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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 patho-physiological 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 μM) and supratherapeutic (10 μM) 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 (↓CYP7A1, ↓CYP27A1) and increased bile efflux (↑ABCB4, ↑ABCB11, ↑OSTA, ↑OSTB). Pleiotropic effects included suppression of TGFβ and IL-6 signaling pathways, and signatures suggestive of HDL suppression (↑SCARB1, ↓ApoAI, ↓LCAT) and LDL elevation (↑ApoB, ↓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.

1. Introduction

Nonalcoholic steatohepatitis (NASH) is part of the progressive non-alcoholic 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, clinicialtrials.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,

Abbreviations: NASH, nonalcoholic steatohepatitis; NAFD, nonalcoholic fatty liver disease; FXR, farnesoid X receptor; FGF19, fibroblast growth factor; DMSO, dimethyl sulfoxide; CYP, cytochrome P450.

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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 and Feldstein, 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 and Schuppan, 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 h 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.

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 >10 μg/million cells/day). The sourcing details and demographics of these donors are included in Supplementary Table 1 (Supplementary material).

2.2. 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 75 mm 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 (50 μg/ml), 0.2% ITS (Cat #MT-25–800CR, Fisher/MediaTech), and dexamethasone (Cat #D4902, Sigma Aldrich, 1 μM at plating and 250 nM 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/cm^2 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% CO_2 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.

2.3. 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 0.5 μM, 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 (10 μM). Cells were exposed to drugs for a period of 48 h following physiologic adaptation in the system as described above in order to measure a toxicological response.

2.4. 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, 2cm^2 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 CYP 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. 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.