

# Characterization of EDP-305, a Highly Potent and Selective Farnesoid X Receptor Agonist, for the Treatment of Non-alcoholic Steatohepatitis

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**Abstract:** Non-alcoholic steatohepatitis (NASH), characterized by hepatocyte injury, inflammation, and fibrosis, is the main cause of chronic liver disease in the Western world. There are currently no approved pharmacological therapies for NASH, underscoring the urgent need for effective treatments. The farnesoid X receptor (FXR) has emerged as an attractive target for the treatment of metabolic and chronic liver diseases. EDP-305 is an FXR agonist currently in phase 2 clinical trials for Primary Biliary Cholangitis (PBC) and NASH. Here, we demonstrate that EDP-305 is a selective and potent FXR agonist that regulates multiple pathways relevant to NASH progression. EDP-305 exhibits anti-fibrotic and anti-inflammatory gene signatures in human macrophage and stellate cell lines, as well as favorable effects on lipid metabolism in hepatocytes, including enhanced low density lipoprotein (LDL)-cholesterol uptake and decreased triglyceride accumulation. The therapeutic potential of EDP-305 was further evaluated in two murine models of NASH: a streptozotocin-high fat diet STAM<sup>TM</sup> model and a dietary induced NASH (DIN) model driven by high fat, cholesterol, and fructose feeding. In both NASH models, EDP-305 significantly decreased hepatocyte ballooning and lowered the non-alcoholic fatty liver disease (NAFLD) activity score. EDP-305 also significantly attenuated hepatic steatosis and dyslipidemia observed in the DIN mouse model. **Conclusion:** EDP-305 is a potent FXR agonist with a favorable gene expression profile for NASH treatment as evidenced by the hepato-protective and anti-steatotic effects observed *in vivo*. The preclinical characterization of EDP-305 presented here suggests that it holds promise for the treatment of NASH.

**Keywords:** Anti-fibrotic, Anti-inflammatory, Low Density Lipoprotein Receptor, Bile Acid, Anti-steatosis

## 1. Introduction

Metabolic syndrome, a cluster of interrelated clinical features including insulin resistance, dyslipidemia, hypertension and visceral obesity, is often manifested in the liver in the form of non-alcoholic fatty liver disease (NAFLD). A significant proportion of patients with NAFLD develop non-alcoholic steatohepatitis (NASH), characterized by hepatocyte injury, inflammation and fibrosis, although the mechanism triggering NASH is still unknown. NAFLD currently affects 20-40% of the general population, with 10-20% of NAFLD patients developing NASH, a leading cause

of cirrhosis and hepatocellular carcinomas. NASH is therefore becoming a major health issue in close association with obesity and diabetes. To date, no approved treatments are available, further underscoring the need for effective therapies [1].

The farnesoid X receptor (FXR), also known as NR1H4, is a member of the nuclear receptor superfamily of ligand activated receptors expressed in the liver, kidney, intestine, and adrenal glands. FXR acts as a bile acid sensor and plays a critical role in the negative feedback regulation of bile acid synthesis via activation of small heterodimer partner (SHP). SHP is a negative regulator of cholesterol 7 $\alpha$ -hydroxylase

(CYP7A1), an enzyme which catalyzes the rate-limiting step of the bile acid synthesis pathway. FXR has emerged as an attractive target for NASH treatment due to its role in protecting hepatocytes against bile acid-induced cytotoxicity, in addition to regulating lipid metabolism and inflammation [1, 2].

The FXR agonist, obeticholic acid (OCA), is a semisynthetic derivative of the primary human bile acid chenodeoxycholic acid (CDCA) [3]. OCA has recently been approved for the treatment of primary biliary cholangitis (PBC) and is currently undergoing a Phase III clinical trial for the treatment of NASH. In recent clinical trials in patients with NASH, as well as healthy volunteers, OCA caused significant dyslipidemia with elevated low density lipoprotein (LDL) cholesterol and reduced high density lipoprotein (HDL) cholesterol [4-7].

In search for novel, safe and more effective FXR agonists, we designed compound EDP-305, a new class of FXR agonists that takes advantage of increased binding interactions with the receptor. EDP-305 lacks the carboxylic acid group, which contributes to the formation of taurine and glycine conjugates - two metabolites that may be associated with liver toxicity [8].

Here, we characterize the potency and efficacy of EDP-305 in the modulation of genes involved in bile acid metabolism *in vitro* and *in vivo*. We also evaluate gene signatures of lipid metabolism, inflammation, and fibrosis that are relevant to NASH progression. Furthermore, the effects of EDP-305 on steatosis and liver injury are reported for two murine models of NASH: a streptozotocin-high fat diet model (STAM<sup>TM</sup>) and a dietary induced NASH model (DIN) driven by high fat, cholesterol, and fructose feeding.

## 2. Materials and Methods

### 2.1. Chemicals

DMSO, CDCA, and lithocholic acid were purchased from Sigma-Aldrich (St. Louis, MO). OCA was synthesized at Enanta Pharmaceuticals (Watertown, MA) with a purity of 99.3%. The structures of OCA and EDP-305 were confirmed by nuclear magnetic resonance spectroscopy.

### 2.2. Potency and Efficacy Analysis of FXR and Takeda G-protein-coupled Receptor 5 (TGR5) Activation in Chinese Hamster Ovary (CHO) Cells and Human Embryonic Kidney (HEK) Cells

EDP-305 was evaluated at a range of concentrations for both potency and efficacy in CHO (INDIGO Biosciences State College, PA) and HEK cells (ATCC Manassas, VA) transfected with a chimeric FXR luciferase reporter and a full-length human FXR luciferase reporter system, respectively. Cells were incubated with the test compounds for 24 hours and potency was assessed using a luciferase substrate. EC<sub>50</sub> values were determined by fitting replicate data to a 9-parameter logistical model using XLfit 4.

EDP-305 was evaluated at a range of concentrations for both potency and efficacy in CHO cells transfected with TGR5 (DiscoverX Fremont, CA). Cells were incubated with the test compounds for 0.5 hour, and potency was assessed using  $\beta$ -galactosidase Enzyme Fragment Complementation technology.

### 2.3. In Vitro Selectivity

*In vitro* selectivity was conducted by INDIGO Biosciences (State College, PA). See Supplemental for details.

### 2.4. In Vitro Gene Expression Studies, Triglyceride Accumulation and LDL Uptake Assays

Gene expression studies were conducted in either HepaRG (Invitrogen Carlsbad, CA), Huh7.5 (JCRB Osaka, Japan), THP-1 (ATCC Manassas, VA) or primary human hepatic stellate cells (ScienCell Research Laboratories, Inc. Carlsbad, CA). Triglyceride accumulation assays were conducted in Huh7.5. See Supplementary Materials and Methods for details.

The human hepatoma cell line, HepG2 cells (ATCC Manassas, VA), were treated in the absence or presence of EDP-305 or OCA at the indicated concentrations in serum-free media for 24 hrs. After 24 hrs, bodipy-LDL (Molecular Probes Eugene, OR) was added at 0.005 mg/mL to each well and incubated at 37°C for 2 hours. Fluorescence was measured at 485/530 (Ex/Em).

### 2.5. Western Blot

Hepatocyte cell and liver lysate (15-30  $\mu$ g) protein (total and membrane-enriched) were separated by 8% SDS-PAGE, transferred to nitrocellulose membrane, and blocked with 5% nonfat dry milk. Membranes were probed with antibodies against the LDL receptor (LDLR) (Abcam) or  $\beta$ -actin (Invitrogen).

### 2.6. Immunofluorescence Staining

Treated Huh7.5 cell monolayers were fixed in 4% paraformaldehyde and rinsed with PBS. Fixed cells were blocked with 3% BSA in PBS, and incubated with primary antibodies against LDLR followed by incubation with goat anti-rabbit Alexa488. Images were taken with 100x objective (EVOS FL microscope).

### 2.7. In Vivo Pharmacodynamical (PD) Model

Male C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). All animals were housed under a standard 12-hr light/dark cycle. All protocols were approved by the institutional animal care and use committee. Mice were maintained on rodent chow, and treated at 8-10 weeks of age with vehicle (0.5% methylcellulose), or EDP-305 at various doses of 3, 10 or 25 mg/kg, via oral gavage for five days. Ileum and liver were harvested four hours post last dose for FXR-targeted gene expression analysis.

## 2.8. Diet Induced NASH (DIN) Mouse Model

The study was carried out according to standard operating procedures in place at the test facility (Physiogenex Laberge, France). All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (revised 1996 and 2011, 2010/63/EU) and French laws. After 6 weeks on a high fat/high cholesterol diet (60% kcal from fat; 0.5 % cholesterol) (Research Diets Inc., NJ, USA) with 10% fructose in drinking water (DIN diet), C57BL/6J mice (Charles River, France) (n=56) were randomized and treated for 10 more weeks with vehicle (0.5 % methylcellulose), OCA at 30 mg/kg, or EDP-305 at 10 or 30 mg/kg, administered in the diet. Plasma chemistry, hepatic total cholesterol, triglycerides and fatty acid levels were measured. Liver samples were also collected for histological analysis (oil red O, H&E) and NAFLD Activity Score (NAS) evaluation.

## 2.9. Streptozotocin-High Fat Diet STAM<sup>TM</sup> Mouse Model

All animals used in the study were housed and treated in accordance with the Japanese Pharmacological Society Guidelines for Animal Use. To induce NASH, male C57BL/6J mice (Japan SLC, Inc., Japan) were injected with a single subcutaneous injection of 200 µg streptozotocin (Sigma-Aldrich Co. LLC., USA) solution 2 days after birth, followed by feeding with a high fat diet (HFD, 57 kcal% fat, cat#: HFD32, CLEA Japan, Inc., Japan) starting at 4 weeks of age. At age 6 weeks, mice were treated for 4 weeks with vehicle (0.5% methylcellulose) OCA (10 mg/kg) or EDP-305 (3 or 10 mg/kg), administered in the high fat diet. Liver samples were collected for histological analysis and NAS evaluation.

**Table 1.** Experimental EC<sub>50</sub> values and efficacy for compounds on chimeric FXR reporter activation in CHO cells. The EC<sub>50</sub> is the concentration of a compound that gives half-maximal luciferase response. Efficacy is normalized to CDCA (set maximal luciferase activity of CDCA as 100%).

Compound	EC <sub>50</sub> ±SD (nM) (% efficacy)		
	FXR activation		TGR5 activation
	Chimeric FXR in CHO cells	Full-length FXR in HEK cells	in CHO cells
OCA	569 ±96 (213%)	130 ±39 (150%)	381 ±102 (72%)
OCA-Glycine	-	301 ±42 (117%)	568 ±139 (175%)
OCA-Taurine	-	275 ±23 (149%)	604 ±7 (177%)
EDP-305	34 ±8 (223%)	8 ±3 (152%)	>15,000

## 3.2. EDP-305 Regulates Genes of Bile Acid Metabolism in Vitro and in Vivo

To determine the *in vitro* effects of the FXR agonist EDP-305 on FXR downstream signaling and gene expression related to bile acid metabolism, human hepatocytes (HepaRG) were treated with EDP-305 for 16 hours. Messenger RNAs (mRNAs) of genes related to bile acid metabolism were quantified by real time PCR. EDP-305 dose-dependently increased the expression of the FXR target gene, *SHP*, and downregulated *CYP7A1* expression in HepaRG hepatocytes (Figure 1C).

Previously, EDP-305 was evaluated in an *in vivo*

## 2.10. Statistics

One-way ANOVA were performed to analyze data among groups of 3 or more. A Student's t test then compared data between control and 1 experimental group. A p-value less than 0.05 was considered statistically significant. Results are expressed as the mean ± 1 standard deviation (SD) unless otherwise noted.

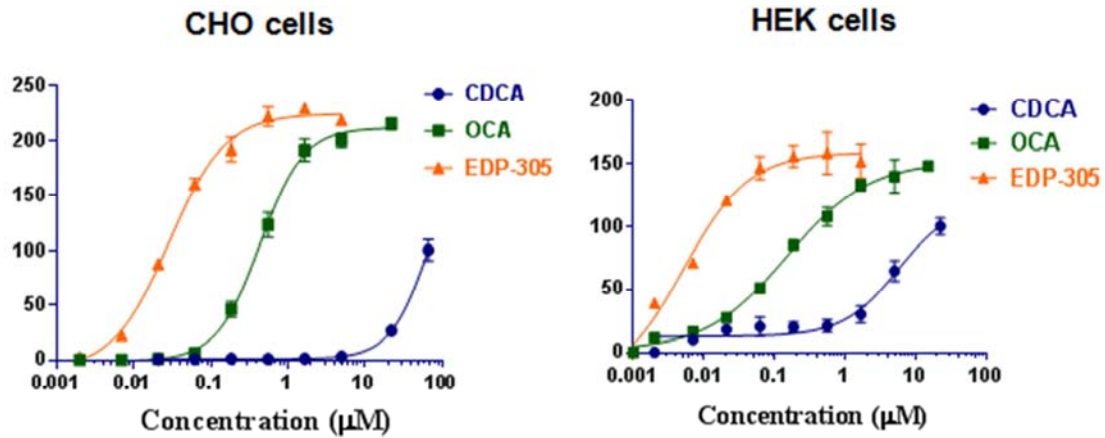
## 3. Results

### 3.1. EDP-305 Is a Potent and Selective FXR Agonist

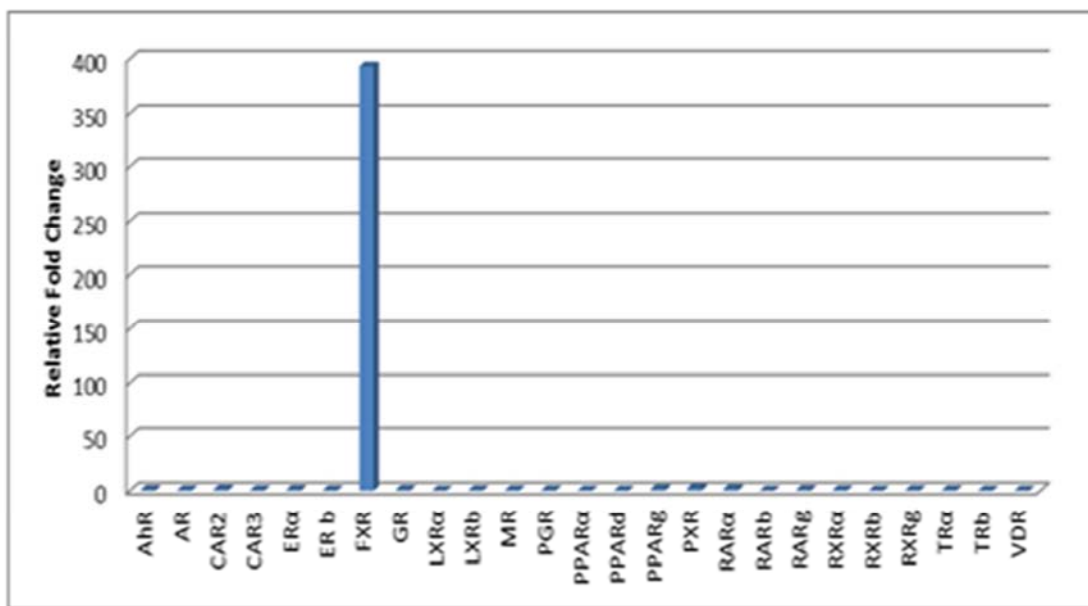
The efficacy and potency of EDP-305 as an FXR agonist were evaluated in both chimeric FXR CHO cell reporter and full-length human-FXR HEK 293 cell reporter assays (Table 1). All efficacy and potency assays for EDP-305 were run in parallel with the endogenous FXR agonist, CDCA, and the CDCA derivative, OCA (Table 1 and Figure 1A). The full-length human FXR HEK293 reporter cell assay demonstrated that EDP-305 is a potent FXR agonist with an EC<sub>50</sub> value of 8 nM, which is 16-fold more potent than OCA (EC<sub>50</sub>: 130 nM) (Table 1).

Profiling of EDP-305 in a broad panel screen of nuclear receptors demonstrated that EDP-305 is highly selective for FXR; EDP-305 activated FXR but did not activate or repress any other nuclear receptors (Figure 1B). Since OCA and its two major metabolites, glycine- and taurine-conjugates, have been demonstrated to possess TGR5 activity [9], we sought to test EDP-305 for TGR5 agonism. The experimental EC<sub>50</sub> value for EDP-305 was greater than 15 µM, which is 100-fold less potent than the TGR5 reference compound, lithocholic acid (LCA), indicating that EDP-305 lacks TGR5 activity (Table 1). This is significant as TGR5 has been reported to induce pruritus in mice [10, 11].

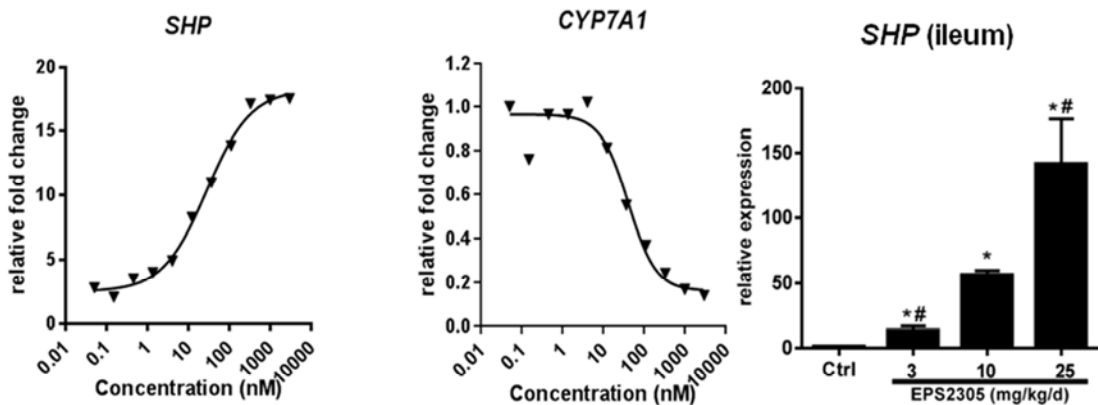
pharmacodynamics model for the regulation of FXR target genes after multiple days of dosing. In the liver, we observed dose dependent regulation by EDP-305 of hepatic *Shp* and *Cyp7A1* mRNA [12]. Here we show that EDP-305 also regulates FXR target gene expression in the ileum. EDP-305 induced a dose-dependent increase in expression of *Shp* (Figure 1D). Together, these results demonstrate that EDP-305 is a potent and selective FXR agonist that robustly regulates FXR down-stream genes involved in bile acid metabolism, both *in vitro* and *in vivo*. (Figure 1)



A



B



C

D

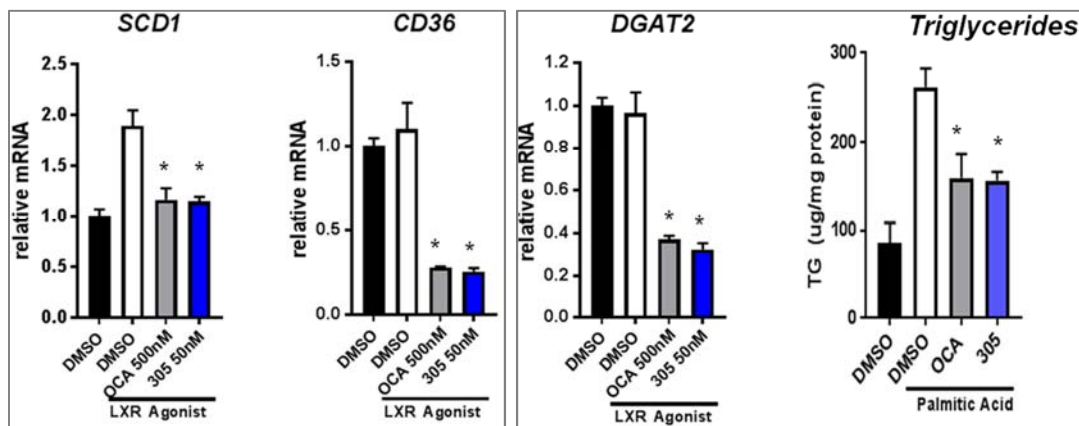
**Figure 1.** EDP-305 is a potent FXR agonist. A. The FXR was activated with increasing concentrations of the indicated compounds in CHO cells (left panel) and in HEK293 cells (right panel). The results show mean  $\pm$  standard deviation, of triplicate samples from a representative experiment performed. B. EDP-305 was examined in a nuclear receptor reporter assay demonstrating selectivity for FXR. C. Gene expression of SHP and CYP7A1 in HepaRG. D. Expression of SHP in the ileum. Values are expressed as fold changes relative to a control group. \*p value < 0.05 between treatment and vehicle control.

### 3.3. EDP-305 Regulates Lipogenic Genes *in Vitro*

NASH is characterized by fat accumulation in the liver which can exacerbate inflammation and fibrosis. FXR activation has anti-lipogenic effects via *SHF* induction. To characterize the *in vitro* pre-clinical profile of EDP-305, we determined the effects of EDP-305 in modulation of lipogenic gene expression, triglyceride accumulation, and LDL uptake. Huh7.5 cells were pre-treated with EDP-305 (50 nM) or OCA (500 nM) followed by treatment with the liver X receptor (LXR) agonist, TO901317, at 1  $\mu$ M to induce lipogenesis. To examine the mechanisms of EDP-305 and OCA independent of their drug potencies, treatments were conducted near their respective EC<sub>50</sub> concentrations. *In vitro*, both EDP-305 and OCA decreased the expression of lipogenic genes at concentrations near their respective EC<sub>50</sub> (Figure 2), including the sterol regulatory element-binding protein 1c (*SREBP1c*) gene target, stearoyl CoA desaturase 1 (*SCD1*) (40% decrease,

respectively). Other genes relevant to lipid metabolism that were decreased in expression by EDP-305 and OCA are cluster of differentiation 36 (*CD36*) involved in fatty acid uptake (77 and 65%, respectively) and the enzyme that catalyzes TG biosynthesis, diacylglycerol O-acyltransferase 2 (*DGAT2*) (68% and 63%, respectively) (Figure 2). Consistent with the observed results in Huh7.5 cells, EDP-305 treatment in HepaRG cells induced dose-dependent decreases in lipogenic gene expression including *SREBP1c*, *SCD1* and *PCSK9* (Supplemental Figure 1A, B and C).

In order to determine the effects of EDP-305 on TG metabolism, Huh7.5 cells were treated with the saturated fatty acid, palmitate, to induce TG synthesis and accumulation. In accordance with the anti-lipogenic gene signature of EDP-305 and OCA, TG accumulation was significantly blunted by 40% (Figure 2) with either FXR agonist treatment. (Figure 2)



**Figure 2.** EDP-305 regulates triglyceride metabolism *in vitro*. Effects of EDP-305 (50 nM) or OCA (500 nM), at concentrations near their respective EC<sub>50</sub>, on mRNA levels of *SCD1* (A), *CD36* (B), and *DGAT2* (C) in Huh7.5 cells. For lipogenic genes, cells were pre-treated with FXR agonists for 40 hours, followed by an FXR-co-treatment with 1  $\mu$ M of LXR agonist (TO901317) for 8h. D. Triglyceride accumulation assay in Huh7.5 cells pre-treated with FXR agonists at 1  $\mu$ M for 16 hr in serum free media, followed by palmitic acid (400  $\mu$ M) for 8h. \* *p* value < 0.05 between FXR agonist treatment and DMSO control in presence of LXR agonist or palmitate.

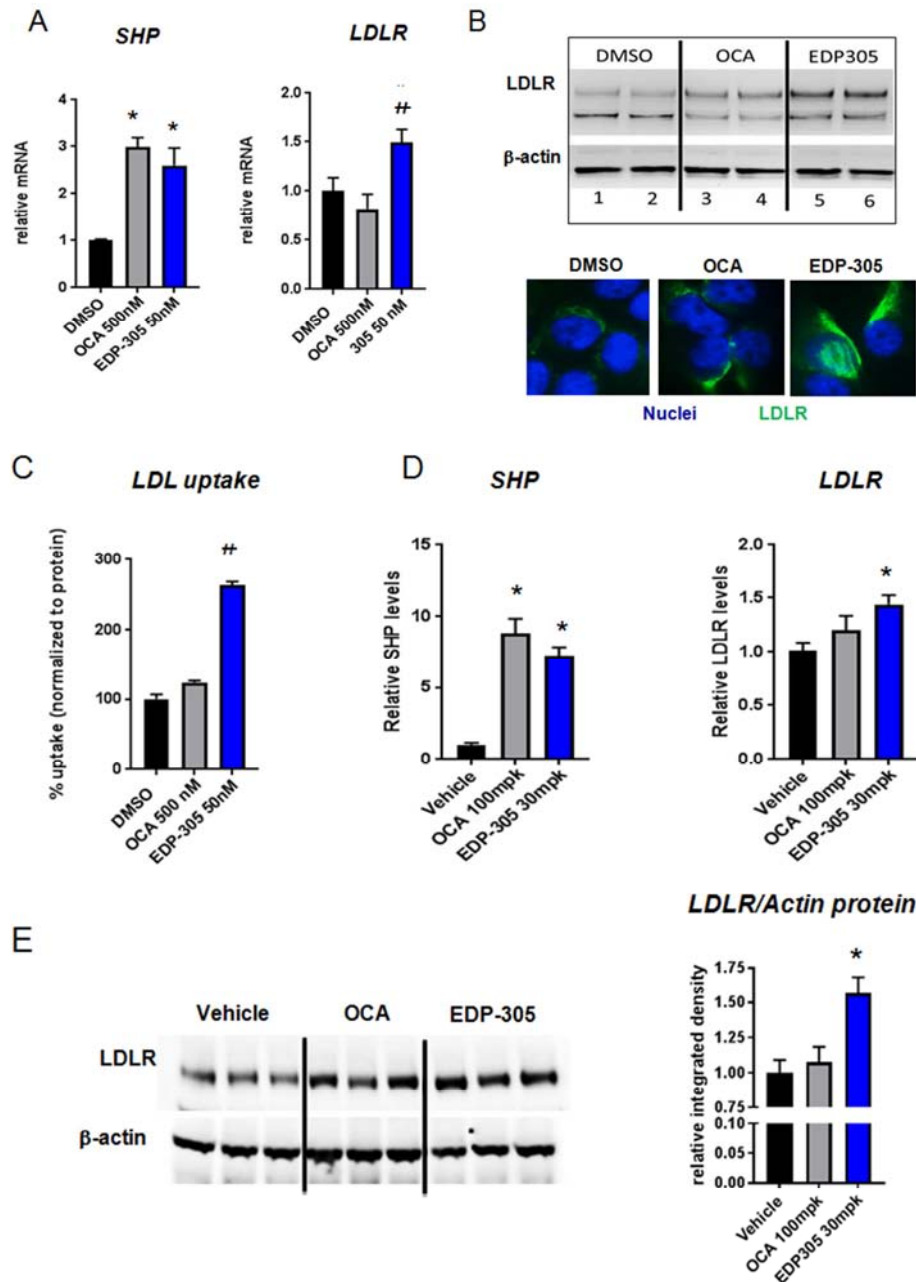
### 3.4. EDP-305 Regulates Lipoprotein Genes

We next sought to evaluate the effects of EDP-305 on genes of lipoprotein metabolism. The LDL receptor (*LDLR*) gene encodes for a membrane receptor highly expressed in hepatocytes that functions to clear LDL-cholesterol from the blood [13]. At concentrations close to their respective EC<sub>50</sub> values, *in vitro* in Huh7.5 cells, EDP-305 (50 nM) significantly increased *LDLR* expression by 35%, while OCA (500 nM) had no effect (Figure 3A). Proprotein convertase subtilisin/kexin type 9 (*PCSK9*), which regulates *LDLR* degradation, and apolipoprotein C3 (*APOC3*), an inhibitor of lipoprotein lipase (*LPL*), which breaks down VLDL-C and IDL-C to generate LDL, were also differentially regulated by EDP-305 (decreased by 67% and 43%, respectively) but not by OCA at their respective EC<sub>50</sub> (Supplemental Figure 1D) [13]. Consistent with the observed increase in *LDLR* mRNA levels, EDP-305 also increased protein levels of membrane *LDLR* as observed by Western blotting and immunofluorescence staining in Huh7.5 cells (Figure 3B).

Since we observed an increase in *LDLR* mRNA and protein levels with EDP-305, we assessed LDL uptake in HepG2 cells by measuring fluorescent BODIPY labeled LDL particles. In accordance with the observed increase in *LDLR* expression, EDP-305 treatment resulted in a significant 2.6-fold increase in LDL uptake by hepatocytes, whereas OCA had no effect (Figure 3C).

To further characterize the effects of EDP-305 on *LDLR* expression, we determined *Ldlr* mRNA and protein levels *in vivo* in C57BL/6 mice treated with EDP-305 or OCA at comparable *in vivo* potencies (EDP-305 30 mg/kg; OCA 100 mg/kg) for three days. Treatment with EDP-305 or OCA induced comparable levels of *Shp* expression in the liver (Figure 3D). At these same concentrations, EDP-305 significantly increased *Ldlr* mRNA and protein levels while OCA did not (Figure 3D and E). Together these results suggest that EDP-305 favorably regulates lipoprotein metabolism by increasing LDL uptake and decreasing triglyceride accumulation in hepatocytes. (Figure 3)





**Figure 3.** EDP-305 favorably regulates lipoprotein metabolism in vitro and in vivo. **A.** Effects of EDP-305 (50 nM) or OCA (500 nM), at concentrations near their respective  $EC_{50}$ , on mRNA levels of SHP and LDLR in Huh7.5 cells after 32hr treatment in 1% FBS. **B.** Western blot and immunofluorescence staining of LDLR in Huh7.5 cells treated with EDP-305 (50 nM) or OCA (500 nM) for 32 hr. **C.** In vitro LDL uptake by EDP-305 (50 nM) or OCA (500 nM) in HepG2 cells. **D.** Expression levels of SHP and LDLR at 4 hours post last dose in the liver of mice treated with EDP-305 (30 mg/kg) or OCA (100 mg/kg) for three days. **E.** Western blot of LDLR in liver of mice treated with EDP-305 (30 mg/kg) or OCA (100 mg/kg) for three days. \*  $p$  value < 0.05 between FXR agonist treatment and DMSO control. #  $p$  value < 0.05 between EDP-305 and OCA.

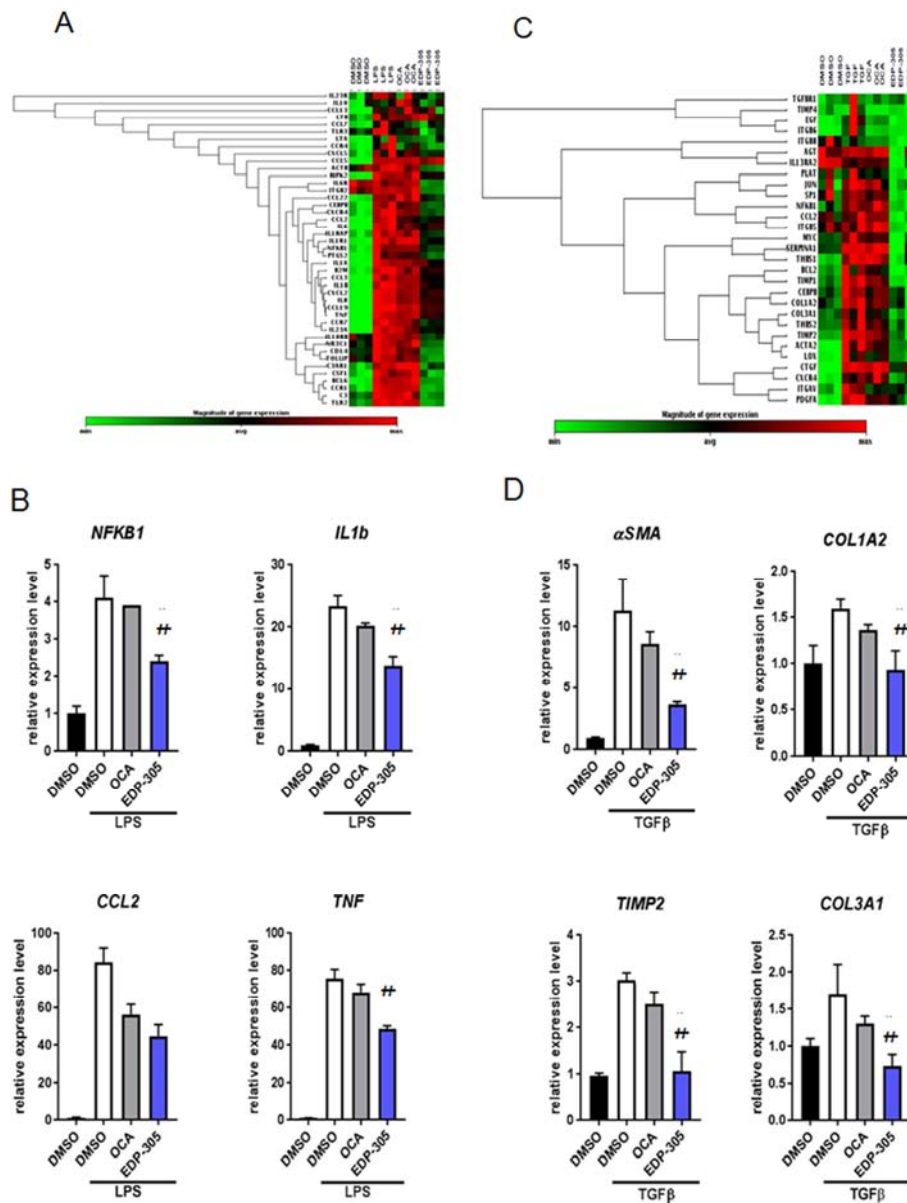
### 3.5. EDP-305 Decreases Expression of Inflammatory Response Genes in LPS-treated Macrophage

Fibrosis, evidenced by excess accumulation and deposition of extracellular matrix proteins, can occur as a result of chronic, non-resolving inflammation which triggers a wound healing process that mitigates tissue destruction, ultimately leading to excessive scarring and organ failure [14]. The recruitment and activation of immune cells at sites of injury

and inflammation is an important event in hepatic fibrosis [14]. To assess the effects of EDP-305 on genes involved in the inflammatory response, THP-1 cells were treated with 50 ng/ml LPS alone, or in combination with either 50 nM of EDP-305 or OCA. Treatment of THP-1 cells with EDP-305 significantly altered the pro-inflammatory gene expression profile induced by LPS treatment and inhibited expression of key genes involved in inflammatory response, such as nuclear factor kappaB ( $NF-\kappa B$ ), tumor necrosis factor  $\alpha$  ( $TNF\alpha$ ), interleukin  $1\beta$  ( $IL-1\beta$ ) and C-C motif chemokine

ligand 2 (*CCL2*) (Figure 4A and B). These receptors and chemokines contribute to the recruitment and activation of inflammatory cells, including monocytes and macrophages, during liver injury. Activation of FXR by EDP-305 may

inhibit the pathogenic inflammatory response by suppressing essential genes involved in inflammation. This reduction in inflammatory cytokine expression may inhibit hepatic stellate cell activation, migration and survival.



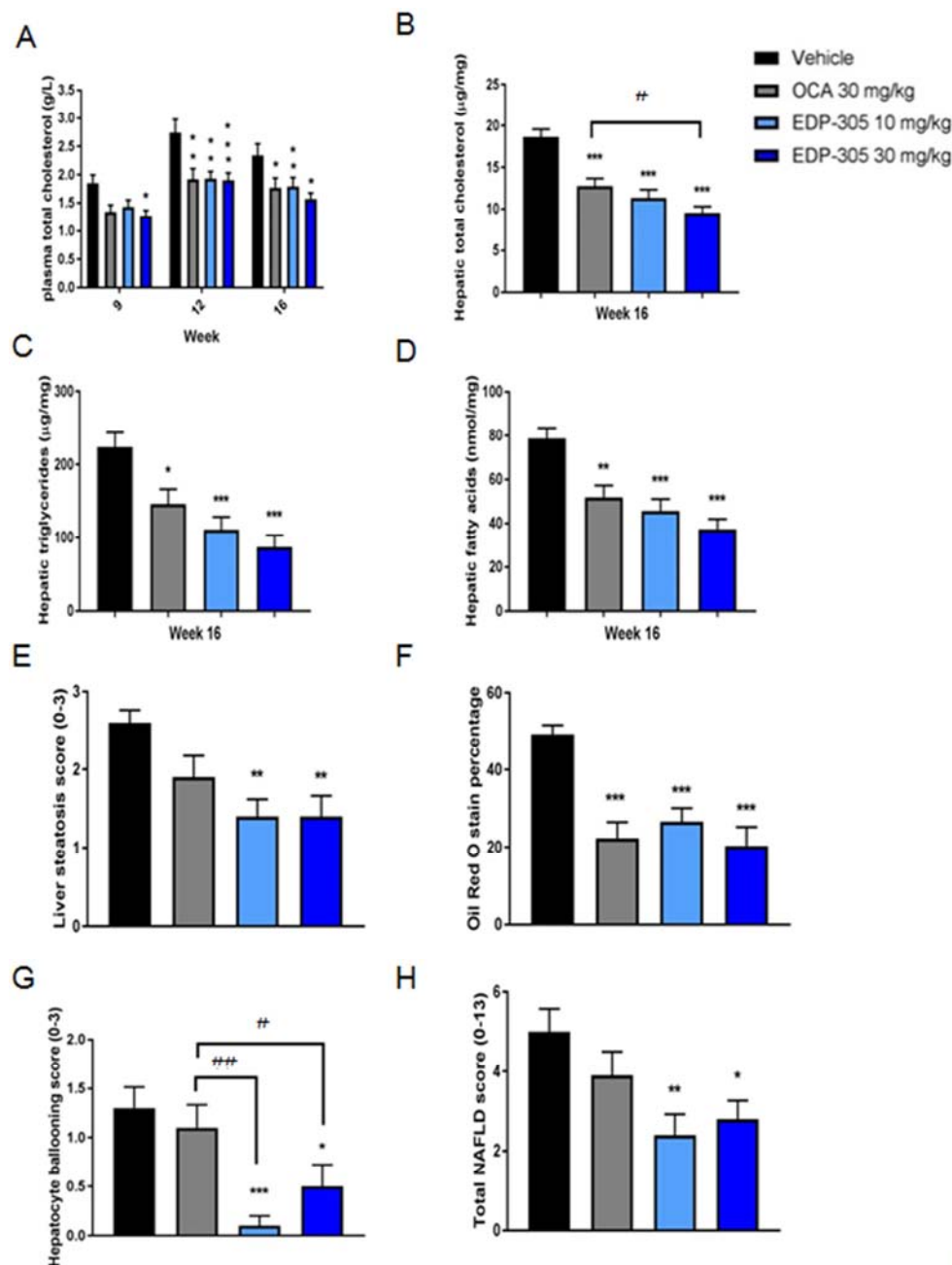
fibrogenesis including collagen type 1  $\alpha 1$  and type 3  $\alpha 1$  (*COL1A1* and *COL3A1*), metalloproteinase inhibitor-2 (*TIMP-2*) and alpha smooth muscle actin ( $\alpha$ -SMA) (Figure 4C and D). (Figure 4)

### 3.7. EDP-305 Reduces Dyslipidemia, Liver Lipid Content, and Liver Enzyme Levels in Vivo

EDP-305 *in vitro* exhibits a favorable gene expression profile for NASH treatment as demonstrated by decreased lipogenic gene expression in hepatocytes, an anti-inflammatory gene profile in macrophages, and decreased expression of fibrogenic genes in stellate cells. Therefore, we

next investigated the effects of EDP-305 on steatosis, hepatocyte injury and NASH development in two murine models of NASH: a STAM<sup>TM</sup> model and a diet induced NASH (DIN) model. The STAM<sup>TM</sup> mouse develops NASH, fibrosis and, at later stages, hepatocellular carcinoma.

In the STAM<sup>TM</sup> model, neonatal male mice exposed to low-dose streptozotocin develop liver steatosis with diabetes one week after starting a high-fat diet (HFD) [18, 19]. This model recapitulates the pathological progression of NASH in patients from steatosis to NASH and fibrosis; STAM<sup>TM</sup> mice manifest NASH at 8 weeks, which progresses to fibrosis at 12 weeks. (Figure 5)



**Figure 5.** EDP-305 reduces plasma and liver lipid content and improves hepatocyte ballooning and NAS in the DIN mouse. A. plasma total cholesterol; B. hepatic total cholesterol; C. hepatic triglycerides (C); D. hepatic fatty acid; E. liver steatosis scoring ; F. Oil Red O staining percentage; G. hepatocyte ballooning score; and H. total NAFLD score were measured in DIN mice treated with vehicle, EDP-305 (10 mg/kg or 30 mg/kg) and OCA 30 mg/kg. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. vehicle with one way ANOVA + Dunnett's post test; # $p < 0.05$  and ## $p < 0.001$  with one way ANOVA + Newman-Keuls post test.



The DIN model mimics the human situation in which diet plays a major role in the development of NAFLD, resembling the human NAFLD/NASH physiopathological context. DIN mice, fed a high fat/cholesterol and high fructose diet, develop liver steatosis in 6 weeks, and early NASH, associated with insulin resistance and obesity, in 17 weeks.

In the DIN mouse model, vehicle-treated animals showed significant increases in plasma total cholesterol and liver lipid content with 16 weeks of high fat/cholesterol and high fructose diet feeding (Figure 5A and B). Treatment with EDP-305 (10 mg/kg or 30 mg/kg) for the next 10 weeks significantly decreased plasma total cholesterol levels (Figure 5A). Consistent with the effects of EDP-305 on triglyceride synthesis and accumulation *in vitro*, treatment with EDP-305 at both doses significantly reduced liver lipids as evidenced by significant reductions in hepatic cholesterol, triglycerides and fatty acids (Figure 5B, C, D) ( $p < 0.001$  versus vehicle for all parameters).

### 3.8. EDP-305 Improves Hepatocyte Ballooning and NAFLD Activity Score *in Vivo*

Histopathologic analysis of H&E stained liver sections revealed severe macrovesicular steatosis and hepatocyte ballooning with minor inflammation and fibrosis in vehicle treated animals on the DIN diet. Consistent with observed reductions in liver lipids, administration of EDP-305 at both doses significantly decreased ( $p < 0.01$ ) liver steatosis while OCA treatment did not have a significant effect (Figure 5E). Morphometric analysis of liver sections stained with Oil Red O revealed significant reduction of liver triglyceride content with EDP-305 at both doses, as well as OCA (Figure 5F). Significantly lower scores for hepatocyte ballooning in EDP-305 treated mice at both doses (the most potent being EDP-305 at 10 mg/kg) were observed (Figure 5G). Overall, total NAFLD score (NAS) was significantly reduced in EDP-305 treated mice at both doses versus vehicle treated mice ( $p < 0.05$ ) (Figure 5H).

**Table 2.** Hepatocyte ballooning score and NAFLD activity score (NAS) in STAMM mice treated with OCA or EDP-305. \*  $p$  value  $< 0.05$  between FXR agonist treatment and vehicle control; \*\*  $p$  value  $< 0.01$  between FXR agonist treatment and vehicle control.

Compound	Dose (mg/kg/day)	N	Hepatocyte ballooning score	NAS
Vehicle	-	7	1.9	5.3
OCA	10	7	1.1	4.3
EDP-305	3	7	1.1	3.7*
EDP-305	10	7	0.7*	3.4**

In the STAM<sup>TM</sup> NASH model, H&E stained liver sections from the vehicle control group exhibited micro- and macrovesicular fat deposition, hepatocellular ballooning and inflammatory cell infiltration. EDP-305 treatment groups (both 3 and 10 mg/kg doses) showed marked improvements in hepatocellular ballooning with significant reductions in NAS when compared with the control group, whereas treatment with OCA did not significantly impact hepatocyte

ballooning or NAS (Table 2). Together, these results suggest that EDP-305 exerts hepato-protective effects against liver injury and NASH development induced by streptozotocin treatment followed by high fat diet, as well as high fat/cholesterol and high fructose diet feeding.

## 4. Discussion

The central homeostatic regulating role of FXR has made it an attractive target for NASH treatment. Besides its chief function as a bile acid sensor, FXR signaling has been implicated in other physiological events, including regulation of lipid and carbohydrate metabolism, glucose homeostasis, liver repair and hepato-protection. In this study, we demonstrated that EDP-305 is a potent and selective FXR agonist that regulates expression of downstream target genes related to bile acid synthesis and transport. Furthermore, EDP-305 treatment resulted in anti-lipogenic, anti-inflammatory, and anti-fibrotic gene signatures favorable for NASH treatment. Consistent with this *in vitro* gene regulation, EDP-305 demonstrated hepato-protective and anti-steatotic effects in murine NASH models, which supports further testing of EDP-305 as a potential therapeutic target for NASH.

The recent FLINT clinical trial demonstrated encouraging results for OCA in improvement of NASH. However, one common side effect following OCA treatment was elevation of LDL-C and reduction of HDL-C [4]. Recently, OCA was found to decrease *APOC3* and *LDLR* expression in a perfused organotypic liver system [20]. Additionally, treatment with OCA in diet-induced NASH hamsters also significantly reduced expression of *LDLR*, contributing to the observed elevations of LDL-C in these animals [21]. Contrary to OCA, EDP-305 has the potential to generate a favorable lipoprotein profile through increased expression of *LDLR*. EDP-305 may lower LDL-C levels as indicated by greater LDL uptake when compared to OCA. EDP-305 exhibits divergent effects from OCA on lipoprotein metabolism. The mechanisms by which EDP-305 differentially modulated lipoprotein metabolism require further elucidation.

Similar to OCA, EDP-305 reduces lipogenic gene expression (*SCD1*, *DGAT2* and *CD36*) and triglyceride accumulation *in vitro* in both Huh7.5 and HepaRG cells. *In vivo*, EDP-305 significantly decreased plasma total cholesterol and liver lipid content in the DIN NASH model. EDP-305 also significantly decreased histopathological scores of steatosis, hepatocyte ballooning and NAS.

Chronic liver inflammation drives hepatic fibrosis through secretion of inflammatory cytokines such as various interleukins (e.g., *IL-1 $\beta$* , *IL-8*) and chemokines like CCL2 which could promote fibrotic processes. In addition, high expression of toll like receptors promotes HSC activation and fibrosis [22]. Activation of FXR inhibits the inflammatory response through repression of key genes contributing to inflammation and inflammatory cytokine secretion, thereby

preventing HSC activation, migration and survival. Furthermore, FXR represses fibrogenesis by remodeling extracellular matrix formation [3]. Consistent with activation of FXR signaling, our results show that EDP-305 represses expression of genes involved in inflammation and fibrogenesis.

EDP-305 treatment exerted hepato-protective effects as evidenced by significantly decreased hepatocyte ballooning scores and NAS *in vivo*. Hepatocellular ballooning is a key feature in the diagnosis of NASH, and is defined as cellular enlargement 1.5-2 times the normal hepatocyte diameter with rarefied cytoplasm. Hepatocyte ballooning has been demonstrated to correlate with fibrosis and injury to the cytoskeleton. A correlation between fat droplet accumulation, cytoskeletal injury and hepatocyte ballooning has also been suggested [23]. Our results demonstrating that EDP-305 significantly reduces hepatocyte ballooning in both the STAM<sup>TM</sup> mouse model and the DIN model suggests that EDP-305 reduces fat droplet accumulation and hepatocyte injury leading to ballooning. It has previously been demonstrated that treatment with EDP-305 significantly reduced collagen deposition and fibrosis progression in two rodent models of fibrosis – choline-deficient high fat diet mice and rats with bile duct ligation [24]. Our present data converge with these findings, underscoring the ability of EDP-305 to attenuate fibrosis in rodent models of fibrosis and NASH.

In conclusion, EDP-305 demonstrates robust potency and efficacy in activating FXR signaling in preclinical testing. Our results indicate that EDP-305 can regulate multiple pathways contributing to the pathogenesis of NASH. EDP-305 acts potently in driving a metabolic gene signature that favors suppression of bile acid production and decreased lipogenesis, in addition to anti-fibrotic and anti-inflammatory responses. While OCA was found to exert an undesirable effect on the lipoprotein profile in the clinic, our present data shows that EDP-305 has the potential to circumvent these effects. Furthermore, our present data demonstrates that EDP-305 exerts beneficial pharmacological effects to prevent steatosis, hepatocyte injury and NASH development *in vivo*. Importantly, EDP-305 reduces hepatocyte ballooning and liver damage in two models of NASH with different pathological mechanisms. With its pleiotropic activities in liver, kidney, and intestine, EDP-305 may be beneficial in a variety of clinical indications, including NASH, obesity, and fibrosis. The potency, selectivity, gene signature profile and *in vivo* efficacy of EDP-305 make it an attractive candidate to advance into clinical studies as a novel therapeutic with potential applications across a range of chronic metabolic and liver diseases.

## 5. Conclusion

EDP-305 is a potent and selective FXR agonist with unique regulatory effects on multiple pathways involved in NASH pathogenesis including lipid and lipoprotein metabolism, inflammation and fibrosis. Additionally, EDP-

305 exerted hepatoprotective effects in two *in vivo* models of NASH. The profile and *in vivo* efficacy of EDP-305 warrant its further investigation as a treatment for chronic metabolic and liver diseases.

## Acknowledgements

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## List of Abbreviations

APOC3, apolipoprotein C3;  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin; CCL2, C-C motif chemokine ligand 2; CD36, cluster of differentiation 36; CDCA, chenodeoxycholic acid; CHO, Chinese hamster ovary; COL1A1, collagen type 1  $\alpha$ 1; COL3A1, collagen type 3  $\alpha$ 1; CYP7A1, cholesterol 7 $\alpha$ -hydroxylase; DGAT2, diacylglycerol O-Acyltransferase 2; DIN, diet induced NASH model; DMSO, dimethyl sulfoxide; FGF15, fibroblast growth factor 15; FXR, Farnesoid X Receptor; H&E, Haematoxylin and Eosin; HDL, high density lipoprotein; HEK, human embryonic kidney; HFD, high fat diet; HSC, hepatic stellate cell; IL-1 $\beta$ , interleukin 1 $\beta$ ; LDL, low density lipoprotein; LDLR, LDL receptor; mRNA, messenger RNA; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; NF- $\kappa$ B, nuclear factor kappa B; OCA, obeticholic acid; PBC, primary biliary cholangitis; PCR, polymerase chain reaction; PCSK9, proprotein convertase subtilisin/kexin type 9; SCD1, stearoyl CoA desaturase; SHP, small heterodimer partner; SREBP1c, sterol regulatory element-binding protein 1c; TG, triglyceride; TGF  $\beta$ , transforming growth factor  $\beta$ ; TGR5, Takeda G-protein-coupled receptor 5; TIMP-2, metalloproteinase inhibitor 2; TNF $\alpha$ , tumor necrosis factor  $\alpha$

## Potential Conflict of Interest

Mary Chau, Yang Li, Manuel Roqueta-Rivera, Kelsey Garlick, Ruichao Shen, Guoqiang Wang, Yat Sun Or, and Li-Juan Jiang are employees of Enanta Pharmaceuticals, Inc.

## Supplementary Materials and Methods

### Plasmids

For expression of human FXR (isoform  $\alpha$ 2, NM\_005123), the coding region was amplified using the following primers: forward primer: 5'-CACCATGGGATCAAAAATGAATCTCA-3' reverse primer: 5'-TCACTGCACGTCCCAGATTTACAGAG-3'. cDNA was cloned to (pcDNA6.2, Life Technologies Woburn, MA). The IR1-type FXR response element from the human IBABP gene was cloned as a 68 bp KpnI-BglII fragment containing two IR1 elements (shown below) into the KpnI-BglII site of pGL4.28-promoter (Promega Madison, WI) resulting in pGL2-IBABPx2.

5'-  
 CcccaGGGTGAaTAACCTcggggctctgtccctccaatcccaGGGTG  
 AaTAACCTcgggA-3' and 5'-  
 GATCTcccAGGTTATTCACCCtgggattggaggacagagcccg  
 AGGTTATCACCCtgggGGTAC-3'. The reporter plasmids  
 (100 ng/well, pGL4.28-IBABPx2) were co-transfected with 5  
 ng full length FXR into HEK293.

#### *In Vitro Selectivity*

*In vitro* selectivity was conducted by INDIGO Biosciences (State College, PA). Briefly, reporter cells used in the various nuclear receptor agonist assays express either the native nuclear receptor or a receptor hybrid in which the native N-terminal DNA binding domain (DBD) is replaced by a yeast Gal4 DBD. A firefly luciferase reporter gene is functionally linked to an upstream receptor-specific genetic response elements (GRE) or a Gal4 upstream activation sequence (UAS). Agonist activity was tested against the following nuclear receptors: AhR, AR, CAR2, CAR3, ER $\alpha$ , ER $\beta$ , FXR, GR, LXR $\alpha$ , LXR $\beta$ , MR, PGR, PPAR $\alpha$ , PPAR $\delta$ , PPAR $\gamma$ , PXR, RAR $\alpha$ , RAR $\beta$ , RAR $\gamma$ , RXR $\alpha$ , RXR $\beta$ , RXR $\gamma$ , TR $\alpha$ , TR $\beta$ , and VDR. Reference compounds were used to confirm assay performance for each nuclear receptor and results are reported as relative activation where the respective reference agonist at EC100 is 100% activity and 0.1% DMSO is 0% activity.

#### *In Vitro* Gene Expression Studies and Triglyceride Accumulation Assay

**Bile Acid Metabolism.** The terminally differentiated human hepatocytes, HepaRG (Invitrogen Carlsbad, CA), were seeded in 96 well collagen-coated plates (Gibco) at a density of 100000 cells per well in complete William's E medium. 72 hours later, cells were treated with an FXR agonist at indicated concentrations in serum-free William's E medium for 16 hours. RNA was then extracted and gene expression was measured using the QIAGEN OneStep RT-PCR Kit. **Lipoprotein Metabolism.** The human hepatoma cell line Huh7.5, from the JCRB Cell Bank (Osaka, Japan) were treated with compounds, EDP-305 or OCA, at indicated concentrations in serum-reduced media (1% FBS) for 32 hours.

**Lipid Metabolism.** For liver X receptor (LXR) agonist

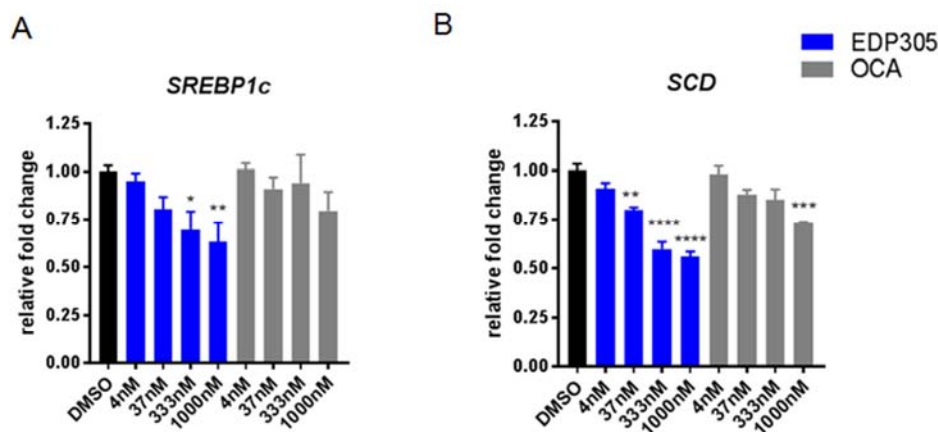
experiments, Huh7.5 cells were pre-treated with EDP-305 or OCA for 40 hours followed by compound co-treatment with an LXR agonist (1 $\mu$ M TO901317) (Sigma-Aldrich St.Louis, MO) for an additional 8 hours. Lipogenic gene expression in HepaRG was evaluated with a 24h FXR agonist pretreatment in William's media without serum, followed by a media switch to high insulin (600nM), high glucose (25mM) William's media for 8h with vehicle (DMSO) or FXR agonists at indicated concentrations. For all HepaRG experiments in a 96 well format, RNA was extracted and gene expression was measured using the QIAGEN OneStep RT-PCR Kit.

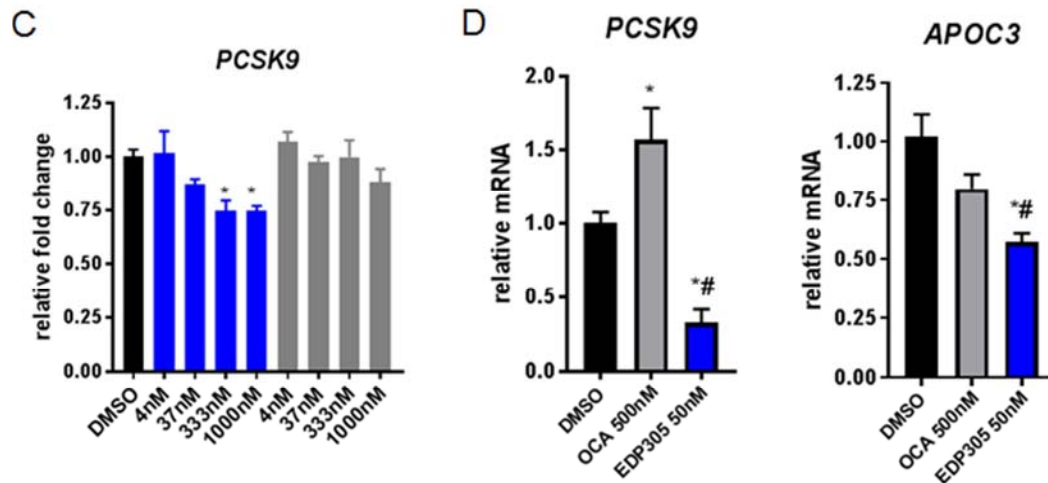
**Inflammation.** Differentiation of human THP-1 monocytes (ATCC Manassas, VA) into macrophages was induced by treatment of cells with phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich St.Louis, MO) at a concentration of 10 ng/ml for 72 hours. Differentiated THP-1 cells were co-treated with lipopolysaccharide (LPS) (50 ng/ml) (Sigma-Aldrich St.Louis, MO) with either 50 nM OCA, 50 nM EDP-305 or DMSO control for 18 hours (n=3 for each treatment). Cells were then harvested for inflammatory gene RNA isolation.

**Fibrosis.** Primary human hepatic stellate cells (ScienCell Research Laboratories, Inc. Carlsbad, CA) were cultured in Stellate Cell Medium supplemented with 2% FBS and 1% penicillin-streptomycin. Cells were activated with 10 ng/mL transforming growth factor beta (TGF $\beta$ ) and co-treated with or without 500 nM OCA, 500 nM EDP-305 or DMSO control diluted in serum free media (n=3 for each treatment) for 18 hours. After 18 hours, cells were harvested for fibrotic gene RNA isolation.

**Triglyceride (TG) accumulation assay.** Huh7.5 cells were treated in the absence or presence of EDP-305 or OCA at the indicated concentrations for 16 hours in serum free media, followed by palmitate (Sigma-Aldrich St.Louis, MO) treatment (400 $\mu$ M) complexed to bovine serum albumin (BSA; 1% final concentration) (Sigma-Aldrich St.Louis, MO) for 8 hours. Cells were then harvested for TG quantification (Wako Kit) (Wako Richmond, VA). TG was normalized to protein.

## Supplemental Figure





Supplemental Figure 1 EDP-305 regulates lipid metabolism-related genes in HepaRG and Huh7.5 cells. Effects of EDP-305 or OCA on mRNA levels of SREBP1c (A), SCD (B) and PCSK9 (C) in HepaRG cells. D. Effects of EDP-305 or OCA on mRNA levels of PCSK9 and APOC3 in Huh7.5 cells. \* p value < 0.05 between FXR agonist treatment and DMSO control; \*\* p value < 0.01 between FXR agonist treatment and DMSO control.

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