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Transcriptional Profiling Suggests That Nevirapine and Ritonavir Cause Drug Induced Liver Injury Through Distinct Mechanisms in Primary Human Hepatocytes

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Abstract

Drug induced liver injury (DILI), a major cause of pre- and post-approval failure, is challenging to predict pre-clinically. Predicting DILI is challenging due to varied underlying direct and indirect mechanisms. Nevirapine, a non-nucleoside reverse transcriptase inhibitor (NNRTI) and Ritonavir, a protease inhibitor, are antiviral drugs that cause clinical DILI with different phenotypes via different mechanisms. Assessing DILI *in vitro* in hepatocyte cultures typically requires drug exposures significantly higher than clinical plasma C_{max} concentrations, making clinical interpretations of mechanistic pathway changes challenging. We previously described a system that uses liver-derived hemodynamic blood flow and transport parameters to restore primary human hepatocyte biology, and drug responses at concentrations relevant to *in vivo* or clinical exposure levels. Using this system, primary hepatocytes from 5 human donors were exposed to approximating clinical therapeutic and supra-therapeutic concentrations levels of Nevirapine (11.3 and 175.0 μ M) and Ritonavir (3.5 and 62.4 μ M) for 48 hours. Whole genome transcriptomics was performed by RNAseq along with functional assays for metabolic activity and function. We observed effects at both doses, but a greater number of genes were differentially expressed with higher probability at the toxic concentrations. At the toxic doses, both drugs showed direct cholestatic potential with Nevirapine increasing bile synthesis and Ritonavir inhibiting bile acid transport. Clear differences in antigen presentation were noted, with marked activation of MHC Class I by Nevirapine and suppression by Ritonavir. This suggests CD8+ T cell involvement for Nevirapine and possibly NK Killer cells for Ritonavir. Both compounds induced several drug metabolizing genes (including CYP2B6, CYP3A4 and UGT1A1), mediated by CAR activation in Nevirapine and PXR in Ritonavir. Unlike Ritonavir, Nevirapine did not increase fatty acid synthesis or activate the respiratory electron chain with simultaneous mitochondrial uncoupling

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supporting clinical reports of a lower propensity for steatosis. This *in vitro* study offers insights into the disparate direct and immune mediated toxicity mechanisms underlying Nevirapine and Ritonavir toxicity in the clinic.

1. Introduction

Drug-induced liver injury (DILI) ranks as the leading cause of liver failure (1) and liver transplantation in western countries, and is a major cause of drug withdrawal and non-approval by regulatory authorities (2). Predicting DILI will help reduce drug attrition and thereby drug development costs. A variety of preclinical assay systems including primary *in vitro* hepatocytes and cell lines are widely used to screen compound libraries for drug metabolism and toxicity(3, 4). While some of these offer advantages of high throughput, economy and simplicity of use, there exist huge challenges in the translatability of results to human clinical effects. DILI also manifests with diverse phenotypes of hepatocellular toxicity, cholestatic or mixed patterns of injury reflecting multiple mechanisms and pathways, that are challenging to study *in vitro*. Levels of cytotoxicity measures like ALT and AST, biomarkers like miR-122 and α -GST, and liver function markers such as albumin are used in preclinical *in vitro* assays (7-12). However they offer no insights into mechanisms of toxicity. Thus, there is a need for an approach using a combination of endpoints to predict and identify mechanisms of liver injury.

In order to develop a robust *in vitro* surrogate for hepatic function, it is critical to mimic *in vivo* physiology. The pharmacokinetics impacting the metabolic fate of drugs, as well as the pharmacodynamics of direct or indirect toxic effects on the liver are tied to retention of hepatocyte-specific structure and function *in vitro*. These depend on adequate levels and appropriate localization of key enzymes (e.g. phase I and phase II metabolic enzymes, surface receptors and transporters), to ensure functionality. Often, the toxicity is caused by a (reactive) metabolite requiring the system to be metabolically competent. However, dedifferentiation and loss of hepatocyte-specific function and metabolic enzyme activity is documented to occur over days in conventional culture models(13-15). This same period is paradoxically critical for the recovery of relevant efflux transporter localization needed for drug elimination kinetics(16). As a result, it is rare for *in vitro* hepatocyte systems to exhibit stable drug responses that capture all aspects of *in vivo* hepatocyte functions over an extended period. Hepatocyte monolayer cultures often lack *in vivo* microenvironmental features such as three-dimensional polarized morphology with resultant biliary canalicular formation, biochemical and mechanical effects of extracellular matrix and localized cytokine/growth factor concentrations. Non-flow systems, by virtue of their static nature, are also limited by the absence of circulation mediated effects such as oxygen and nutrient transport. Another factor undermining the physiological nature of drug response in these systems is that drug concentrations used are usually very different, often orders of magnitude higher, than the corresponding *in vivo* plasma or tissue concentrations achieving similar effects (18). The static nature of these systems also means that metabolites produced by the hepatocytes could build up over time in the interval between medium changes, and may not be reflective of physiological responses *in vivo*.

To address these issues, we developed a hepatocyte system that restores critical aspects of sinusoidal and interstitial fluid dynamics resulting in solute transport analogous to that achieved with *in vivo* liver circulation. The system is based on a cone-and-plate viscometer technology and was initially designed to re-establish *in vivo* blood vessel cell phenotypes by reproducing the exposure of vascular endothelial cells to *in vivo*-like human hemodynamic blood flow forces *in vitro*(19, 20). We previously described a configuration of this flow-based culture system where primary hepatocytes are shielded from direct effects of flow, as they would be *in vivo*, but perfusion, nutrient gradients and interstitial fluid movement are maintained via a synthetic sinusoidal membrane. After a prescribed time period, *in vivo* like biology is restored in the hepatocytes *in vitro*, resulting in drug and hormone responses within the system at *in vivo*-relevant concentrations(21, 22). We note restoration of *in vivo* like phenotype, seen by differentiated transcriptomic signatures, polarized morphology, transporter localization and metabolic function in primary human hepatocytes(23). The differentiated state results in restoration of liver-like responsiveness that allows us to culture the cells at near-physiological levels of glucose and insulin(24), unlike other culture systems that need up to 20,000 times higher levels of insulin in the culture media(25, 26). More importantly, this elicits induction, efficacy or toxicity responses to drugs at clinically relevant therapeutic and toxic concentrations(27). Through an NIH NIDDK funded SBIR project (R44 DK091104), we have now assessed over 30 drugs in the system at clinically relevant concentrations for mechanisms of toxicity.

Hepatotoxicity is very common with anti-HIV medications, and occurs across most classes of anti-retroviral molecules(28). Nevirapine, a non-nucleoside reverse transcriptase inhibitor (NNRTI), is the most common antiretroviral drug causing serious, clinical liver injury and has a black box warning for liver toxicity. Some patients on nevirapine can present with severe, life-threatening hepatotoxicity, sometimes exhibiting cholestatic features and often also associated with rash (Nevirapine hypersensitivity syndrome)(29) and immune involvement is suspected. We chose to evaluate Nevirapine due to its complicated mechanism for hepatotoxicity. We simultaneously assessed Ritonavir, a protease inhibitor, as it is a hepatotoxic antiretroviral with a different profile of adverse effects. Ritonavir, is a substrate, inhibitor and inducer of CYP3A enzymes(30) and can cause potentially serious and/or life threatening reactions due to changes in the metabolism of other co-administered drugs, leading to a black box warning for drug-drug interactions (31). Treatment with Ritonavir can also lead to substantial increases in the concentration of total cholesterol and triglycerides. Ritonavir causes clinically apparent acute liver injury that manifests in ~15% of patients with fatty liver changes, and increased serum levels of various liver enzymes(32). We chose to assess a comparative response of Nevirapine to Ritonavir to determine whether the system can distinguish between diverse mechanisms of drug toxicity at clinically relevant concentrations. Lower therapeutic (safer) concentrations (C_{Av}) were calculated approximating the minimum human therapeutic plasma concentration required for virologic response and the higher supra-therapeutic (toxic) concentrations approximated a 5 to 10-fold multiple of the peak human serum concentration ($5-10 C_{max}$) following steady-state dosing with the highest FDA-approved therapeutic dose. Here we show that, we can demonstrate differences in signatures, suggestive of direct and indirect effects on signaling mechanisms

and metabolism/toxicity pathways, using an *in vitro* system that retains physiological parameters and hepatocyte responsiveness.

2. Materials and Methods

2.1. Donor Selection and Quality Assessment

Human cryopreserved hepatocytes from various healthy donors were screened and tested to select 5 donors that met the following quality control criteria: 1. Post-thaw viability >85%, 2. Plating efficiency >75%, 3. Polarized morphology and 4. Albumin rates > 10ug/million cells/day). The sourcing details and demographics of these donors are included in Table S2 (supplementary material)

2.2. Cell Culture and Device Operating Conditions

Primary hepatocytes from 5 individual donors were thawed and separately plated in a collagen gel sandwich configuration on the undersurface of the membranes of 75 mm polycarbonate transwells (Corning) using previously described protocols(22). The cultures were left overnight in maintenance medium (MM) that consisted of DMEM/F-12 supplemented with fetal bovine serum (10% at the time of plating), gentamycin (50 µg/ml), 0.2% ITS (Fisher/MediaTech MT-25–800CR), and dexamethasone (Cat# D4902, Sigma Aldrich, St. Louis, MO, 1 µM at plating and 250 nM thereafter). On the 2nd day, the transwells were set up within HemoShear devices in a configuration to allow for control of hemodynamics and transport as described previously(22). A proprietary hepatocyte flow medium (HFM), modified from MM but with significantly lower levels of key hormones and growth factors, was continuously perfused on both sides while shear stress was applied on the top surface based on the calculations described below. The devices were housed in a controlled environment at 37°C with 5% CO₂ mixed with air. For the flow experiments described in this study the shear stress of 0.6 dyn/cm² was applied based on calculations using the following equation for pressure driven flow of a Newtonian fluid through a cylinder;

$$\tau = \Delta P r^2 l$$

whereby the reference values for the pressure gradient across the sinusoid (ΔP), the radius of sinusoids (r), and the length of the sinusoids (l) were obtained from the literature(33, 34). Based on our prior experiments, hepatocytes were cultured for 7 days under controlled hemodynamics to restore a stable *in vivo* like phenotype prior to drug treatment.

2.3. Drug preparation and hepatocyte drug exposure

Nevirapine (Cat# SML0097, Sigma Aldrich, St. Louis, MO) and Ritonavir (Cat# 13872, Cayman Chemical, Ann Arbor, MI) were dissolved in tissue culture grade dimethyl sulfoxide (DMSO, Cat# D2650, Sigma Aldrich, St. Louis, MO), stored under nitrogen gas, and frozen at –80°C until use. The final concentration of DMSO was 0.1% and all drug-treated conditions were compared to the appropriate vehicle controls. For Nevirapine, the therapeutic and supra-therapeutic concentrations were 11.3 µM and 175.0 µM (35)

respectively and for Ritonavir the therapeutic and supra-therapeutic concentrations were 3.5 μ M and 62.4 μ M(36) respectively. Cells were exposed to drugs for a period of 48 hrs following physiologic adaptation in the HemoShear system as described above.

2.4. Functional Assay of Phase I/Phase II Enzyme Activity

The potential of Nevirapine and Ritonavir to induce or inhibit Phase I enzyme activity was assessed using specific cytochrome P450 (CYP) enzyme-selective probe substrates. Bupropion was used to measure CYP2B6 activity. To assess CYP3A4 activity, two probe substrates were used (testosterone and midazolam) since the activity of this important CYP enzyme is known to be substrate-dependent. At the conclusion of each experiment 2 cm² segments of the transwell membrane, were removed from each experimental device (N=3/device), washed twice with phosphate-buffered saline (PBS), and placed into 24-well plates. Pre-warmed serum-free media (0.5 mL) containing the Phase I enzyme substrates was added and the samples incubated for 60 min at 37°C and gently agitated using a nutator mixer. The media was then removed and quenched with an equal volume of ice-cold acetonitrile, vortexed for 2 min and stored at -80°C. The samples were sent to QPS, LLC (Newark, DE) for the quantitation of substrate metabolites by LC-MS.

2.5. RNA Preparation and RT-PCR for Quality Control (QC) Gene Panel

Hepatocyte cell pellets were collected and RNA isolated using the Invitrogen Purelink RNA Mini kit (Cat# 12183018A) according to the manufacturer's instructions. RNA concentrations were determined with the Nanodrop and RNA integrity was determined using the Agilent 2100 Bioanalyzer and the Agilent RNA 6000 kit (Cat# 5067-1511) according to the manufacturer's instructions. RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Expression of our internal QC genes CYP1A1, CYP2C9, CYP3A4, GSTP1, UGT1A1, GADD45A, GSR, CXCL2, HMGB1 and FGF21 (primer sequences in Supplementary Table S3) was determined by RT-PCR using iQ SYBR Green Supermix (Bio-Rad) and a CFX96 Real-Time System (with C1000 Thermal Cycler; Bio-Rad) and normalized to endogenous expression of β 2-microglobulin and 40S ribosomal protein S11.

2.6. RNA Deep Sequencing

Samples with a minimum RNA Integrity Number >7.0 were used for further transcriptomics processing. 1 μ g RNA per sample was submitted to Expression Analysis, Inc., A Quintiles Company, North Carolina, USA, for Illumina-based RNA deep sequencing. Approximately 20 million 50 base paired-end reads were generated per sample. For each treatment, 5 different human hepatocyte donors were used.

2.7. Data Analysis and Statistics

Our rigorous data analysis algorithms have been previously published(37). Briefly, following RNA deep sequencing, the reads were aligned to a standard hg19 human transcriptome. Alignment was done using Bowtie 0.12.9 with the final gene counts calculated using RSEM 1.2.0. RNA deep sequencing quality control measurements were performed to detect outliers and batch effects associated with the dataset by: 1) false color heatmaps of distance between

samples, 2) principal component analyses, and 3) overlapping density estimates. Outliers were excluded and batch effects, which consisted of human donor variability, removed. The RNA deep sequencing dataset counts were then analyzed to determine differentially expressed genes (DEGs). Methods for DEG determination were implemented using the open source R/BioConductor software (<http://www.bioconductor.org>) and the edgeR package. Genes that passed a threshold of more than 2 counts per million in at least 5 human samples were included for further analysis. A gene was determined to be a DEG by passing an FDR threshold of 10% (using the Benjamini & Hochberg FDR correction). Select genes of relevant biological processes were chosen to build response heatmaps that depict changes in gene expression based on a log₂fold scale of -2 to 2: blue = downregulation, red = upregulation, white = no change; the intensity of the color reflects the magnitude of change; a white dot indicates a statistically significant change in the expression of the corresponding gene (FDR<10%). To compare the effects of two different treatments or conditions, individual gene fold-changes were plotted on x- and y-axes. If two conditions stimulated identical responses, all the data would fall on the identity line of a gene-by-gene scatterplot. The Response Similarity Index (RSI) was calculated for each gene and determines the degree to which the two conditions alter gene expression in the same (RSI>0.2, purple dots) or opposite (RSI<-0.2, green dots) manner.

Qiagen's Ingenuity Pathway Analysis (IPA) layered transcriptomic data onto canonical pathways and simulates the downstream consequences of gene regulation. The color of the lines (edges) reflect the expected direction of effect between the two molecules with blue lines implying inhibition and orange lines suggesting activation. The color of the molecule/enzyme reflects the z-score calculated from the dataset. This could indicate upregulation (red) and predicted activation (orange) or downregulation (blue) and predicted inhibition (blue) of that molecule. The shade of color is darker when the z-score is higher reflecting a greater significance.

A global protein-protein interaction (PPI) network, derived from publicly available high-confidence interactions defined by the STRING database was used for overlaying our transcriptomic data (<http://string-db.org>). The goal of the protein-gene network is to identify centers of dysregulation in response to drug treatment. Connections between nodes were determined by evidence of functional protein-protein interactions (as defined by STRING). Edges were weighted by the probability that their incident nodes were differentially expressed. Connections with low weight were filtered, substantially reducing the number of proteins in the network; the resulting network of interactions reflects the strongest evidence of regulation. The size of any given node is proportional to its weighted connectivity and the weighted connectivity of its neighbors, thus, node size is an indicator of the relative importance of each node. Protein communities, comprised of more densely connected nodes, likely represent coordinated biological activities that contribute to similar signaling pathways.

3. Results

We assessed the toxicity of a 48 hour exposure of Nevirapine and Ritonavir on primary hepatocytes from 5 human donors maintained under controlled hemodynamics and transport.

For Nevirapine, the concentrations were 11.3 μM (Low) and 175.0 μM (High) and for Ritonavir the concentrations were 3.5 μM (Low) and 62.4 μM (High). At the end of the 48 hours of exposure, the cultures were imaged for morphology and cellularity, and MTT assays were performed to ensure that the viability was over 60% (data not shown). RNA was isolated from the cells and 13,102 genes (about 46%) passed the quality threshold of more than 2 counts per million in the 5 human donors and these were included in the transcriptomic analysis. Live assays were done to assess drug metabolizing enzymes..

3.1. Global responses: Dose Relationships, Similarities and Differences

Global changes in gene expression induced by the drugs were assessed by transcriptomic analysis of the RNA following 48-hour exposure to the drugs at both concentrations. Volcano plots (Figure 1A) depict the overall global responses to the therapeutic and toxic levels of Nevirapine and Ritonavir. A greater number of genes were differentially expressed with a higher probability at the toxic concentrations for both drugs. An unbiased analysis of the transcriptomic data by comparative Response Similarity Index (RSI) analysis identified similar and differential regulation of key pathways between Ritonavir and Nevirapine (Figure 1B). Significant responses of similar direction (purple dots) included drug and xenobiotic metabolizing enzymes (up-regulated) and cytokines and chemokine responses (down-regulated). Responses of opposite direction (green dots) were seen for mitochondrial uncoupling and unfolded protein response (UPR) and endoplasmic reticulum (ER) stress. Analysis of RSI between low and high doses of both Nevirapine and Ritonavir (Supplementary Figure S1) revealed that a majority of genes significantly regulated by both doses of drug moved in the same direction (purple dots – 81% for Nevirapine and 99% for Ritonavir). A much smaller number of genes significantly regulated by both doses moved in opposite directions (green dots – 19% for Nevirapine and 1% for Ritonavir). The overall response was much greater for Ritonavir, with a larger number of genes showing either significant upregulation or downregulation at both doses (1659 genes for Ritonavir vs. 367 for Nevirapine). For many additional genes regulated by Nevirapine (including some of the toxicity responses described below), the response manifested as a trend with the lower dose, attaining significance only at the higher dose. The lack of significance at lower doses of Nevirapine could be attributed to the biological variability across the relatively small number of subjects (5 subjects) included in the study, and subtle response to the drug. Hence the data presented in the subsequent sections mostly focuses on the significant effects seen at the higher dose though similar trends were seen in the lower dose.

3.2. Drug and Xenobiotic metabolism

Constitutive androstane receptor (CAR) and pregnane X receptor (PXR) act as sensors of drugs and xenobiotic substances upregulating the expression of metabolizing enzymes, such as cytochrome P450, phase II enzymes and transporters responsible for metabolism and excretion(38-41). They play a major role in the detoxification and reactive metabolite generation pathways for endobiotics, and xenobiotics, and their regulation by a drug thus has implications for interactions with concomitantly administered drugs.

Unbiased pathway analysis of the transcriptomic data indicates that various phase I, phase II enzymes and transporters are induced by Nevirapine. These include various CYP enzymes

(CYP2A, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4, CYP3A5 and CYP3A7), phase II enzymes (PAPS, SULT, GST) and transporters (MRP2 and MRP4). The overall pattern of induction is predictive of the activation of key nuclear receptor pathways such as CAR-RXR signaling (Figure 2A). Additional induction of genes coding for OATP8, PLTP and NR0B2 is reflective of some amount of activation of FXR-RXR signaling. Ritonavir up-regulates most of the same phase I, phase II genes and transporters, but unbiased pathway analysis predicts it to be due to activation of PXR-RXR signaling (Figure 2B). Measurement of the activity of CYP enzymes using different drug substrates revealed that Nevirapine marginally induced CYP 2B6 and 3A4 activities but unlike the up-regulation at the gene level, the increase was not significant. On the other hand, Ritonavir significantly induced CYP2B6 activity (6.7-fold increase in the formation of hydroxyl bupropion, $p < 0.05$) and significantly inhibited CYP3A4 activity as evidenced by two separate drug substrates (8.8-fold decrease in the formation of hydroxy midazolam and a 4.4-fold decrease in the hydroxylation of testosterone, $p < 0.01$). This is consistent with known clinical effects of Ritonavir that contribute to the black box warning for drug-drug interactions.

3.3. Bile Acid Synthesis and Transport

Cholestasis is a disturbance in bile formation and accumulation and is a reported side effect of both Nevirapine and Ritonavir(44), with a buildup of intracellular bile acids in hepatocytes that can lead to hepatotoxic injury.

We found that the pathways involved in bile acid synthesis were significantly regulated by both Nevirapine (FDR 0.1, $p = 0.03$) and Ritonavir (FDR 0.1, $p = 0.002$). Nevirapine significantly up-regulated the expression of SCP2 gene, which encodes for Sterol carrier protein2 and is known to enhance 7 α -hydroxycholesterol formation (Seltman et al 1985). The mRNA for cholesterol 7- α -hydroxylase (CYP7A1), the rate limiting step in bile acid synthesis, also shows a trend towards up-regulation following treatment with Nevirapine, along with activation of other downstream genes of the pathway (cholestanetetraol 26-dehydrogenase, Propanoyl-CoA C-acyltransferase). The predicted biological effect in our analysis suggests (Figure 3A) an up-regulation of the downstream products of the pathway (cholic acid, taurocholate, glycocholate, chenodeoxycholic acid, taurochenodeoxycholate and glycochenodeoxycholate) consistent with FXR activation. However, there is no accompanying compensatory up-regulation of genes for bile acid efflux transporters (Fig 3A inset), which is suggestive of the potential for an intracellular build-up of bile acids, and potential cholestatic changes. Following treatment with Ritonavir, negative regulators of bile acid synthesis (FXR and FGF19) are significantly up-regulated at the transcriptional level, while CYP7A1 is suppressed. Ritonavir further significantly down-regulated the expression of SCP2 and CYP8B1 genes confirming the suppression of the classical pathway of bile synthesis. The predicted biological effect of Ritonavir treatment (Fig 3B) is the down-regulation of the end products of bile acid synthesis (cholic acid, taurocholate, glycocholate, chenodeoxycholic acid, taurochenodeoxycholate and glycochenodeoxycholate). Furthermore, genes for the bile acid transporters are regulated by Ritonavir. The α subunit of the organic solute transporter (SLC51A, OST- α), the Na⁺ taurocholate co-transporting polypeptide (SLC10A1, NTCP) and ABCB4 (MDR3), which is responsible for Progressive Familial Intrahepatic Cholestasis 3(45), are all down-regulated

by Ritonavir(Fig 3B inset). The data thus suggest that the cholestatic potential of Nevirapine may be related to increased bile acid synthesis via classical pathway rather than transporter inhibition, while that of Ritonavir is more likely related to the inhibition of bile acid transporters.

3.4. MHC class I and II Presentation

Major histocompatibility complexes (MHCs) are surface molecules that bind antigens and display them for recognition by appropriate T cells mediating immunological responses. MHC class I, expressed on all nucleated cells while MHC class II are expressed on antigen presenting cells (APC) and to a lesser extent on hepatocytes.

Unbiased pathway analysis indicates that Nevirapine (FDR 0.1, $p=0.01$) regulates the antigen presentation pathway and is predicted to increase the expression of both MHC class I and II (Figure 4A). NOD-like receptor family CARD domain containing 5 (NLRC5), a major regulator of MHC I, is up-regulated by Nevirapine resulting in downstream gene up-regulation of the MHC1a and MHC1b subunits as well as processes of peptide and antigen presentation to CD8+ cytotoxic T-lymphocytes. Additionally, class II, major histocompatibility complex class II transactivator (CIITA), a major regulator of MHC II is also moderately up-regulated by Nevirapine along with MHC1a and MHC1b subunits resulting in antigen presentation in the context of MHC II to the CD4+ T-Helper cells. The transcriptomic data for Nevirapine was overlaid on a functional protein-protein interaction network (as defined by the STRING database) to identify centers of dysregulation in response to drug treatment (Figure 4C). In the context of the global biological response, antigen processing and MHC I antigen presentation emerge as one of the most central and most highly connected communities (Figure 4C, inset). This supports our interpretation of the transcriptomic data and suggests that the pathways are significantly perturbed following treatment with Nevirapine. In contrast, Ritonavir was predicted to down-regulate expression of MHC class I while upregulating the expression of MHC II (Figure 4B). NLRC5, was down-regulated by Ritonavir resulting in downstream gene down-regulation of the MHC1a and MHC1b subunits as well as processes of peptide and antigen presentation to CD8+ cytotoxic T-lymphocytes. However, CIITA was strongly up-regulated by Ritonavir than Nevirapine, along with MHC1a and MHC1b subunits, and antigen presentation in the context of MHC II to the CD4+ T-Helper cells.

3.5. Cellular Stress, Unfolded Protein Response and Proteasomal Degradation

The unfolded protein response (UPR) is a cellular stress response seen with many hepatotoxic drugs in response to an accumulation of unfolded or misfolded proteins in the lumen of the endoplasmic reticulum (ER). Unsuccessful or prolonged activation of the UPR can directly induce apoptosis via mitochondria-dependent and mitochondria-independent pathways involving CHOP, JNK, and caspase-4(47).

An unbiased analysis of the transcriptomic data by comparative Response Similarity Index (RSI) analysis identified differential regulation the UPR and ER stress pathways as a key difference between Ritonavir and Nevirapine (Figure 1B). Ritonavir was predicted to activate the unfolded protein response along with up-regulation of EIF2a, ATF4 and CANX.

Additionally, pathway analysis predicted activation of ATF4 downstream pathways leading to apoptosis. Supporting the evidence of a strong unfolded protein response was the induction of ERO1-L β and ER oxidoreductases, such as PDI as well as various heat shock proteins such as HSPH1, HSP70, HSP40 (Figure 5B). The activation of the unfolded protein response by Ritonavir was dose-dependent (Heatmap, Supplementary Figure 1). In contrast Nevirapine, inhibited EIF2 signaling and was predicted to suppress downstream ATF4 and CHOP related apoptosis pathway as well as PDI and heat shock responses (Figure 5A). The suppression of the UPR pathway related genes was more noticeable at the toxic concentration (Heatmap, Supplementary Figure 1).

3.6. Lipid Metabolism/Respiration/Steatotic Potential

An increase in fatty acid biosynthesis can contribute to lipid accumulation, increased lipid peroxidation, mitochondrial dysfunctions and cell death. Nevirapine and Ritonavir had differing effects on fatty acid synthesis and mitochondrial fatty acid β -oxidation. Treatment with Nevirapine had no observed effect on either pathway (Figure 6A). However, after Ritonavir treatment, we observed an up-regulation of the rate limiting genes and initiation steps of fatty acid biosynthesis (Figure 6D). In addition, we observed an up-regulation of genes involved in the rate limiting steps of fatty acid β -oxidation. Acyl-CoA Synthetase Long-Chain enzyme family (ACSL) plays a key role in lipid biosynthesis and fatty acid degradation. Increased fatty acid β -oxidation can contribute to mitochondrial dysfunctions, uncoupling and cell death.

The electron transport chain (ETC) composed of Complexes I-IV, generates a proton gradient across the inner mitochondrial membrane, and is coupled to the activity of adenosine triphosphate (ATP) synthetase (Complex V) which utilizes the electrochemical gradient to make ATP via oxidative phosphorylation. In certain drug toxicities, the electron transport chain can become uncoupled from ATP synthetase(48, 49). Long term mitochondrial uncoupling results in thermogenesis and depletion of ATP, resulting in cell death(48, 49).

Nevirapine and Ritonavir differentially regulated genes of the respiratory electron transport chain. Pathway analysis based on gene expression changes predicted an increase in the activity of Complexes I through IV in response to Nevirapine along with an increase of the ATP synthesis genes in complex V (Figure 6B) suggesting that Nevirapine treatment increases cellular respiration without inducing mitochondrial uncoupling. On the other hand, Ritonavir increased the activity of Complexes I and IV (the major electron producers in the ETC) while strongly downregulating ATP Synthetase (Complex V) (Figure 6C). This could be suggestive of mitochondrial uncoupling and proton leakage that leads to dissipation of H⁺ ions preventing ATP synthesis.

3.7. Inflammatory responses (chemokine cytokine secretion and Interferon signaling)

Interferons are cytokines that are key regulators of inflammatory responses, and released from cells in response to infection or injury. Interferons bind to their receptors, initiating JAK-STAT signaling and ultimately prompting interferon responsive gene transcription and orchestration of immune responses(51).

Multiple upstream regulators involved in inflammatory responses (NFkB, IFN- α and IFN- λ signaling) are predicted by our pathway analysis to be inhibited by the toxic dose of Nevirapine. (Figure 7A and 7B). Nevirapine at that dose down-regulates or shows a trend towards decreasing chemokine (CXCL1, 6, 10 and CCL2, 4, 20) and Interleukin (IL8, 32, LIF) gene expression. Interferon-stimulated genes ISG15, ISG20 IFI44, IFI6, IRF7, DDX58, DDX60 and OSA1, 2, 3 were down-regulated. Ritonavir had a similar, but more profound effect on inhibition of inflammatory cytokines and chemokines (data not shown). Ritonavir at the high dose down-regulated genes expression of chemokines (CXCL1, 2, 5, 6, 10, 12 and CCL2, 3, 4, 20) and interleukins (IL7, 8, 32, LIF)

4. Discussion

This study aimed to generate hypothesis about mechanisms driving differences in the DILI signatures of two hepatotoxic antiviral drugs with distinct clinical safety outcomes. We used concentrations reflective of therapeutic and toxic clinical plasma exposures of the drugs, in a system that retains an *in vivo*-like phenotype by restoration of physiological parameters. Our observations identified signaling pathways that were predictive of distinct causative mechanisms of cholestasis in each case; increased bile synthesis with Nevirapine, and decreased bile acid efflux with Ritonavir. Differences in MHC presentation patterns also pointed to divergent immunologically mediated downstream cellular involvement between the drugs (CD8+ in Nevirapine and CD4+ response in Ritonavir). We predicted a higher propensity of steatosis and unfolded protein response with Ritonavir. Collectively, the data provided understanding into both direct and indirect mechanisms of hepatotoxicity, and mechanistic insights confirming and supporting differing clinical safety issues with the drugs. This has not been shown before *in vitro*.

4.1. Uniqueness of Approach (Dosing, endpoints, analysis)

The demonstration of a high degree of fidelity with *in vivo* phenotype and response to stimuli is key to the predictivity of cell-based assays and their utility in studying drug pharmacodynamics. A critical and novel aspect of this study was the use of clinically-relevant drug concentrations. The lower of the two concentrations chosen approximated the plasma threshold for clinical efficacy, and the higher dose was a low multiple of the C_{max} . These were significantly below those which many culture systems typically use in their toxicity assessments(18). Fang and Beland(18) estimated an IC_{50} of 813.7 μ M for a 48 hour of Nevirapine which was almost six times the higher toxic concentration used in our study. Such high concentrations are useful for cytotoxicity metrics but may not reveal subtler pathway perturbations reflective of early mechanisms. We chose to do this to avoid saturating the subtle responses with overwhelmingly high drug concentrations. Our approach is supported by evidence that hepatocytes retained a more differentiated (and thereby responsive) state under the influence of controlled hemodynamics and the continuous perfusion allowed us to overcome dosing limitations associated with static culture.

The primary endpoint of these studies was RNAseq to capture a global snapshot of responses that would help generate hypothesis around mechanisms causing toxicity. Since the volume of data generated is enormous, we approached the analysis in both an unbiased

and biased manner. The strength of an unbiased analysis is that it provides a complete characterization of the transcriptome dynamics in response to drug treatment. It is designed to detect marked and subtle differences in an unbiased manner. This was evidenced by the identification of unfolded protein response and stress response pathways being clearly different between the two drugs by unbiased RSI analysis (Figure 1B). However, our analytics approach also allows us to focus on specific biological pathways that we expect or hypothesize may be modulated following drug treatment. Our analytical toolset also allows us to go beyond looking at individual genes and understand differences in regulation at an upstream level. For instance, though there was a considerable overlap in the regulation of genes coding for drug metabolizing enzymes between the two drugs, biological pathway analysis revealed that Nevirapine up-regulated CAR while Ritonavir up-regulated PXR and suppressed CAR, consistent with previous reports in literature(52-54). In some instances, transcriptomics needs to be supplemented by functional end points as evidenced by our data with Ritonavir. While the drug was seen to up-regulate the expression of various CYPs, including CYP3A4, consistent with PXR up-regulation, we noticed a suppression of CYP3A4 at the activity level, which was confirmed by two different probes (midazolam and testosterone). This interesting observation of both mRNA induction and functional inhibition of CYP3A4 by Ritonavir has been previously described in literature(55). The overall effect will be concentration dependent. However, Ritonavir has a black box warning for drug-drug interactions owing to its propensity to inhibit or induce the CYP enzymes.

4.2. Mechanistic insights: Direct vs. indirect toxicity

DILI can arise from a variety of mechanisms that involve multiple nuclear receptors and their signaling cascades, resulting in a diverse set of phenotypic effects that may trigger compensatory protective responses. When these responses are overwhelmed, cell death could occur via apoptotic or necrotic pathways. Though the effects of the drug can be directly mediated by the drugs or their metabolites on hepatocytes, often there could be indirect immunological mechanisms involving the recruitment of circulating immune cells via antigen presenting mechanisms resultant from adduct formation with reactive intermediates. Our transcriptomic approach allowed us to look at a wide range of cellular effects to dissect and understand direct and indirect mechanisms.

While cholestasis is clinically associated with both Ritonavir and Nevirapine, our findings allowed us to propose different underlying mechanisms predicting intracellular increases of bile acids with either drug. The observed up-regulation of the bile acid synthesis pathway via the classical CYP7A1-mediated pathway and the absence of a compensatory up-regulation of efflux bile acid transporters is suggestive of the cholestatic potential of Nevirapine due to increase in accumulation of intracellular bile acids and salts. Toxic bile acid accumulation can enhance cholestasis and hepatotoxicity, and inhibit metabolizing enzymes like CYPs by their detergent effect(56). However, the reciprocal relationship between bile acid metabolism and nuclear receptors like PXR, CAR, LXR, and FXR is complex (57-60). Compensatory effects on bile acid metabolism can also be regulated by CYPs, e.g. CYP3A4, which can be protective. Vitamin D Receptor (VDR)-mediated up-regulation of intestinal CYP3A4 expression in transgenic mice increases bile acid metabolism, lowering toxic bile acid (like lithocholic acid, LCA) concentrations and reduces

hepatotoxicity(61). In our study, we noted a concomitant upregulation of CYP3A4 by Nevirapine, which could be a protective response. In contrast, in Ritonavir treated conditions, cholestasis may result from the inhibition of various efflux bile acid transporters including basolaterally located SLC51A and canalicular transporter ABCB4 (MDR3) responsible for Progressive Familial Intrahepatic Cholestasis 3. Previous studies by McRae and colleagues support the finding that protease inhibitors including Ritonavir, but not a NNRTI like Nevirapine, inhibit bile acid transport in human and rat hepatocytes(62). Similarly the difference in signatures of endoplasmic reticular stress and unfolded protein response between the two drugs in our study highlights how different underlying cellular response mechanisms could impact toxicity outcomes. Protease inhibitors, including Ritonavir, have been previously shown to activate the unfolded protein response (UPR) in multiple cell types including macrophages, adipocytes and primary hepatocytes(63, 64). We noted a clear activation of the UPR genes by Ritonavir at both doses (Supplementary Figure S1) confirming this effect. However there are no published reports of the impact of Nevirapine, if any, on the unfolded protein response. Our transcriptomic analysis suggests for the first time that it suppresses the unfolded protein response at high doses. These findings could be meaningful in the context of the overall metabolic effects and cellular responses seen with Nevirapine and could have implications for other conditions that could benefit from inhibition of the unfolded protein response, e.g. neurodegenerative diseases. However, this observation would first need to be confirmed in future studies.

In vitro hepatocyte cell cultures are typically challenged in capturing or predicting indirect and idiosyncratic mechanisms of toxicity either due to the lack of extrinsic mechanisms mediated by non-parenchymal cells or by altered baseline inflammatory conditions of the hepatocytes themselves as a culture artifact that may diminish responsiveness to drugs. Uetrecht and colleagues have demonstrated that Nevirapine hypersensitivity is related to the 12-hydroxylation of Nevirapine which can be converted to a reactive quinone methide and bind to hepatic proteins causing toxicity(66). With the expectation that metabolically competent hepatocytes in our system would generate the necessary reactive metabolites and consequent haptens, we looked at the regulation of MHC class I and II by Nevirapine as a possible mechanism of toxicity. The up-regulation of the expression of MHC class II that we noted with Nevirapine has long been known to induce immune-mediated toxicity by recruitment and activation of CD4⁺ helper T cells. However, the simultaneous and marked up-regulation of MHC class I on hepatocytes lends support to the generation and clearance of endogenous (cytosolic) antigens. Thus, we hypothesized the recruitment, activation and expansion of CD8⁺ cytotoxic T cells in the *in vivo* context. In support of this, a recent study by Philips and colleagues in patients also confirmed that though HLA Class I restricted CD8⁺ and Class II restricted CD4⁺ T cells are implicated in the pathogenesis of Nevirapine hypersensitivity, CD8⁺ cells may play a greater role than previously believed(67). Consistent with the MHC class I activation is the observed simultaneous suppression of the upstream inflammatory regulators like NOD-like receptor family CARD domain containing 5 (NLRC5), known to negatively regulate the NF-kappaB and type I interferon signaling pathways(68, 69). We detected the major hydroxy metabolites of Nevirapine including 12-hydroxy Nevirapine in the effluent medium from the system (data not shown, separate manuscript in progress). Our system allowed us to demonstrate a distinct response from

Ritonavir which clearly down-regulated MHC class I indicating that CD8⁺ cells do not contribute to Ritonavir induced liver injury. This confirms previous animal model experiments that have demonstrated Ritonavir blocks antigen presentation to CD8⁺ T-lymphocytes(70). It is reported that down-regulation of MHC class I resulting from ER stress could activate natural killer T (NKT) cells(71). This is also consistent with the ER stress response noted with Ritonavir, suggesting an alternate mechanism of immune-mediated injury. Additionally we also saw a marked up-regulation of MHCII expression that indicates a predominant CD4⁺ T cell role in Ritonavir-mediated injury.

4.3. Importance of physiological milieu on drug effect on metabolism and respiration

Energy substrate source utilization in hepatocytes is highly milieu dependent. Traditional hepatocyte culture systems use maintenance media that are unphysiological in terms of both glucose, which is often at diabetic levels (10-15 mM), as well as insulin levels of around 3-5 μ M, which is almost 20,000 times higher than plasma levels(25, 26). High insulin concentrations are known to repress insulin receptor expression and signaling in hepatocytes cultured *in vitro*(72) making the baseline metabolic state likely not reflective of *in vivo* carbohydrate and lipid metabolism. The differentiated phenotype restored in our system allowed us to culture hepatocytes at physiological levels of glucose and near physiological levels of insulin making it suitable to study the metabolic effects of the drugs.

Nevirapine and Ritonavir revealed clear differences in how they impact lipid and carbohydrate metabolism and ultimately mitochondrial dysfunction. In the Ritonavir-treated cells, pathway analysis predicted up-regulation of the genes responsible for the rate limiting steps in fatty acid biosynthesis and β -oxidation. This is consistent with previous reports in rats(65) and may be related to the UPR activation and inhibition of proteasomal proteolysis described above, which has been previously shown to extend the half-life of SREBP, and increase synthesis of hepatic lipids(73, 74). This was accompanied by activation of complexes I to IV of the electron transport chain with simultaneous decoupling and inhibition of the mitochondrial complex V. This is suggestive of an increased propensity for steatohepatic changes with the drug and correlates with published clinical observations(75). Additionally this can contribute to mitochondrial dysregulation ultimately leading to necrosis and apoptosis of hepatocytes, and is corroborated by clinical data that nucleoside analogs induce mitochondrial dysfunctions(76, 77). Conversely, Nevirapine activates the rate-limiting enzyme of the pentose phosphate pathway suggesting a protective effect against oxidative stress by an increase of reduced glutathione in cells, and supporting the concept of reversion of nucleoside-associated mitochondrial toxicity by Nevirapine seen in the clinic(77). Pathways differentially regulated by Nevirapine relative to Ritonavir, such as fatty acid synthesis and activation of mitochondrial electron chain without simultaneous mitochondrial uncoupling, also suggest a lower propensity for steatosis associated with Nevirapine. This is also consistent with the suppression of the unfolded protein response (UPR), an up-regulation of which has been associated with metabolic effects and fatty liver(46, 65).

Our findings collectively shed light on direct and indirect toxicity mechanisms underlying early cholestatic features and delayed hypersensitivity reported with Nevirapine, and

differential drug responses to Ritonavir that confirm distinct hepatotoxicity outcomes, thereby helping understand the DILI causing potential of these drugs in a clinical context.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

Nevirapine and Ritonavir hepatotoxicity was profiled in an organotypic liver model.

Data predict differences in MHC antigen presentation and downstream T-cell responses.

Intracellular bile acid accumulation and cholestasis mechanisms are different.

Drug metabolizing enzymes are regulated via different receptors (CAR vs. PXR).

Lipid metabolism and respiratory chain effects suggest different steatotic potential.

Figure 1. Global Responses of Differentially Expressed Genes

(A). Volcano plots of differentially expressed genes (DEGs) of the therapeutic doses toxic doses are depicted. The dots represent collections of DEGs. Along the x-axis, genes on the left of the midline of each panel are down-regulated while those on the right are up-regulated. The Y-axis represents the probability of genes being differentially expressed with the dotted red line being the threshold for 50% chance of being a DEG. The intensity of the color of each dot is related to the number of differentially expressed genes. Response Similarity Index (RSI) analysis of Nevirapine and Ritonavir (B). The RSI analysis of the transcriptomic data identifies major pathways that are similarly regulated (in the same direction) by both drugs (purple dots) and those that are differently regulated in opposite directions (green dots).

Figure 2. Nevirapine and Ritonavir regulate key drug metabolizing enzymes

Ingenuity pathway analysis of transcriptomic signatures following 48 hour toxic exposure of Nevirapine (A) predicts activation of CAR/RXR signaling consistent with upregulation of multiple phase I, phase II enzymes and transporters. However 48-hour toxic exposure Ritonavir (B) is associated with metabolic gene activation consistent with PXR-RXR signaling. Assessment of CYP activity (C) indicates modest (< 2-fold) induction of CYP 3A4 activity by Nevirapine using testosterone and midazolam substrates. Ritonavir significantly induces CYP2B6 ($p < 0.01$) while suppressing CYP3A4 activity ($p < 0.01$).

Figure 3. Cholestatic potential of Nevirapine and Ritonavir are mediated by different ways
 Unbiased analysis of transcriptomics reveals that a 48-hour toxic exposure Nevirapine significantly regulates bile acid synthesis (FDR 0.1, $p < 0.05$) resulting in increased expression of several key genes. (A) Ingenuity pathway analysis of predicts an increase of bile acids and bile salts suggestive if intracellular accumulation (Inset). A 48-hour toxic exposure to Ritonavir (B) also regulates bile acid synthesis (FDR 0.1, $p < 0.01$) but downregulates the expression of the same genes with a predicted decrease of bile acids and bile salts. Ritonavir significantly inhibits key efflux transporters like ABCB4 indicating a different mechanism of intracellular bile acid accumulation (Inset).

Figure 4. Distinct Antigen Presentation Patterns induced by Nevirapine and Ritonavir
Ingenuity Pathway analysis following 48 hour exposure to toxic concentration of Nevirapine indicates regulation of the antigen presentation pathway (FDR 0.1, $p=0.01$) predicting increase in the expression of both MHC class I and II (A). In contrast, Ritonavir was predicted to down-regulate expression of MHC class I while upregulating the expression of MHCII (B) suggesting differences in secondary immune cell involvement. An overlay of the Nevirapine data on a functional protein-protein interaction(C) network in the context of the global biological response reveal antigen processing and MHC I antigen presentation as one of the most central and most highly connected communities (C, inset)

Figure 5. Nevirapine and Ritonavir Have Differing Effects on ER Stress and Unfolded Protein Response

Exposure to toxic concentrations of Nevirapine downregulates BIP, CALR, GRP94, HSP70 and SEL1L genes and IPA analysis predicts suppression of the unfolded protein responses and downstream pathways of ATF4 signaling and apoptosis (A). However Ritonavir clearly activates the unfolded protein response along with upregulation of EIF2a, ATF4 and CANX, and IPA analysis predicted activation of ATF4 downstream pathways leading to apoptosis.

Figure 6. Nevirapine and Ritonavir Differentially Regulate Fatty Acid Synthesis and Mitochondrial Dysfunction

Exposure to toxic concentration of Nevirapine for 48 hours is associated with no significant effect on fatty acid synthesis (A) but increases mitochondrial respiratory activity by upregulating complex I through V (Complex V shown in figure B) of the electron transport chain (FDR = 0.1). However Ritonavir upregulates the key initiation steps of fatty acid biosynthesis (D) and stimulates Complex I through IV of mitochondrial electron transport while suppressing ATP synthesis in Complex V (C), indicative of simultaneous mitochondrial uncoupling. Taken together, this may be suggestive of a greater steatotic potential with Ritonavir.

Figure 7. Effect on Inflammatory responses

Based on downregulation of multiple inflammatory genes, IPA predicts inhibition of key upstream regulators of inflammatory responses (A) INF-alpha, (z-score = -3.3, p<0.001) and (B) NFkB (z-score = -2.7, p<0.05) following 48-hour exposure to toxic levels of Nevirapine.