differences by sex (Braeckman et al., 1995; Vega et al., 2017). Mice were kept in a specific pathogen free status standard barrier animal room and monitored monthly using in-room sentinel animals (bioexclusion criteria upon receipt are located at <https://www.jax.org/strain/009376> (last accessed March 31, 2020)). Animals were acclimated on arrival for a minimum of 2 weeks; during this time, animals were weighed and randomized into test groups to minimize experimental bias and assigned a unique animal identification number. The large number of zileuton-treated animals utilized in this study was intended to adequately power genetic association studies which are the focus of a subsequent manuscript in preparation. For downstream mechanistic investigations, a more balanced study design with approximately 15 animals per group was utilized as described below.

Zileuton ($C_{11}H_{12}N_2O_2S$; $\geq 98\%$ purity) was purchased from LKT Labs (St. Paul, Minnesota). Testing cohorts were approximately 50 mice/cohort and included representation for both vehicle and zileuton treatment. The dosing volume was 10 ml/kg. Gavage needles were dipped into 1 g/ml sucrose solution prior to intragastric instillation to reduce gavage stress (Hoggatt et al., 2010). Staggered cohorts of female DO mice received oral instillation of zileuton (300 mg/kg, $N = 400$) or vehicle (sterile distilled water; $N = 50$) once daily for 7 consecutive days. The dose of zileuton was selected based on prior unpublished data from the Harrill lab showing a lack of morbidity or mortality in 34 inbred mouse strains. The 300 mg/kg dose in mice is equivalent to 1.68 g/day using the FDA 2005 guidance for allometric scaling to the human equivalent dose (FDA, 2005); the dose used in the study presented here is less than the 2.4 g/day maximum prescribed to adult patients for maintenance treatment of asthma (1200 mg twice/day extended release tablets) (Nelson et al., 2007).

Body weight was measured daily during the dosing interval and at time of necropsy. The ages of animals at time of necropsy ranged from 10 to 20 weeks. Animals were housed in polycarbonate cages in the animal facility at the University of Arkansas for Medical Sciences (UAMS) at a density of up to 4 mice per cage. The room temperature remained between 18 and 26°C and humidity remained approximately 30%–70%. Envigo 8640-fixed formula, nonautoclavable pelleted rodent chow (22% crude protein, 5.5% fat, 40.6% available carbohydrate, 3.9% crude fiber; Madison, Wisconsin) and reverse osmosis distilled water were available *ad libitum*. All animals were used in accordance to the “Guide for the Care and Use of Laboratory Animals” under a protocol approved by the UAMS Institutional Animal Care and Use Committee.

Tissue and serum collection. Necropsy was conducted at the same time of day for each cohort to avoid introducing cohort variability caused via diurnal variation. Animals were euthanized under CO₂ anesthesia 24 h following the final exposure day, followed by exsanguination via cardiac puncture. Blood was collected into serum separation tubes (Sarstedt, Numbrecht, Germany). Whole blood was processed into serum by centrifugation (20 min, 10 000 rpm) prior to storage at –20°C. Livers were excised and a section of the left liver lobe was fixed in 10% neutral buffered formalin for 24 h prior to storage in 70% ethanol. The remainder of the liver was separated into left, median, and caudate lobes, flash frozen separately in microcentrifuge tubes in liquid nitrogen and stored at –80°C. Only the left liver lobe was utilized for analyses in this study to maintain consistency across endpoints. Liver and body weights for individual mice are available via download at <https://doi.org/10.22427/NTP-DATA-002-00076-0001-0000-6> (last accessed March 31, 2020).

Tissue pathology. Formalin-fixed tissues were paraffin embedded and were routinely processed according to standard procedures by the UAMS Histology Research Service Center. Briefly, tissues were fixed in 10% neutral buffered formalin for 24 h, and then transferred to 70% ethanol prior to paraffin embedding. Five µm thick slices of the left lateral liver lobe were sectioned and stained with hematoxylin and eosin (H&E). Random sampling and comparison between H&E stained sections of the left lateral and median lobes did not indicate the presence of lobular specificity in tissue findings. H&E stained liver sections were submitted to Experimental Pathology Laboratories, Inc (EPL) for pathology. All slides were evaluated under light microscopy by an EPL veterinary pathologist blinded to the treatment conditions.

Findings for each animal were graded 1–5 depending on severity. The severity grading scale was: minimal = 1, mild = 2, moderate = 3, moderately severe = 4, or severe = 5. Histopathological findings in selected animals guided downstream analyses into mechanisms that underlie each major finding. Liver histopathology findings and severity scores for individual mice are available via download at <https://doi.org/10.22427/NTP-DATA-002-00076-0001-0000-6> (last accessed March 31, 2020).

Clinical chemistry. Clinical chemistry analyses were performed in serum samples collected at necropsy for each animal. Tests included: alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, and blood urea nitrogen (BUN). Serum samples were assayed by Antech Diagnostics GLP (Morrisville, North Carolina) using standard methods. Clinical chemistry data points for individual

mice are available via download at <https://doi.org/10.22427/NTP-DATA-002-00076-0001-0000-6> (last accessed March 31, 2020).

Subsetting representative tissue samples for mechanistic analysis. To investigate potential molecular mechanisms underlying zileuton hepatotoxicity in DO mice, a subset of samples was selected for further analysis. The subset of animals included randomly selected vehicle controls (*n* = 15), randomly selected zileuton-exposed mice that lacked histologic findings in the liver related to exposure (*n* = 15), all zileuton-exposed mice with hepatic findings of mitosis (*n* = 14), and all zileuton-exposed mice with findings of hepatocellular necrosis (*n* = 8). Two mice with findings of necrosis also had concurrent findings of fatty change. Two additional groups included a random sample of zileuton-exposed animals with findings of fatty change (absent necrosis) that were in the upper quartile (*n* = 15) and in the lowest quartile (*n* = 15) of serum ALT values at necropsy because we initially hypothesized that molecular mechanism underlying necrosis may be separate from those underlying fatty change. However, our analysis suggested that there was no difference in immunohistochemical markers between the 2 groups (*p* > .05); thus, the 2 sets were subsequently combined into a single fatty change group for visualization and analysis (*n* = 30).

Triglyceride assay. Concentration of triglycerides in selected liver samples was performed using the Abcam Triglyceride Quantification Kit (Cambridge, Massachusetts) according to the manufacturer’s instructions. Briefly, 40 mg of frozen tissue from the left liver lobe was sectioned and homogenized using the Bullet Blender homogenizer (Atkinson, New Hampshire) along with Rhino homogenization beads (Kennesaw, Georgia) in a 5% NP-40/ddH₂O solution. The supernatant was removed and stored in 1.5 ml tubes. Reaction mix was added following a 20 min incubation with lipase. Following a 60 min incubation, absorbance was read at OD 570 nm using BioTek Cytation 3 Imaging reader (Winooski, Vermont).

Immunohistochemistry: Nitrotyrosine, inducible nitric oxide synthase, and proliferating cell nuclear antigen. Immunohistochemistry was conducted on 5 µm sections of the left liver lobe as described for tissue pathology. Nitrotyrosine (NT) and inducible nitric oxide synthase (iNOS) were detected using the DAKO K4010 kit (Via Real, Carpinteria, California). The primary polyclonal antibody used was from Millipore (Cat. No. 06-284) (Billerica, Massachusetts). The optimized dilution for the NT antibody was 1:1000. NT antibody was preincubated with 1 mM nitrotyrosine to establish nonspecific binding to serve as a block. For iNOS, the rabbit polyclonal antibody used was from Millipore Cat. No. 06-573 at an optimized dilution of 1:1000. Proliferating cell nuclear antigen (PCNA) (Cat. No. PC-10) (Santa Cruz, Dallas, Texas) antibody was utilized at an optimized dilution of 1:400. First the liver tissue slides were deparaffinized and rehydrated through a wash series of xylene, ethanol, and ddH₂O. Antigen unmasking was performed by washing the slides in 10 mM sodium citrate buffer with pH 6.0. Next, Pierce Peroxidase Suppressor was added to each slide and incubated for 15 min. Each slide was then washed twice in TBS (Tris Buffered Saline) and dried, followed by a 20 min incubation with DAKO protein block. Primary antibody was added and each slide was incubated at 4°C overnight in a sealed container. The next day, slides were equilibrated to room temperature in TBS containing 0.05% Tween 20 (TBST), and then washed with TBST and TBS. Next, the biotinylated secondary antibody from the DAKO kit was added and the

slides were incubated for 30 min. Streptavidin peroxidase was then added to each slide and incubated for 15 min. Each slide was then washed in TBST and TBS. Buffered substrate and DAB chromogen solution was added to each slide and incubated for 3 min. To counterstain, DAKO Mayer's Hematoxylin (Lillie's Modification, Santa Clara, California) was added to each slide. The slides were then immersed in ammonia blue solution 10 times, incubated for 2 min, then washed in ddH₂O. Dehydration and CytoSeal 60 (Richard-Allan Scientific, Kalamazoo, Michigan) mounting was performed on each slide.

Image analysis. iNOS and NT: For image analysis of liver sections stained for iNOS and NT detection, ImageJ (v 6.00) was utilized. Five random fields of the left liver lobe were photomicrographed using the Nikon DS-Ri2 light microscope at a magnification of $\times 100$. Within each field, percent area stained was calculated. In each photomicrograph, DAB color was separated from hematoxylin counterstain with the ImageJ Colour Deconvolution filter. The photomicrograph was calibrated using a step tablet and gray-scale values were converted to optical density. The area of each tissue was measured using ImageJ. The percent area stained was averaged across the 5 fields for each slide to generate a single composite score for each animal.

PCNA: For each slide, photomicrographs were taken of 5 random fields using the Nikon DS-Ri2 light microscope at $\times 200$ magnification. Each nucleus was counted manually, and the percent PCNA stained nuclei over the total nuclei present in the field was calculated. Similar to iNOS and NT, a composite score per animal was generated as the average of percent staining over 5 random fields.

Tissue adipokine analysis. Adipokine concentrations were measured in left liver lobe tissue using the Mouse Adipokine Magnetic Bead Panel Endocrine Multiplex Assay (Millipore, MADKMAG-71K). This kit includes reagents to assay for insulin, leptin, and resistin. All reagents were prepared and the assay was run according to the manufacturer's instructions except as noted. Tissue collected from the left liver lobe, (25 mg) was homogenized in phosphate buffered saline with Roche protease inhibitor cocktail tablets (Indianapolis, Indiana). Samples were centrifuged for 5 min at 10 000 rpm, the supernatant was removed and the samples were stored at approximately -80°C . At time of assay, liver extracts were incubated with color-coded microspheres with a specific capture antibody and complexed to 2 fluorescent dyes. Following analyte bead capture, a biotinylated detection antibody was introduced and the reaction mixture incubated with a streptavidin-PE conjugate reporter molecule. Multiplexed fluorescence intensities for each analyte based on its individual reporter signal were measured using the Luminex MAGPIX model instrument (Austin, Texas) with xPONENT software (v 4.2) (Luminex).

Statistical analysis. All statistical tests were performed using GraphPad Prism version 6.00 (La Jolla, California). Differences between groups were compared using a Mann-Whitney test or 1-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test as appropriate. Analysis of the liver enzyme levels ALT, ALP, and AST was performed using R (version 3.6.1) and RStudio (version 1.2.1335) to fit a 2-way ANOVA to the natural log-transformed biomarker data as a function of zileuton exposure and animal age at time of necropsy for each of the 3 endpoints. The statistical significance was assessed as $p < .05$. Outlier analysis was conducted using Grubbs' test with $\alpha = .05$.

RNA-seq analysis. A randomly selected subset of liver samples (44 vehicle-treated and 51 zileuton-treated) was processed to perform RNA-sequencing (RNA-seq). RNA was isolated from 25 mg of liver using the RNEasy Mini Kit according to the manufacturer's protocol (Qiagen, Valencia, California). RNA purity and quality were assessed using the 2100 Bioanalyzer (Agilent, Santa Clara, California) and RNA concentration was quantified using a Cytation 3 spectrophotometer (BioTek). Samples with an RNA Integrity Number < 8 or $260/280 < 1.8$ were excluded from further processing. Extracted total RNA was used to prepare RNA-seq libraries using the TruSeq RNA Library Preparation kit v2 according to the manufacturer's protocol, and then sequenced using the Illumina Mouse WG-6 v2.0 Expression BeadChip (Illumina, San Diego, California). Samples with a read count lower than 1.5 million were excluded from further analyses. Output files in fastq format were uploaded to Partek Flow (Partek, St. Louis, Missouri) and underwent prealignment quality control. Then the reads were trimmed and aligned using the STAR method. Aligned reads were quantified to obtain the raw gene counts and then assessed by the principal component analysis (PCA) to identify samples with poor data quality which were also excluded from further analyses, leaving 41 vehicle- and 49 zileuton-exposed samples that passed quality control remaining for analysis. The RNA-seq data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE141089 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE141089>, last accessed March 31, 2020).

Differential gene expression and pathway analysis. DESeq2 differential gene expression analysis was performed on the raw gene counts in Partek Flow (Partek). The PCA was performed on all genes using DESeq2-normalized gene counts to compare the transcriptomic profiles of 2 treatment groups. Significant differences in gene expression between the treatment groups were determined following the Wald test as adjusted p values $< .01$ after false discovery rate step-up correction. Hierarchical clustering was performed on these significantly different genes to visualize the distinct expression patterns between 2 treatment groups. The DESeq2-analyzed dataset was imported into Ingenuity Pathway Analysis (IPA, Qiagen, Germantown, Maryland) to investigate the pathways associated with DEGs. Each gene identifier was mapped to the gene annotation in the Ingenuity Pathway Knowledge Base. The DEGs were evaluated by the Core Analysis to explore the key Canonical Pathways, Diseases and Bio Functions, and Tox Functions and Tox Lists related to the pathological changes observed in our study.

RESULTS

In-life Observations, Body, and Organ Weights

DO mice were dosed for 7 days with either sterile water (vehicle) or zileuton (300 mg/kg per day). During the dosing interval, the animals experienced no identifiable changes in health status and the drug was well tolerated with respect to a lack of outward signs of morbidity. Animals remained well groomed throughout the study until time of necropsy. Liver-to-body weight ratio was modestly elevated in the zileuton-treated animals as a group (average increase in 5.46%) compared with the vehicle-treated animals (average increase in 4.48%) ($p < .05$).

There was no significant effect of zileuton on body weight in DO mice when comparing the change in body weight measured

Table 1. Incidence of Liver Histopathology

Treatment	Vehicle (Sterile Water)	Zileuton (300 mg/kg)
Number of animals	50	400
Extramedullary hematopoiesis	19 (38%) [1.0]	110 (27.5%) [1.0]
Fatty change (microvesicular; midzonal, centrilobular, or panlobular)	0	81 (20.3%) [1.1–1.4]
Centrilobular hypertrophy	0	12 (3.0%) [1.0]
Increased mitosis	0	14 (3.5%) [1.0]
Inflammation; focal or multifocal	0	2 (0.4%) [1.0]
Necrosis: hepatocyte; focal or multifocal	1 (2.0%) [1.0]	8 (2.0%) [1.0]
Polyploidy	0	1 (0.2%) [1.0]

Shown are the total number of mice which displayed specific histopathological findings, the percentage of animals with the finding (), and the mean group severity score [].

prior to dosing on the first and last days of exposure (Supplementary Figure 1A). There was a marked and significant effect of zileuton to increase the liver-to-body weight ratio when compared with vehicle-exposed animals (Supplementary Figure 1B) and no significant effect on the spleen or right kidney weight with respect to body weight (Supplementary Figs. 1C and 1D).

Liver Histopathology and Clinical Chemistry

The histopathological findings of the left liver lobe for vehicle-treated animals versus those treated with zileuton are summarized in Table 1. Of the 400 animals treated with zileuton, 81/400 (20.3%) exhibited microvesicular fatty change; findings of microvesicular fatty change were absent in the vehicle treated group. On average, the severity of the microvesicular fatty change was minimal.

A small fraction of the zileuton-treated DO mice—8/400 (2.0%)—displayed focal or multifocal hepatocellular necrosis of minimal severity. In contrast, a single animal in the control group displayed hepatocellular necrosis (minimal). Additional findings that occurred solely in zileuton-treated animals were evidence of mitosis in 14/400 (3.5%) animals, focal or multifocal inflammation in 2/400 (0.5%) of mice, and a single animal that displayed polyploidy. Although 19/50 (38%) of vehicle-treated DO mice displayed extramedullary hematopoiesis, this percentage was not significantly different in zileuton-treated DO mice 110/400 (27.5%); this finding was likely a background finding and unlikely to have resulted from drug exposure.

After adjusting for the age range at time of necropsy, zileuton exposure was associated with overall increases in ALT, AST, and ALP compared with control animals (Figure 1). Because animal age can affect interpretation of biomarker levels, a 2-way ANOVA was conducted on log-normalized data with the factors exposure and age. The elevation in ALT was statistically significant ($F_{1,424} = 15.913$, $p = 7.8 \times 10^{-5}$) for the zileuton-exposed animals. There were no age-related trends on serum ALT, AST, or ALP levels with increasing animal age (Supplementary Figure 5) and the interaction effect between exposure and age was not statistically significant for the liver biomarkers. The DO mice displayed interindividual variability in biomarker levels in the zileuton group. Among zileuton-treated DO mice, there were extreme responders with greater serum levels of ALT, AST, and ALP compared with other DO mice in the same group (Figure 1). There was a single outlier animal in the vehicle control group that displayed a high ALT value outside of the established DO reference range (Figure 1A) (Harrill et al., 2018). Although there were individuals in the zileuton-exposed group that experienced a large increase in total bilirubin, there was overall not a significant treatment-related effect in the zileuton group within

this short-term dosing paradigm (data not shown; $p = .50$). There were no significant elevations in BUN, suggesting that zileuton did not cause kidney injury (data not shown; $p = .38$).

Mechanistic Analysis on Histopathological Findings Associated With Zileuton Treatment

A subset of animals was selected for mechanistic analysis based on the histopathological findings to investigate toxicity modes of action in zileuton-treated animals that displayed: (1) no adverse liver histopathology, (2) microvesicular fatty change, (3) evidence of mitosis within hepatocytes, or (4) hepatocellular necrosis. Representative photomicrographs of these pathological changes are shown in Figure 2A. Of the mice that experienced liver necrosis, 2 mice also exhibited microvesicular fatty changes, whereas none of the mice in the subsetted group with fatty change had necrosis.

We sought to confirm that the microvesicular fatty change observed by the study pathologist was indeed composed of lipid droplets and not a function of alternative etiologies such as phospholipidosis or fluid accumulation. To further characterize this pathology, triglyceride concentrations were measured in the liver tissue. A significant increase in triglyceride concentration was observed in the subsets of zileuton-treated DO mice that experienced fatty change or necrosis (Figure 2B). There was no significant elevation in triglycerides in the zileuton-treated animals that experienced no pathology or in those that solely experienced hepatocellular mitosis in the absence of histopathological lesions.

In rat liver microsomal systems, zileuton was previously shown to be metabolized to 2-acetylbenzothiophene, which is subsequently bioactivated to reactive intermediates (both singly and doubly oxidized species) with the potential to generate oxidative damage (Joshi et al., 2004). Furthermore, the reactive intermediates have been shown to form conjugates with glutathione (GSH) and with N-acetylcysteine, suggesting a reactive potential for formation of protein adducts (Joshi et al., 2004). To explore this potential pathway of oxidant-mediated liver injury in the DO mice, NT-protein adducts were measured as a biomarker of collective nitrosative stress. As indicated by the immunohistochemistry staining, NT-protein adducts were clearly present in the liver of mice that experienced hepatocellular necrosis, yet were absent from livers of zileuton-treated mice with lesser pathological findings of mitosis and fatty change (Figure 2A). This NT staining was 3-fold greater in the necrosis group as compared with the vehicle group (Figure 2B). NT staining was diffuse throughout the liver section and absent when the NT antibody was preincubated with 1 mM NT to evaluate nonspecific binding. To investigate a possible source of nitric oxide, liver sections were stained for iNOS (Figure 2A). Similar to

Figure 1. The serum levels of liver biomarkers in DO mice after zileuton treatment. DO mice were treated with either vehicle ($n=50$) or zileuton (300 mg/kg, $n=400$) i.g. daily for 7 days and serum levels of ALT (A), ALP (B), and AST (C) were measured at Day 8. Raw data for individual mice are shown. A 2-way ANOVA was performed to the log-transformed values (not shown) of these biomarkers as function of the treatment and age to assess differences between the treatment groups (statistical significance at $p < .05$ [*]).

NT staining, iNOS protein was clearly visible in the necrosis group with a 3.3-fold increase respective to vehicle, and there was no appreciable accumulation in mice with other pathological manifestations (Figure 2B). Importantly, there was no NT or

iNOS staining evident in the livers of mice that were treated with zileuton, yet experienced no adverse liver pathology.

A key question, with respect to the mitosis pathology subset, was whether the observation of mitosis reflected repair following an initial nitrosative injury, or whether mitosis occurred independently of these processes. To address this question, liver sections from each pathology subset were evaluated for the presence of PCNA, a marker of cellular division and proliferation (Figs. 2A and 2B). Not surprisingly, PCNA was elevated by 2-fold in the livers of mice presenting mitosis compared with the control. Interestingly, PCNA abundance was reduced in zileuton-treated mice with the fatty changes and trended downward in those with no pathology.

Linkage to Energy Metabolism

Metabolic syndrome is a risk factor for nonalcoholic fatty liver disease and factors that influence energy balance are increasingly implicated as risk factors for DILI (as has been recognized for methotrexate-induced liver injury [Shetty et al., 2017]). Because a high percentage of DO mice exposed to zileuton exhibited microvesicular fatty change (20.3%) and because this group was inclusive of mice that also experienced hepatocellular necrosis, we sought to investigate whether metabolic risk factors are associated with susceptibility to zileuton-induced hepatocellular necrosis.

The levels of insulin, leptin, and resistin were measured as local concentrations within the liver tissue (Figure 3). We hypothesized that hormone levels would be preferentially disrupted in those mice that experienced fatty change with or without necrosis. To our surprise, DO mice that were exposed to zileuton, but experienced no adverse liver pathology, exhibited a significant increase in hepatic concentrations of insulin and leptin, and a concomitant decrease in resistin concentration. Across the hormones tested, zileuton-treated mice that experienced overt pathology (mitosis, fatty change, necrosis) exhibited tissue concentrations that were not different from the vehicle-treated mice.

Analysis of Global Liver Transcriptional Profiles in DO Mice

To investigate molecular pathways altered by zileuton exposure, RNA-seq and differential gene expression analysis was performed on a subset of samples. After excluding any outliers and the samples with poor amplification on RNA-seq, a total of 41 vehicle-treated samples and 49 zileuton-treated samples were carried forward for further analyses. There was no apparent effect of animal age at time of necropsy on the overall transcriptional profile between individuals (Supplementary Figure 6). The histopathological presentations and the clinical chemistry profile of this subset of samples are shown in Supplementary Table 1 and Figure 2, respectively. Generally, the subset is reflective of the pathological changes observed in the full set of samples: fatty changes and mitosis were observed, and ALT values were significantly elevated in the zileuton group compared with the controls ($p < .05$). The differences in the expression of 18 223 mapped transcripts between vehicle- and zileuton-treated groups were visualized in the PCA plot (Figure 4A). The plot shows that samples within the 2 treatment groups were spatially separated, indicating a distinct transcriptional profile of DO mice exposed to zileuton. Transcripts (2765) were found to have significantly different expressions between the treatment groups, as determined by the adjusted p value. Of these DEGs, about half (1507 genes) were upregulated and the other half (1258 genes) were downregulated in zileuton-treated animals, as reflected in the heatmap generated from the

Figure 2. Histopathological presentations of DO mice treated with zileuton. A, Representative photomicrographs are shown for vehicle- or zileuton-treated DO mice exhibiting varied pathology in response to drug exposure. Shown in rows are liver sections stained with H&E, nitrotyrosine-protein adducts (Nitrotyrosine), iNOS, and PCNA. Original objective $\times 40$. B, Triglyceride concentrations in the liver and IHC % area of NT stained tissue, of iNOS stained tissue, and of PCNA stained nuclei were measured in control group (vehicle-treated DO mice) and different histopathological subgroups of zileuton-treated DO mice. Data were presented as boxplots with median and interquartile range (IQR) and analyzed by 1-way ANOVA to compare between the control and different subgroups. Statistical significance was set at $p < .05$ (*).

hierarchical cluster analysis on the DEGs (Figure 4B). A clear distinction between 2 treatment groups was visualized in the heatmap.

Pathways Associated With Zileuton-exposure Across DO Mice

To identify the transcriptomic markers of injury and subsequently infer the modes of toxicity associated with ZILI in DO

Figure 3. Hepatic insulin, leptin, and resistin levels of DO mice treated with zileuton. Liver concentrations insulin, leptin, and resistin were measured in control group (vehicle-treated DO mice) and histopathological subgroups of zileuton-treated DO mice. Data were presented as boxplots with median and IQR and analyzed by 1-way ANOVA to compare between the control and different subgroups. For resistin, the lower limit of detection was at 5.4 ng/mL. Statistical significance was set at $p < .05$ (*).

mice, pathway analysis was performed on the DEGs using IPA core analysis. Significant Diseases and Tox Functions associated with the DEGs were in line with histopathological findings observed in our samples (Supplementary Figs. 3 and 4). Among the top 20 Diseases and Bio Functions were cell death and survival, lipid metabolism, molecular transport, cell cycle, and hepatic system disease, which reflect fatty changes, mitosis, and necrotic presentations of affected DO mouse livers. Ten out of the top 20 IPA Tox Functions reflected by the DEGs were consistent with the observed pathological and IHC features, including liver hyperproliferation, liver steatosis, liver cell death, cirrhosis, cholestasis, and hyperbilirubinemia.

The complete lists of Canonical Pathways and Tox Lists associated with the DEGs are provided in Supplementary Tables 2 and 3. There was substantial overlap between these 2 lists.

Various pathways were linked to oxidative stress and glutathione conjugation, and this finding agrees with our results from tissue analysis supporting the possibility of oxidant-mediated liver injury. In addition, several statistically significant pathways ($p < .05$) were related to fatty acid metabolism and many of the associated molecules were genes for key proteins in mitochondrial fatty acid oxidation (FAO), including *Cpt1a* (carnitine palmitoyltransferase 1a), and *Acox1* (acyl-CoA oxidase 1). Furthermore, some of the significant Tox Lists were related to mitochondrial toxicity (decreased transmembrane potential of mitochondria and mitochondrial membrane, decreased permeability transition of mitochondria and mitochondrial membrane, and mitochondrial dysfunction). Selected pathways that are relevant for zileuton toxicity and associated genes are compiled in Table 2. Altogether, these results point to a key role of mitochondrial functionality in mediating the zileuton-induced liver toxicity, and this finding merits follow-up investigations.

Numerous transcription factors or regulators were also shown to be significantly altered, including farnesoid X receptor (FXR), aryl hydrocarbon receptor (AhR), peroxisome proliferator-activated receptor- α (PPAR α), pregnane X receptor (PXR), and liver X receptor (LXR). Of these, the most significant identified pathway (as determined by the p value) was FXR, which can regulate many genes involved in fatty acid metabolism as well as bile homeostasis, both of which are commonly disrupted in DILI (Figure 5). Interestingly, 2 transcriptional targets of FXR, *Abcb11* (encoding for the bile salt export pump or Bsep) and *Slc51b* (encoding for organic salt transporter beta or Ost β), were shown to be modestly downregulated in the zileuton group, suggesting a compromised function of FXR among mice treated with zileuton. Lastly, a large number of genes encoding the cytochrome P450 (CYP) enzymes, including *Cyp1a2*, were significantly altered, which may reflect metabolic alterations more generally.

DISCUSSION

The use of zileuton has been limited due to associated idiosyncratic liver toxicity. The rarity of this hepatotoxicity, and the absence of an adequate laboratory model, posed a challenge to our understanding of the mechanisms underlying the susceptibility to ZILI. The DO mouse population possesses broad genetic variability that can reflect interindividual differences among humans in response to chemical exposure. Successes of DO mice as a translational model for rare chemical toxicities and associated genetic predispositions have been previously shown. In this study, we aimed to model ZILI in DO mice and investigate the modes of action underlying the susceptibility to ZILI-associated hepatotoxicity. Our findings demonstrated that (1) differential toxicity outcomes were observed across the individuals of a DO mouse population exposed to zileuton; (2) nitrosative stress and mitochondrial toxicity may play key roles in mediating ZILI in sensitive individuals; and (3) interindividual differences in transcriptional expression of genes involved in metabolism and oxidative defense pathways may influence one's susceptibility to ZILI.

Various histopathological and clinical presentations were observed in DO mice treated with zileuton, reflecting the population's diverse genetic composition. As in clinical populations, only a small fraction of zileuton-treated animals experienced overt hepatocellular injury as represented by elevations in serum liver enzyme levels and hepatocellular necrosis. In a subset of mice treated with zileuton, microvesicular fatty changes were concurrently present with necrosis, indicating that different pathological changes may not necessarily be independent,

Figure 4. Transcriptome profiling of DO mice after zileuton treatment. Liver tissues of a subset of DO mouse samples were processed for RNA-seq analysis. A, A PCA plot of transcriptomic data of DO mice treated with vehicle ($n = 41$) or zileuton ($n = 49$) revealed the differential gene expression profiles between 2 treatment groups. B, Hierarchical clustering of DEGs (adjusted p value $< .01$) in each treatment group.

and represent a continuum of cellular injury processes. These fatty changes were accompanied by significant increases in triglyceride levels, indicating that the lipid balance was altered in the affected mice.

Cellular proliferation which was observed in zileuton-treated mice presenting with mitosis may indicate a repair in response to hepatocellular injury that occurred earlier in the dosing interval. Alternatively, zileuton may have prompted increased cellular turnover via an as yet to be identified mechanism. Further work is necessary to elucidate the mechanistic underpinnings for these mitotic changes. Decrease in the marker for cellular proliferation, PCNA, was observed in zileuton-treated mice with fatty changes, but a decrease in PCNA below baseline levels is generally not toxicologically meaningful.

As expected from their differential histopathological and clinical presentations, zileuton-treated DO mice presented with distinct liver transcriptomic profiles. However, the subgrouping

among zileuton-treated mice as observed for histopathological endpoints was not seen with transcriptional changes. This may be because the sample size of the subset used for the transcriptomic analysis was not big enough to see clear subdivisions across the individuals. Yet, the diversity of the population was evident in the hierarchical clustering of DEGs which showed varying extents of changes among individuals. Diseases and toxicological functions associated with DEGs were in line with the histopathological and clinical findings, suggesting that the transcriptional changes can serve as good predictive markers for ZILI.

Zileuton-exposed mice that experienced hepatocellular necrosis uniquely exhibited high levels of NT adducts and iNOS which were not observed in zileuton-treated mice which experienced no adverse effects. This result establishes that nitrosative stress was unique to those DO mice that were genetically sensitive to zileuton-induced necrosis. Often, interindividual differences in drug metabolism and clearance are key factors determining

Table 2. Pathways and Associated Molecules Relevant to Liver Toxicity of Zileuton in DO Mice

Functional Category	Pathway	Genes in the Dataset
Fatty acid homeostasis	Fatty acid β -oxidation/metabolism	Acaa1, Acaa1b, Acad11, Acadm, Acadvl, Acox1, Acox2, Acsl4, Adhfe1, Akr1a1, Aldh1a1, Aldh1a3, Aldh3a2, Auh, Cpt1a, Cyp1a1, Cyp1a2, Cyp2a5, Cyp2b23, Cyp2b10, Cyp2c55, Cyp2c40, Cyp2c29, Cyp2c65, Cyp2d26, Cyp2e1, Cyp3a59, Cyp3a41b, Cyp3a13, Cyp4a31, Cyp4a14, Cyp4a12a, Cyp4f15, Dhrr9, Ech1, Eci1, Eci2, Eci3, Ehhadh, Hadha, Hadhb, Hsd17b4, Iws1, Sds, Slc27a1, Slc27a2
	γ -Linolenate biosynthesis	Acsl4, Cyb5a, Fads1, Fads2, Slc27a1, Slc27a2
	Stearate biosynthesis	Acot2, Acot3, Acot4, Acot8, Acsl4, Cyp2e1, Cyp4a31, Cyp4a12a, Dhcr24, Elovl2, Mboat7, Slc27a1, Slc27a2
Mitochondrial toxicity	Decreased mitochondrial transmembrane potential and permeability transition	Ap2a2, Atpif1, B2m, Bard1, Bcl2l2, Birc5, Bnip3, Casp2, Chek2, Cisd1, Dffa, Hspa5, Immt, Irf5, Irs1, Krt8, Lcn2, Ldha, Map2k4, Mapk9, Myc, Mycn, Nfkb1, Ogdh, Pawr, Pla2g6, Shc1, Tgm2, Timp3, Trp53, Ucp2
	Mitochondrial dysfunction	Aco1, Aco2, Atp5c1, Cox17, Cox6a1, Cox8a, Cpt1a, Cyb5a, Furin, Gpx4, Gpx7, Lrrk2, Map2k4, Mapk9, Ndufa10, Ndufab1, Ndubf3, Ndufs1, Ogdh, Pdha1, Prdx3, Psen2, Psenen, Trak1, Ucp2, Uqcrl10, Uqcrls1, Vdac2
Transcription factors/regulators	FXR/RXR activation	A1bg, Abcb11, Abcc2, Abcg5, Abcg8, Ahsg, Alb, Ambp, Apoa1, Apoc4, Apof, Apoh, Apom, Baat, C3, C4b, C9, Fga, Fgfr4, Foxa2, G6pc, Hnf1a, Il1rn, Itih4, Lipc, Map2k4, Mapk9, Nr1i2, Pcyox1, Pltp, Saa2, Scarb1, Sdc1, Serpina1e, Serpinf2, Slc22a7, Slc4a2, Slc51b, Srebf1, Tf, Ttr, Vtn
	AhR signaling	Aldh1a1, Aldh1a3, Aldh1l2, Aldh3a2, Arnt, Atr, Ccna2, Ccnd3, Ccne1, Ccne2, Cdk6, Cdkn1b, Chek1, Chek2, Cttd, Cyp1a1, Cyp1a2, Cyp2c55, Cyp2c40, Cyp2c54, Cyp2c29, Cyp2c65, Cyp3a59, Cyp3a41b, Cyp3a13, E2f1, Esr1, Gm3776, Gstm1, Gstm2, Gstm3, Gstm4, Gstp2, Gstt2, Hsp90b1, Jun, Mgst1, Myc, Nfia, Nfib, Nfkb1, Nqo1, Rxrg, Tgm2, Tp53
	PPAR α /RXR α signaling	Acaa1a, Acadl, Acox1, Adcy1, Adcy6, Adcy9, Adipor1, Ap2a2, Apoa1, Cd36, Chuk, Clock, Cyp2c55, Cyp2c40, Cyp2c54, Cyp2c29, Cyp2c65, Dut, Ehhadh, Fabp1, Ghr, Gna11, Gnas, Got2, Hsd17b4, Hsp90b1, Il1rn, Irs1, Itgb5, Jun, Kras, Map2k4, Map2k6, Me1, Med24, Myc, Nfkb1, Ngfr, Notum, Pdga, Pdgc, Pdgrb, Pdia3, Plcb3, Plcg1, Ppard, Prkaa1, Prkaa2, Prkar1a, Raf1, Rap2a, Rras, Rras2, Shc1, Slc27a1, Stat5a, Stat5b, Tgfb2, Traf2
	PXR/RXR activation	Abcb11, Abcb9, Abcc2, Abcc3, Alas1, Aldh1a1, Aldh3a2, Cpt1a, Cyp1a2, Cyp2a5, Cyp2b10, Cyp2c29, Cyp2c65, Cyp3a41b, Cyp3a13, Foxo3, G6pc, Gstm1, Nr1i2, Prkar1a, Ugt1a1, Ugt1a9
	LXR/RXR activation	A1bg, Abcg5, Abcg8, Ahsg, Alb, Ambp, Apoa1, Apoc4, Apof, Apoh, Apom, C3, C4b, C9, Cd36, Fga, Hmgcr, Il1rn, Itih4, Ldlr, Nfkb1, Ngfr, Pcyox1, Pltp, Rxrg, Saa2, Serpina1e, Serpinf2, Srebf1, Trf, Ttr, Vtn
	Hypoxia-inducible factor signaling	Arnt, Cops5, Creb1, Egl3, Eif1ax, Eif2b5, Elavl1, Hif1a, Jun, Kras, Ldha, Mapk9, Mmp11, Mmp14, Nqo1, Pdgc, Pik3cb, Plcg1, Prok1, Rap2a, Rras, Rras2, Shc1, Slc2a1, Sumo1, Trp53, Ube2c, Ube2e3, Ube2n, Ube2s, Vegfb
Cell cycle regulation	Cell cycle control of chromosomal replication	Cdc45, Cdc6, Cdk1, Cdk18, Cdk20, Cdk6, Cdk8, Cdt1, Chek2, Dbf4, Dna2, Lig1, Mcm2, Mcm3, Mcm4, Mcm5, Mcm6, Mcm8, Orc1, Pole, Rpa2, Top2a, Top2b
	Cell cycle phase/cyclin regulation	ATR, Ccna2, CCNB1, CCNB2, Ccnd3, Ccne1, Ccne2, Ccnh, Cdc25a, CDC25B, CDC25C, Cdc34, CDK1, Cdk6, Cdkn1b, Cdkn2c, CHEK1, CHEK2, CKS1B, CKS2, CUL1, E2f1, E2f4, Hdac5, PKMYT1, PLK1, Ppp2r5a, Ppp2r5d, Ppp2r5e, PTPMT1, Raf1, SKP1, Suv39h1, TOP2A, TOP2B, Trp53, WEE1, YWHAH, YWHAQ
	Mitotic roles of polo-like kinase	Anapc13, Anapc5, Ccnb1, Ccnb2, Cdc20, Cdc25a, Cdc25b, Cdc25c, Cdk1, Chek2, Espl1, Hsp90b1, Kif11, Kif23, Pkmyt1, Plk1, Plk3, Plk4, Ppp2r5a, Ppp2r5d, Ppp2r5e, Prc1, Smc3, Wee1
Stress signaling	Acute phase response signaling	Ahsg, Alb, Ambp, Apoa1, Apoh, C3, C4b, C9, Cebpb, Chuk, Cp, F2, Fga, Fgb, Fgg, Ftl1, Hnf1a, Hnmpk, Il1rn, Il6r, Irak1, Itih2, Itih3, Itih4, Jun, Klkb1, Kras, Map2k4, Map2k6, Map3k5, Mapk9, Mtor, Nfkb1, Ngfr, Pik3cb, Plg, Raf1, Rap2a, Rras, Rras2, Saa1, Serpina1e, Serpine1, Serpinf2, Shc1, Trf, Traf2, Ttr, Vwf
	Oxidative stress	Cyp2e1, Gpx2, Gpx4, Gss, Gm3776, Gstm2, Jun, Me1, Mgst1, Nfkb1, Nqo1, Prdx1, Prdx3, Trp53, Vcam1
	Endoplasmic reticulum stress pathway	Atf4, Calr, Dnajc3, Hsp90b1, Hspa5, Map3k5, Mbtps2, Taok3, Traf2
	NRF2-mediated oxidative stress response	Abcc2, Abcc4, Actb, Akr1a1, Aox1, Atf4, Bach1, Cdc34, Cyp1a1, Cyp1a2, Cyp2a5, Cyp2b10, Cyp2c55, Cyp2c40, Cyp2c54, Cyp2c29, Cyp2c65, Cyp2d26, Cyp2e1, Cyp3a25, Cyp3a41b, Cyp3a13, Cyp4a31, Cyp4a14, Cyp4a12a, Dnajb11, Dnajb12, Dnajc13, Dnajc16, Dnajc3, Dnajc9, Ephx1, Fkbp5, Fth1, Ftl, Gpx2, Gm3776, Gstm2, Gstm3, Gstm4, Gstm1, Gstp2, Gstt2, Hacd3, Hsp90b1, Jun, Kras, Maff, Mafk, Map2k4, Map2k6, Map3k5, Mapk9, Mgst1, Nqo1, Pik3cb, Prdx1, Prkce, Raf1, Rap2a, Rras, Rras2, Scarb1, Sqstm1, Txn, Ube2e3

Table 2. (continued)

Functional Category	Pathway	Genes in the Dataset
Metabolism	Xenobiotic metabolism signaling	Abcc2, Abcc3, Adhfe1, Akr1c6, Aldh1a1, Aldh1a3, Aldh1l2, Aldh3a2, Arnt, Ces1, Ces1g, Cyp1a1, Cyp1a2, Cyp2a5, Cyp2b6, Cyp2b23, Cyp2c29, Cyp2c40, Cyp2c54, Cyp2c55, Cyp2c65, Cyp2d26, Cyp2e1, Cyp3a25, Cyp3a41b, Cyp3a13, Cyp4f15, Dhfr9, Ephx1, Ephx2, Fmo2, Fmo3, Fmo5, Ftl1, Gal3st1, Gm3776, Gstm2, Gstm3, Gstm4, Gstm1, Gstp2, Gstt1, Gstt2, Gstt3, Hdac5, Hs2st1, Hsp90b1, Kras, Map2k4, Map2k6, Map3k4, Map3k5, Map3k6, Mapk9, Mgst1, Ndst1, Nfkb1, Nqo1, Nr1i2, Pik3cb, Ppp2r5a, Ppp2r5d, Ppp2r5e, Prkce, Raf1, Rap2a, Rras, Rras2, Sult1a1, Sumo1, Ugt1a1, Ugt1a9, Ugt1a7c, Ugt2b34, Ugt2b1, Ugt2b5, Ugt2b35
Liver injury and regeneration	Glutathione detoxification reaction/depletion	Abcc3, Aldh1a1, Anpep, Ccng1, Cyp2b10, Ephx1, Gm3776, Gstm2, Gstm3, Gstm4, Gstm1, Gstp2, Gstt1, Gstt2, Gstt3, Gpx2, Gpx4, Gpx7, Mgst1, Por, Tkt, Ugt1a9
	Liver steatosis	Arnt, Atf4, C3, Cd36, Cidec, Cnot3, Crtc3, Cyp2e1, Ddc, Elovl2, Epas1, Fgf21, Gps2, Grb14, Jun, Lipc, Lpin1, Map3k5, Mark2, Mfsd2a, Mogat2, Noct, Nr1i2, Pdgfc, Plk1, Pnrc2, Srebf1
	Liver necrosis/cell death	ARNT, ASAH2, ATP7B, ATR, BIRC2, BUB1, C3, CBS, CDKN1B, CEBPB, CHUK, CTH, CTNNB1, CYP2E1, E2F1, ENTPD5, ESR1, FAIM, FGA, FOXO3, G6PC, GADD45B, GIMAP3, HADHA, HSPD1, IGF1, IL1RN, IL6Ra, INHBA, IQGAP2, IRF5, ITIH4, JUN, KRT8, LDLR, MAML1, MAP2K4, MAPK9, MNT, MTOR, Mug2, MYC, MYCN, NCL, NCOA5, NFKB1, NGF, NGFR, PAFAH2, PLG, PLK1, PRKAA2, PROS1, RAF1, RBCK1, SAA2, SELP, SERPINE1, SH3BP5, SHC1, SLC20A1, SLC25A5, STK4, TIMP3, Trp53, USP2
	Liver proliferation/hyperplasia	ACTN4, Arnt, Atf4, BIRC2, BIRC5, BUB1, C3, Cbs, Ccnd3, CCNE1, CCNG1, CDCA8, CDKN1B, Cebpb, CHUK, Csf2rb, CTNNB1, Csc12, Dlc1, E2F1, Entpd5, F2, FAM120A, FOXM1, Gfer, HIF1A, HMGCR, HMMR, Hnf1a, HSPA5, Igf1, Igf2, IGFBP2, Il1m, Il4r, Il6r, Inhba, Iqgap2, ITIH4, JUN, Lgals1, LTB, LTBR, Map2k4, Map2k6, MAPK9, Mmp14, MYC, Ncoa5, Nfatc3, NFKB1, Ngfr, Nr1i2, ODC1, PIM3, Plg, Pml, Ppard, Prkaa1, Pros1, Raf2, RRM2, SDR9C7, Slc20a1, Smarcb1, TERT, TGFA, TGFB2, Trf, Trp53, TPX2, Yod1
	Hepatic cholestasis	Abcb11, Abcc2, Abcc3, Abcg5, Abcg8, Adcy1, Adcy6, Adcy9, Chuk, Clcf1, Esr1, Fgfr4, Hnf1a, Il1m, Irak1, Jun, Ltb, Map2k4, Map3k4, Mapk9, Nfkb1, Ngfr, Nr1i2, Prkar1a, Prkce, Slc22a7, Slc4a2, Srebf1, Tjp2, Traf2

varying susceptibility to the toxicity of chemicals. Therefore, a possible explanation for nitrosative stress in susceptible animals is a higher tissue drug exposure due to reduced clearance, disruption of hepatic transport of the parent compound or metabolites, or an altered capacity for Phase I or II metabolism (Krautbauer et al., 2017). In CD-1 mice, zileuton has previously been shown to modestly induce CYP2B and CYP4A abundance and monooxygenase activity (Rodrigues and Machinist, 1996). A potential avenue for increased toxicity in susceptible individuals could be a hyper-efficient induction of CYP enzymes that catalyze conversion of the parent drug to reactive intermediates. Alternatively, a reduced capacity of CYP enzymes such as CYP1A2 which transform zileuton into nonreactive metabolites can lead to an increased formation of reactive intermediates and therefore a greater risk for toxicity. Indeed, several genes and pathways related to CYP enzymes and their transcriptional regulators were shown to be significantly altered in zileuton-treated mice. From this result, we can speculate that interindividual variability in zileuton toxicity may be partly due to different capacity for metabolism. Further analyses on measuring the activities of different CYP enzymes in liver tissues would better elucidate the relationship between the metabolic activity and the extent of zileuton toxicity.

Differences in plasma C_{max} or clearance of the compound may also affect the degree of hepatotoxicity sustained in individual animals. In the current analysis, interindividual differences in tissue exposure and pharmacokinetics of zileuton were not investigated. Although its half-life in the DO mouse population is unknown, zileuton is known to be rapidly cleared in

humans, with the mean terminal half-life at 2.5 h. Therefore, it is unlikely that zileuton would be present at detectable levels in the blood at time of necropsy, 24 h following the last dose. Future studies that investigate variation in pharmacokinetics of zileuton oral dosing in DO mice are needed to address the extent to which tissue exposure may play a role in differential susceptibility. It is important to note however that in prior mouse population-based analyses of drug-induced liver or kidney injury, there was no significant correlation between either C_{max} or of tissue concentration of the tested compound (or active metabolite) with the incidence or severity of organ damage (Harrill et al., 2012; Mosedale et al., 2014). This finding does not downplay the importance of metabolic differences in contribution to overall toxicity outcomes, but does suggest that interindividual differences in pharmacodynamic processes play an important role in the toxicity outcome.

Differential capacity in protection against zileuton-induced nitrosative stress may also lead to the population variability in liver injury. Glutathione is a key antioxidant and individuals with reduced glutathione capacity experience a greater degree of cellular damage caused by nitrosative stress. In this study, tissue levels of glutathione were not measured because the timing of tissue collection (24 h postdosing) and the low level of hepatic injury observed were unlikely to enable observation of transient glutathione reductions. Yet, the pathway analysis revealed that the expression of genes involved in glutathione-mediated detoxification including glutathione peroxidase was significantly increased in DO mice exposed to zileuton. This observation implies that glutathione may be a key defense

Figure 5. Interaction of FXR signaling and pathways relevant for zileuton-induced hepatotoxicity. DEGs as identified by RNA-seq analysis of transcriptomic data from DO mice were analyzed using IPA to discover top enriched pathways relevant for ZILI. The molecular relationships of the FXR signaling pathway, which was the most significant canonical pathway, and pathways relevant for the liver toxicity outcomes are depicted.

mechanism in ZILI. Therefore, impaired functionality of glutathione-mediated defense pathways may contribute to the sensitivity to zileuton-induced hepatotoxicity.

Interestingly, DO mice resistant to zileuton toxicity experienced marked disruptions in hepatic tissue concentrations of hormones that regulate energy metabolism including

Figure 6. Proposed modes of action underlying the susceptibility to zileuton-induced liver toxicity in individual DO mice. Briefly, zileuton enters hepatocytes and is metabolized into reactive metabolite(s) as well as nonreactive metabolites that are excreted. Reactive metabolite(s), whether directly or indirectly, lead to nitrosative stress, as evidenced by generation of iNOS and presence of NT-protein adducts, which are found in sensitive DO mice that experience necrosis. Nitrosative stress causes mitochondrial damage which then impairs FAO and energy production. Compromised defense mechanisms against these liver toxicities, such as FXR signaling and glutathione-mediated detoxification, can render DO mice susceptible to ZILI. The figure is the composite of previous findings and the results from this study.

adipokines like leptin and resistin (Buechler et al., 2017). Leptin, which can prevent lipid accumulation in the liver and other tissues via insulin-independent pathways (Petersen et al., 2002), was elevated in the resistant DO mice. This finding supports that an impaired lipid regulation and liver steatosis are key processes in the progression of ZILI. Also, increased levels of leptin in adipose tissue and in plasma have been associated with over-feeding (Kolaczynski et al., 1996). Thus, it is also possible that the resistant DO mice had an increased food consumption which could lead to greater levels of protective glutathione. However, it should be noted that body weights were not affected by zileuton treatment and food consumption was not monitored in this study. Conversely, resistin, another adipokine, was decreased in the livers of resistant DO mice. Increased resistin levels were previously shown to be associated with reduced fatty acid β -oxidation (He et al., 2018; Ikeda et al., 2013). Lower levels of resistin in resistant DO mice also imply the importance of lipid metabolism in determining the susceptibility to ZILI.

Further supporting these clinical observations, the results from the pathway analysis found several canonical pathways

and molecules related to the fatty acid metabolism and mitochondrial FAO to be significantly altered in response to ZILI. Mitochondria are key regulators of the fatty acid metabolism and energy production. Mitochondrial impairment has been known to mediate DILI and nonalcoholic fatty liver disease. A good example is acetaminophen-induced liver toxicity where a reactive metabolite of acetaminophen disrupts mitochondrial functionality. Similarly, reactive species generated from ZILI and subsequent nitrosative stress may cause damage to mitochondria, altering FAO to induce fatty changes and inhibiting energy production and causing hepatic necrosis. Nuclear receptors such as FXR and PPAR α regulate genes involved in the fatty acid metabolism and maintain the lipid homeostasis. FXR was noted the most significant canonical pathway in our analysis. As depicted in Figure 5, FXR closely interacts with numerous genes involved in fatty acid metabolism and bile acid secretion, and this indicates that FXR may be an important feedback mechanism in response to altered FAO after zileuton treatment. A predicted decrease in FXR activity among ZILI-treated DO mice implies that compromised feedback regulation via FXR is

potentially a key susceptibility factor for ZILI. An important caveat to consider when studying a diverse population (such as DO mice or humans) is that there may be a difference in the underlying basal expression of transcripts caused by polymorphisms in genomic regulatory elements (eg, expression quantitative trait loci, eQTL). In principle, the effect of eQTL on the observations in the drug-exposed group is limited by including larger sample size and a balanced case-control design.

It is important to note that the observations in this study reflect a snapshot of ZILI. Therefore, it is difficult to definitively differentiate the initiating events from the secondary events of the injury. A future study exploring the effects of zileuton over different time points will provide further mechanistic insights by elucidating the temporal relationships of the events. In addition, the dosing duration of zileuton in this study is shorter from that in the clinical settings, and likely contributes to the different degrees of injury between the DO mice and the patients. In the DO mice, a low level of liver injury was observed after 7 days of exposure whereas patients who exhibit ZILI typically develop elevated liver enzymes and jaundice after several weeks to months of zileuton therapy (Watkins et al., 2007). A potential consideration is that sensitive patients may experience low-level liver injury that is not apparent until a larger accumulated injury is made manifest via jaundice and associated symptoms. It may be beneficial to expose the DO mice to zileuton for a longer duration to better mimic the timeline of ZILI manifestation in the clinic.

In conclusion, our data suggest that zileuton-induced hepatotoxicity in genetically sensitive individuals is mediated by nitrosative stress and a subsequent impairment in key functions of mitochondria which then result in fatty changes and cellular necrosis in the liver. The proposed mechanism is illustrated in Figure 6 (Ananthanarayanan et al., 2001; Bessone et al., 2018; Cipriani et al., 2010; Figge et al., 2004; Heni et al., 2013; Iorga et al., 2017; Joshi et al., 2004, 2009; Liu et al., 2018; Makishima et al., 1999; Nassir and Ibdah, 2014; Natarajan et al., 2006; Plass et al., 2002; Preidis et al., 2017; Sinal et al., 2000; Walters, 2000; Watanabe et al., 2004; Zhang et al., 2012). This figure synthesizes the previous findings and new results from our study. This study demonstrated the utility of DO mice to investigate ZILI in the population. Our results provide important first clues regarding differential modes of action underlying susceptibility to zileuton toxicity. Follow-up investigations are necessary to confirm the mechanisms proposed in this paper, but this work provides an important first step toward understanding the mechanisms of ZILI, which may inform future efforts toward personalized prescribing of zileuton and protection of genetically sensitive patients.

DATA AVAILABILITY

Supplementary data are available at: <https://doi.org/10.22427/NTP-DATA-002-00076-0001-0000-6> (last accessed March 31, 2020).

RNA-seq data: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE141089> (last accessed March 31, 2020).

ACKNOWLEDGMENTS

The authors sincerely thank Karen Cimon of Experimental Pathology Laboratories, Inc for pathology support, Shaoke Luo, Todd Fite, and Haixia Lin of UAMS for technical assistance, and Jennifer James in the UAMS Histology Research Service Center that provided support. We greatly

appreciated helpful discussions with Daniel Acosta, Mark Avigan, Donna Mendrick, William Slikker, and of the FDA National Center for Toxicological Research throughout the conduct of the research. Sincere thanks also go to Pierre Bushel, Stephen Ferguson, Arun Pandiri, and Keith Shockley of NIEHS and Lei Guo of NCTR for critical review of the manuscript. Lastly, the authors would like to acknowledge Mike C. Conway of NIEHS for programming support and the statistical support from Social & Scientific Systems, Inc (Contract HHSN273201600011C).

FUNDING

This work was supported by The Food & Drug Administration, The National Institute of Environmental Health Sciences, The Burroughs Wellcome Fund Innovation in Regulatory Science Award, the Arkansas Biosciences Institute, and the University of Arkansas for Medical Sciences Translational Research Institute (UL1TR000039) through the NIH National Center for Research Resources and National Center for Advancing Translational Sciences. L.E.L.-C. Jr was supported as a predoctoral fellow by NIH (T32 GM106999), as well as the Southern Regional Education Board. The content of this manuscript is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or of the Food & Drug Administration.

DECLARATION OF CONFLICTING INTERESTS

G.J.L. is a paid contractor for the NIEHS to provide statistical support services.

REFERENCES

- Ananthanarayanan, M., Balasubramanian, N., Makishima, M., Mangelsdorf, D. J., and Suchy, F. J. (2001). Human bile salt export pump promoter is transactivated by the farnesoid X receptor/bile acid receptor. *J. Biol. Chem.* **276**, 28857–28865.
- Bailie, M. B., Dahm, L. J., Peters-Golden, M., Harris, R. R., Carter, G. W., and Roth, R. A. (1995). Leukotrienes and alpha-naphthylisothiocyanate-induced liver injury. *Toxicology* **100**, 139–149.
- Bessone, F., Dirchwolf, M., Rodil, M. A., Razori, M. V., and Roma, M. G. (2018). Review article: Drug-induced liver injury in the context of nonalcoholic fatty liver disease—A physiopathological and clinical integrated view. *Aliment. Pharmacol. Ther.* **48**, 892–913.
- Braeckman, R. A., Granneman, G. R., Locke, C. S., Machinist, J. M., Cavanaugh, J. H., and Awni, W. M. (1995). The pharmacokinetics of zileuton in healthy young and elderly volunteers. *Clin. Pharmacokinet.* **29**, 42–48.
- Buechler, C., Haberl, E. M., Rein-Fischboeck, L., and Aslanidis, C. (2017). Adipokines in liver cirrhosis. *Int. J. Mol. Sci.* **18**, pii: E1392.
- Church, R. J., Gatti, D. M., Urban, T. J., Long, N., Yang, X., Shi, Q., Eaddy, J. S., Mosedale, M., Ballard, S., Churchill, G. A., et al. (2015). Sensitivity to hepatotoxicity due to epigallocatechin gallate is affected by genetic background in Diversity Outbred mice. *Food Chem. Toxicol.* **76**, 19–26.

- Churchill, G. A., Gatti, D. M., Munger, S. C., and Svenson, K. L. (2012). The Diversity Outbred mouse population. *Mamm. Genome* **23**, 713–718.
- Cipriani, S., Mencarelli, A., Palladino, G., and Fiorucci, S. (2010). FXR activation reverses insulin resistance and lipid abnormalities and protects against liver steatosis in Zucker (fa/fa) obese rats. *J. Lipid Res.* **51**, 771–784.
- Edgar, R., Domrachev, M., and Lash, A. E. (2002). Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res.* **30**, 207–210.
- FDA. (2005). *Guidance for Industry: Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers*. Available at: <https://www.fda.gov/downloads/drugs/guidances/ucm078932.pdf>. Accessed March 31, 2020.
- Figge, A., Lammert, F., Paigen, B., Henkel, A., Matern, S., Korstanje, R., Shneider, B. L., Chen, F., Stoltenberg, E., Spatz, K., et al. (2004). Hepatic overexpression of murine Abcb11 increases hepatobiliary lipid secretion and reduces hepatic steatosis. *J. Biol. Chem.* **279**, 2790–2799.
- Gatti, D. M., Weber, S. N., Goodwin, N. C., Lammert, F., and Churchill, G. A. (2017). Genetic background influences susceptibility to chemotherapy-induced hematotoxicity. *Pharmacogenomics J.* **18**, 319–330.
- Harrill, A. H. (2016). Mouse population-based toxicology for personalized medicine and improved safety prediction. In *Drug Discovery Toxicology: From Target Assessment to Translational Biomarkers* (Y. Will, Ed.), pp. 314–329. John Wiley & Sons, Inc., Hoboken, NJ.
- Harrill, A. H., Borghoff, S., Zorrilla, L., Blystone, C., Kissling, G. E., Malarkey, D., Shockley, K., Travlos, G., and DeVito, M. J. (2018). In *NTP Research Report on Baseline Characteristics of Diversity Outbred (J:DO) Mice Relevant to Toxicology Studies: Research Report 6*, Durham, NC. Available at: <https://doi.org/10.22427/NTP-RR-6>. Accessed March 31, 2020.
- Harrill, A. H., Desmet, K. D., Wolf, K. K., Bridges, A. S., Eaddy, J. S., Kurtz, C. L., Hall, J. E., Paine, M. F., Tidwell, R. R., and Watkins, P. B. (2012). A mouse diversity panel approach reveals the potential for clinical kidney injury due to DB289 not predicted by classical rodent models. *Toxicol. Sci.* **130**, 416–426.
- He, F., Jin, J. Q., Qin, Q. Q., Zheng, Y. Q., Li, T. T., Zhang, Y., and He, J. D. (2018). Resistin regulates fatty acid beta oxidation by suppressing expression of peroxisome proliferator activator receptor gamma-coactivator 1alpha (PGC-1alpha). *Cell. Physiol. Biochem.* **46**, 2165–2172.
- Heni, M., Wagner, R., Ketterer, C., Bohm, A., Linder, K., Machicao, F., Machann, J., Schick, F., Hennige, A. M., Stefan, N., et al. (2013). Genetic variation in NR1H4 encoding the bile acid receptor FXR determines fasting glucose and free fatty acid levels in humans. *J. Clin. Endocrinol. Metab.* **98**, E1224–E1249.
- Hoggatt, A. F., Hoggatt, J., Honerlaw, M., and Pelus, L. M. (2010). A spoonful of sugar helps the medicine go down: A novel technique to improve oral gavage in mice. *J. Am. Assoc. Lab. Anim. Sci.* **49**, 329–334.
- Ikeda, Y., Tsuchiya, H., Hama, S., Kajimoto, K., and Kogure, K. (2013). Resistin affects lipid metabolism during adipocyte maturation of 3T3-L1 cells. *FEBS J.* **280**, 5884–5895.
- Iorga, A., Dara, L., and Kaplowitz, N. (2017). Drug-induced liver injury: Cascade of events leading to cell death, apoptosis or necrosis. *Int. J. Mol. Sci.* **18**, 1018.
- Joshi, E. M., Heasley, B. H., Chordia, M. D., and Macdonald, T. L. (2004). In vitro metabolism of 2-acetylbenzothioephene: Relevance to zileuton hepatotoxicity. *Chem. Res. Toxicol.* **17**, 137–143.
- Joshi, E. M., Heasley, B. H., and Macdonald, T. L. (2009). 2-ABT-S-oxide detoxification by glutathione S-transferases A1-1, M1-1 and P1-1: Implications for toxicity associated with zileuton. *Xenobiotica* **39**, 197–204.
- Kolaczynski, J. W., Nyce, M. R., Considine, R. V., Boden, G., Nolan, J. J., Henry, R., Mudaliar, S. R., Olefsky, J., and Caro, J. F. (1996). Acute and chronic effects of insulin on leptin production in humans: Studies in vivo and in vitro. *Diabetes* **45**, 699–701.
- Krautbauer, S., Haberl, E. M., Eisinger, K., Pohl, R., Rein-Fischboeck, L., Rentero, C., Alvarez-Guita, A., Enrich, C., Grewal, T., Buechler, C., et al. (2017). Annexin A6 regulates adipocyte lipid storage and adiponectin release. *Mol. Cell. Endocrinol.* **439**, 419–430.
- Lazarus, S. C., Lee, T., Kemp, J. P., Wenzel, S., Dube, L. M., Ochs, R. F., Carpentier, P. J., and Lancaster, J. F. (1998). Safety and clinical efficacy of zileuton in patients with chronic asthma. *Am. J. Manag. Care* **4**, 841–848.
- Lewis, R. A., Austen, K. F., and Soberman, R. J. (1990). Leukotrienes and other products of the 5-lipoxygenase pathway. Biochemistry and relation to pathobiology in human diseases. *N. Engl. J. Med.* **323**, 645–655.
- Liu, Y., Song, A., Yang, X., Zhen, Y., Chen, W., Yang, L., Wang, C., and Ma, H. (2018). Farnesoid X receptor agonist decreases lipid accumulation by promoting hepatic fatty acid oxidation in db/db mice. *Int. J. Mol. Med.* **42**, 1723–1731.
- Makishima, M., Okamoto, A. Y., Repa, J. J., Tu, H., Learned, R. M., Luk, A., Hull, M. V., Lustig, K. D., Mangelsdorf, D. J., and Shan, B. (1999). Identification of a nuclear receptor for bile acids. *Science* **284**, 1362–1365.
- Mosedale, M., Wu, H., Kurtz, C. L., Schmidt, S. P., Adkins, K., and Harrill, A. H. (2014). Dysregulation of protein degradation pathways may mediate the liver injury and phospholipidosis associated with a cationic amphiphilic antibiotic drug. *Toxicol. Appl. Pharmacol.* **280**, 21–29.
- Nassir, F., and Ibdah, J. A. (2014). Role of mitochondria in nonalcoholic fatty liver disease. *Int. J. Mol. Sci.* **15**, 8713–8742.
- Natarajan, S. K., Eapen, C. E., Pullimood, A. B., and Balasubramanian, K. A. (2006). Oxidative stress in experimental liver microvesicular steatosis: Role of mitochondria and peroxisomes. *J. Gastroenterol. Hepatol.* **21**, 1240–1249.
- Nelson, H., Kemp, J., Berger, W., Corren, J., Casale, T., Dube, L., Walton-Bowen, K., LaVallee, N., and Stepanians, M. (2007). Efficacy of zileuton controlled-release tablets administered twice daily in the treatment of moderate persistent asthma: A 3-month randomized controlled study. *Ann. Allergy Asthma Immunol.* **99**, 178–184.
- Petersen, K. F., Oral, E. A., Dufour, S., Befroy, D., Ariyan, C., Yu, C., Cline, G. W., DePaoli, A. M., Taylor, S. I., Gorden, P., et al. (2002). Leptin reverses insulin resistance and hepatic steatosis in patients with severe lipodystrophy. *J. Clin. Invest.* **109**, 1345–1350.
- Plass, J. R., Mol, O., Heegsma, J., Geuken, M., Faber, K. N., Jansen, P. L., and Muller, M. (2002). Farnesoid X receptor and bile salts are involved in transcriptional regulation of the gene encoding the human bile salt export pump. *Hepatology* **35**, 589–596.
- Pohl, R., Haberl, E. M., Rein-Fischboeck, L., Zimny, S., Neumann, M., Aslanidis, C., Schacherer, D., Krautbauer, S., Eisinger, K., Weiss, T. S., et al. (2017). Hepatic chemerin mRNA expression is reduced in human nonalcoholic steatohepatitis. *Eur. J. Clin. Invest.* **47**, 7–18.
- Preidis, G. A., Kim, K. H., and Moore, D. D. (2017). Nutrient-sensing nuclear receptors PPARalpha and FXR control liver energy balance. *J. Clin. Invest.* **127**, 1193–1201.

- Rodrigues, A. D., and Machinist, J. M. (1996). Hepatic peroxisomal and drug metabolizing activity in CD-1 mice after oral treatment with a novel 5-lipoxygenase inhibitor. *Toxicol. Appl. Pharmacol.* **137**, 193–201.
- Shetty, A., Cho, W., Alazawi, W., and Syn, W. K. (2017). Methotrexate hepatotoxicity and the impact of nonalcoholic fatty liver disease. *Am. J. Med. Sci.* **354**, 172–181.
- Sinal, C. J., Tohkin, M., Miyata, M., Ward, J. M., Lambert, G., and Gonzalez, F. J. (2000). Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. *Cell* **102**, 731–744.
- Somogyi, A. A., and Phillips, E. (2017). Genomic testing as a tool to optimise drug therapy. *Aust. Prescr.* **40**, 101–104.
- Sorkness, C. A. (1997). The use of 5-lipoxygenase inhibitors and leukotriene receptor antagonists in the treatment of chronic asthma. *Pharmacotherapy* **17**, 50S–54.
- Vega, M., Verma, M., Beswick, D., Bey, S., Hossack, J., Merriman, N., Shah, A., and Navarro, V., and Drug Induced Liver Injury, N. (2017). The incidence of drug- and herbal and dietary supplement-induced liver injury: Preliminary findings from gastroenterologist-based surveillance in the population of the state of Delaware. *Drug Saf.* **40**, 783–787.
- Walters, J. R. (2000). Bile acids are physiological ligands for a nuclear receptor. *Gut* **46**, 308–309.
- Wang, W., Lockwood, K., Boyd, L. M., Davidson, M. D., Movafaghi, S., Vahabi, H., Khetani, S. R., and Kota, A. K. (2016). Superhydrophobic coatings with edible materials. *ACS Appl. Mater. Interfaces* **8**, 18664–18668.
- Watanabe, M., Houten, S. M., Wang, L., Moschetta, A., Mangelsdorf, D. J., Heyman, R. A., Moore, D. D., and Auwerx, J. (2004). Bile acids lower triglyceride levels via a pathway involving FXR, SHP, and SREBP-1c. *J. Clin. Invest.* **113**, 1408–1418.
- Watkins, P. B., Dube, L. M., Walton-Bowen, K., Cameron, C. M., and Kasten, L. E. (2007). Clinical pattern of zileuton-associated liver injury: Results of a 12-month study in patients with chronic asthma. *Drug Saf.* **30**, 805–815.
- Zhang, Y., Li, F., Patterson, A. D., Wang, Y., Krausz, K. W., Neale, G., Thomas, S., Nachagari, D., Vogel, P., Vore, M., et al. (2012). Abcb11 deficiency induces cholestasis coupled to impaired beta-fatty acid oxidation in mice. *J. Biol. Chem.* **287**, 24784–24794.