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Articles

Antihyperlipidemic Activity of Gut-Restricted LXR Inverse Agonists

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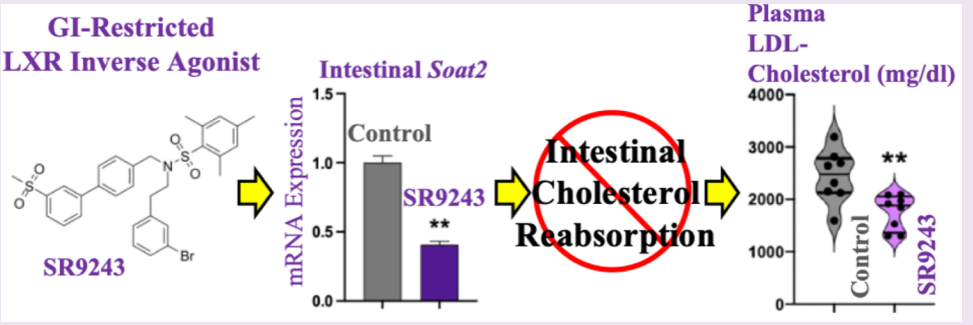

ABSTRACT: Hyperlipidemia and increased circulating cholesterol levels are associated with increased cardiovascular disease risk. The liver X receptors (LXRs) are regulators of *de novo* lipogenesis and cholesterol transport and have been validated as potential therapeutic targets for the treatment of atherosclerosis. However, efforts to develop LXR agonists to reduce cardiovascular diseases have failed due to poor clinical outcomes-associated increased hepatic lipogenesis and elevated low-density lipoprotein (LDL) cholesterol (C). Here, we report that LXR inverse agonists are effective in lowering plasma LDL cholesterol and triglycerides in several models of hyperlipidemia, including the *Ldlr* null mouse model of atherosclerosis. Mechanistic studies demonstrate that LXR directly regulates the expression of *Soat2* enzyme in the intestine, which is directly responsible for the re-uptake or excretion of circulating lipids. Oral administration of a gut-specific LXR inverse agonist leads to reduction of *Soat2* expression in the intestine and effectively lowers circulating LDL cholesterol and triglyceride levels without modulating LXR target genes in the periphery. In summary, our studies highlight the therapeutic potential of the gut-restricted molecules to treat hyperlipidemia and atherosclerosis through the intestinal LXR-*Soat2* axis.

INTRODUCTION

Cardiovascular disease (CVD) is a leading cause of death worldwide and often arises due to chronically elevated plasma cholesterol levels. Risk factors including poor diet, low activity levels, obesity, smoking, and diabetes are associated with an increase in circulating cholesterol levels and enhance the risk for more dangerous complications, including atherosclerosis, heart attack, and stroke. Current statin-based therapies for lowering LDL cholesterol levels are effective, but many patients do not reach target LDL-cholesterol target on statin therapy alone, and, additionally, many are unable to use statins due to significant side effects. Furthermore, individuals with familial hypercholesterolemia are resistant to the effects of hepatic LDLR-targeted therapies such as statins and PSCK9 inhibitors. Thus, additional therapeutics that effectively reduce LDL-cholesterol are needed.

The liver X receptors, LXRα and LXRβ, are nuclear receptors. LXRα is primarily expressed in the liver, kidneys, intestines, and adipose tissue, while LXRβ is widely expressed. They function as ligand-sensitive transcription factors and bind directly to specific DNA sequences known as LXR response elements (LXREs). Although they were discovered before their cognate ligands were identified, oxidized cholesterol metabolites were soon characterized as direct ligands (agonists) for both receptor proteins. Unliganded LXRs selectively recruit corepressors such as NCOR1 and SMRT to form repressor complexes at LXR target gene promoters.[1,2] Through this mechanism, LXRs silence the expression of many target genes in the absence of agonists. Conversely, LXR agonist binding induces dissociation of corepressor and recruitment of coactivators.

LXRs have been shown to regulate cholesterol efflux and transport, in addition to stimulating lipogenesis.[3−5] Synthetic

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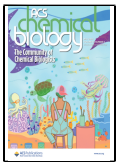

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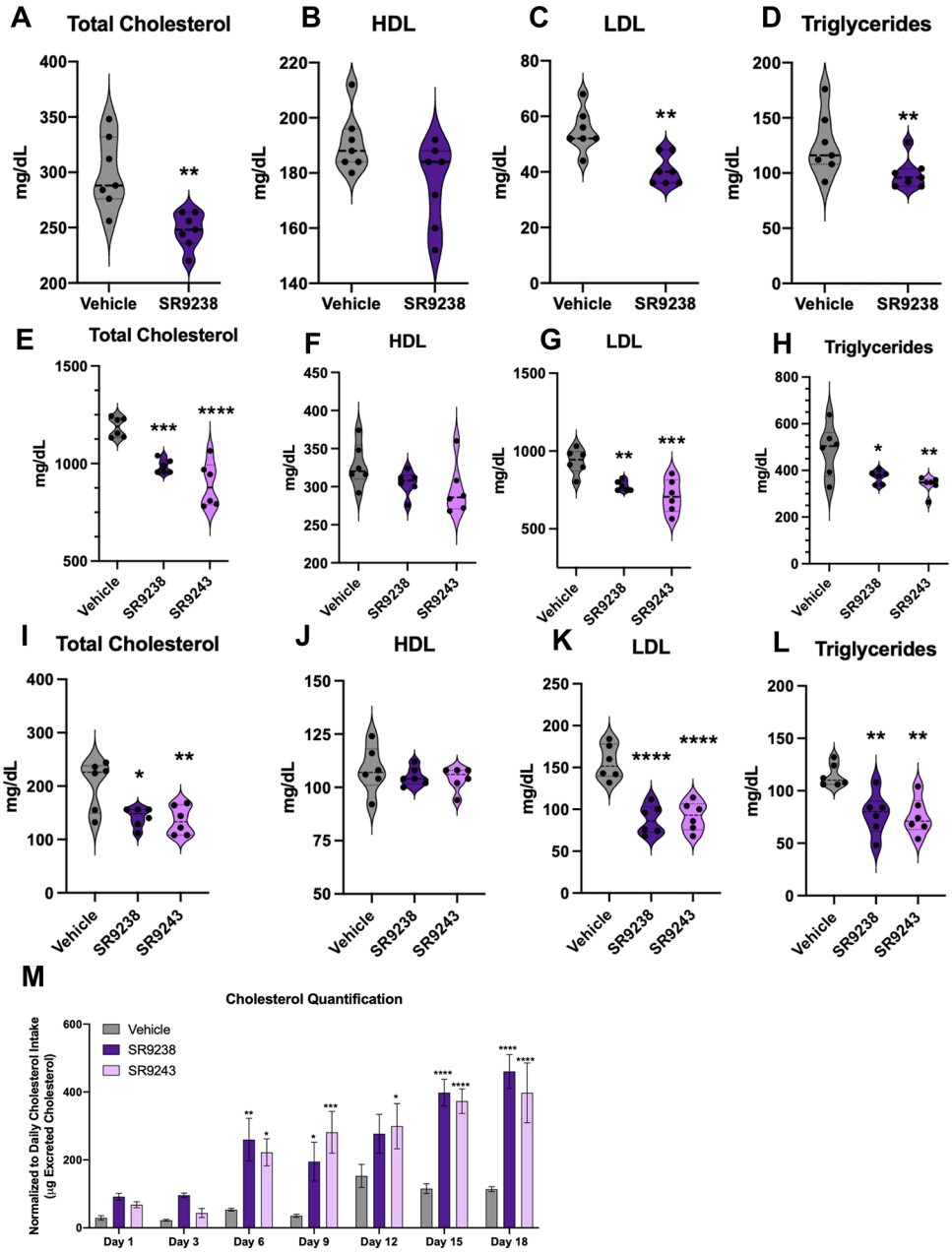

Figure 1. LXR inverse agonists reduce LDL and total cholesterol in mouse models of metabolic disease. In an obese mouse (*ob*/*ob*) fed standard chow, the LXR inverse agonist SR9238 (30 mg/kg, i.p., q.d.; $n = 7$) suppressed total cholesterol levels (A) compared to vehicle-treated mice (10% DMSO; 10% Tween-80; PBS). HDL levels (B) were unaffected by SR9238 treatment; however, LDL (C) and plasma triglyceride levels (D) were significantly reduced. Significance was assessed by Student's *t*-test (2-tailed). We then tested the effects of the LXR inverse agonists on *Ldlr* null mice fed a HCD. Similar to our previous study, the *Ldlr* null mice dosed with SR9238 or SR9243 (30 mg/kg, i.p., q.d.; $n = 8$) had reduced total cholesterol (E) levels. HDL was unaffected by the LXR inverse agonists (F). LDL cholesterol levels (G) and triglyceride levels (H) were also significantly reduced in the *Ldlr* null mouse. Significance was assessed by student's t-test (2-tailed). Plasma lipid level effects were then confirmed in a third model. C57Bl/6J mice fed a HCD and subsequently treated with vehicle (10% DMSO; 10% Tween-80; PBS; $n = 6$), SR9238 (30 mg/kg, i.p., q.d.; $n = 6$), or SR9243 (30 mg/kg, i.p., q.d.; $n = 6$). Terminal blood plasma was analyzed by clinical chemistry and confirmed the previous results. Total cholesterol (I) was significantly lowered in both drug-treated groups. While HDL remained unaffected by drug administration (J), both LDL (K) and plasma triglyceride levels (L) were also significantly reduced. Significance was assessed by one-way ANOVA with Dunnett's multiple comparisons test. Cholesterol fecal excretion was quantified using the Amplex Red system over a 3-week period from these same C57Bl/6J mice. Both LXR inverse agonists SR9238 and SR9243 significantly increased the amount of excreted cholesterol compared to vehicle-treated controls by Day 6 (M). Significant fecal cholesterol excretion in the test groups was consistent through the end of the study. Data was normalized to daily cholesterol intake as measured by food intake and assessed for significance using two-way ANOVA with Tukey's post-hoc. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, and $^{****}p \leq 0.0001$.

LXR agonists display antiatherogenic activity in animal models of cardiovascular disease due to their effects on reverse cholesterol transport mediated by increased cholesterol efflux from the periphery via stimulation of the expression of cholesterol transporter genes such as *Abca1* and *Abcg1*[6−9] (e.g., leading to increased high-density lipoprotein (HDL)-C).

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However, activation of LXR by synthetic agonists also results in a significant deleterious effect due to increased hepatic lipogenesis,[10,11] which has led to the inability to develop LXR agonists for the treatment of atherosclerosis. Stimulation of hepatic lipogenesis by LXR agonists is due to increased expression of key lipogenic enzymes such as *sterol regulatory element binding protein 1c (SREBP1c)* and *fatty acid synthase (FASN)* that are direct target genes of the LXRs.

Several years ago, we developed a new class of synthetic LXR ligands that actively recruit corepressors rather than coactivators, leading to the active repression of LXR target genes.[12] These inverse agonists are effective in repressing the expression of *SREBP1c* and *FASN* as well as other key genes driving lipogenesis via LXR.[12] We developed these compounds as potential anticancer compounds targeting inhibition of *de novo* lipogenesis, which is a key metabolic process preferentially utilized by cancer cells. SR9243 (Supporting Information Figure 1A), a high-affinity LXRα/β inverse agonist, was the tool compound used to test this hypothesis and indeed suppressed *de novo* lipogenesis in tumors both in vitro and in vivo and was an efficacious anticancer agent.[1] SR9243 was not optimized for oral delivery but was administered intraperitoneally (i.p.) to provide systemic exposure. Elevated *de novo* lipogenesis is also a key component of nonalcoholic steatohepatitis (NASH), so we hypothesized that this may also be a target for LXR inverse agonists. However, systemic exposure would likely suppress reverse cholesterol transport, leading to increased atherosclerosis that would be unacceptable for a NASH therapeutic (a side effect that would likely be acceptable in oncology). Thus, we developed SR9238 (Supporting Information Figure 1A), a "soft" drug that, after administered i.p., would be able to act on hepatic LXR but be rapidly metabolized to a nonactive form with no systemic exposure.[12] SR9238 was very efficacious in mouse models of NASH and had no systemic exposure as predicted.[12] As we examined both compounds in mouse models, we noted a pronounced ability of the compounds to reduce plasma LDL-cholesterol levels; however, we did not understand the mechanism.

Alternative approaches to reducing plasma cholesterol levels include targeting the *sterol O-acyltransferase* (also called *Acyl-CoA cholesterol acyltransferase*) enzyme (encoded by the *Soat2* gene) with inhibitors to suppress intestinal cholesterol reabsorption. SOAT2 is sometimes incorrectly called ACAT2, but it is not to be confused with *acetyl-coenzyme A acetyltransferase*, whose official name is ACAT2. Importantly, SOAT2 is a cholesterol esterification enzyme exclusively expressed in the intestine and liver and plays a key role in intestinal cholesterol reabsorption.[13,14] SOAT1, in contrast, is widely expressed and plays a critical role in cholesterol esterification in the macrophage and brain.[13,14] *Soat2* null mice are resistant to diet-induced hypercholesterolemia and are also resistant to developing atherosclerosis.[15,16] Conversely, *Soat1* null mice display either no effect on the development of atherosclerosis[17] or significantly enhanced atherosclerosis in the macrophage-specific *Soat1* deletion.[18] SOAT2 is a key target for the development of anti-hypercholesterolemia therapeutics (although commonly, but incorrectly, called ACAT2 inhibitors).[13,14] However, the development of these compounds has been problematic due to the difficulty in designing specific SOAT2 inhibitors lacking SOAT1 inhibition as well as substantial problems with the lack of solubility of the inhibitors themselves (they are typically lipids).[13,14]

Here, we demonstrate that LXR inverse agonists directly suppress the expression of intestinal *Soat2*, leading to reduced cholesterol reabsorption and plasma LDL-cholesterol levels. Furthermore, we show that oral administration of a gut-restricted LXR inverse agonist displays the ability to reduce LDL-cholesterol levels without widely affecting LXR target gene expression, suggesting that such compounds could be used to treat atherosclerosis.

RESULTS AND DISCUSSION

LXR Inverse Agonists Reduce Plasma LDL-Cholesterol Levels by Inhibiting Cholesterol Reabsorption. LXR agonists were originally developed for their potential to treat atherosclerosis due to their ability to enhance reverse cholesterol transport.[19−21] Unfortunately, these agonists also enhanced the expression of several genes within the *de novo* lipogenesis pathway, leading to hepatic steatosis and limiting the development of LXR agonists. We saw the effective regulation of *de novo* lipogenesis by LXR agonists as an opportunity to develop LXR inverse agonists that could recruit corepressors to the key genes that direct lipogenesis and thus inhibit this pathway that is critical for the pathogenesis of nonalcoholic fatty liver disease (NAFLD)/NASH and cancer.[1,2] Our efforts led to the development of two tool compounds, SR9238 and SR9243. When administered to mice i.p., SR9243 displays wide exposure, while SR9238 only has exposure to the intestine and liver.[1,2] Neither compound was found to have any oral exposure (plasma levels following oral administration).[1,2] We and others had noted in several studies that both of these compounds reduced total cholesterol, and, in the *ob/ob* mouse on a HF diet, LDL-C levels were substantially reduced.[1,2,12,22] The substantial reduction in LDL-C levels was an unexpected result, and, given that these effects were observed with SR9238 whose exposure is limited to the intestine and liver, we hypothesized that the effects were mediated by LXR either in the liver and/or intestine.

First, we confirmed our previous results showing that these compounds reduced LDL cholesterol using three distinct models. Uncharacteristically high circulating LDL-cholesterol levels (>250 mg/dL) are displayed in *ob/ob* mice fed standard chow.[2,12] We administered 30 mg/kg SR9238 i.p, (once per day (q.d.)) or vehicle for 30 days to these mice, followed by an assessment of plasma lipids. We observed that total and LDL-C levels (−11 and −25%, respectively), as well as triglyceride (TG) levels (−18%), were also significantly reduced (Figure 1A,C,D). Interestingly, HDL was unaffected in the SR9238 (Figure 1B). Since NAFLD is often a comorbidity of cardiometabolic disorders, including atherosclerosis, which is characterized by high cholesterol levels leading to arterial plaque formation, we further investigated the effects of SR9238 on plasma cholesterol levels. We utilized the *Ldlr* null mouse model that displays very elevated levels of cholesterol and is commonly used as a model for studying atherosclerosis. These mice were administered 30 mg/kg SR9238 or SR9243 (i.p, q.d.) or vehicle for 30 days to these mice, followed by an assessment of plasma lipids. As illustrated in Figure 1E−H, substantial decreases in plasma lipids were noted. Both compounds displayed similar effects, reducing total cholesterol (TC) (−15 to −22%), LDL-cholesterol (−24 to −27%), and TG (−25 to −30%), while HDL-C was unaffected. In C57Bl/6J mice fed a high-cholesterol diet, administration of 30 mg/kg day SR9238, SR9243, or vehicle for 19 days led to similar results as well. TC (−38 to −40%), LDL-cholesterol (−43 to

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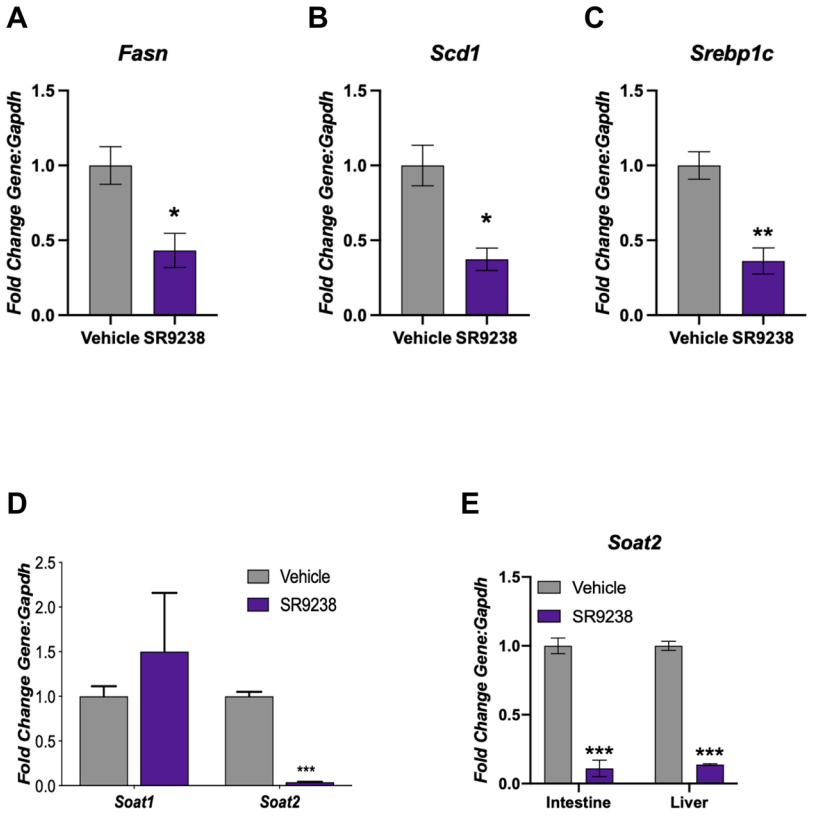

Figure 2. Gene expression investigation of cholesterol pathways suggests that LXR ligands modulate *Soat2* expression. Liver gene expression from the chow-fed *ob*/*ob* mice treated with either vehicle (10% DMSO; 10% Tween-80; PBS; $n = 7$) or SR9238 (30 mg/kg, i.p., q.d.; $n = 7$) for 30 days shows that classic LXR target genes *Fasn* (A), *Scd1* (B), and *Srebp1c* (C) are down-regulated with inverse agonist treatment. (D) Gene expression of *soat1* and *soat2* from the liver of the *ob*/*ob* chow-fed mice. SR9238 does not appear to affect the expression of *soat1*; however, it significantly represses the expression of *soat2* compared to the vehicle-treated mice. (E) Liver and intestine expression of *soat2* in a separate NASH mouse model (*ob*/*ob* mice fed high trans-fat, high cholesterol, high fructose diet; weight ≥58 g) demonstrated that SR9238 treatment for 30 days significantly reduced the expression of *soat2* in both the liver and the intestine. RT-QPCR significance was determined using Student's *t*-test (2-tailed). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$.

−50%), and TGs were reduced (−32 to −41%) while HDL-cholesterol was unaffected (Figure 1I−L).

To test our hypothesis that gut cholesterol absorption was altered by LXR inverse agonists, we also collected fecal samples from C57Bl6 mice on the HCD described above to evaluate whether the LXR inverse agonists affected cholesterol absorption/excretion from the gut. Fecal samples were collected daily, and cholesterol was extracted utilizing the Folch method[23−25] and quantitated. As shown in Figure 1M, by the sixth day of treatment, LXR inverse agonist-treated groups excreted significantly more cholesterol than the vehicle-treated group. This increased excretion of cholesterol was continuous throughout the experiment, suggesting that LXR inverse agonists suppress cholesterol reabsorption in the gut.

LXR Inverse Agonists Directly Suppress Sterol O-Acyltransferase 2 Expression to Inhibit Cholesterol Reabsorption. To further our understanding of the increased cholesterol excretion with the LXR inverse agonist-treated animals, we performed a targeted expression analysis for genes involved in cholesterol reabsorption, excretion, and metabolism using both hepatic and intestinal tissue from SR9238-treated mice. Many of the classical LXR pathway genes were identified, including *Fasn, Scd1*, and *Srebp1c* (Figure 2A−C); however, of particular interest was the identification of the *Soat2* gene as substantially repressed by the LXR inverse agonist SR9238 (Figure 2D−E). SOAT2 is an enzyme that converts cholesterol to cholesterol esters and plays an important role in intestinal cholesterol absorption, lipoprotein assembly, and cholesterol metabolism. Another isoform, SOAT1 (sometimes incorrectly called ACAT1), is much more widely expressed and plays a critical role in macrophages.[26,27] Based on its role in cholesterol reabsorption, SOAT2 was identified as a therapeutic target for atherosclerosis, as increased expression of *Soat2* is an indicator of increased risk of development of CVD.[28−31] While increased *Soat2* expression is associated with increased risk of CVD, the opposite appears to be the case for *Soat1*. Knockout of *Soat1* in macrophages leads to increased foam cell formation and atherosclerosis, while knockout of *Soat2* in mice fed a high-fat and high-cholesterol diet are resistant to the development of hypercholesterolemia or atherosclerosis. Development of SOAT2-specific inhibitors has proven to be difficult, particularly with the general poor chemical and physical properties of the inhibitors and none have progressed to approval. Specific inhibition of SOAT2 activity is a preferential path toward novel therapeutics, and to confirm the regulation of *Soat2* expression by LXR inverse agonists, as well as to determine the relative specificity for *Soat2* over *Soat1*, we also examined their expression in additional mouse models. We re-examined liver tissue samples from a previous study[2] in which *ob*/*ob* mice were treated with SR9238 or vehicle for *Soat1 and 2* gene expression, and as shown in Figure 2D, we observed

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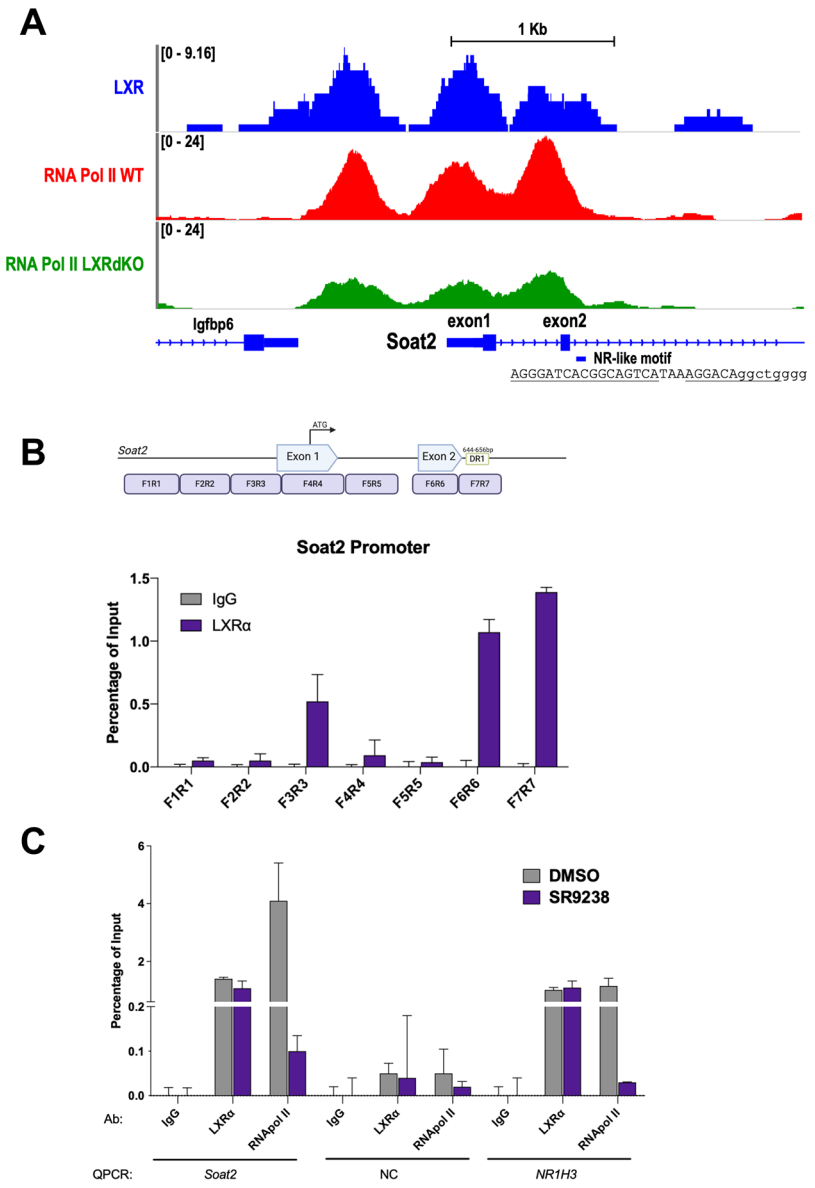

Figure 3. LXR α can bind to a putative binding site in *Soat2*. (A) Published ChIP-Seq data demonstrates that LXRα occupies a region of the *Soat2* promoter near the transcriptional start site as well as exon 2 in wild-type mouse liver. A sequence resembling the NR-binding motif was present in the LXR peak at exon 2, which is conserved with the human *SOAT2* gene. In the LXR double knockout mouse tissue, RNA poll II occupancy is reduced, suggesting that LXR may directly regulate the activation of this gene. To investigate whether LXRα also occupied the promoter region of human *Soat2*, ChIP-QPCR was performed on HepG2 cells. (B) We first designed several primer sets to amplify the promoter region of *Soat2*, as shown in the schematic. LXRα pull-downs in naïve HepG2 cells were analyzed using each of the primer sets (compared to IgG control pull-down), and the regions of amplification are shown as a percentage of input DNA. (C) HepG2 cells were treated with either DMSO or 10 μM SR9238 for 24 h; the ChIP-QPCR was performed utilizing the F7R7 primer set identified in the previous pull-down study. We demonstrate that the *Soat2* region containing the putative DR1 (LXRE) binding site has LXRα occupancy in the DMSO-treated cells. RNA poll II was used as the positive control pull-down, and its binding to *Soat2* was assayed at the transcription start site. Upon treatment with the LXR inverse agonist, SR9238, LXRα continues to occupy the promoter region; however, RNA pol II occupancy is reduced. This coincides with our previous work demonstrating that the inverse agonists suppress the expression by recruiting corepressors and remain bound to the target DNA. LXRα regulates itself in the same way, and therefore it was used as a positive control in this assay. Like the *Soat2* data, RNA pol II binding is decreased at the NR1H3 (*LXRα*) promoter when the cells are treated with SR9238. NC = control (background) antibody.

specific repression of *Soat2* expression. Treatment with SR9238 for 30 days significantly reduced the expression of *Soat2* (>95%) but did not affect the expression of *Soat1* (Figure 2D). We also re-examined hepatic and intestinal tissues from another previous study in *ob*/*ob* mice, where we fed the mice a high trans-fat, high cholesterol, high fructose diet and treated them with SR9238 or vehicle in a similar manner.[12] We found that in both liver and intestine, *Soat2* expression was significantly suppressed in the SR9238-treated animals (~85 to 90%) (Figure 2E). These data illustrate efficacious suppression of *Soat2* expression in the liver and intestine, suggesting a novel therapeutic strategy for lowering cholesterol and treating atherosclerosis.

LXR Directly Regulates *Soat2* Gene Expression. Our data clearly suggests that LXR inverse agonists suppress cholesterol absorption in the gut, leading to lowered circulating

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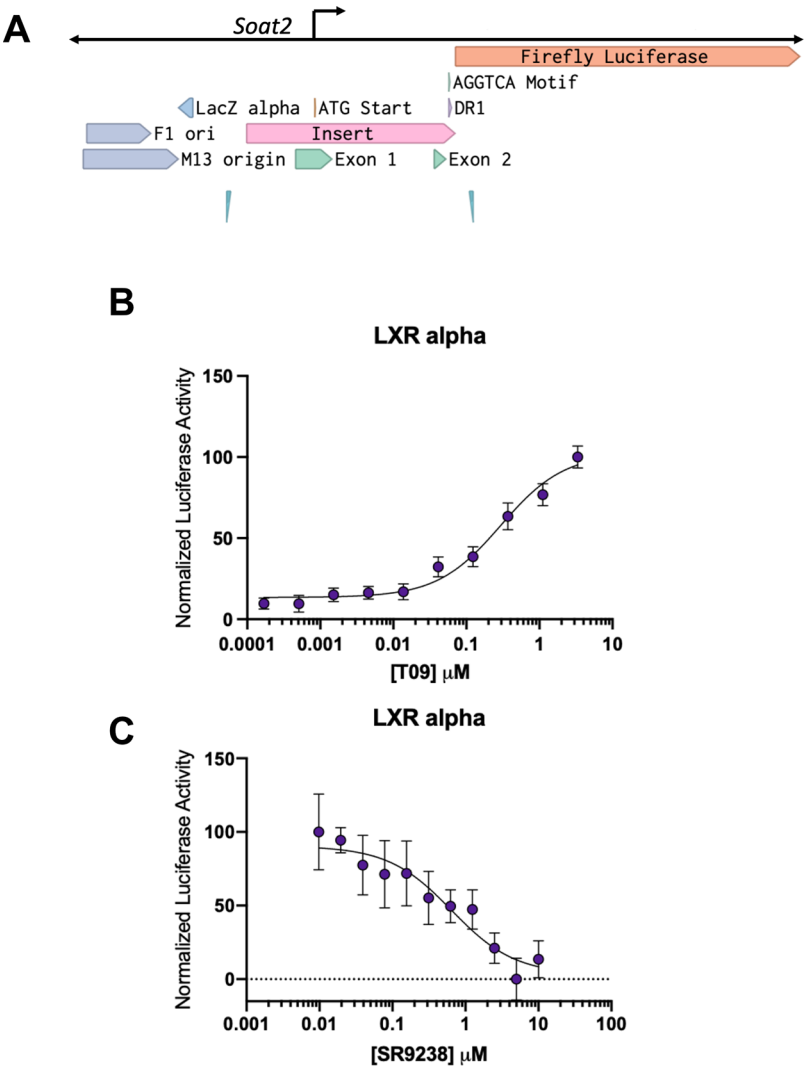

Figure 4. LXRα regulates *Soat2* activity in a cell-based co-transfection assay. Based on the ChIP-QPCR data demonstrating that LXRα binds the promoter region of *Soat2* in HepG2 cells, we wanted to validate that LXRα also regulated the activity of this gene. (A) We generated a luciferase reporter construct of the *Soat2* promoter region, as shown in the schematic. (B) We first validated the LXRα-*Soat2* regulation by co-transfecting the HEK293t cells with full-length LXRα and the *Soat2*-luciferase construct. The cells were subsequently treated with increasing concentrations of the LXR agonist T0901317, and luciferase activity was read and normalized to DMSO. The reporter appears to have an agonist-dependent increase in luciferase activity, suggesting that LXRα directly regulates *Soat2* transcription. (C) The same assay was performed using the *Soat2*-luciferase construct and the LXR inverse agonist SR9238 and demonstrated the suppression of luciferase in a dose-dependent manner. For each concentration of the compound, 4 replicates were averaged and normalized to DMSO. Mean ± SEM is shown.

cholesterol levels. Furthermore, we identified *Soat2*, a critical regulator of cholesterol reabsorption, as a gene whose expression was substantially suppressed by LXR inverse agonist treatment. To determine if *Soat2* is a direct LXR target gene, we first examined published LXR ChIP-Seq data from mouse liver.[32] We observed significant LXRα occupancy of the *Soat2* promoter and transcriptional start site, as well as an exon 2 site, which features a nearby nuclear receptor (NR)-binding motif (Figure 3A), consistent with *Soat2* as a direct LXR target gene. The results also showed LXR-dependent recruitment of RNA pol II (Figure 3A), indicating that the binding by LXR is functionally important. Although we identified *Soat2* as a putative LXR target gene based on repression mediated by LXR inverse agonists, we anticipated that agonists might elevate its expression. We then investigated whether LXR occupied the *Soat2* promoter in the HepG2 cell line. LXRα ChIP showed a strong LXRα occupancy of a region upstream of the TSS/exon 1 as well as near exon 2 of human *SOAT2* (Figure 3B,C). Consistent with LXR inverse agonism expected from SR9238 treatment, we observed a decrease in RNA pol II binding at the *SOAT2* and *NR1H3* (positive control) promoters in the treated HepG2 samples (Figure 3C). This is consistent with our previous work, where we found that upon LXR inverse agonist treatment in cells, LXR continues to occupy its target gene while recruiting corepressors (NCOR/SMRT) to repress transcription.[1,2] Since LXR regulates the same genes in a variety of cell and tissue types, we assumed that LXRα would also occupy the promoter of *Soat2* in intestinal epithelial cells.

Since the ChIP-QPCR data suggested that LXRα occupies a putative binding element in *Soat2* in HepG2 cells, we sought to determine if this region of *Soat2* would confer LXR responsiveness to a reporter gene in a co-transfection assay system. For this experiment, we generated a *Soat2*-luciferase

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expression construct consisting of the region near the TSS and the putative LXRE at Exon 2 (Figure 4A). The insert was designed to target the region of the *Soat2* promoter that displayed LXR occupancy in the ChIP-QPCR experiments. To determine if the *Soat2* promoter fragment conferred LXR responsiveness to the luciferase reporter construct, we transfected them into HEK293T cells along with LXRα and treated the cells with increasing concentrations of the LXR agonist T0901317. As shown in Figure 4B, the *Soat2* promoter fragment conferred LXR agonist responsiveness in a dose-dependent manner, suggesting that this region of the *Soat2* gene does indeed direct LXRα binding. Based on our data with LXR inverse agonists that allowed us to identify *Soat2* as an LXR target gene, we predicted that SR9238 should suppress luciferase activity in the co-transfection assay. Indeed, the *Soat2* promoter fragment conferred dose-dependent LXR inverse agonist (SR9238)-mediated suppression of luciferase activity (Figure 4C). Thus, these data, taken together with the ChIP and gene expression data, indicate that *Soat2* is a direct LXR target gene.

LXR Double Knockout Mouse Tissue Expression Confirms LXR Regulation of *Soat2* Expression in the Intestine. In the cell-based assays, we verified that LXR occupies the promoter of *Soat2* by ChIP-QPCR (Figure 3A–C) and confirmed that LXR can regulate the activity of the *Soat2* promoter in the co-transfected HEK293t cells (Figure 4B,C). To investigate the mechanism of LXR inverse agonists' suppression of *Soat2* expression *in vivo* (examining the intestine), we compared the effects of pharmacological driven repression of LXR target genes (C67Bl/6J mice on HCD) with SR9238 to loss of both LXRα and LXRβ in the double KO.[7,33] In the intestine, the SR9238-treated and LXR DKO mice displayed significantly reduced expression of *Soat2* (Figure 5).

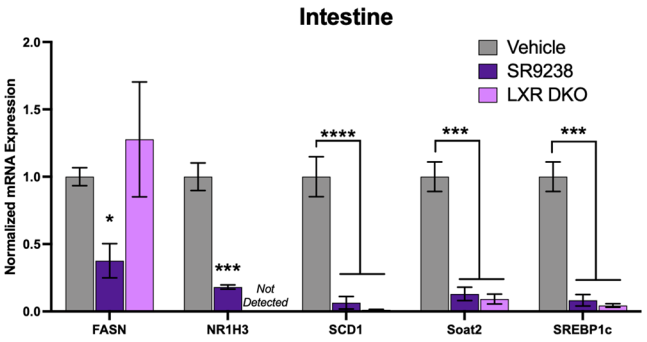

Figure 5. LXR inverse agonist treatment in mouse models and the LXR double knockout (DKO) mouse have suppressed *Soat2* expression. Gene expression data from C57Bl/6J mice on a high-cholesterol diet treated with either vehicle (10% DMSO; 10% Tween-80; PBS) or SR9238 (30 mg/kg, i.p., q.d.) and LXR DKO mice in the intestine. SR9238 treatment and the LXR DKO mice display significantly reduced expression of *Soat2* in both the liver and the intestine. Significance was determined by Student's *t*-test (2-tailed). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$.

Expression of LXR target genes (*Scd1, SREBP1c*) were significantly lower in both the SR9238-treated animals and the LXR DKO animals. There were some cases where inverse agonist treatment reduced the expression of a target gene, whereas loss of LXR expression had no effect (e.g., FASN). We should note that the inverse agonists induce functional repression of target genes via the active recruitment of corepressors to those genes, but in the case of loss of LXR, there would be simply the loss of LXR-mediated regulation. Thus, it is not unreasonable that we may find that in some cases, the inverse agonist drives repression of gene transcription that is not matched by the LXR KO.

Oral Administration of a Gut-Restricted LXR Inverse Agonist in *Ldlr*$^{-/-}$ Mice Reduces LDL-Cholesterol and May Be Atheroprotective. The previous data show that intraperitoneal administration of LXR inverse agonists suppresses *Soat2* expression in both the liver and intestine and significantly reduces the circulating cholesterol levels in the treated mice. As hypercholesterolemia is a significant health issue worldwide and leads to common cardiovascular diseases, including atherosclerosis, we wanted to evaluate the efficacy of these compounds in a mouse model of hypercholesterolemia. SR9238 has limited exposure following i.p. administration (only liver and intestine) and was designed to have such a limited distribution based on engineering a labile ester group into the structure that, when hydrolyzed, yields an inactive metabolite.[2] Given the rapid hydrolysis of such compounds in the gastric environment, we expected no active compound would be available to the intestine. We focused on SR9243, which we found had systemic exposure if administered via i.p. methods but had no plasma exposure when administered orally. However, we found substantial intestinal exposure when the compound was administered orally (Supporting Information Figure 1B), allowing us to test the hypothesis that a compound with gut-restricted exposure would be able to suppress the intestinal *Soat2* expression and limit cholesterol reabsorption, leading to reduced plasma LDL cholesterol levels. Importantly, SR9243 plasma levels were at the lower limit of quantitation and 3 orders of magnitude lower than what was observed in the intestine. Furthermore, the fact that we did not observe the expected time-dependent reduction of levels of the drug in these tissues confirms that these concentrations were below the quantitative limits of the assay. We believe gut restriction is necessary to avoid suppressing reverse cholesterol transport in the periphery leading to conflicting effects of such compounds toward the treatment of atherosclerosis.

To determine if SR9243 retained its ability to suppress *Soat2* expression and reduce plasma LDL-cholesterol when administered orally, we utilized the *Ldlr* null mouse model, which is commonly used as a model for atherosclerosis and displays very high plasma LDL-cholesterol levels. We fed *Ldlr*$^{-/-}$ mice a high-cholesterol diet for 10 days prior to the start of dosing. The mice continued the HCD and were administered 30 mg/kg SR9243 once daily by oral gavage for 10 days. Following the tenth day of gavage, tissues and blood were collected for analysis. Administration of the compound did not affect body weight or food intake (Figure 6A). Orally administered SR9243 significantly reduced LDL-cholesterol in these mice by ~25% (Figure 6B). Total cholesterol was also significantly reduced (Figure 6C), but HDL-cholesterol levels were unaffected (Figure 6D). Plasma TGs were also significantly reduced in the SR9243-treated animals (Figure 6E). To validate that the oral administration of SR9243 was targeting LXR in the gut, we performed gene and protein expression analysis for *Soat2* in the intestine. Consistent with the efficacy of LXR inverse agonist on reduction in plasma LDL-cholesterol levels, we observed a significant decrease in intestinal *Soat2* gene expression (Figure 6F). Protein expression also confirms the SR9243-mediated reduction of

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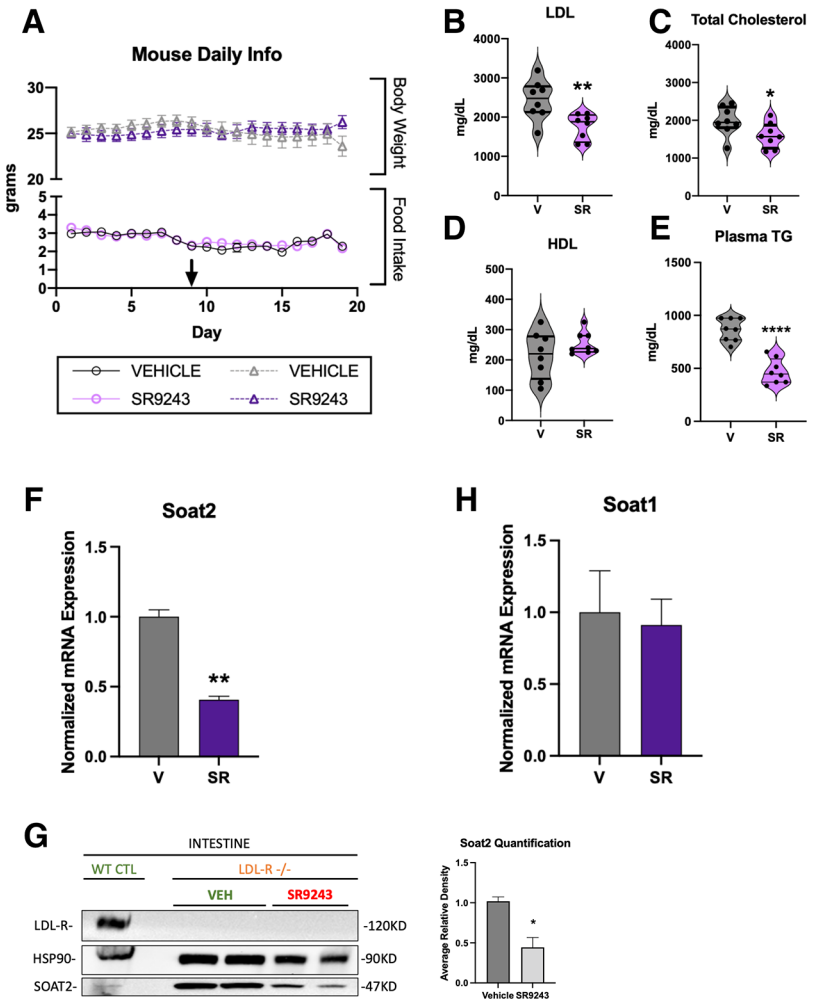

Figure 6. Gut-restricted exposure of SR9243 reduces LDL cholesterol in a mouse model of hypercholesterolemia. The effect of oral delivery of SR9243 (30 mg/kg, p.o., q.d.) in *ldlr* null mice fed a HCD diet was evaluated. (A) Mouse daily weight and food intake data showing no differences among the two treatment groups were observed. The arrow indicates when oral gavage of vehicle or SR9243 began. After 10 days, the mice were euthanized, and clinical chemistry analysis was performed, which subsequently showed a reduction in LDL (B) and total cholesterol levels (C) in the SR9243 group compared to the vehicle control group. Like the intraperitoneal injection data from previous studies, the HDL levels were unaffected (D). Interestingly, plasma triglycerides were also significantly reduced (E). We then evaluated the gene (F) and protein (G) expression of *Soat2* in the intestine. A western blot of LDLR was also performed to confirm the mouse genotype, with a C57Bl/6J mouse intestinal control run in the first lane as an antibody control. Expression of *Soat1* remained unaffected by SR9243 administration in the intestine (H). Significance was determined by Student's *t*-test (2-tailed). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$.

Soat2 in the intestine (Figure 6G) compared to the vehicle-treated *Ldlr* null mice. *Soat1* expression in the intestine was unaffected by drug treatment (Figure 6H). Importantly, LXR-regulated genes in other tissues were unaffected by the treatment (Supporting Information Figure 2).

In accordance with these observations in the *Ldlr* null mice as well as the previous mouse studies, LXR inverse agonists (both SR9238 and SR9243) significantly suppress the expression of *Soat2* in the gut via the LXRα regulation. These *in vivo* studies indicate that utilizing gut selective LXR inverse agonists pharmacologically suppress *Soat2* to drive cholesterol excretion, thus lowering the circulating plasma lipid levels (LDL and total cholesterol). Since the LXR inverse agonists have no effect on *Soat1*, it further supports the notion of targeting LXR with gut-restricted small molecules for the treatment of hypercholesterolemia and/or atherosclerosis.

CONCLUSIONS

Activation of LXR leads to enhanced reverse cholesterol transport, and thus, many pursued the development of synthetic agonists for the treatment of atherosclerosis as well as other diseases.[19,34−37] Another critical function of LXR is the regulation of *de novo* lipogenesis, and synthetic agonists were shown to increase plasma TGs and induce hepatic steatosis, thus limiting the clinical development of LXR agonists. Based on studies demonstrating that LXR is such an efficacious inducer of *de novo* lipogenesis and others showing that under certain circumstances, LXRs can recruit corepressors,[1,2] we hypothesized that if we could optimize LXR inverse agonists that increase LXR corepressor recruitment, we could effectively suppress *de novo* lipogenesis. Such compounds could potentially hold utility for the treatment of cancer or hepatic steatosis. We indeed developed two LXR inverse agonist tool compounds, SR9243 and SR9238, and showed that they effectively suppressed the expression of LXR target genes that drive lipogenesis (i.e., *Fasn*, *Srebp1c*, and *Scd*) in

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models of NAFLD, NASH, alcoholic steatohepatitis (ASH), and cancer.[1,2,12,22,38] Our NAFLD and NASH models (using *ob*/*ob* mice) displayed significantly elevated LDL cholesterol levels compared to either WT diet induced obese mice due to the combination of the leptin-deficient induced obesity and a diet containing 2% cholesterol in addition to high fat and fructose. This model enabled us to observe that LXR inverse agonists not only suppressed LXR target genes that drive hepatic steatosis in the liver (NAFLD, NASH and ASH models) but it also had an unexpected beneficial effect of lowering plasma LDL-cholesterol.

We were able to observe that administration of LXR inverse agonists resulted in the suppression of plasma TC, LDL-C, and TG levels in *ob*/*ob* mice, *Ldlr* null mice, and WT mice fed an HCD. Furthermore, we observed that the LXR inverse agonists increased fecal elimination of cholesterol, suggesting that the compounds suppressed intestinal cholesterol reabsorption. We identified *Soat2* as a novel, direct target gene of LXR using an integrated approach combining ChIP-seq analysis, ChIP-QPCR, cell-based luciferase reporter assays, and animal physiology. As we investigated this reduction in total and LDL-C by determining that the cholesterol was not reabsorbed in the gut but rather excreted in the feces of the treated mice, it was clear that it was through an LXR-mediated mechanism.

Given the need for additional therapeutics that can reduce LDL-C levels to treat CVD, we hypothesized that a gut-restricted LXR inverse agonist may provide such an opportunity. If the LXR inverse agonist only acted in the intestines, it could effectively suppress *Soat2* expression, reduce cholesterol reabsorption, and reduce plasma LDL-C levels without adversely affecting reverse cholesterol transport. We had observed during the initial characterization of SR9243 that it had no oral bioavailability but based on the predicted chemical and physical properties of the compound, we expected it to retain intestinal exposure. Indeed, when we administered SR9243 by oral gavage, we had substantial intestinal exposure but barely any detectable levels of the compound in the plasma. We show that in just 10 days of oral administration, LDL-C and total cholesterol levels were significantly reduced in this model, while LXR target genes outside the intestine were not affected. HDL-C was not significantly altered ($p < 0.05$) in any of the models; however, it is important to note that we observed a trend toward a decrease in HDL-C with SR9238 treatment in the *ob*/*ob* mice fed a normal chow diet ($p = 0.0593$) (Figure 1B) although this was not consistent when other mouse models were used (*ldlr* null mice/HCD; SR9238 $p = 0.3201$/SR9243 $p = 0.1257$; Figure 1F, WTC57Bl6 mice/HCD; SR9238 $p = 0.6656$/SR9243 $p = 0.5162$; Figure 1J, and *ldlr* null mice/HCD 9243 oral $p = 0.222$; Figure 6D).

Importantly, *Soat1* expression is not adversely affected by treatment with the LXR inverse agonists. Although SOAT inhibitors have been evaluated as potential antihyperlipidemic agents, SOAT2 specificity is required based on data suggesting that *Soat1* is atheroprotective.[39] Isoform specificity issues along with the problem that SOAT2 inhibitors typically were extremely hydrophobic and did not display druglike chemical and physical properties limited their development. Thus, a gut-restricted LXR inverse agonist may be one method to inhibit SOAT2 activity specifically, albeit indirectly.

While more exhaustive studies to determine the effects of these LXR inverse agonists on atherosclerotic plaques remain to be performed, it is important to note that the FDA currently does not require plaque reduction as a clinical output for a drug to be labeled as atheroprotective. Current guidelines suggest that simply a reduction in LDL cholesterol is sufficient, paving the way for these LXR inverse agonists to be further explored.

METHODS

Materials Availability Statement.

1. Plasmids in this study have been deposited to Addgene.
2. LXR inverse agonists are available from the lead contact without restriction.

Mouse Studies. All animal studies were run in accordance with the approved IACUC protocols at Saint Louis University and Washington University in St. Louis School of Medicine. Six-week-old B6 V-Lep^{ob}/J (*ob*/*ob*) male mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed individually in standard cages. The mice were maintained on standard chow diet for 6 weeks prior to treatment and handled daily to become acclimated to the researcher. A separate cohort used for gene expression studies only was fed a NASH Diet (Research Diets; D09100301; 40% kcal from primex, 20% fructose 2% cholesterol), as previously described;[12] however, the experimental parameters were identical. Groups were weight-matched prior to beginning the treatment. The mice were treated with either 30 mg/kg SR9238 q.d. i.p. (10% DMSO; 10% Tween-80; 80% PBS) or vehicle for 30 days. Body weight and food intake were monitored daily, blood glucose was determined weekly using a hand-held glucometer, and a final fasting blood glucose was collected at termination. At the termination of dosing, blood was collected by cardiac puncture and analyzed using clinical chemistry (Roche cobas c311). The liver was collected, weighed, and a portion was immediately flash-frozen in liquid nitrogen for RNA, as previously described.[12] For the cholesterol excretion experiment, C57Bl/6J mice were purchased from Jackson Laboratories and immediately housed in individual cages with aspen chip bedding and placed on a high-cholesterol diet (HCD; Teklad; TD.96121(21% milkfat, 1.25% cholesterol)). Cages were changed daily, and feces were collected into individual 1/2 dram vials for analysis. The mice were grouped randomly and treated with either 30 mg/kg SR9238, 30 mg/kg SR9243, or vehicle (as described above). At the end of the experiment, the mice were euthanized, and tissues were collected, as previously described. For evaluating the effect of SR9243 on plasma cholesterol levels by oral administration, *Ldlr* null mice were purchased from Jackson Labs (Bar Harbor, ME) and allowed to acclimate to our facility for 7 days. Upon receipt, mice were placed on a HCD (Teklad; TD.96121), and this diet was continued for the entire study. To reduce stress due to oral gavage, all mice were given an oral gavage of PBS for 1 week prior to the start of treatment and weighed daily by the researchers. The mice were randomly grouped at the start of dosing and were given a daily gavage of either vehicle or SR9243 (5% DMSO, 10% Cremophore EL, 85% PBS) at 30 mg/kg based on body weight at a dosing volume of 5 μL/g. At the termination of the study, the mice were euthanized, and tissues were collected, as previously described.

Fecal Cholesterol Extractions. Briefly, for the analysis of fecal lipids, feces were collected from the mice housed individually in metabolic cages over a 24-h period. Typically, 100 mg aliquots of feces were cleaned and dried for 1 h at 70 °C, incubated with 2 mL of chloroform-methanol (2:1) for 30 min at 60 °C with constant agitation, and then centrifuged. Water (1 mL) was added to the supernatant, and following vortexing, phase separation was induced by low-speed centrifugation (2000 rpm for 10 min). The lower chloroform phase was then removed and transferred to a new tube, and the sample was evaporated to dryness. Samples were then resuspended in 500 μL of chloroform-1% Triton X-100, evaporated to dryness, and finally resuspended in 500 μL of water so that the final solvent was 1% Triton X-100 in water. Cholesterol was then measured using the Amplex Red Cholesterol kit (Thermo Fisher Scientific

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Table 1. Primer Information

Human Positive Control Primer Set Gapdh (ChIP)		active motif	71004
ChIP Assay: hSoat2 ChIP RNApol F1	5′- CTTTGGAGCTGTCACCTGAGCTGA-3′	IDT DNA	Reference Number 209259373
ChIP Assay: hSoat2 ChIP RNApol F2	5′- AGCTGGGCATCTGGATGGTGGT-3′	IDT DNA	Reference Number 209259374
ChIP Assay: hSoat2 ChIP RNApol F3	5′- CAAGCTTCTGAGAGGCAAAGTTC-3′	IDT DNA	Reference Number 209259375
ChIP Assay: hSoat2 ChIP RNApol F4	5′- TGAGTAGCACAGTGCCAACCCC-3′	IDT DNA	Reference Number 209259376
ChIP Assay: hSoat2 ChIP RNApol R1	5′- AGCCCTTCTGTCCTCTGCAGACGC-3′	IDT DNA	Reference Number 209259377
ChIP Assay: hSoat2 ChIP RNApol R2	5′- ACTAAAGTCCTTTCAGGAGGAGGG-3′	IDT DNA	Reference Number 209259378
ChIP Assay: hSoat2 ChIP RNApol R3	5′- TCACTCTGCTGTCTGTCGCAGG-3′	IDT DNA	Reference Number 209259379
ChIP Assay: hSoat2 ChIP RNApol R4	5′- ATGGCTGGCCTTCCAGTCTCTC-3′	IDT DNA	Reference Number 209259380
hSREBP1c ChIP FWD Set 1 (Control)	5′- GCCTTGACAGGTGAAGTCG-3′	IDT DNA	Reference Number 209259381
hSREBP1c ChIP REV Set 1 (Control)	5′- TACCTTCGAAAGTGCAATCCAT-3′	IDT DNA	Reference Number 209259382
hLXRa ChIP RTF	5′- ACAATCTCGGCTCACTGCAA-3′	IDT DNA	Reference Number 203662214
hLXRa ChIP RTR	5′- GTTGAGCCAGCAATGGTGTG-3′	IDT DNA	Reference Number 203662215
hSoat2 ChIP RTF (F7)	5′- AGGAAACACTGAGACGCACA-3′	IDT DNA	Reference Number 203662219
hSoat2 ChIP RTR (R7)	5′- CACTCCAGCCTGAGCAACAG-3′	IDT DNA	Reference Number 203662220
hSoat2 ChIP F1	5′- CCAGGGTTGTATTAGACTTGAGTAG-3′	IDT DNA	Reference Number 195085967
hSoat2 ChIP R1	5′- GATAGGCTGAGGGCAGATTAAG-3′	IDT DNA	Reference Number 195085968
hSoat2 ChIP F2	5′- GTCTTTAGGCACAGAGGAAGAG-3′	IDT DNA	Reference Number 195085969
hSoat2 ChIP R2	5′- AGGAGAAGGAGAGGTCAGAA-3′	IDT DNA	Reference Number 195085970
hSoat2 ChIP F3	5′- CTCCTACATCCGTTGCTCTTC-3′	IDT DNA	Reference Number 195085971
hSoat2 ChIP R3	5′- CTCAGCTATCTGTGTGGCATAA-3′	IDT DNA	Reference Number 195085972
hSoat2 ChIP F4	5′- GCATCAGAATCCTCCGGAGGGC-3′	IDT DNA	Reference Number 195085973
hSoat2 ChIP R4	5′- GCTGTCACAGGAAGGACTAAAG-3′	IDT DNA	Reference Number 195085974
hSoat2 ChIP F5	5′- GTTGGAAAGAGTGTTGGTGTTG-3′	IDT DNA	Reference Number 195085975
hSoat2 ChIP R5	5′- GTCAGGGAGGAGTCAGAAATG-3′	IDT DNA	Reference Number 195085976
hSoat2 ChIP F6	5′- GAGCTGAACGCACTGATACA-3′	IDT DNA	Reference Number 195085977
hSoat2 ChIP R6	5′- CATCTCCACAGGGTTGGC-3′	IDT DNA	Reference Number 195085978
Mouse Nr1h2 (LXR β) QPCR Primer Set		Bio-Rad	qMmuCED0003744
Mouse Nr1h3 (LXR α) QPCR Primer Set		Bio-Rad	qMmuCID0014005
Mouse Soat1 QPCR Primer Set		Bio-Rad	qMmuCED0046076
Mouse Soat2 QPCR Primer Set		Bio-Rad	qMmuCID0015332

A12216) as per manufacturer's protocol. Data were normalized to daily cholesterol ingestion (calculated from daily food intake values).

ChIP-Seq. Published ChIP-Seq datasets for LXR and Pol II (SRR403083, SRR403093, SRR403094, SRR403099, SRR403100)[32] were downloaded from the NIH SRA server. SRR403083 is the LXR ChIP-Seq derived from wild-type (WT) mouse liver tissue. SRR403093 and SRR403094 are Pol II ChIP-Seq derived from WT mouse liver tissue. SRR403099 and SRR403100 are Pol II ChIP-Seq derived from LXR α and LXR β double knockout (DKO) mouse tissue. The results were analyzed from the raw data, which were in the FASTQ format. The FASTQ files were aligned to the mm10 mouse reference genome and subsequently analyzed using HOMER software, as previously described.[40]

Cell Culture and Luciferase Assays. HepG2 cells were purchased from ATCC and maintained in Gibco Advanced MEM media supplemented with 10% FBS (Gemini Bio) and L-glutamine (Gibco) at 37 °C with 5% CO_2, as previously described.[2] HEK293t cells were purchased from ATCC and maintained in DMEM GlutaMAX media (Gibco) supplemented with 10% FBS (Gemini Bio) and L-glutamine (Gibco) at 37 °C, with 5% CO_2, as previously described.[2]

ChIP-qPCR. The assays were carried out on HepG2 cells treated with either SR9238 or DMSO using the ChIP-IT Express Enzymatic kit (Active Motif) as per the manufacturer's protocol. Immunoprecipitation was performed on the prepared chromatin by incubation with antibodies (Active Motif) on an end-to-end rotator overnight at 4 °C. Following elution, ChIP DNA was treated with Proteinase K and heated for 2.5 h to reverse cross-links and then purified on a column using proprietary Active Motif DNA elution buffer. A 2 mL aliquot of purified ChIP DNA was used for each qPCR reaction. Standard curves of input DNA ranging from 0.005 to 50 ng of DNA were generated for each of the primer sets used. Primer information is given in Table 1. Assays were carried out on a standard cycle using SYBR Select (Applied Biosystems) on an Applied Biosystems QuantStudio 7 Flex instrument (Thermo Fisher Scientific).

Gene Expression. HepG2 cells were plated at 100,000 cells/well in 12-well plates and allowed to settle for 24 h. The media was removed and replaced with standard media containing either DMSO or the compound at 10 μM. Cells were placed back into the incubator for an additional 24 h, then RNA was isolated using a column homogenizer and RNA-column-based kit (Qiagen Kits) as per manufacturer's protocol. Bio-Rad iScript was used to generate cDNA, and the expression of pertinent genes was analyzed by QPCR, as previously described.[2] In the case of the RNA-seq studies, RNA isolation from HepG2 cells was performed as described above. The RNA was further purified using a second ethanol precipitation and reconstituted in nuclease-free water, placed immediately on dry ice, and transported to GTAC facility for bioanalysis and sequencing. For mouse studies, tissues were flash-frozen in liquid nitrogen and stored at −80 °C. RNA was isolated from the tissues using the trizol method and reconstituted in nuclease-free water as previously described.[2] Following RNA isolation, the process to generate and assay cDNA was identical to the process described above for HepG2 cells.

Pharmacokinetics Study of SR9243 Delivered by Oral Gavage. Twelve[12] 8-week-old male mice were purchased from Jackson Labs (Bar Harbor, ME) and housed under controlled conditions (22 ± 4°C; relative humidity 30−70%; 12 h L:D) and allowed to acclimate for 10 days to IVC housing. The animals always had free access to standard chow and water. The mice were given a single oral gavage of SR9243 (5% DMSO, 10% Cremophor EL, 85% PBS) at 30 mg/kg based on body weight at a dosing volume of 5 μL/g. Groups of four mice were euthanized at 1, 2, or 4 h post-gavage, and tissues were immediately collected and flash-frozen and stored at −80°C until mass spectrometry analysis. Blood samples were collected into lithium-heparin-containing tubes and kept on wet ice

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prior to processing. Samples were centrifuged at 2000*g* for 10 min at 4 °C. Plasma was collected into Eppendorf safe-lock tubes and stored at −80 °C until analysis. For LC-MS/MS analysis, tissue samples were homogenized using a bead beater in acetonitrile:water (3:1) at 250 mg/mL. Naïve tissues were used to prepare standard, quality control (QC), and blank samples in a tissue matrix. Homogenized tissue or plasma samples were kept on ice and spiked with internal standard (IS). Analytes were extracted using protein precipitation techniques with acetonitrile, and samples were then analyzed using a SCIEX Triple Quad 5500+ system−QTRAP Ready linked to an ExionLC AD-HPLC system. Data analysis was performed using SCIEX OS software.

Statistical Analysis. GraphPad Prism was used to perform statistical analysis of all gene expression, cell-based, and animal studies. Statistical details, including group sizes and analyses performed, are detailed in each figure legend. For ChIP-Seq peak calling, default cutoffs were applied, including FDR (false discovery rate) ≤0.001, Poisson *p*-value threshold relative to local tag count ≤0.001, and *p*-value threshold relative to input tag count ≤0.001. Fold enrichment over input tag count ≥4.0; fold enrichment over local tag count ≥ 4.0. For all experiments, significance was measured by *p*-value: $*p \le 0.05$; $**p \le 0.01$; $***p \le 0.001$; and $****p \le 0.0001$.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.2c00057.

> Supporting Information contains two supplementary figures illustrating the structures of SR9243, pharmacokinetics of SR9243, and gene expression following SR9243 treatment (PDF)

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K.G.: Conceptualization, funding acquisition, methodology, investigation, and writing (original draft, editing, and review); M.H. and R.S.: investigation and methodology (mouse models, cholesterol excretion experiments, and luciferase assays); B.E.: supply of key reagents (SR9243 and SR9238); G.B.-D. and M.P.B.: investigation, methodology, and formal analysis (mouse models, gene expression studies); K.A.: investigation and methodology (pharmacokinetic experiments); T.K.: methodology (luciferase constructs and assays); J.Z.: investigation and formal analysis (ChIP-Seq); I.S. resources, writing (review and editing); and T.P.B. conceptualization, resources, formal analysis, supervision, and writing (review and editing).

Notes

The authors declare no competing financial interest.

The published article includes all NIH SRA datasets SRR403083, SRR403093, SRR403094, SRR403099, and SRR403100 analyzed during this study (32).

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