Published in final edited form as:

Toxicol In Vitro. 2017 March ; 39: 93–103. doi:10.1016/j.tiv.2016.11.014.

Pharmacotoxicology of Clinically-Relevant Concentrations of Obeticholic Acid in an Organotypic Human Hepatocyte System

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Abstract

Nonalcoholic steatohepatitis (NASH) is an emerging health crisis with no approved therapies. Obeticholic acid (OCA), a farnesoid X receptor (FXR) agonist, shows promise in NASH trials. However, the precise mechanisms mediating OCA effects and impact on cholesterol metabolism are not fully understood. We explored the pharmaco-toxicological effects of OCA on pathophysiological pathways in hepatocytes using a previously described perfused organotypic liver system that allows culture in near-physiological insulin/glucose milieus, and exhibits drug responses at clinically-relevant concentrations. Primary hepatocytes experienced 48 -hour exposure to OCA at concentrations approximating therapeutic (0.5 μ M) and supratherapeutic (10 μ M) levels. Global transcriptomics by RNAseq was complimented by cellular viability (MTT), CYP activity assays, and secreted FGF19 levels in the media. Dose-dependent, transcriptional effects suggested suppression of bile acid synthesis ($\downarrow$ CYP7A1, $\downarrow$ CYP27A1) and increased bile efflux ($\uparrow$ ABCB4, $\uparrow$ ABCB11, $\uparrow$ OSTA, $\uparrow$ OSTB). Pleiotropic effects included suppression of TGF β and IL-6 signaling pathways, and signatures suggestive of HDL suppression ($\uparrow$ SCARB1, $\downarrow$ ApoAI, $\downarrow$ LCAT) and LDL elevation ($\uparrow$ ApoB, $\downarrow$ CYP7A1). OCA exhibited direct FXR-mediated effects with increased FGF19 secretion. Transcriptomics revealed regulation of metabolic, anti-inflammatory, and anti-fibrotic pathways beneficial in NASH, and predicted cholesterol profiles consistent with clinical findings. Follow-up studies under lipotoxic/inflammatory conditions would corroborate these effects in a disease-relevant environment.

Keywords

Liver; Flow; FXR; NASH; Transcriptomics; Obeticholic

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Introduction

Nonalcoholic steatohepatitis (NASH) is part of the progressive nonalcoholic fatty liver disease (NAFLD) spectrum, which affects a third of the general adult population, 75% of type 2 diabetics and almost 95% of obese individuals (Angulo, 2002). Early steatotic changes in the liver can progress to NASH and eventually lead to cirrhosis or end-stage liver disease. Cirrhosis due to NASH is projected to become the most common cause of liver transplantation within the next decade (Vernon et al. 2011). This is one of the most active areas in drug discovery with over 35 drugs in the preclinical and clinical phase of development, but none to date have advanced beyond Phase III clinical trials or been approved for therapy (Ratziu et al. 2015). A promising new drug for NASH is obeticholic acid (OCA). This semi-synthetic derivative of chenodeoxycholic acid (CDA) is a farnesoid X receptor (FXR) agonist and nearly 100-fold more potent than CDA (Pellicciari et al. 2002). Bile acid-induced activation of FXR in enterocytes induces secretion of fibroblast growth factor 19 (FGF19) leading to feedback inhibition of the bile acid synthesis enzyme CYP7A1 via FGF receptor 4 (FGFR4) and small heterodimer partner (SHP) (Song et al. 2009). The drug has been shown to improve insulin sensitivity and ameliorate the histological findings of NASH in the recently evaluated multicenter, double-blinded, randomized Phase IIb FLINT trial (Neuschwander-Tetri et al. 2015). Intercept Pharmaceuticals has recently initiated the REGENERATE study, a Phase III clinical trial for OCA (NCT02548351, clinicaltrials.gov), which will provide further insight into OCA efficacy. However, there is controversy regarding the role of FXR and its relevance in hepatocytes as a target for NASH (Fang et al. 2015; Tanaka et al. 2015). OCA is also associated with an increase in LDL-cholesterol (LDL-C) and a decrease in HDL-cholesterol (HDL-C) levels in patients with NASH, a population already at higher cardiovascular risk (Mudaliar et al. 2013). However, the molecular basis for these effects is not well established, and mechanistic understanding would aid future efforts for FXR-related therapeutics.. Finally, the potential for off-target toxicity in the liver is not fully known.

A major impediment to understanding NASH and reliably evaluating therapies arises from the lack of adequate human-relevant preclinical models. There are over 40 NASH rodent models described in the literature, and while some of them serve as good surrogate models to induce disease-related changes (Adkins et al. 2013; Kohli et al. 2011), inter-species genetic differences may contribute to gaps in mapping the human response and prevent meaningful translational success of therapeutic targets. Primary human hepatocytes and liver-derived cell lines offer a tool to interrogate human specific responses. However, many of these systems are challenged by rapid dedifferentiation in culture, resulting in a fibroblastic phenotype and responses that do not reflect *in vivo* hepatocyte response (Baker et al. 2001; Boess et al. 2003; Rodriguez-Antona et al. 2002). The meaningfulness of these *in vitro* hepatocyte systems for NAFLD/NASH drug discovery programs is further compromised by their requirement of nonphysiological levels of insulin (as high as 10,000 times the physiological levels) and diabetic levels of glucose to maintain hepatocyte survival in static culture (Nelson et al. 2013). This not only impairs their responsiveness to these factors, but also reflects a milieu that is far from physiological, limiting their utility in drug discovery decision-making for a metabolic disease like NASH. More importantly, drug

concentrations required to elicit pharmacological or toxicological effects in these systems are typically much higher than therapeutic free drug concentrations making *in vivo* correlations challenging. *Ex vivo* approaches such as precision cut liver slices (PCLS) offer the advantage of retaining native liver microarchitecture and heterotypic cellular interactions, and are being used for metabolic clearance, disease, and toxicity applications (Graaf et al. 2007; Olinga et al. 2013; Smith et al. 1985; Westra et al. 2014). However, progressive necrosis of the outer surfaces and rapid loss of differentiated liver function limits their utility for drug exposures beyond the order of hours (Graaf et al. 2007; , Neupert et al. 2003). For instance, a recent study evaluating OCA in a human PCLS system failed to demonstrate regulation of CYP7A1 and CYP8B1, key metabolic genes that should be suppressed by FXR activation, attributing it to the rapid loss of metabolic gene expression over the 24 hour period of the study (Ijssennagger et al. 2016).

We previously described a system that cultures primary hepatocytes in a three-dimensional context with the application of sinusoidal hemodynamic flow, perfusion, and transport (Dash et al. 2013a; Feaver et al. 2016). Hepatocytes retain an *in vivo*-like, stable, mature differentiated phenotype reflected by stable polarized morphology, *in vivo*-like transcriptomic signatures distinct from static cultures, consistently higher liver-specific function such as the production of albumin and urea, as well as drug and xenobiotic metabolizing enzyme activity over 2 weeks (Dash et al. 2013b). More importantly, hepatocytes in this system exhibit induction and toxicity responses to drugs at levels that closely approximate therapeutic concentrations (Terelius et al. 2016), and are cultured in a milieu that is reflective of normal glucose and near physiological insulin levels (Dash et al. 2013c). We recently showed the functional impact of OCA in a lipotoxic system of NASH (Feaver et al. 2016). In this study, we chose to gain a mechanistic understanding of the pharmaco-toxicological effects of OCA in this system at clinically-relevant concentrations and to assess the risk of off-target toxicities in the liver.

Materials and Methods

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 Supplementary Table 1 (Supplementary Material).

Cell Culture and Device Operating Conditions

Primary hepatocytes from 5 individual donors were separately plated in a collagen gel sandwich configuration on the undersurface of the membranes of 75mm polycarbonate transwells (Corning) using previously described protocols (Dash et al. 2013a; Terelius et al. 2016). The transwells were incubated overnight in maintenance medium (MM) that consisted of DMEM/F-12 supplemented with fetal bovine serum (10%), gentamycin (50µg/ml), 0.2% ITS (Cat #MT-25-800CR, Fisher/MediaTech), and dexamethasone (Cat# D4902, Sigma Aldrich, 1µM at plating and 250nM thereafter). On the 2nd day, the transwells were

set up within the previously described devices. 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 of 0.6 dyn/cm² was applied on the top surface ((Dash et al. 2013a; Terelius et al. 2016). The devices were housed in a controlled environment at 37°C with 5% CO₂ mixed with air. 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.

Drug Preparation and Hepatocyte Drug Exposure

Obeticholic acid (OCA; Cat# AG-CR1-3560-M025, Adipogen) was dissolved in tissue culture grade dimethyl sulfoxide (DMSO, Cat# D2650, Sigma Aldrich), 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 control. The therapeutic concentration of OCA used was 0.5μM, and was estimated from blood levels published in previous human studies (Hirschfield et al. 2015; Sanyal et al. 2009). We also assessed a supratherapeutic concentration that was 20 fold higher (10μM). Cells were exposed to drugs for a period of 48 hours following physiologic adaptation in the system as described above in order to measure a toxicological response.

Functional Assay of Phase I/Phase II Enzyme Activity

The potential of OCA to induce or inhibit key phase I enzyme activities was assessed using specific cytochrome P450 (CYP) enzyme-selective probe substrates for CYP1A2 and CYP3A4 activity (phenacetin and testosterone, respectively) and quantifying the expected metabolites (acetaminophen and 6β-hydroxytestosterone respectively). At the conclusion of each experiment, 2cm² 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 (0.5mL) containing the phase I CYP enzyme substrates was added and the samples incubated for 60 minutes 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 minutes and stored at -80°C. The samples were sent to QPS, LLC (Newark, DE) for the quantitation of substrate metabolites by LC-MS.

FGF19 Assay

Media effluent was collected at the end of the experiment and assessed by a colorimetric ELISA for secreted protein levels of FGF19 (Abcam, Cat #ab193703) using the manufacturer's protocol.

Immunostaining

At the end of the experiment, segments from the device membranes (~2cm²) containing cells (N=3/device) were excised and washed gently with PBS, followed by fixation with 4% paraformaldehyde for 30 minutes. The samples were first permeabilized with 0.1% Triton-X for 20 minutes, washed with PBS and blocked with 5% goat serum. The incubation with primary antibodies (HNF-4α Cat # sc-8987, Santa Cruz, E-cadherin: Cat # sc-71009, Santa

Cruz, and MRP2: Cat # ab3373, Abcam) was at a dilution of 1:100 for 1 hour. After 3 washes with PBS with 1% BSA, the secondary antibody was added at a dilution of 1:500 for 1 hour. The samples were then washed with PBS with 1% BSA and then mounted for imaging on a Nikon C1+ Confocal System microscope.

MTT Assay

At the conclusion of each experiment, segments from the device membranes (~2cm²) containing cells (N=3/device) were immediately excised and transferred to standard 24 well plates. The following MTT assay was immediately performed *ex situ*, in the absence of perfusion and hemodynamics. The cells were incubated with 500µl of Thiazolyl Blue Tetrazolium Bromide (MTT) solution in culture medium at a final concentration of 1mg/ml. After 1 hour, the MTT containing medium was replaced by 500µl of DMSO and the plates were agitated on a shaker for 10 minutes. The purple supernatants were then transferred to 96-well plates and assayed for absorbance at 595nm using a spectrophotometer.

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

Hepatocyte cell pellets were collected and RNA isolated using the Purelink RNA Mini kit (Cat# 12183018A, Invitrogen) 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 (Cat # 4368814, Applied Biosystems). Expression of our internal QC genes CYP1A1, CYP2C9, CYP3A4, GSTP1, UGT1A1, GADD45A, GSR, CXCL2, HMGB1 and FGF21 was determined by RT-PCR using iQ SYBR Green Supermix (Cat # 170-8885, 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.

RNA Deep Sequencing

Samples with a minimum RNA Integrity Number >7.0 were used for further transcriptomics processing. 1µg 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.

Data Analysis and Statistics

Our data analysis algorithms have been previously published (Cole et al. 2015; Feaver et al. 2016). Briefly, following RNA deep sequencing, the reads were aligned to a standard hg19 human transcriptome using Bowtie 0.12.9 (Langmead and Salzberg, 2012) and the final gene counts calculated using eXpress v1.5.1 (Roberts et al. 2013). 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 (Robinson et al. 2010). 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 = down-regulation, red = up-regulation, white = no change; the intensity of the color reflects the magnitude of change; a dot indicates a statistically significant change in the expression of the corresponding gene (FDR<10%). To compare the effects of two different concentrations of OCA, 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 determined the degree to which the two conditions altered gene expression in the same (RSI>0.2, purple dots) or opposite (RSI<-0.2, green dots) manner (Supplementary Figure S1) (Cole et al. 2015; Feaver et al. 2016). Summary radar plots were plotted to quantitatively depict the average FDR-corrected gene expression fold-change for the indicated pathways relative to the vehicle control. Each gene fold-change was multiplied by (1-FDR) in order to down-weight less significant genes. These FDR-corrected gene expression fold-changes were then averaged over a panel of genes that represent a specific biological process or pathway (i.e. bile acid synthesis). ROAST, a gene set test tool (Wu et al. 2010), was used to evaluate whether the pathway was significantly differentially expressed in a particular direction when comparing OCA treatment to the vehicle.

For the functional data (FGF19 and CYP activity assays), we used the 'lme4' package for building mixed effects models with device as a random effect. We used the 'multcomp' package for performing all pairwise comparisons for treatment effects, with p-values adjusted for multiple comparisons (Hothorn et al. 2008). Donor 4 was excluded from the functional endpoints as data values were outside the limit of detection of the assays used.

For assessing FXR DNA binding sites, FXR ChIP-seq reads were downloaded from the NCBI GEO database, accession number GSE57312 (Zhan et al. 2014). Reads were aligned to the human genome, build GRCh37/hg19, using bowtie2 version 2.2.9 (Langmead and Salzberg, 2012). Peaks were called using the MACS tool for both the stimulated and unstimulated conditions, which were merged into a single set of binding sites (Zhang et al. 2008). The coordinates of these sites were intersected with those of protein coding genes plus a 1kb region upstream of each transcription start site using the bedtools software package (Quinlan and Hall, 2010). The significance of association between the genes that intersected FXR binding sites and the set of differentially expressed genes was calculated using Fisher's exact test.

Results

The pharmacotoxicology of a 48-hour exposure of obeticholic acid (OCA) on primary hepatocytes was evaluated in the *in vitro* liver system that maintained cells under controlled

hemodynamics and transport conditions. OCA was evaluated using 5 human donors and at two concentrations—a lower therapeutic concentration of 0.5 μ M and a higher supratherapeutic concentration of 10 μ M (Figure 1). Following drug exposure, the cultures were imaged for morphology and cell number and MTT assays were performed to ensure viability comparable to controls. RNA was isolated for transcriptomic and pathway analysis. Live cell assays were performed to assess the drug metabolizing enzymes, CYP1A2 and CYP3A4.

OCA Did Not Induce Significant Toxicity and Exhibited Global Transcriptomic Signatures Reflecting On-Target Drug Effects at Both Doses

The average MTT activity measured from the hepatocytes following 48 hours of OCA exposure was lower than vehicle controls at both doses (Supplementary Figure S2); however, these values were not statistically significant ($p=0.24$ for 0.5 μ M, $p=0.71$ for 10 μ M) indicating that no severe cytotoxicity was noted. The yield and integrity of RNA from the drug-treated conditions also passed our quality control (data not shown) confirming that the samples were suitable for transcriptomic profiling. Global transcriptomic changes in gene expression induced by OCA at both doses are depicted in the volcano plots (Figure 2). The distribution and spread of data points reflects a greater number of genes that were differentially expressed at the supratherapeutic concentration (Total: 4954, Up-regulated: 2539, Down-regulated: 2415; $p<0.01$, FDR<10%) than the therapeutic concentration (Total: 2373 Up-regulated: 1110, Down-regulated: 1263; $p<0.01$). Analysis of the transcriptomic responses at both doses using the relative similarity index (RSI) methods described previously (Cole et al. 2015; Feaver etl. 2016), revealed that a majority of the differentially expressed genes (DEGs) were regulated in the same direction (2238 genes; $RSI>0.74$) and only a small number (3 genes; $RSI<-0.74$) were regulated in opposite directions (Supplementary Figure S1). Analysis of the top 25 up-regulated and down-regulated genes following 48-hour exposure to the therapeutic dose (0.5 μ M) of OCA reveals on-target drug effects with FGF19 being the most up-regulated gene and CYP7A1 being the most down-regulated gene (Table 1).

Drug Metabolizing Enzymes are Regulated by OCA at the Gene and Activity Level

Analysis of the transcriptomic data revealed that OCA regulated the expression of the CYP enzyme genes (Figure 3A) at both doses. Of particular interest was the suppression of the enzymes CYP1A2 and CYP3A4, two key phase I enzymes comprising almost half of the hepatic CYP pool and metabolizing almost 50% of all drugs (Guengerich et al. 2003). The gene expression results were confirmed by the measurement of the enzyme activities. This was reflected by the suppression of acetaminophen formation (primary metabolite of the probe substrate phenacetin for CYP1A2—Figure 3B) which attained significance at the higher dose relative to DMSO controls (0.79 vs. 2.89nM/ μ g protein, $p<0.01$). The lower dose showed a trend of suppression of CYP1A2 activity as well (0.79 vs. 1.79nM/ μ g protein, $p=0.077$). We also noted a marked suppression of formation of 6 β -hydroxytestosterone (primary metabolite of the probe substrate testosterone for CYP3A4—Figure 3C) that was significantly lower at both doses than the DMSO control (1.45 vs. 6.01nM/ μ g protein, $p<0.001$ and 1.82 vs. 6.01nM/ μ g protein, $p<0.01$). The genes for various phase II enzymes were also regulated by the drug at both doses (Supplementary Figure S3).

OCA Exerts On-Target FXR-Mediated Effects

OCA exposure resulted in a gene expression pattern in hepatocytes consistent with its expected pharmacology as an FXR agonist (Ding et al. 2015), at both the therapeutic and supratherapeutic concentrations in a dose-dependent manner. The up-regulated genes (red) included FGF19 and NR0B2 (SHP), as well as bile acid efflux transporters such as ABCB4 (MDR3), ABCB11 (BSEP) and SLC51A/B (OSTA and OSTB) (Figure 4A). Down-regulated (blue) genes included FGFR4 receptor as well as bile synthesis genes such as CYP7A1 and CYP27A1 (Figure 4A). Furthermore, we identified DNA binding sites for FXR in hepatocytes from ChIP-seq data generated by Zhan et al. (2014). These binding sites were associated with 1200 of the 12532 protein coding genes included in our differential expression analysis. Genes differentially expressed at 1% FDR in response to both the low and high dose of obeticholic acid were highly enriched for FXR binding sites (p-values = $1e-20$ and $2e-45$, respectively), strongly suggesting that a significant portion of the differential transcriptomic response is indeed a direct result of FXR regulation. Finally, as with FGF19 gene expression, dose-related increases of secreted FGF19 protein levels were measured in the media effluent (97.97pg/ml at 0.5 μ M and 1029.62pg/ml at 10 μ M) that were significantly higher than the DMSO control (24.37pg/ml, $p < 0.001$) (Figure 4B). The FGF19 levels were also significantly different between the two doses ($p < 0.001$). These changes are depicted in the schematic (Figure 4C).

The Transcriptomic Signature of OCA on Lipid and Cholesterol Metabolism Support its Efficacy in Reducing Steatosis in NASH as well as the Altered Cholesterol Profile Observed Clinically

The lipid lowering effect of OCA is supported by the down-regulation (blue) of various genes involved in fatty acid and triglyceride synthesis at both the therapeutic and supratherapeutic concentrations (Figure 5A–upper panel). These genes that are down-regulated, such as ACACA and FASN, are important in mediating *de novo* lipogenesis. Interestingly, there are some genes that are up-regulated, such as some fatty acid elongases (ELOVL1,2,7) and acyl-CoA synthetases (ACSL4,5,6) which are further downstream of the fatty acid synthesis pathway and may represent a compensatory response. However, DGAT2 represents a gene in the final step of lipogenesis and is down-regulated by OCA. A simultaneous up-regulation (red) in fatty acid beta-oxidation genes is also noted, particularly at the higher concentration (Figure 5A–lower panel). These changes are depicted in the schematic (Figure 5C). OCA down-regulates (blue) genes involved in cholesterol synthesis (HMGCS1, HMGCR) along with transcription factors SREBF1 and 2, particularly at the higher concentration (Figure 5B–upper panel). The effect on lipoprotein metabolism genes is more complex with up-regulation of ApoA2, ApoB, ApoC2 and SCARB1 and down-regulation of LDLR, LIPC, ApoA1, ApoC3 and ApoE. (Figure 5B–lower panel). These changes would be predictive of an overall decrease in HDL and increase in LDL and VLDL and are depicted in the schematic (Figure 5C).

OCA Suppresses Gluconeogenesis Pathway Genes While Up-regulating Glycogen Synthesis Genes Predictive of a Beneficial Effect on Glucose Sensitivity

OCA was noted to transcriptomically regulate carbohydrate metabolism in the system. We observed an up-regulation (red) of key glycogen synthesis-related genes such as UGP2 and GYS2 (Supplementary Figure S4A) along with simultaneous down-regulation (blue) of important pathway genes associated with gluconeogenesis such as FBP1, PCK1 and PC (Supplementary Figure S4B). These changes could collectively predict a decrease in hepatic glucose output and are depicted in the schematic (Supplementary Figure S4C).

Impact of OCA on TGF β Signaling, Inflammation, Oxidative Stress and Apoptosis Pathway Genes Suggest Benefits Beyond Lipid and Carbohydrate Metabolism Effects

The expression of various genes associated with a Nrf2 anti-oxidant response (TXN, PRDX, GPX2, SOD2 and GSTP1) were significantly down-regulated (blue) by OCA relative to the vehicle controls, reflective of a lower level of oxidative stress (Figure 6A–upper panel). The supratherapeutic concentration of OCA significantly down-regulated (blue) the expression of TGF β signaling genes, including but not limited to, TGF β 1 and TGF β R1 genes as well as COL1A1 suggestive of a potential effect on TGF β signaling and extracellular matrix remodeling (Figure 6A–lower panel). This could be related to up-regulation of PPAR- γ or the suppression of Nrf2 response described earlier and potentially reflects a lower level of fibrotic pathway activation. OCA also exhibited anti-inflammatory effects, as evidenced by the down-regulation (blue) of IL-6 signaling pathway including IL6R as well as downstream genes like A2M, SERPINA1, MYC and CRP (Figure 6B–top panel). Additionally, other inflammatory and stress-related pathways such as TNF, Myd88 and Nrf2 signaling were also modestly down-regulated (Supplementary Figure S5). At the higher dose, OCA was associated with down-regulation (blue) of the pro-apoptotic gene BID and up-regulation (red) of the anti-apoptotic gene BCL2, which may be indicative of a protective effect (Figure 6B–bottom panel). These changes are depicted in the schematic (Figure 6C).

Discussion

The farnesoid X receptor (FXR) is involved in a gut-liver axis feedback that regulates lipid and glucose homeostasis and thus is particularly important in NASH. In the two decades since its discovery, the development of OCA, a ‘first-in-class’ selective FXR agonist, has gone from the laboratory to clinical studies with extensive pre-clinical and clinical characterization of its on-target effects (Hirschfield et al. 2015; Ijssennagger et al. 2016; Maneschi et al. 2013; Mudaliar et al. 2013; Neuschwander-Tetri et al. 2015; Sanyal et al. 2009; Verbeke et al. 2014). In this study, we separate the effects of OCA mediated by its intestinal exposure to those that are directly mediated on hepatocytes by studying physiologically-relevant doses in an *in vitro* system at near physiological conditions. The study confirmed the on-target pharmacology of OCA associated with FXR activation both at the gene and secreted protein level. Transcriptomics revealed downstream effects on multiple other cellular pathways supporting observed metabolic clinical effects in addition to decreased bile synthesis (Mudaliar et al. 2013; Neuschwander-Tetri et al. 2015). The down-regulation of fatty acid and triglyceride synthesis pathway with simultaneous up-regulation of fatty acid beta-oxidation genes predicts an overall decrease in intracellular lipids and

supports the resolution of the steatotic pathology seen clinically in NASH. Up-regulation of glycogen synthesis-related genes with simultaneous down-regulation of key gluconeogenesis genes could help explain its benefit in improving glucose sensitivity in diabetic patients of NASH. We also noted a suppression of stress, inflammatory and pro-apoptotic signaling, suggestive of additional beneficial effects of the drug. These effects have been summarized in the radar plot in Figure 7, which is a quantitative representation of the transcriptomic effects on the above-mentioned key pathways (FDR<0.01, ROAST). Importantly, we also showed for the first time at clinically-relevant concentrations that the FXR-mediated effects of OCA on FGF19 may operate at an autocrine level within hepatocytes in addition to the more widely appreciated paracrine enterocyte-hepatocyte mechanism described in the literature.

An important aspect of OCA that could impact its eventual success as a therapy for NASH, is its effect on the lipoprotein profile. Results from the FLINT study, examining OCA in non-cirrhotic, NASH patients, have led to concern over abnormalities of increased total cholesterol and LDL and decreased HDL seen in OCA-treated patients compared to placebo (Mudaliar et al. 2013; Neuschwander-Tetri et al. 2015). In our study, we found that OCA inhibited cholesterol synthesis pathways in a manner that was dose-dependent, an effect possibly mediated by a suppression of HMGCR that was observed at a gene level. An increase in scavenger receptor class B type I (SCARB1) expression could suggest an up-regulation of reverse cholesterol transport explaining the decrease in HDL. This hypothesis is also supported by studies on FXR knockout mice that exhibit down-regulation of SCARB1, a key receptor for hepatic clearance of cholesterol from HDL, leading to a marked increase in HDL (Sinal et al. 2000). Similarly, SCARB1 knockout mice show elevated HDL levels (Rigotti et al. 1997). Additional mechanisms that mediate the HDL-lowering effect could be the observed decreases in HDL-cholesterol esterifying enzyme, lecithin-cholesterol acyl transferase (LCAT), and apolipoprotein A-I (ApoA1) gene expression in our study, both of which have been previously demonstrated to be regulated by FXR in animal studies (Claudel et al. 2002). Hypercholesteremia and increased plasma LDL have been tied to a deficiency of cholesterol 7 α -hydroxylase (CYP7A1) in patients having a polymorphism in the CYP7A1 gene (Pullinger et al. 2002). Conversely, overexpression of CYP7A1 in animal models is associated with a large decrease in the level of LDL and protection from diet-induced atherosclerosis (Li et al. 2011). It is unclear whether this is related to the role of CYP7A1 in the conversion of cholesterol to bile acids. In our study the suppression of both CYP7A1 and CYP27A1 genes and the overall bile acid synthesis pathway is reflective of the reduced cholesterol conversion. Additionally, we note an up-regulation in ApoB, the gene that encodes Apolipoprotein B, suggesting increased levels of the primary apolipoprotein of LDL cholesterol. We have recently described a similar finding when we examined the functional impact of OCA in a lipotoxic system of NASH (Feaver et al. 2016).

Current understanding suggests that the effects of FXR activation are mediated via the secretion of FGF19 from the ileum, which is stimulated by post prandial bile secretion, thereby setting up a paracrine gut-liver axis to regulate bile acid homeostasis (Song et al. 2009). FGF19 interacts with FGFR4 in human hepatocytes and is linked to not just the suppression of bile acid synthesis, but also to complex effects on carbohydrate and lipid metabolism. An underappreciated aspect is the direct induction of FGF19 in hepatocytes that

could be as important as the paracrine effects. We demonstrate *in vitro* for the first time, the ability of physiologically-relevant concentrations of OCA to induce FGF19 in human hepatocytes at a transcriptional level and at the level of secreted protein. This suggests an additional autocrine role of FGF19 via FXR-mediated signaling within the hepatocytes. Our results are consistent with findings from a recently published study using human precision cut liver slices (Ijssennagger et al. 2016). In that study however, though FGF19 was the most highly up-regulated gene by OCA, Ijssennagger et al. (2016) noted the lack of on-target FXR response on key metabolic enzymes such as CYP7A1 and CYP8B1, and attributed it to the rapid loss of expression of these genes in the model. In our system, we noted a clear regulation of these genes by the drug over 48 hours with FGF19 being the most up-regulated gene and CYP7A1 being the most down-regulated gene (Table 1). This confirms and highlights the retention of metabolic phenotype in our system. Additionally, we detected a far greater number of genes from pathways implicated in NASH pathogenesis that were differentially regulated by OCA at both concentrations, suggesting that sensitivity to the global drug response in the system is preserved.

A crucial clinical endpoint of NASH is the development of extracellular matrix and fibrotic changes that eventually lead to cirrhosis when untreated. In clinical studies, the effect of OCA on fibrosis reduction was modest though significant (35% OCA vs. 19% placebo) (Neuschwander-Tetri et al. 2015). We demonstrated in our study that although OCA has a suppressive effect on TGF β genes and extracellular matrix reorganization, the overall effect on TGF β signaling and stellate cell activation pathway genes is likely minimal and less pronounced than its metabolic effects. This highlights an opportunity for considering the combination of a more direct anti-fibrotic therapy along with OCA to simultaneously impact the metabolic, inflammatory and fibrotic processes and achieve a synergistic effect on disease resolution.

The transcriptomic approach employed in this study helps us gain insights into underlying pathway perturbations that contribute to the pharmaco-toxicological effects seen with the drug, and help explain mechanisms driving efficacy and toxicity. However gene effects do not always translate into function as they may reflect compensatory responses, or activity may be dependent upon downstream post-translational protein modifications. Confirmation of the predicted pathway effects by the measurement of downstream functional endpoints would further enhance our confidence in the findings. We did this in the case of FGF19, which was measured as a secreted protein in the media effluent. Another important activity confirming our transcriptomic data was the demonstration of significant inhibition of CYP1A2 and CYP3A4 enzyme activities, as evidenced by the suppression of the formation of metabolites from their respective substrates (Figure 3). Since these phase I enzymes comprise almost half of the hepatic CYP pool and metabolize around 50% of all drugs (Guengerich et al. 2003), this could have potential implications for resulting drug-drug interactions, and requires further evaluation.

Clinical trial results with OCA, though promising, also suggest that the effects are not consistent across the overall population. For example, although twice as many OCA-treated patients met the primary treatment outcome of improvement in the histological score of biopsies as compared to the placebo group (43% in OCA vs. 21% in placebo), the overall

percentages seem to suggest a high number of non-responders (Mudaliar et al. 2013; Neuschwander-Tetri et al. 2015). An interesting observation of a difference in histological response between OCA-treated patients with or without diabetes relative to placebo groups (odds ratio of 4.5 in diabetics and 2.0 in non-diabetics) suggests that insulin responsiveness could be a factor that influences the outcome of OCA treatment (Musso et al. 2016). This difference in response would need to be confirmed in wider studies that are stratified to include larger non-diabetic NASH patient populations. Additionally, evaluating OCA in responsive human *in vitro* systems that reproduce disease-like altered insulin sensitivity under lipotoxic milieus may provide insights into mechanisms that drive these differences in outcomes. To this end, we have generated an *in vitro* lipotoxic model that reflects the NASH phenotype and will be useful in interrogating novel therapeutic approaches to treat NASH and similar diseases (Feaver et al. 2016).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to express thanks to Michael Simmers, Andrew Pryor, Nathan Day, Joshua Thomas, Chelsi Snow, Christin Hamilton, Diana Berry, Sandi Walton, and Crystal Passmore for technical assistance. This study was funded by a NIH NIDDK funded SBIR grant (R44 DK091104) to HemoShear Therapeutics, Charlottesville, VA.

List of Abbreviations

NASH	nonalcoholic steatohepatitis
NAFLD	nonalcoholic fatty liver disease
FXR	farnesoid X receptor
FGF19	fibroblast growth factor
DMSO	dimethyl sulfoxide
CYP	cytochrome P450

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Highlights

- Clinical concentrations of obeticholic acid were tested in an organotypic liver model.
- Transcriptomics reveal metabolic pathway effects beneficial in fatty liver disease.
- Changes predictive of lipoprotein abnormalities were also noted.
- Cytochrome p450 effects raise concern for potential drug-drug interactions.

Figure 1. Device configuration and experimental paradigm

Based on the microarchitecture of the hepatic sinusoid (A), hepatocytes are configured in a collagen gel sandwich on the underside of a porous polycarbonate membrane (B) while controlling media flow and solute transport via inflow/outflow tubes and rotation of the cone (red). Stable, polarized and differentiated morphology of day 10 hepatocytes is reflected by peripheral green E-cadherin staining and red nuclear HNF-4 α staining (C) along with simultaneous canalicular localization of efflux transporters such as MRP-2 (D). The experimental design (E) involves restoration of biology under flow and transport in the system over 7 days, followed by a 48-hour exposure to obeticholic acid at a therapeutic (0.5 μ M) or supratherapeutic (10 μ M) concentration, prior to assessment of cellular and media endpoints.

Figure 2. Global transcriptomic responses

Volcano plots of differentially expressed genes (DEGs) of the therapeutic and supratherapeutic doses are depicted. The colored dots represent collections of DEGs. The x-axis represents log₂ fold gene expression, where 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 colored dotted lines being the threshold for the indicated % chance of being a DEG. The intensity of each dot is related to the number of differentially expressed genes. N=5 donors.

Figure 3. Drug metabolizing enzymes are regulated by obeticholic acid (OCA) at the gene and activity level

Various cytochrome P450 genes (including CYP1A2 and CYP3A4) involved in the metabolism of drugs and xenobiotics, are regulated by OCA (A). The inhibition of activity of key CYP enzymes was also reflected by suppression of metabolite formation following incubation with the substrates (B) for CYP1A2 (substrate Phenacetin, metabolite Acetaminophen) at the higher dose ($p < 0.01$ for 10 μ M). (C) CYP3A4 activity (substrate Testosterone, metabolite Hydroxy-testosterone) was significantly suppressed at both doses ($p < 0.001$ for 0.5 μ M, $p < 0.01$ for 10 μ M). N=5 donors for transcriptomic; N=4 donors for CYP assays (each donor datapoint represented by a different shape).

Figure 4. On-target FXR-mediated effects of obeticholic acid (OCA)

(A) OCA effects a gene expression pattern in hepatocytes consistent with FXR agonism at therapeutic and supratherapeutic concentrations and is predictive of suppressed synthesis and increased metabolism and export of bile acids. (B) Dose-related increases in levels of secreted FGF19 protein are measured in the media effluent (97.97pg/ml at 0.5 μ M and 1029.62pg/ml at 10 μ M) that were significantly higher than the DMSO control (24.37pg/ml, p<0.001). (C) The schematic reflects the hypothesized effects where OCA binding to FXR leads to increased FGF19 expression as well as increased BSEP and MDR3 (ABCB4). FGF19 inhibits CYP7A1 expression and thus bile acid synthesis, while BSEP and MDR3 are bile acid efflux transporters that will promote bile acid secretion. N=5 donors for transcriptomics; N=4 donors for FGF19 assay (each donor datapoint represented by a different shape).

Figure 5. Effects of obeticholic acid (OCA) on lipid and cholesterol metabolism support its therapeutic effect

The transcriptomic signature of OCA reveals suppression of fatty acid and triglyceride synthesis with simultaneous increase in fatty acid metabolism supporting its observed anti-steatotic clinical effect (A). Cholesterol synthesis is suppressed along with a lipoprotein metabolism signature that supports a profile associated with a reduction of HDL and increase of LDL/VLDL. (B) These effects are depicted in the schematic (C). N=5 donors.

Figure 6. Effect of obeticholic acid (OCA) on inflammation, oxidative stress, TGF β signaling and apoptosis

Transcriptomic analysis of hepatocytes exposed to reveals down-regulation of genes involved with Nrf2-mediated anti-oxidant response as well as TGF β signaling and extracellular matrix genes (A). Simultaneously, multiple genes of the IL-6 signaling pathway are down-regulated, reflective of an anti-inflammatory effect (B). The net effect on apoptosis is suppression due to down-regulation of pro-apoptotic genes and up-regulation of the anti-apoptotic gene BCL2 (B). These effects are depicted in the schematic (C). N=5 donors.

Figure 7. Summary of obeticholic acid (OCA) effects on pathways relevant to NASH

The radar plot summarizes the main effects of therapeutic (dashed black line) and supratherapeutic (bold black line) concentrations of OCA on various cellular processes depicted along each axis (see Materials and Methods for further details). The values on the axes are the FDR-adjusted average gene expression fold-change for each pathway (based on publicly available KEGG and reactome databases). The grey area represents the vehicle effects at 1.0 and the drug effects are shown relative to the vehicle. Pathway enrichment statistics: *, FDR<10% for #0.5uM OCA and *10uM OCA. N=5 donors.

Top 25 Up-regulated and down-regulated genes following 48-hour exposure to obeticholic acid (0.5µM) (N=5 donors).

Table 1

Top Up-regulated Genes					Top Down-regulated Genes				
Rank	Gene	Description	Fold Change	Gene	Description	Fold Change	Gene	Description	Fold Change
1	FGF19	Fibroblast Growth Factor 19	5.86	CYP7A1	Cytochrome P450, Family 7, Subfamily A, Polypeptide 1	-6.94			
2	FOXP3	Forkhead Box P3	5.81	BC020644	Homo sapiens cDNA clone IMAGE:4809499.	-6.29			
3	Z24749	H.sapiens polyA site DNA sequence	5.33	LIME1	Lck Interacting Transmembrane Adaptor 1	-4.44			
4	SLC51B	Solute Carrier Family 51, Beta Subunit	4.47	NGS-17	TAP Binding Protein (Tapasin)	-4.34			
5	UBXN10	UBX Domain Protein 10	4.22	PTBLP	Polypyrimidine Tract Binding Protein 2	-3.56			
6	LINC00870	Long Intergenic Non-Protein Coding RNA 870	4.18	HIST1H2BJ	Histone Cluster 1, H2bj	-3.54			
7	INHBB	Inhibin, Beta B	3.65	NPW	Neuropeptide W	-2.68			
8	LGI4	Leucine-Rich Repeat LGI Family, Member 4	3.4	S100A3	S100 Calcium Binding Protein A3	-2.34			
9	FETUB	Fetuin B	3.3	IGFALS	Insulin-Like Growth Factor Binding Protein, Acid Labile Subunit	-2.32			
10	MFSD2A	Major Facilitator Superfamily Domain Containing 2A	3.3	FLJ00322	Polycystic Kidney Disease 1 (Autosomal Dominant) Pseudogene 1	-2.31			
11	SLC51A	Solute Carrier Family 51, Alpha Subunit	3.15	CRC11	Cysteine-Rich C- Terminal 1	-2.25			
12	UROCI	Urocanate Hydratase 1	3.08	METRNL	Meteorin, Glial Cell Differentiation Regulator-Like	-2.22			
13	RTP3	Receptor (Chemotaxis) Transporter Protein 3UDP	2.96	C5AR2	Complement Component 5a Receptor 2	-2.21			
14	UGT2B10	Glucuronosyltransferase 2 Family, Polypeptide B10	2.93	ADAMTSL2	ADAMTS-Like 2	-2.18			
15	CA14	Carbonic Anhydrase XIV	2.92	UGT1A7	UDP Glucuronosyltransferase 1 Family, Polypeptide A7	-2.17			
16	AK094117	Homo sapiens cDNA FLJ36798 fis, clone ADRGL2007283	2.84	CYP2E1	Cytochrome P450, Family 2, Subfamily E, Polypeptide 1	-2.08			
17	ROS1	ROS Proto-Oncogene 1, Receptor Tyrosine Kinase	2.74	CYP4F22	Cytochrome P450, Family 4, Subfamily F, Polypeptide 22 Solute Carrier Family 13	-2.06			
18	KIAA1932	Leukocyte Receptor Cluster (LRC) Member 8	2.71	SLC13A5	(Sodium-Dependent Citrate Transporter), Member 5	-2.02			
19	MLLT11	Myeloid/Lymphoid Or Mixed-Lineage Leukemia; Translocated To, 11	2.62	KRT16P2	Keratin 16 Pseudogene 2	-1.96			
20	AF047486	Amine Oxidase, Copper Containing 4, Pseudogene	2.61	ADH1C	Alcohol Dehydrogenase 1C (Class I), Gamma Polypeptide	-1.95			
21	CTAGE15	CTAGE Family, Member 15	2.54	LOC100507537	uncharacterized LOC100507537	-1.94			
22	AL832937	SUZ RNA binding domain containing 1 (SZRD1), transcript variant 1, mRNA.	2.5	C14orf80	Chromosome 14 Open Reading Frame 80	-1.89			
23	GSTT1	Glutathione S-Transferase Theta 1 Transcription	2.47	ASPG	Asparaginase	-1.88			
24	TCEA2	Elongation Factor A (SII), 2	2.44	KRT16P3	Keratin 16 Pseudogene 3	-1.87			

Top Up-regulated Genes					Top Down-regulated Genes				
Rank	Gene	Description	Fold Change	Gene	Description	Fold Change	Gene	Description	Fold Change
25	AK124179	cDNA FLJ42185 fis,clone THYMU2032080		MT1H	Metallothionein 1H	2.42			-1.84