Published in final edited form as:

Biochem Pharmacol. 2019 August ; 166: 212–221. doi:10.1016/j.bcp.2019.05.023.

A dual farnesoid X receptor/soluble epoxide hydrolase modulator treats non-alcoholic steatohepatitis in mice

Md. Abdul Hye Khan^a, Jurema Schmidt^b, Anna Stavniichuk^a, John D. Imig^{a,1}, Daniel Merk^{b,c,*},¹

^aDepartment of Pharmacology & Toxicology, Medical College of Wisconsin, Milwaukee, WI, USA

^bInstitute of Pharmaceutical Chemistry, Goethe University, Frankfurt, Germany ^cInstitute of Pharmaceutical Sciences, Swiss Federal Institute of Technology (ETH) Zurich, Switzerland

Abstract

Non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) are the most prevalent metabolic liver disorders and a serious global health burden. NAFLD/NASH pathogenesis and progression are highly multi-factorial and likely demand a combination of multiple mechanisms to provide a more effective treatment. We have developed a dual farnesoid X receptor agonist (FXRA)/soluble epoxide hydrolase inhibitor (sEH) to simultaneously address two validated and complementary modes of action in NASH treatment. Here we report the *in vivo* profiling for this FXRA/sEH in toxin- and diet-induced rodent NASH models. In streptozotocin-induced NASH as a proof-of-concept study, the experimental FXRA/sEH drug robustly prevented hepatic steatosis and fibrosis, and improved lipid homeostasis as well as biochemical markers of liver health. In methionine-choline-deficient high-fat diet-induced NASH, FXRA/sEH treatment reduced hepatic steatosis and fibrosis to levels similar to healthy animals and demonstrated anti-inflammatory activity confirming that dual FXRA/sEH modulation produces a triad of complementary anti-NASH effects. Our results validate dual FXRA/sEH modulation as an effective therapeutic strategy to treat NASH and advocates for a combinational drug therapeutic approach for multifactorial liver diseases.

Keywords

Non-alcoholic fatty liver; NASH; Polypharmacology; Multi-target ligand; Partial FXR agonist

*Corresponding author at: Institute of Pharmaceutical Chemistry, Goethe University, Frankfurt, Germany. merk@pharmchem.uni-frankfurt.de (D. Merk).

¹J.D.I. and D.M. contributed equally to senior authorship and are listed in alphabetical order.

Author contributions

Md.A.H.K and A.S. performed experiments with the diet-induced NASH model, J.S. synthesized the study compound, D.M. developed the dual modulator concept and designed the FXRA/sEH, J.D.I. and D.M. supervised the project, and analyzed and interpreted the data. Md.A.H.K., J.D.I. and D.M. wrote the manuscript. All authors have given approval to the final version of the manuscript.

Declaration of interest

The development of the dual modulator has been financially supported by Fortress Biotech (New York City, U.S.).

1. Introduction

Non-alcoholic steatohepatitis (NASH) constitutes a major challenge to healthcare with an alarming global prevalence estimated at 3–15% of adults worldwide [1,2]. Although the global prevalence is increasing, there is a lack of pharmacological treatment options. NASH has its roots in non-alcoholic fatty liver disease (NAFLD) which is the hepatic manifestation of metabolic syndrome and currently NAFLD is the most prevalent chronic liver disease affecting around 30% of the worldwide population [1,3]. From the reversible NAFLD condition, NASH manifests with hepatic inflammation and fibrosis that can progress to liver cirrhosis or hepatocellular carcinoma [1]. Development and progression of NAFLD to NASH depends on several hallmarks. NAFLD originates from excessive caloric intake, lack of physical exercise, and insulin resistance leading to increased free fatty acids and lipotoxic lipids that accumulate in the liver to trigger oxidative stress, mitochondrial dysfunction, and hepatic inflammation [1,2,4]. Hepatitis and oxidative stress damage in hepatocytes ultimately results in apoptosis to cause replacement by irreversibly fibrotic tissue and NASH [1,2,4].

Several therapeutic strategies and molecular targets have been proposed to address the various factors contributing to NAFLD/NASH [5]. One target is the bile acid activated transcription factor farnesoid X receptor (FXR) that provides crucial liver protection. FXR activation prevents hepatocyte damage and reduces hepatic fat accumulation. FXR activators are leading the NASH pipeline and have already revealed therapeutic efficacy [5–7]. In addition, several other experimental strategies to counter steatosis, fibrosis, or inflammation in NASH are being investigated in clinical trials [8,9].

The promise for FXR activation to treat NASH is dampened by potential side-effects. Although efficacy of obeticholic acid (OCA) in clinical trials has validated FXR as target to treat NASH, the drug has revealed FXR mechanism-based side-effects [6,10]. OCA causes disturbances in cholesterol homeostasis with increased total cholesterol levels and a decrease in the HDL-c/non-HDL-c ratio [10]. This adverse effect of OCA can be ascribed to FXR-mediated repression of cholesterol 7 α -hydroxylase (CYP7A1), the rate-limiting enzyme for cholesterol conversion to bile acids [11–14]. Partial FXR activation appears to be a suitable approach to avoid this putative FXR mechanism-based side-effect [15,16].

Another emerging therapeutic target for NASH and other metabolic disorders is the enzyme soluble epoxide hydrolase (sEH). The sEH enzyme acts on the CYP branch of the arachidonic acid cascade metabolizing epoxyeicosatrienoic acids (EETs) to their corresponding diols with actions that can treat NAFLD/NASH [17,18]. EETs are important lipid mediators in inflammation, mitochondrial function, and cardiovascular homeostasis [17]. Inhibiting sEH increases EETs and sEH inhibitors have been studied in clinical trials for their anti-diabetic effects [17,19]. Experimental studies with sEH inhibition have revealed anti-inflammatory activity in liver and have demonstrated efficacy of sEH knockout or inhibition with small molecules in NASH animal models [20–22]. In addition, sEH inhibition was found to prevent fibrosis in fibrotic disease animal models [23,24]. Together these results point to great therapeutic value for sEH inhibition in NASH as it could counter the two key disease factors of hepatic inflammation and fibrosis.

NAFLD/NASH represent a multifactorial complex disease involving hepatic fat accumulation, oxidative stress, hepatic inflammation, hepatocyte apoptosis, and liver fibrosis. Therefore, we hypothesized that a dual mode of action involving partial FXR activation and sEH inhibition would generate additive therapeutic efficacy in NASH by combining anti-steatotic, anti-fibrotic, and anti-inflammatory actions [25]. Moreover, as this approach involves modest FXR activation, it should avoid FXR mediated disruption of cholesterol homeostasis.

2. Materials & methods

2.1. Materials

All chemicals were purchased from Sigma-Aldrich unless mentioned otherwise. The dual modulator FXRA/sEHi was prepared at the Institute of Pharmaceutical Chemistry, Goethe University Frankfurt, Germany according to the previously reported procedure [16].

2.2. Methods

2.2.1. Animal studies—Two sets of animal studies involving two separate NASH models were carried out to assess the ability for an FXRA/sEHi to prevent NASH progression. Efficacy for the FXRA/sEHi on NASH was studied using several biochemical, molecular biological, and histopathological techniques to analyze serum and liver tissues.

2.2.1.1. Streptozotocin-induced mice NASH model.: This animal study was carried out in accordance with the Japanese Pharmacological Society Guidelines for Animal Use. Animals were housed in the SMC laboratories (Tokyo, Japan) with a 12/12 h light-dark cycle and free access to water and chow. Experiments were carried out in 32 adult male C57BL/6 mice. Two days after birth, NASH was induced by a single injection of a 200 µg streptozotocin solution followed by high-fat diet (HFD, CLEA Japan, Japan) feeding started at 4 weeks of age. At 6 weeks of age the mice started to receive FXRA/sEHi, obeticholic acid or vehicle treatments for 4 weeks. In the treatment protocol, the drugs were prepared in vehicle (0.99% HPMC and 0.01% Tween 80). The NASH-mice were divided into four groups (n = 8/group). Group 1 received two doses of vehicle daily. Group 2 received FXRA/sEHi (3 mg/kg, thrice daily), Group 3 received FXRA/sEHi (10 mg/kg, thrice daily), and Group 4 received obeticholic acid (OCA, 10 mg/kg, once daily). All drugs were administered orally and the volume of drug solution was 10 ml/kg for all drugs. At the end of the study, the animals were euthanized with an overdose of isoflurane and exsanguinated through direct cardiac puncture. Liver tissue was collected, weighed and processed for histology studies. Non-fasting blood was collected for serum and plasma.

2.2.1.2. Choline-deficient high-fat diet induced mice NASH model.: Animals were housed at the Medical College of Wisconsin with a 12/12 h light-dark cycle and free access to water and chow. This study was approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee that coincides with the National Institutes of Health Guidelines. Experiments were carried out in 18 (n = 6/group) adult (8–12 weeks old) male C57BL/6J mice. A diet-induced mouse NASH model was evaluated. Mice were fed a commercially available methionine-choline deficient (MCD) diet (D518810, Dyets Inc.,

Bethlehem, PA) or a composition-matched methionine-choline replete control diet (D518754) for 4 weeks. Dietary depletion of methionine and choline leads to hepatic steatosis, liver injury, inflammation, and fibrosis, and is a widely used preclinical model of NASH [26]. NASH mice were treated with vehicle (0.01% DMSO and 0.05% cyclodextrin) or FXRA/sEHi (10 mg/kg/d) in drinking water for 4 weeks. Water intake was monitored daily to ensure a proper dosing. FXRA/sEHi concentration was adjusted if water consumption changed during the protocol. Mice were euthanized with an overdose of isoflurane using an induction chamber until the animal was unresponsive to a toe pinch or other stimuli followed by thoracotomy. Liver tissue was collected and snap-frozen in liquid nitrogen and processed for histology and mRNA expression studies.

2.2.2. Biochemical analysis

Serum alanine aminotransferase (ALT) levels were measured by FUJI DRI-CHEM 7000 (Fujifilm Corporation, Japan). Non-fasting serum total triglyceride, total cholesterol, HDL-cholesterol, LDL-cholesterol and VLDL-cholesterol were quantified by HPLC (Skylight Biotech Inc., Japan).

2.2.3. Histological analysis

Liver sections were fixed in Boin's solution or in 10% buffered formalin. The fixed liver sections were paraffin embedded and cut into sections for histological analysis. The sections were stained with Lillie-Mayer's Hematoxylin (Muto Pure Chemicals Co, Japan) and eosin solution (Wako Pure Chemical Industries, Japan). NAFLD activity score (NAS) was calculated according to the criteria of Kleiner et al. [27]. To study collagen accumulation, the sections were stained with Picrosirius Red (PSR, Waldeck, Germany) and examined in bright field images at a magnification of 200 $\times$ using a digital camera (DFC295, Leica, Germany) and ImageJ software (National Institute of Health, USA). Hepatic steatosis was assessed using frozen liver sections (10 μ m) stained with Oil Red O dye using the Oil Red O staining (Abcam, Cambridge, MA, USA). To assess the extent of steatosis, the percent of liver tissue area covered by oil-red positive lipid droplets was calculated as described previously [28]. The percentage area positive for lipid (red color) was calculated from the mean of 20 fields (200 $\times$) for each animal using Nikon NIS Elements Software (Nikon Instruments Inc., Melville, NY, USA). All histological analysis was carried out by at least two blinded observers.

2.2.4. Real-time PCR analysis

Hepatic mRNA expression of fibrotic marker α -smooth muscle actin (α -SMA, *Acta2*), fibroblast specific protein-1 (FSP-1, *Atf1*) and fibronectin (*Fn1*) were studied using Real Time-PCR (RT-PCR) analysis. RT-PCR analysis was also used to study the hepatic mRNA expression of inflammatory markers tumour necrosis factor- α (TNF- α , *Tnf- α*), interleukin 1beta (IL-1 β , *Il1b*) and transforming growth factor- β (TGF- β , *tgfb1*). Additionally, we further studied hepatic chemokine mRNA expression of C-X-C motif chemokine receptor CXCR3 (*Cxcr3*) and its ligands CXCL9 (*Cxcl9*) and CXCL10 (*Cxcl10*) using RT-PCR. Each sample was run in triplicate, and the comparative threshold cycle (C_t) method was used to quantify the change in the expression of target genes and the detail of the RT-PCR

analysis is described earlier. The primer sequences of the genes used in this study are provided in Table 1 [29].

2.2.5. Statistical analysis

All data are expressed as mean $\pm$ S.E.M. To determine statistical significance between groups, data was analyzed using GraphPad Prism 7 (*GraphPad Software Inc.*, USA) as follows: Normality of data distribution was analyzed by Shapiro-Wilk's test ($p < 0.05$ for normal distribution). For normally distributed data one-way ANOVA with Dunnett's T3 test was performed. For not normally distributed data, Kruskal-Wallis one-way analysis of variance was applied followed by Dunn's test to calculate significance. P values < 0.05 were considered as statistically significant. P values > 0.1 were assumed as trend or tendency.

3. Results

3.1. FXRA/sEHi characterization

We have recently developed a fatty acid mimetic with dual FXR agonist/sEH inhibitor activity (FXRA/sEHi) that partially activates FXR ($35 \pm 1\%$ efficacy) with an EC_{50} value of 20 ± 4 nM and simultaneously inhibits sEH with an IC_{50} value of 4.1 ± 0.4 nM (Fig. 1) [16,30]. Beyond these two selected molecular targets, FXRA/sEHi turned out highly selective and non-toxic. Extensive *in vitro* characterization of the FXRA/sEHi revealed attractive modulation of FXR regulated gene expression with partial efficacy compared to full FXR activators such as OCA and GW4064 [16]. Moreover, the FXRA/sEHi produced anti-inflammatory effects in HepG2 cells involving reduced NF- κ B expression and diminished release of inflammatory cytokines (TNF- α , IL-12p70) [16]. These effects partly exceeded the activities of a reference FXR agonist (OCA) and a reference sEH inhibitor (CIU) suggesting potential synergy of the two modes of action [16].

Initial evaluation of the FXRA/sEHi in mice confirmed pharmacodynamic modulation of FXR and sEH targets *in vivo* and revealed favorable pharmacokinetic properties rendering the compound suitable for disease animal studies [16]. A single 10 mg/kg oral dose of the FXRA/sEHi produced significant induction of FXR regulated gene expression in mice and shifted EET/DHET ratios in mouse plasma towards higher EET levels [16]. Here we report the activity of the innovative dual modulator in two common rodent models of NASH, streptozotocin induced NASH in mice and methionine choline-deficient high-fat diet induced NASH in mice [26].

3.2. FXRA/sEHi prevented hepatic steatosis in toxin-induced NASH

As a proof-of-concept model, we studied the FXRA/sEHi in streptozotocin-induced NASH in mice compared to OCA (10 mg/kg) as reference. Based on previously reported pharmacokinetic data and the observation that a single oral dose of 10 mg/kg produced strong pharmacodynamic effects *in vivo*, we selected 3 mg/kg and 10 mg/kg as treatment doses of the FXRA/sEHi [16]. The study drugs FXRA/sEHi and OCA were well tolerated and neither FXRA/sEHi nor OCA demonstrated any adverse effects on the mice health and behavior. Body and liver weights for the mice were similar across the experimental groups (Fig. 2). NASH mice demonstrated liver injury with marked fibrosis, steatosis and

hepatocyte ballooning, and other microscopic hepatocyte damage. The FXRA/sEH_i treated mice had reduced or no hepatocyte ballooning, liver fat accumulation, liver fibrosis, and microscopic hepatocyte damage (Fig. 3). These liver actions for the FXRA/sEH_i were dose-dependent. The low dose (3 mg/kg) diminished steatosis and fibrosis but hepatocyte damage was still observed in the histological samples. At 10 mg/kg, the FXRA/sEH_i prevented liver damage and histological samples were like that observed in mice treated with a well-known full FXR agonist, OCA (10 mg/kg). NASH mice demonstrated liver dysfunction and hyperlipidemia with elevated ALT, plasma triglyceride, and total cholesterol (Fig. 4). The FXRA/sEH_i dose-dependently reduced ALT and plasma lipids in the NASH mice. FXRA/sEH_i (10 mg/kg) treatment caused an improvement in cholesterol homeostasis with an increase in HDL- to non-HDL-cholesterol while this beneficial effect was not present in mice receiving OCA. Additionally, we demonstrated elevated NAFLD activity score (NAS) in the NASH mice. Both FXRA/sEH_i (10 mg/kg) and OCA (10 mg/kg) improved NAS and had a beneficial effect in preventing NASH.

3.3. FXRA/sEH_i prevented hepatic fibrosis and steatosis in diet-induced NASH

With the encouraging efficacy of the FXRA/sEH_i in treating toxin-induced NASH, we induced NASH in mice by methionine-choline deficient (MCD) diet to further characterize the drug in a complementary model. Based on the strong effects observed for the 10 mg/kg FXRA/sEH_i dose in toxin-induced NASH, this dose was maintained for the diet-induced model and compared to vehicle as well as animals receiving healthy replacement diet. In the MCD diet-induced NASH model mice developed hepatic fibrosis with 45% higher collagen positive hepatic area compared to control (Fig. 5a, b). Moreover, the hepatic fibrosis in this model was associated with a 2–5-fold higher liver mRNA expression of fibrotic markers α -SMA, fibronectin, and FSP-1 compared to control (Fig. 5c). Treatment with the FXRA/sEH_i markedly blunted hepatic fibrosis development and the treated mice had a 50% lower collagen positive area and 50–70% lower fibrotic marker mRNA expression compared to vehicle (Fig. 5). NASH mice also revealed a 50% increase in hepatic lipid accumulation compared to control indicative of hepatic steatosis. Treatment with the dual modulator FXRA/sEH_i markedly reduced hepatic steatosis development in NASH mice and hepatic lipid accumulation was 60% lower compared to vehicle (Fig. 6).

3.4. FXRA/sEH_i blunted hepatic inflammation in NASH

Liver mRNA expression of C-X-C motif chemokine receptor 3 (CXCR3) and its ligands CXCL9 and CXCL10 were 2.5–5-fold higher in vehicle treated NASH mice compared to control. In NASH mice, FXRA/sEH_i treatment decreased the expression of these three genes by almost 60% compared to NASH vehicle treated mice (Fig. 7a–c). Major inflammatory genes associated with CXCR3/CXCL-CXCL10 axis were increased in the liver of vehicle treated NASH mice compared to healthy control animals. Hepatic TNF- α , IL-1 β , and TGF- β mRNA expression was 2–4-fold higher compared to control mice. FXRA/sEH_i treatment prevented hepatic inflammation in NASH, and the expression of inflammatory markers was 45–75% lower compared to NASH mice with vehicle treatment (Fig. 7d–f).

4. Discussion

Considered as the hepatic manifestation of the metabolic syndrome, NAFLD and NASH arise as an enormous health burden with alarming prevalence and serious consequences. Despite intensive research for novel drugs to counter the metabolic liver disease, there is no pharmacotherapy available to date and the frontrunner in the NASH pipeline obeticholic acid while providing therapeutic efficacy could have limiting adverse effects according to current data [6,10]. The need for effective and safe NASH therapies, therefore, remains very high.

We hypothesized that a dual mode of action strategy would be a superior approach for two reasons. First, NAFLD and NASH are a complex multifactorial disease with several hallmarks contributing to its manifestation and progression. Second, FXR has been validated as a suitable target for NASH treatment but strong FXR activation appears to cause serious disturbances in cholesterol homeostasis as a side effect. Partial FXR activation, however, could exploit the beneficial effects of FXR activation while avoiding marked effects on cholesterol metabolism. To interfere with NASH progression at several points and to support partial FXR activation, we have chosen soluble epoxide hydrolase inhibition as the secondary mode of action and developed a FXRA/sEHi that simultaneously hits both molecular targets. Such multi-target designed drugs provide numerous advantages and as a key characteristic can spare side-effects by modestly interfering with individual targets but through synergies or additive effects retain high therapeutic efficacy [25]. Our dual FXR/sEH modulator FXRA/sEHi possesses low nanomolar dual potency and we have demonstrated previously that it engages on both targets *in vivo*. Initial evaluation of the FXRA/sEHi in mice revealed that the compound after a single oral dose (10 mg/kg) causes a robust shift of EET/DHET (sEH substrate/product) ratios *in vivo* towards higher substrate levels [16]. Moreover, we observed marked modulation of FXR regulated gene expression in mouse livers [16]. Encouraged by this verification of target engagement on both FXR and sEH *in vivo*, we have applied the FXRA/sEHi to further *in vivo* evaluation and here characterize its therapeutic efficacy in two NASH rodent models. Based on the FXRA/sEHi's favorable pharmacokinetics and the observation that 10 mg/kg of the FXRA/sEHi produced robust pharmacodynamic effects in the initial *in vivo* evaluation, we selected this effective dose and a lower dose of 3 mg/kg for treatment in the NASH models.

In a common toxin-induced NASH model where the disease is established by application of streptozotocin to mice two days after birth followed by high-fat diet, the FXRA/sEHi was well tolerated and appeared safe. FXRA/sEHi treatment for four weeks markedly improved liver histopathologies in a dose-dependent manner compared to animals receiving vehicle. Toxin-induced NASH mice that were vehicle treated revealed strong fat accumulation, hepatocyte damage/ballooning, and fibrosis. These histopathological hallmarks of NAFLD/NASH were improved by 3 mg/kg FXRA/sEHi and fully prevented by 10 mg/kg FXRA/sEHi as well as the FXR agonist obeticholic acid (10 mg/kg), which was used as positive control. Moreover, our multi-target agent at 10 mg/kg significantly reduced serum ALT levels suggesting beneficial effects on liver health, which was not observed for the positive control OCA.

Further biochemical analysis revealed that the FXRA/sEHi dose-dependently improved metabolic parameters including lower plasma triglycerides and lower total cholesterol levels. These positive FXRA/sEHi (10 mg/kg) effects on lipid profiles were not present in toxin-induced NASH mice receiving the positive control OCA. On cholesterol homeostasis, which is known to be disturbed by long-term OCA treatment in men, the FXRA/sEHi had a beneficial impact, too. In addition to the trend towards lower total cholesterol levels, the FXRA/sEHi (10 mg/kg) increased the HDL-c/non-HDL-c ratio indicating improved cholesterol balance. OCA had no effect on the HDL-c/non-HDL-c ratio. These data demonstrate that FXRA/sEHi treated toxin-induced NASH and has superior therapeutic potential compared to FXR activation by OCA.

Evaluation of NAFLD activity scores (NAS) in all three groups showed modest improvements for both study drugs (FXRA/sEHi and OCA) that failed to reach statistical significance probably due to the relatively mild disease achieved in this toxin-induced NASH model. Closer evaluation suggested that the FXRA/sEHi (10 mg/kg) had a stronger effect on the steatosis score compared to OCA which could arise from synergies between FXR and sEH modulation to counter hepatic fat accumulation. The ballooning score, which depicts hepatocyte damage was more improved by OCA which is a more effective liver cell protector FXR agonist. Overall, the streptozotocin induced NASH study confirmed therapeutic efficacy for the FXRA/sEHi, suggested that partial FXR activation supported by sEH inhibition as secondary mode of action is suitable to treat NASH *in vivo*, and encouraged further *in vivo* evaluation of the FXRA/sEHi.

We, therefore, expanded the *in vivo* profiling of the FXRA/sEHi (10 mg/kg) to a diet-induced NASH model that better reflects the pathophysiological development and condition of the disease. NASH was induced in mice by a methionine choline-deficient high-fat diet, which is a common NASH rodent model. Compared to healthy mice receiving normal diet, NASH mice developed strong liver steatosis and fibrosis accompanied by hepatocyte damage. Treatment with the FXRA/sEHi for four weeks prevented this manifestation of NASH. In mice receiving the FXRA/sEHi, liver histology, collagen positive area and steatosis area at the end of the study were not distinguishable from the healthy controls. Hence, the FXRA/sEHi was also effective in preventing diet induced NASH. Because fibrosis is a key hallmark of NASH and currently the strongest predictor for disease progression, we evaluated the effects of the FXRA/sEHi on fibrosis markers. In congruence with its strong efficacy in preventing fibrosis, the FXRA/sEHi reduced liver mRNA expression of fibrosis markers α -SMA, FSP-1, and fibronectin to or below baseline levels while these genes were significantly induced in NASH mice. The dual strategy involving inhibition of sEH was particularly designed to add anti-inflammatory activity to the known anti-steatotic and anti-fibrotic effects of FXR activation in NASH. Therefore, we studied effects of FXRA/sEHi in the mouse livers to investigate its anti-inflammatory activity on NASH development.

CXC chemokines play an important role in NASH pathophysiology that is characterized by an inflamed and fibrotic liver [31,32]. One chemokine receptor that importantly contributes to chronic liver inflammation is CXCR3 a G protein-coupled receptor for the chemokines CXCL9 and CXCL10 [31,33]. In the present study, we observed an elevation in hepatic

CXCR3 mRNA expression and its ligands CXCL9 and CXCL10 in MCD diet-induced NASH mice. This finding is consistent with reports of upregulated liver mRNA and protein CXCR3 levels in pre-clinical NASH models and NAFLD patients [31]. Elevated levels of CXCR3 ligands, CXCL9 and CXCL10 are functionally linked to hepatic injury, inflammation, and fibrosis [34]. CXCL9 and CXCL10 are associated with NF- κ B activation and the induction of pro-inflammatory cytokines including TNF- α and IL-1 β in NASH [31,33,35,36]. Previous studies with the FXRA/sEHi have observed reduced NF- κ B expression and decreased TNF- α generation in HepG2 cells [16]. Accordingly, in this study we demonstrate that MCD-diet induced NASH mice displayed significantly increased hepatic expression of inflammatory cytokines TNF- α , IL-1 β , and TGF- β . Dual FXR and sEH targeting with FXRA/sEHi reduced chemokine CXCR3, CXCL9, and CXCL10 expression and inflammatory cytokines TNF- α , IL-1 β , and TGF- β in NASH mice. Our findings clearly demonstrate marked anti-fibrotic and anti-inflammatory actions of FXRA/sEHi in NASH and link these effects to reduced chemokine CXCR3 and inflammatory cytokine activation as part of the FXRA/sEHi's molecular mechanism of action. The FXRA/sEHi prevented the inflammatory response in NASH and reduced cytokine expression to a level similar to that of healthy control mice.

In summary, common toxin- and diet-induced NASH models have clearly confirmed the therapeutic efficacy of our dual FXRA/sEHi modulator highlighting designed multi-target drugs as a potentially safer and more effective treatment strategy in the multifactorial condition of NAFLD/NASH. The simultaneous partial FXR activation and sEH inhibition resulted in a triad of anti-NASH effects with anti-steatotic, anti-fibrotic, and anti-inflammatory activity in the liver. Additionally, the FXRA/sEHi improved metabolic parameters with reduced plasma triglycerides and a favorable cholesterol profile. This provides compelling evidence that the partial FXR activation at least in mice avoids the mechanism-based side effect of disturbed cholesterol homeostasis arising from strong FXR activation. Thereby, the second rationale for applying a multi-target drug to treat NASH is equally confirmed and supported by the results.

The results from our *in vivo* profiling of a dual FXR activator and sEH inhibitor confirm therapeutic efficacy of this multi-target strategy with a triad of anti-NASH effects. This highlights the potential of designed polypharmacology in the treatment of multifactorial metabolic diseases and advocates the use of multi-targeted strategies for a safer and more effective NASH treatment. With its highly promising *in vivo* profile, our FXRA/sEHi appears suitable for further preclinical evaluation and development towards clinical trials.

Acknowledgements

Technical assistance provided by Lauren Kolb of the Department of Pharmacology & Toxicology, Medical College of Wisconsin, Milwaukee, WI 53226, USA is gratefully acknowledged. The authors thank Jason Wang and Martin Raditsch for their support in the project.

Funding information

JDI was funded by a National Institute of Health (NIH) grant (DK103616) and Dr. Ralph and Marian Falk Medical Research Trust Bank of America, N.A., Trustee grant. DM was supported by an ETH Zurich Fellowship (Grant 16-2 FEL-07). Fortress Biotech financially supported the streptozotocin-induced NASH model.

Abbreviations:

ALT	alanine aminotransferase
α-SMA	α -smooth muscle actin
CXCR3	C-X-C motif chemokine receptor 3
CXCL9	chemokine (C-X-C motif) ligand 9
CXCL10	chemokine (C-X-C motif) ligand 10
CYP7A1	cholesterol 7 α -hydroxylase
FGF-19	fibroblast growth factor 19
FSP-1	fibroblast specific protein-1
FXR	farnesoid X receptor
HDL-c	high density lipoprotein cholesterol
HFD	high-fat diet
IL-1β	interleukin 1beta
LDL-c	low density lipoprotein cholesterol
MCD	methionine-choline deficient
NAFLD	non-alcoholic fatty liver disease
NAS	NAFLD activity score
NASH	non-alcoholic steatohepatitis
OCA	obeticholic acid
PSR	Picrosirius Red
sEH	soluble epoxide hydrolase
SHP	small heterodimer partner
TGF-β	transforming growth factor β
TNF-α	tumour necrosis factor- α
VLDL-c	very low density lipoprotein cholesterol

References

- [1]. Neuschwander-Tetri BA, Non-alcoholic fatty liver disease, BMC Med 15 (2017) 45, 10.1186/s12916-017-0806-8. [PubMed: 28241825]
- [2]. Bessone F, Razori MV, Roma MG, Molecular pathways of nonalcoholic fatty liver disease development and progression, Cell. Mol. Life Sci (2018), 10.1007/s00018-018-2947-0.

- [3]. Lucas C, Lucas G, Lucas N, Krzowska-Firych J, Tomasiewicz K, A systematic review of the present and future of non-alcoholic fatty liver disease, *Clin. Exp. Hepatol* 4 (2018) 165–174, 10.5114/ceh.2018.78120. [PubMed: 30324141]
- [4]. Buzzetti E, Pinzani M, Tsochatzis EA, The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD), *Metabolism* 65 (2016) 1038–1048, 10.1016/j.metabol.2015.12.012. [PubMed: 26823198]
- [5]. Oseini AM, Sanyal AJ, Therapies in non-alcoholic steatohepatitis (NASH), *Liver Int* 37 (2017) 97–103, 10.1111/liv.13302. [PubMed: 28052626]
- [6]. Neuschwander-Tetri BA, Loomba R, Sanyal AJ, Lavine JE, Van Natta ML, Abdelmalek MF, Chalasani N, Dasarthy S, Diehl AM, Hameed B, Kowdley KV, McCullough A, Terrault N, Clark JM, Tonascia J, Brunt EM, Kleiner DE, Doo E, Farnesoid X nuclear receptor ligand obeticholic acid for non-cirrhotic, non-alcoholic steatohepatitis (FLINT): a multicentre, randomised, placebo-controlled trial, *Lancet* 385 (2014) 956–965, 10.1016/S0140-6736(14)61933-4. [PubMed: 25468160]
- [7]. Sepe V, Distrutti E, Fiorucci S, Zampella A, Farnesoid X receptor modulators 2014-present: a patent review, *Expert Opin. Ther. Pat* 28 (2018) 351–364, 10.1080/13543776.2018.1459569. [PubMed: 29649907]
- [8]. Gellrich L, Merk D, Therapeutic potential of peroxisome proliferator-activated receptor modulation in non-alcoholic fatty liver disease and non-alcoholic steatohepatitis, *Nucl. Recept. Res* 4 (2017) 101310, 10.11131/2017/101310.
- [9]. Ratzu V, Harrison SA, Francque S, Bedossa P, Leher P, Serfaty L, Romero-Gomez M, Boursier J, Abdelmalek M, Caldwell S, Drenth J, Anstee QM, Hum D, Hanf R, Roudot A, Megnien S, Staels B, Sanyal A, Mathurin P, Gournay J, Nguyen-Khac E, De Ledinghen V, Larrey D, Tran A, Bourliere M, Maynard-Muet M, Asselah T, Henrion J, Nevens F, Cassiman D, Geerts A, Moreno C, Beuers UH, Galle PR, Spengler U, Bugianesi E, Craxi A, Angelico M, Fargion S, Voiculescu M, Gheorghe L, Preotescu L, Caballeria J, Andrade RJ, Crespo J, Callera JL, Ala A, Aithal G, Abouda G, Luketic V, Huang MA, Gordon S, Pockros P, Poordad F, Shores N, Moehlen MW, Bambha K, Clark V, Satapathy S, Parekh S, Reddy RK, Sheikh MY, Szabo G, Vierling J, Foster T, Umpierrez G, Chang C, Box T, Gallegos-Orozco J, Elafibranor, an agonist of the peroxisome proliferator-activated receptor- α and - δ , induces resolution of non-alcoholic steatohepatitis without fibrosis worsening, *Gastroenterology* 150 (2016), 10.1053/j.gastro.2016.01.038 1147–1159e5. [PubMed: 26874076]
- [10]. Mudaliar S, Henry RR, Sanyal AJ, Morrow L, Marschall HU, Kipnes M, Adorini L, Sciacca CI, Clopton P, Castelloe E, Dillon P, Pruzanski M, Shapiro D, Efficacy and safety of the farnesoid x receptor agonist Obeticholic acid in patients with type 2 diabetes and nonalcoholic fatty liver disease, *Gastroenterology* 145 (2013) 574–582.e1, 10.1053/j.gastro.2013.05.042. [PubMed: 23727264]
- [11]. Flesch D, Cheung S-Y, Schmidt J, Gabler M, Heitel P, Kramer JS, Kaiser A, Hartmann M, Lindner M, Lüddens-Dämgen K, Heering J, Lamers C, Lüddens H, Wurglics M, Proschak E, Schubert-Zsilavecz M, Merk D, Non-acidic farnesoid X receptor modulators, *J. Med. Chem* 60 (2017) 7199–7205, 10.1021/acs.jmedchem.7b00903. [PubMed: 28749691]
- [12]. Merk D, Steinhilber D, Schubert-Zsilavecz M, Medicinal chemistry of farnesoid X receptor ligands: from agonists and antagonists to modulators, *Future Med. Chem* 4 (2012), 10.4155/fmc.12.47.
- [13]. Liu H, Pathak P, Boehme S, Chiang JYL, Cholesterol 7 α -hydroxylase protects the liver from inflammation and fibrosis by maintaining cholesterol homeostasis, *J. Lipid Res* 57 (2016) 1831–1844, 10.1194/jlr.M069807. [PubMed: 27534992]
- [14]. McMillin M, Grant S, Frampton G, Petrescu AD, Kain J, Williams E, Haines R, Canady L, DeMorrow S, FXR-mediated cortical cholesterol accumulation contributes to the pathogenesis of type A hepatic encephalopathy, *Cell. Mol. Gastroenterol. Hepatol* 6 (2018) 47–63, 10.1016/j.jcmgh.2018.02.008. [PubMed: 29928671]
- [15]. Merk D, Lamers C, Ahmad K, Carrasco Gomez R, Schneider G, Steinhilber D, Schubert-zsilavecz M, Gomez RC, Schneider G, Steinhilber D, Schubert-zsilavecz M, Extending the structure-activity relationship of anthranilic acid derivatives as farnesoid X receptor modulators:

development of a highly potent partial farnesoid X receptor agonist, *J. Med. Chem* 57 (2014) 8035–8055, 10.1021/jm500937v. [PubMed: 25255039]

- [16]. Schmidt J, Rotter M, Weiser T, Wittmann S, Weizel L, Kaiser A, Heering J, Goebel T, Angioni C, Wurglics M, Paulke A, Geisslinger G, Kahnt A, Steinhilber D, Proschak E, Merk D, A dual modulator of farnesoid X receptor and soluble epoxide hydrolase to counter nonalcoholic steatohepatitis, *J. Med. Chem* 60 (2017) 7703–7724, 10.1021/acs.jmedchem.7b00398. [PubMed: 28845983]
- [17]. Imig JD, Prospective for cytochrome P450 epoxigenase cardiovascular and renal therapeutics, *Pharmacol. Ther* 192 (2018) 1–19, 10.1016/j.pharmthera.2018.06.015. [PubMed: 29964123]
- [18]. Imig JD, Epoxides and soluble epoxide hydrolase in cardiovascular physiology, *Physiol. Rev* 92 (2012) 101–130, 10.1152/physrev.00021.2011. [PubMed: 22298653]
- [19]. He J, Wang C, Zhu Y, Ai D, Soluble epoxide hydrolase: a potential target for metabolic diseases, *J. Diab* 8 (2016) 305–313, 10.1111/1753-0407.12358.
- [20]. Schuck RN, Zha W, Edin ML, Gruzdev A, Vendrov KC, Miller TM, Xu Z, Lih FB, DeGraff LM, Tomer KB, Jones HM, Makowski L, Huang L, Poloyac SM, Zeldin DC, Lee CR, The cytochrome P450 epoxigenase pathway regulates the hepatic inflammatory response in fatty liver disease, *PLoS One* 9 (2014) e110162, 10.1371/journal.pone.0110162. [PubMed: 25310404]
- [21]. Liu Y, Dang H, Li D, Pang W, Hammock BD, Zhu Y, Inhibition of soluble epoxide hydrolase attenuates high-fat-diet-induced hepatic steatosis by reduced systemic inflammatory status in mice, *PLoS One* 7 (2012) e39165, 10.1371/journal.pone.0039165. [PubMed: 22720061]
- [22]. López-Vicario C, Alcaraz-Quiles J, García-Alonso V, Rius B, Hwang SH, Titos E, Lopategi A, Hammock BD, Arroyo V, Clària J, Inhibition of soluble epoxide hydrolase modulates inflammation and autophagy in obese adipose tissue and liver: role for omega-3 epoxides, *Proc. Natl. Acad. Sci* 112 (2015) 536–541, 10.1073/pnas.1422590112. [PubMed: 25550510]
- [23]. Kim J, Imig JD, Yang J, Hammock BD, Padanilam BJ, Inhibition of soluble epoxide hydrolase prevents renal interstitial fibrosis and inflammation, *Am. J. Physiol. Physiol* 307 (2014) F971–F980, 10.1152/ajprenal.00256.2014.
- [24]. Kim J, Yoon SP, Toews ML, Imig JD, Hwang SH, Hammock BD, Padanilam BJ, Pharmacological inhibition of soluble epoxide hydrolase prevents renal interstitial fibrogenesis in obstructive nephropathy, *Am. J. Physiol. Physiol* 308 (2015) F131–F139, 10.1152/ajprenal.00531.2014.
- [25]. Proschak E, Stark H, Merk D, Polypharmacology by design: a medicinal chemist's perspective on multitargeting compounds, *J. Med. Chem* 62 (2019) 420–444, 10.1021/acs.jmedchem.8b00760. [PubMed: 30035545]
- [26]. Takahashi Y, Soejima Y, Fukusato T, Animal models of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis, *World J. Gastroenterol* 18 (2012) 2300, 10.3748/wjg.v18.i19.2300. [PubMed: 22654421]
- [27]. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Ferrell LD, Liu Y-C, Torbenson MS, Unalp-Arida A, Yeh M, McCullough AJ, Sanyal AJ, Nonalcoholic Steatohepatitis Clinical Research Network, Design and validation of a histological scoring system for nonalcoholic fatty liver disease, *Hepatology* 41 (2005) 1313–1321, 10.1002/hep.20701. [PubMed: 15915461]
- [28]. Hye Khan MA, Kolb L, Skibba M, Hartmann M, Blöcher R, Proschak E, Imig JD, A novel dual PPAR- γ agonist/sEH inhibitor treats diabetic complications in a rat model of type 2 diabetes, *Diabetologia* 61 (2018) 2235–2246, 10.1007/s00125-018-4685-0. [PubMed: 30032428]
- [29]. Skibba M, Hye Khan MA, Kolb LL, Yeboah MM, Falck JR, Amaradhi R, Imig JD, Epoxyeicosatrienoic acid analog decreases renal fibrosis by reducing epithelial-to-mesenchymal transition, *Front. Pharmacol* 8 (2017) 406, 10.3389/fphar.2017.00406. [PubMed: 28713267]
- [30]. Proschak E, Heitel P, Kalinowsky L, Merk D, Opportunities and challenges for fatty acid mimetics in drug discovery, *J. Med. Chem* 60 (2017) 5235–5266, 10.1021/acs.jmedchem.6b01287. [PubMed: 28252961]
- [31]. Zhang X, Han J, Man K, Li X, Du J, Chu ESH, Go MY, Sung JJY, Yu J, CXC chemokine receptor 3 promotes steatohepatitis in mice through mediating inflammatory cytokines,

macrophages and autophagy, *J. Hepatol* 64 (2016) 160–170, 10.1016/j.jhep.2015.09.005. [PubMed: 26394162]

- [32]. Xu L, Kitade H, Ni Y, Ota T, Roles of chemokines and chemokine receptors in obesity-associated insulin resistance and nonalcoholic fatty liver disease, *Biomolecules* 5 (2015) 1563–1579, 10.3390/biom5031563. [PubMed: 26197341]
- [33]. Tacke F, Zimmermann HW, Berres M-L, Trautwein C, Wasmuth HE, Serum chemokine receptor CXCR3 ligands are associated with progression, organ dysfunction and complications of chronic liver diseases, *Liver Int* 31 (2011) 840–849, 10.1111/j.1478-3231.2011.02504.x. [PubMed: 21645215]
- [34]. Marra F, Tacke F, Roles for chemokines in liver disease, *Gastroenterology* 147 (2014) 577–594.e1, 10.1053/j.gastro.2014.06.043. [PubMed: 25066692]
- [35]. Manousou P, Kolios G, Drygiannakis I, Koulentaki M, Pyrovolaki K, Voumvouraki A, Notas G, Bourikas L, Papadaki HA, Kouroumalis E, CXCR3 axis in patients with primary biliary cirrhosis: a possible novel mechanism of the effect of ursodeoxycholic acid, *Clin. Exp. Immunol* 172 (2013) 9–15, 10.1111/cei.12032. [PubMed: 23480180]
- [36]. Berres M-L, Asmacher S, Lehmann J, Jansen C, Görtzen J, Klein S, Meyer C, Strunk HM, Fimmers R, Tacke F, Strassburg CP, Trautwein C, Sauerbruch T, Wasmuth HE, Trebicka J, CXCL9 is a prognostic marker in patients with liver cirrhosis receiving transjugular intrahepatic portosystemic shunt, *J. Hepatol* 62 (2015) 332–339, 10.1016/j.jhep.2014.09.032. [PubMed: 25457205]

Fig. 1.

Structure (a) and pharmacological profile (b/c) of the dual FXR agonist/sEH inhibitor (FXRA/sEHi, 1). (b) The FXRA/sEHi possesses low nanomolar potency on FXR with partial activation efficacy compared to the reference FXR agonist obeticholic acid (OCA) and the endogenous FXR agonist CDCA. The compound robustly induced hepatic FXR-regulated gene expression in mice after a single oral dose (10 mg/kg). (c) The FXRA/sEHi inhibits sEH with low nanomolar potency and is clearly more active than the reference sEH inhibitor CIU. In mice, the FXRA/sEHi shifted sEH substrate/product ratios to higher substrate levels indicating sEH inhibition. For details refer to the original publication [16]. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 2. Body and liver weight of animals in the streptozotocin-induced NASH model. The study drugs FXRA/sEHl (3 mg/kg or 10 mg/kg) and OCA (10 mg/kg) were well tolerated and had no effect on body weight or liver/body weight ratio in mice. All data expressed as mean $\pm$ S.E.M.; $n = 8$ /group.

Fig. 3. Liver histological analysis (Oil Red-O staining and Sirius Red staining) at the end of the streptozotocin-induced NASH model confirmed dose-dependent efficacy for the FXRA/sEH in preventing hepatic steatosis and fibrosis. Compared to vehicle treated mice, animals receiving low-dose (3 mg/kg) FXRA/sEH displayed improvements in liver histology in terms of diminished steatosis and fibrosis. High-dose (10 mg/kg) FXRA/sEH strongly reduced steatosis, fibrosis and hepatocyte ballooning. The FXR agonist obeticholic acid (OCA, 10 mg/kg) was equally active.

Fig. 4.

Biochemical analysis at the end of the streptozotocin-induced NASH model. (a) Treatment with the FXRA/sEHi reduced serum alanine transferase (ALT) levels in a dose dependent manner compared to vehicle treated animals as marker for improved liver health. High-dose (10 mg/kg) FXRA/sEHi treatment resulted in significant ALT reduction. OCA (10 mg/kg) had no effect. (b) Treatment with the FXRA/sEHi (10 mg/kg) reduced serum triglyceride and total cholesterol levels and improved cholesterol balance indicated by an increased HDL-c/non-HDL-c ratio. Low-dose (3 mg/kg) FXRA/sEHi and OCA had no effect on lipid homeostasis. (c) The FXRA/sEHi had a dose-dependent effect on the NASH activity score (NAS). Both the FXRA/sEHi (10 mg/kg) and OCA (10 mg/kg) achieved a modest NAS improvement wherein the FXRA/sEHi was more effective in reducing steatosis while OCA mainly lowered the ballooning score. All data expressed as mean $\pm$ S.E.M.; $n = 8/\text{group}$; * $p < 0.05$, # $p < 0.1$.

Fig. 5.

Evaluation of hepatic fibrosis at the end of the MCD diet-induced NASH model. (a) Representative photomicrograph depicting collagen positive area (arrows) and (b) percentage of liver collagen positive area in all experimental groups (n = 5/group) at the end of the experimental protocol. (c) Liver mRNA expression of fibrotic markers α -smooth muscle actin (α -SMA), fibronectin, and fibroblast specific protein-1 (FSP-1). All data expressed as mean $\pm$ S.E.M.; n = 6/group; *p < 0.05.

Fig. 6.

Evaluation of hepatic steatosis at the end of the MCD diet-induced NASH model. (a) Percentage of liver Oil-Red positive area indicating hepatic steatosis in different experimental groups at the end of the *in vivo* study. (b) Representative photomicrograph depicting Oil-Red positive area. All data expressed as mean $\pm$ S.E.M.; $n = 6/\text{group}$; $*p < 0.05$.

Fig. 7.

Hepatic inflammation in MCD diet-induced NASH mice and anti-inflammatory effect of FXRA/sEH. Liver mRNA expressions of CXC motif chemokine receptor CXCR3 and its ligands CXCL9 and CXCL10 in different experimental groups (a-c). mRNA expression of TNF- α , IL-1 β and TGF- β in the mouse liver from all three experimental groups (d-f). All data expressed as mean $\pm$ S.E.M.; n = 6/group; *p < 0.05.

Table 1

Sequences of the primers used in RT PCR experiment.

Gene	Forward Sequence	Reverse Sequence
TNF- α (<i>Tnf-α</i>)	CGACTGACAAAGCCTGTAGCC	GAGAACCTGGGAGTAGACAAGG
IL1- β (<i>Il1-β</i>)	AAGGAGAACCAAGCAACGAC	AACTCTGCAGACTCAAACTCCAC
TGF- β 1 (<i>Tgf-β1</i>)	AGTGTGGAGCAACATGTGGAA	CACTCAGGCGTATCAGTGGG
Fibronectin (<i>Fn1</i>)	C ACTGCAGAAACCAGAGGAGG	ATGGCGTAAATGGGAAACCCGT
α -SMA (<i>Acta2</i>)	CTACGAACTGCCCTGACGGG	GCTGTTATAGGTGGTTTCGTGG
FSP-1 (<i>Ahl1</i>)	TCAGCACATTCTCTCTCTTGGT	AGCTTGAAACTTGTCACCCCTCT
GAPDH (<i>Gapdh</i>)	AGGAGTAAGAAACCCCTGGAC	CTGGGATGGAAATTGTGAG
PGK-1 (<i>pgk1</i>)	GGAAGCGGGTCGTGATGA	GCCTTGATCCTTTGGTTGTTTG
CXCR3 (<i>Cxcr3</i>)	TCTCGTTTTTCCCCATAATCG	AGCCAAAGCCATGTACCTTGA
CXCL9 (<i>Cxcl9</i>)	CGAGGCACGATCCACTACAA	GAGTCCGGATCTAGGCAGGT
CXCL10 (<i>Cxcl10</i>)	ACTGCATCCATATCGATGAC	TTCATCGTGGCAATGATCTC