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RESEARCH PAPER

A novel potent synthetic steroidal liver X receptor agonist lowers plasma cholesterol and triglycerides and reduces atherosclerosis in LDLR$^{-/-}$ mice

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BACKGROUND AND PURPOSE

Potent synthetic nonsteroidal liver X receptor (LXR) agonists like T0901317 induce triglyceridaemia and fatty liver, effects not observed with some natural and synthetic steroidal, relatively weak agonists of LXR. To determine if potency is responsible for the lack of side effects with some steroidal agonists, we investigated the *in vivo* effects of a novel steroidal LXR agonist, ATI-111, that is more potent than T0901317.

EXPERIMENTAL APPROACH

Eight week old male LDLR$^{-/-}$ mice fed an atherogenic diet were orally treated with vehicle or ATI-111 at 3 and 5 mg·kg^{-1}·day^{-1} for 8 weeks, and effects on plasma and liver lipid levels, expression of genes involved in lipid metabolism and on atherogenesis were analysed.

KEY RESULTS

ATI-111 increased the expression of genes involved in lipid transport, such as ABCA1, ABCG1 and ABCG5/G8, in intestine and macrophages; decreased ABCG1, apoE; and slightly increased ABCA1 and ABCG5/G8 expression in liver. ATI-111 markedly increased sterol regulatory element-binding protein (SREBP)-1c mRNA in some tissues, whereas acetyl-coenzyme A carboxylase and fatty acid synthase expression was unaffected or only slightly increased in intestine and liver. ATI-111 inhibited the conversion of SREBP-1c precursor form to its active form. Compared with vehicle-treated mice, the levels of hepatic lipids and liver-secreted nascent lipoproteins were not altered, while a significant decrease in plasma cholesterol and triglyceride levels was observed in ATI-111-treated mice. ATI-111 significantly inhibited atherogenesis in three separate vascular sites.

CONCLUSIONS AND IMPLICATIONS

ATI-111 is a promising candidate for further development as a treatment of certain vascular diseases as it lacks the significant side effects associated with nonsteroidal LXR agonists, the induction of fatty liver and hypertriglyceridaemia.

Abbreviations

25-HC, 25-hydroxycholesterol; 36B4, acidic ribosomal phosphoprotein; ABCA1, ABCG1, ABCG5, ABCG8, ATP-binding cassette transporter A1 or G1 or G5 or G8; ACC, acetyl-coenzyme A carboxylase; AIM, apoptosis inhibitor expressed by macrophages; apoCII, apolipoprotein CII; apoE, apolipoprotein E; AR, androgen receptor; ArgII, arginase II; ATI-111, 3α, 6α, 24-trihydroxy-22-en-24, 24-di(trifluoromethyl)-5β-cholane; ATI-829, 3α, 6α, 24-trihydroxy-24, 24-di(trifluoromethyl)-5β-cholane; CAR, constitutive androstane receptor; CD36, cluster of differentiation 36; CYP7A1, cytochrome P450, family 7, subfamily A, polypeptide 1; DR, direct repeat; EM, electron microscopy; ER, oestrogen

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receptor; FAS, fatty acid synthase; FPLC, fast protein liquid chromatography; FXR, farnesoid X receptor; GR, glucocorticoid receptor; GW3965, 3-[3-[N-(2-chloro-3-trifluoromethylbenzyl)-(2,2-diphenylethyl)amino]propyloxy] phenylacetic acid hydrochloride; HDL, high density lipoprotein; HL, hepatic lipase; IDL, intermediate density lipoprotein; IL, interleukin; iNOS, inducible nitric oxide synthase; LDL, low density lipoprotein; LDLR-/-, low density lipoprotein receptor deficiency; LPL, lipoprotein lipase; LPS, lipopolysaccharide; LXR, liver X receptor; MCP-1, monocyte chemotactic protein-1; MMP-9, matrix metallopeptidase 9; NPC1L1, Niemann-pick C1 like 1; PCR, polymerase chain reaction; PLTP, phospholipid transfer protein; PXR, pregnane X receptor; RAR, retinoic acid receptor; SREBP-1c, sterol regulatory element binding protein 1c; T0901317, N-(2,2,2-trifluoroethyl)-N-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]-benzenesulfonamide; TNFα, tumour necrosis factor α; TR, thyroid hormone receptor; VDR, vitamin D receptor; VLDL, very low density lipoprotein

Introduction

Atherosclerosis, a chronic inflammatory disease often associated with a disorder of lipid metabolism, is the most common cause of death in the world. Plaque rupture and thrombosis result in the acute clinical complications of myocardial infarction and stroke. As modulators of both lipid metabolism and immune responses, macrophages play a central role in the atherogenic process. The accumulation of cholesterol-loaded macrophages in the arterial wall is the hallmark of the early atherosclerotic lesion. In response to lipid loading, which is facilitated by high plasma cholesterol levels, macrophages activate a compensatory pathway for cholesterol efflux mediated by genes such as ABCA1, ABCG1 and apoE that are transcriptionally regulated by the oxysterol-activated nuclear receptors, liver X receptors α and β (LXRα and LXRβ) (Cao *et al.*, 2004). Recent studies on Toll-like receptors that mediate microbial pathogen–induced innate immunity indicate that bacterial and viral pathogens can attenuate macrophage cholesterol efflux by antagonizing LXR transcriptional activity and consequently lowering the expression of ABCA1, ABCG1 and apoE in macrophages. This suggests that pathogen-mediated modulation of the LXR pathway may disrupt the balance between cholesterol uptake and efflux in macrophages and facilitate atherogenesis (Castrillo *et al.*, 2003). Thus, targeting LXRs is a promising strategy to prevent and treat atherosclerosis, especially in patients who have normal plasma cholesterol levels.

The pharmaceutical development of LXR agonists has been limited by LXR agonist–induced hepatic steatosis and hypertriglyceridaemia. These effects occur predominantly through the induction of sterol regulatory element-binding protein (SREBP)-1c, which is the master regulator of genes involved in *de novo* lipogenesis (Schultz *et al.*, 2000; Grefhorst *et al.*, 2002; Horton *et al.*, 2002). Therefore, a key challenge for the further therapeutic evolution of LXR agonists is to dissociate the favourable effects on cholesterol homeostasis from the unfavourable effects on fatty acid metabolism. We and others recently observed that certain synthetic steroidal LXR agonists that are more potent than natural steroidal LXR agonists, but less potent than the nonsteroidal LXR agonist T0901317, exhibit tissue selectivity in gene transactivation and minimally activate hepatic lipogenesis (Quinet *et al.*, 2004; Peng *et al.*, 2008; Kratzer *et al.*, 2009). LXR tissue-selective gene transcription may be dependent on the relative levels of nuclear receptor coactivators and corepressors in a given cell as well as ligand-specific interactions of LXR with coactivators and corepressors. Partial LXR agonists, such as the natural steroidal ligand 22(R)-hydroxycholesterol and the weak nonsteroidal ligand GSK418224, differentially recruit coactivators and corepressors compared with full LXR agonists, such as the potent nonsteroidal ligand T0901317 (Albers *et al.*, 2006; Phelan *et al.*, 2008).

One drawback of oxysterols relates to their potential cytotoxicity especially when used at higher concentrations. Some of the natural oxysterols display pro-oxidant and pro-inflammatory characteristics at high concentrations (Kölsch *et al.*, 2001; Lemaire-Ewing *et al.*, 2005). Therefore, a useful oxysterol LXR agonist may require high potency, which would allow lower doses and thus avoid potential cytotoxicity. However, whether highly potent synthetic steroidal LXR agonists still have as minimal adverse effects as their weaker relatives is still not clear. To address this question, we designed and synthesized a new steroidal LXR agonist ATI-111, which is even more potent than T0901317 in activating LXRα. Activation of LXR target gene expression was characterized in different mouse tissues. The impact of ATI-111 on liver and plasma lipid levels and plasma lipoprotein profile and on the development and progression of atherosclerosis in three arterial regions was examined in male low density lipoprotein receptor deficient ($LDLR^{-/-}$) mice. These studies confirm the preliminary promise of this agent and indicate its potential in pharmaceutical development.

Methods

Chemicals

T0901317 was purchased from Cayman Chemical Company (Ann Arbor, MI). 25-Hydroxycholesterol (25-HC) was purchased from Steraloids Inc (Newport, RI). GW3965 was purchased from Sigma-Aldrich (St. Louis, MO). ATI-829 was synthesized as described previously (Peng *et al.*, 2008). ATI-111 was synthesized from 3α,6α-dihydroxy-23,24-bisnorcholanic acid and structure and purity (>99%) were confirmed by ^{1}H, ^{13}C nuclear magnetic resonance spectroscopy and high-resolution mass spectrometry. Details on the synthesis of ATI-111 are presented in Supporting Figures S1 and S2.

Cell culture

Human embryonic kidney (HEK) 293 cells, human Caco-2 cells and human HepG2 cells were obtained from the American Type Culture Collection (Manassas, VA) and

cultured at 37°C in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 U·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin (Mediatech Inc., Hemdon, VA). HEK293 cells were plated on 48-well plate 24 h before ligand or vehicle addition. Cells were treated with LXR ligands, dissolved in dimethyl sulphoxide (DMSO), for 24 to 48 h. The activity of LXR ligands was analysed by a *trans*-activation assay as described previously (Song and Liao, 2001). Briefly, cells were transiently transfected with an expression vector for the indicated human nuclear receptor and the appropriate luciferase-based reporter gene using a calcium phosphate coprecipitation method. Each well contained 0.58 ng phRL-TK (normalization reporter, Promega, Madison, WI), 214 ng pBS-SK$^+$II (carrier DNA, Stratagene, La Jolla, CA), 58 ng pSG5-based expression vector for the indicated nuclear receptor and 58 ng of the appropriate pGL3-derived reporter gene. Nuclear receptor expression vectors were constructed from polymerase chain reaction (PCR)-amplified cDNAs for the indicated nuclear receptor using various human cDNA libraries as starting material. PCR-derived cDNA was subcloned into the *Eco*RI site of pSG5 (Stratagene), and accuracy of the construct was determined by sequencing the entire inserted cDNA. Cells were cultured at 37°C, and after 4 h of exposure to the precipitate, media was aspirated and new media containing the indicated concentration of compound was added. All compounds were dissolved in DMSO, and controls contained equal amounts of solvent. After 48 h of exposure to compounds, media was removed, and cells were lysed with 0.1 mL of passive lysis buffer (Promega). Firefly and sea pansy luciferase activity in cell lysates was measured using a dual luciferase assay (Promega) using a Monolight luminometer. Peritoneal macrophages were obtained from thioglycollate-injected C57BL/6 mice (1.5 mL of 4% thioglycollate per mouse, and mice were left for another 4 days to allow macrophage stimulation by thioglycollate in intraperitoneal cavity; Castrillo *et al.*, 2000). Each well of a 24-well plate was seeded with 10^6 cells in DMEM supplemented with 10% FBS and allowed to adhere for 2 h at 37°C in 5% CO_2. Nonadherent cells were removed by vigorous washing, and adherent macrophages were utilized immediately for further studies in serum-free DMEM medium supplemented with 0.2% BSA.

Analysis of SREBP-1 processing

The effect of different compounds on SREBP-1 processing in Caco-2 and HepG2 cells was analysed as described previously (Adams *et al.*, 2004). Cells were cultured as described above, and when cells reached approximately 80% confluency, media was removed and cells were washed twice with PBS. Culture media was changed to DMEM/F-12 containing 5% newborn calf lipoprotein-deficient serum, 1% (w/v) hydroxypropyl-*β*-cyclodextrin (Acros Organics, Morris Plains, NJ), 50 μM sodium compactin (EMD Chemicals, Gibbstown, NJ) and 50 μM sodium mevalonate (Sigma-Aldrich) (medium A), and cells were then incubated for 1 h at 37°. Solutions of sodium compactin and mevalonate were prepared as previously described (Brown *et al.*, 1978). The media was changed to media A without hydroxypropyl-β-cyclodextrin containing the indicated compounds or vehicle (DMSO), and cells were incubated for 6 h at 37°C (Adams *et al.*, 2004). After the 6 h incubation, cells were washed twice with cold PBS and lysed on the plate with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Cell lysates were heated at 100°C for 7 min and stored at –20°C until use. Cell lysates containing 50 μg protein were separated on an 8% SDS-PAGE gel, and the level of full-length and processed SREBP-1 was measured by immunoblot analysis with antibody against SREBP-1 (sc-8984, Santa Cruz Biotechnology, Santa Cruz, CA). Monoclonal anti-actin antibody was purchased from Millipore (mAB 1501, Bedford, MA).

Animals and diets

All work with mice followed National Institutes of Health guidelines for care and use of animals in experimentation. Animal work was reviewed and approved by the University of Chicago Institutional Animal Care and Use Committee. Male LDLR$^{-/-}$ mice on a C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and fed an atherogenic diet (TD94059, 15.8% fat and 1.25% cholesterol, Harlan TEKLAD, Madison, WI) for 8 weeks starting at the age of 8 weeks for atherosclerosis studies. Mice were administered ATI-111 for 8 weeks at doses of 3 and 5 mg·kg^{-1} by gavage daily in a 20% microemulsion (Gao *et al.*, 1998) starting with initiation of the atherogenic diet. The control group (vehicle) received a microemulsion only. At 16 weeks of age, the mice were deprived of food for 4 h, anaesthetized with ketamine (100 mg·kg^{-1}) and xylazine (10 mg·kg^{-1}), and a blood sample was removed from the retro-orbital plexus. The mice were then perfused transcardially with PBS followed by paraformaldehyde, and the heart and upper vasculature were removed and prepared for histology as described previously (Reardon *et al.*, 2001).

Eight week old male LDLR$^{-/-}$ mice were fed the atherogenic diet described above and treated with vehicle or ATI-111 as described above for 8 weeks to analyse LXR target gene expression in tissues, lipid levels in liver and lipase activity in plasma. Resident peritoneal macrophages were obtained by flushing the peritoneal cavity with ice-cold PBS and pelleted by centrifugation at 500× *g* for 10 min. Cell pellets were resuspended in RNAlater (Ambion, Austin, TX) for storage prior to RNA isolation. Intestine and liver samples were also stored in RNAlater prior to RNA isolation. Liver samples for lipid analysis were stored frozen at –70°C. Blood was collected from the retro-orbital plexus 5 min after injection of 500 U·kg^{-1} body weight of heparin via the retro-orbital sinus for plasma lipase activity analysis.

RNA Isolation and gene expression analysis

Total RNA from mouse macrophages, intestine and liver was isolated by using the RNeasy Mini System (QIAGEN, Valencia, CA). First-strand cDNA was synthesized by utilizing SuperScript III System (Invitrogen Life Technologies, Carlsbad, CA). Sequences of gene-specific primers and probes are shown in Supporting information and were purchased from IDT (Coralville, IA). Quantitative real-time PCR was performed by using 'TaqMan Universal PCR Master Mix' from Applied Biosystems (Foster City, CA, USA). The results were normalized to 36B4 mRNA.

Lipid and lipoprotein analysis

Two hundred microlitres of plasma was fractionated on tandem Superose 6 fast protein liquid chromatography

(FPLC) columns (Reardon *et al.*, 2001). Cholesterol and triglyceride in the even-numbered FPLC fractions and in plasma samples were measured by using commercial kits (Stanbio Laboratory, Boerne, TX). Very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL)/LDL and high density lipoprotein (HDL) lipids were analysed from fractions 6–14, 15–35 and 36–52 respectively. Liver samples were homogenized, and the lipids were extracted as described previously (Bligh and Dyer, 1959) and measured as described above.

Electron microscopy (EM) of lipoproteins

VLDL and IDL/LDL particles from pooled FPLC fractions were placed on a carbon-coated EM grid and negatively stained with 1% uranyl acetate. Particles were examined with a FEI Tecnai F30 electron microscope (FEI Company, Hillsboro, OR). OpenLab software version 3.1.5 (Improvision Inc., Lexington, MA) was used in the measurement of particle size.

Lipase analysis

Mice were deprived of food for 4 h and then injected with 500 U·kg^{-1} body weight of heparin via the retro-orbital sinus. After 5 min, mice were bled from the contralateral retro-orbital plexus, and post-heparin plasma was prepared. Total and hepatic lipase activity were measured by using a CONFLUOLIP™ total or hepatic lipase kit (RDI division of Fitzgerald Industries Intl., Concord, MA) according to the manufacturer's instructions by using a Wallac Victor 1420 (Perkin-Elmer, Boston, MA) fluorescent plate reader.

Nascent lipoprotein analysis

Eight week old LDLR$^{-/-}$ male mice fed the atherogenic diet described above were gavaged daily with vehicle, 3 and 5 mg·kg^{-1} ATI-111 for 8 weeks. After being deprived of food for 16 h, mice were anaesthetized as described above and injected with 500 mg·kg^{-1} body weight of Triton WR1339 (Tyloxapol, Sigma-Aldrich) (15% w/v Triton WR1339 in 0.9% w/v NaCl) via the retro-orbital sinus. Three hours after injection, mice were bled from the contralateral retro-orbital plexus. The plasma samples were then fractionated by FPLC for lipoprotein lipid analysis.

Histology and lesion analysis

Lesions in the innominate artery were quantified from four digitally captured oil red O–stained 10 μm sections, each separated by 100 μm and located between 150 and 450 μm distal to the branch point of the innominate artery from the aortic arch. Lesions in the ascending aorta were assessed from three oil red O–stained sections, each separated by 100 μm and located between 100 and 300 μm below the apex of the lesser curvature of the aortic arch. Aortic sinus lesions were evaluated from three sections, each separated by 100 μm, beginning at the site of appearance of the coronary artery. OpenLab software version 3.1.5 was used in the quantification.

Statistical analysis

Values are presented as means ± SEM and were analysed by using one-way ANOVA and Fisher's protected least significant difference *post hoc* test. A statistically significant difference is set at $P < 0.05$.

Results

In vitro *characterization of ATI-111*

Previously, we modified the side chain of hyodeoxycholic acid by introducing the acidic bis-trifluoromethyl carbinol present in T0901317 to create ATI-829 (Figure 1A), an LXR-specific agonist, which has around 30% of the potency of the nonsteroidal synthetic LXR agonist T0901317 (Peng *et al.*, 2008). Here we introduced a double bond into the side chain of ATI-829 to create ATI-111 (Figure 1A). T0901317, GW3965, ATI-829 and ATI-111 activated a luciferase reporter gene containing LXR response elements in HEK293 cells expressing LXRα or LXRβ (Figure 1B, C). Reporter gene transactivation by LXR agonists in HEK293 cells was dependent on cotransfection of plasmids encoding LXRα or LXRβ (Supporting Figure S3). ATI-111 was the most potent agonist of LXRα (Figure 1B) with EC50 ≈ 60 nM. With LXRβ, GW3965 was the most potent agonist, while ATI-829 was the weakest; T0901317 and ATI-111 had similar potency with LXRβ (Figure 1C) with ED_{50} ≈ 600 and 700 nM respectively. ATI-111 generally was an LXR-specific agonist and did not activate various other nuclear receptors with the exception of a slight (1.5-fold) activation of farnesoid X receptor (FXR) at the higher (10 μM) concentration (Figure 1D). In the positive control, known agonists activated the various receptors tested (Supporting Figure S4), and the synthetic FXR agonist GW4064 activated FXR more than 70-fold at 10 μM (data not shown). Overall, ATI-111 was a more potent LXRα agonist than either T0901317 or GW3965 and preferentially activated LXRα.

The expression of LXR target genes, ABCA1, ABCG1, apoE, SREBP-1c, ACC, FAS, AIM and ArgII, was also evaluated in thioglycollate-elicited peritoneal mouse macrophages treated with T0901317, GW3965 and ATI-111 (Figure 2A). In comparison with vehicle (DMSO)-treated macrophages, treatment with 0.1 and 1 μM of each of the three compounds robustly induced the expression of ABCA1 up to five- to sevenfold and for SREBP-1c up to six- to ninefold, while both doses of these compounds only mildly raised ABCG1 mRNA up to two- to threefold and did not induce the expression of ArgII mRNA in macrophages. Only 1 μM of GW3965 slightly but significantly induced the expression of apoE mRNA in macrophages. T0901317 and ATI-111 were not able to stimulate the expression of apoE mRNA at these concentrations. Both 0.1 and 1 μM of T0901317 slightly but significantly induced the expression of ACC mRNA, while only 1 μM of GW3965 and ATI-111 slightly raised ACC mRNA. Both doses of T0901317 and GW3965 mildly raised FAS mRNA up to two- to fourfold, while ATI-111 only doubled the expression of FAS mRNA at the same doses. All three compounds at 1 μM doubled AIM mRNA expression. However, at 0.1 μM, T0901317 was inactive in the induction of AIM mRNA expression, and GW3965 significantly but only slightly raised AIM mRNA in macrophages, while ATI-111 doubled apoptosis inhibitor expressed by macrophages (AIM) mRNA levels compared with the vehicle treatment in macrophages.

The anti-inflammatory properties of T0901317, GW3965 and ATI-111 were also tested in thioglycollate-elicited peritoneal mouse macrophages (Figure 2B). Both 1 and 10 μM of each of the three compounds remarkably and

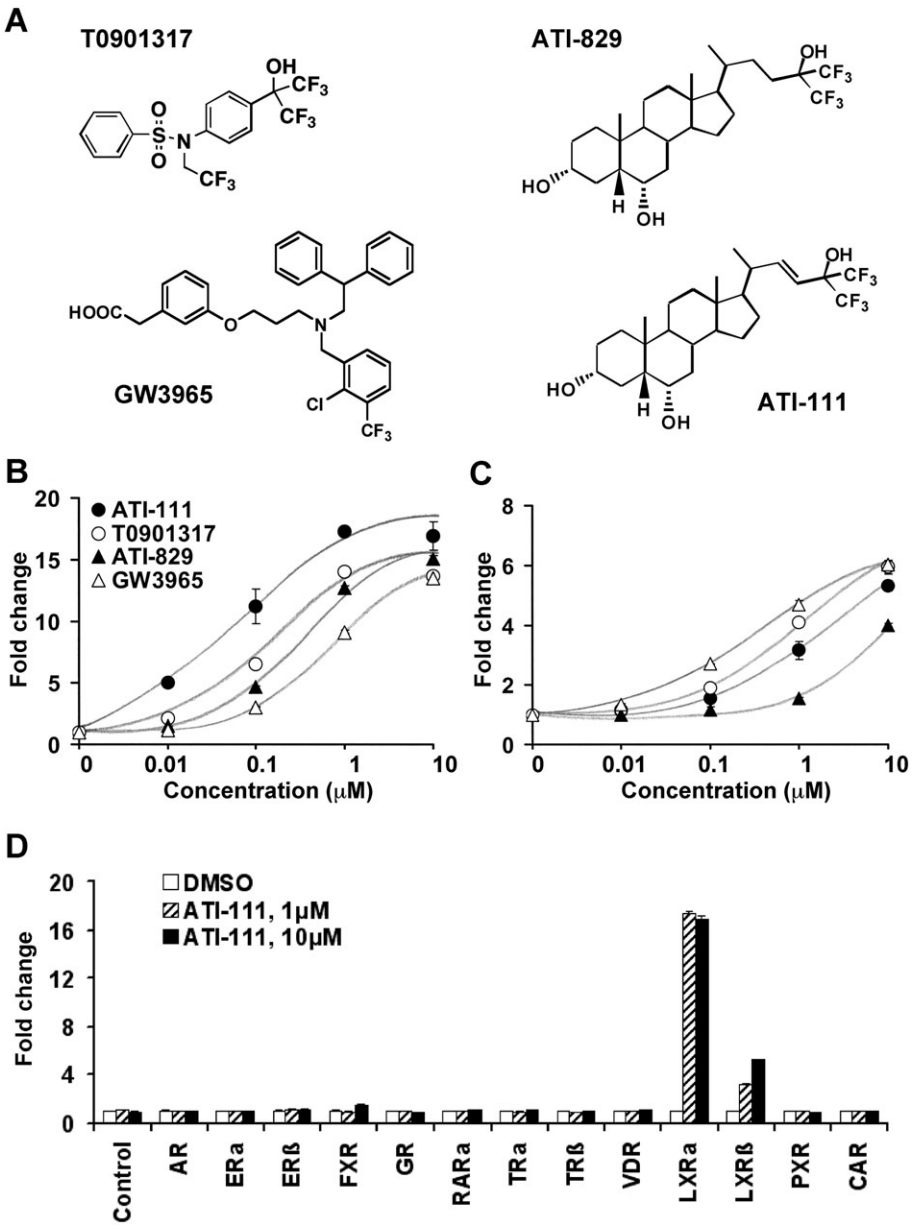

Figure 1

Chemical structures of LXR agonists T0901317, GW3965, ATI-829 and ATI-111 (A); effect of T0901317, GW3965, ATI-829 and ATI-111 on transactivation of a luciferase reporter gene in HEK293 cells expressing LXRα (B) or LXRβ (C) and cotransfected with a pGL3-derived reporter gene containing four copies of an LXR response element and a c-fos minimal promoter; and nuclear receptor–specific transactivation by ATI-111 (D). For receptor-specific transactivation, HEK293 cells were cotransfected with various nuclear receptors and their cognate luciferase reporters and then treated with ATI-111. Firefly luciferase expression was normalized using a cotransfected sea pansy luciferase expression vector and normalized firefly luciferase expression is presented relative to luciferase expression in transfections without added agonist. $n = 6$. LXR, liver X receptor.

dose-dependently repressed the lipopolysaccharide (LPS)-induced expression of inducible nitric oxide synthase (iNOS) mRNA in macrophages, while little or no inhibitory effect on LPS-induced expression of tumour necrosis factor α (TNFα) and matrix metallopeptidase 9 (MMP-9) mRNA was observed. The higher dose (10 μM) of each of the three compounds markedly inhibited the LPS-induced COX2, IL-1β and IL-6 mRNA expression, while the lower dose (1 μM) had no effect. The higher dose of GW3965 and ATI-111 also exerted significant suppression of LPS-induced IL-12 and MCP-1 mRNA expression in macrophages, while this dose of T0901317 only significantly repressed LPS-induced MCP-1 mRNA expression. In addition, a lower dose (1 μM) of ATI-111 significantly inhibited LPS-induced MCP-1 mRNA expression in macrophages. Overall, GW3965 and ATI-111 had a more pronounced anti-inflammatory activity on macrophages compared with T0901317. For some inflammatory genes (iNOS, IL-1β, IL-6, IL-12 and MCP-1), the higher concentration of GW3965

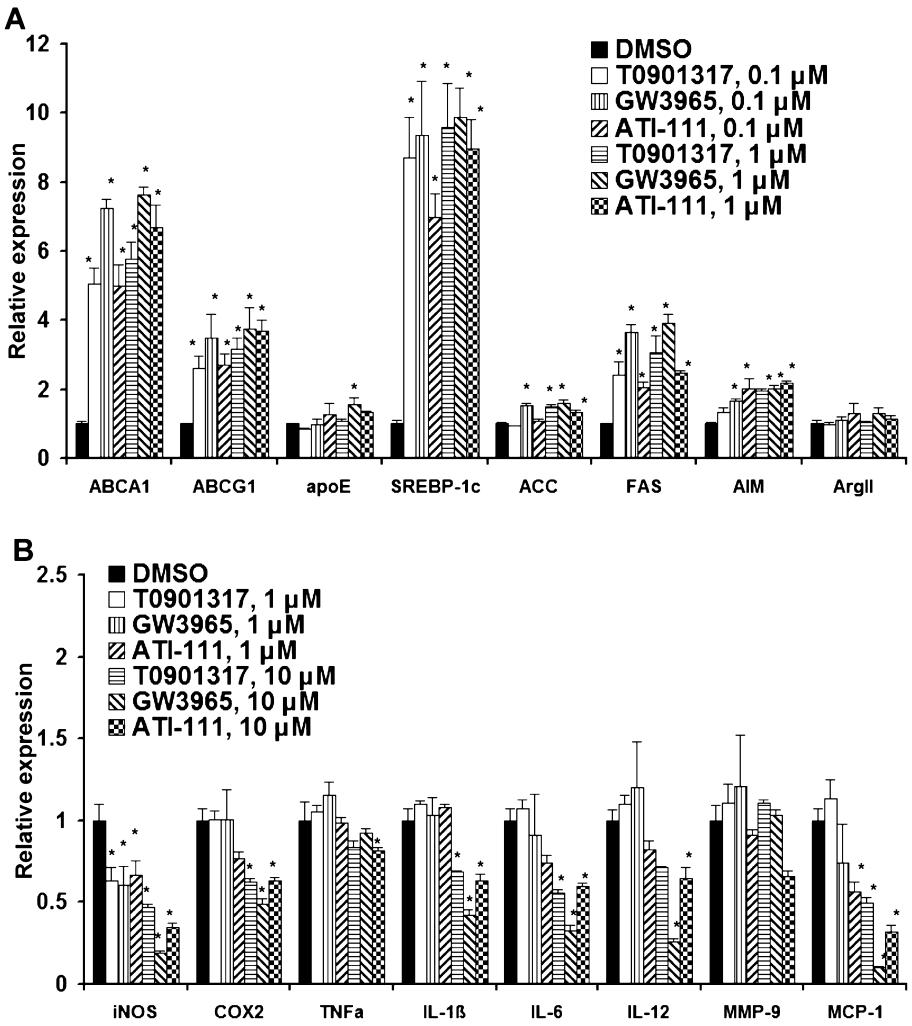

Figure 2

Expression of LXR target genes (A) and inflammatory genes (B) in mouse peritoneal macrophages treated with T0901317, GW3965 and ATI-111. Elicited peritoneal macrophages from C57BL/6 mice were cultured with 0.1 and 1 µM of T0901317, GW3965 and ATI-111 for 24 h to evaluate the effect of LXR agonists on expression of LXR target genes (A). Peritoneal macrophages were also cultured with 1 and 10 µM of T0901317, GW3965 and ATI-111 for 18 h, and then an LPS (400 ng mL^{-1}) was added for another 6 h to evaluate the effect of LXR agonists on expression of various inflammatory genes (B). Specific mRNA levels were measured by quantitative RT-PCR, normalized with 36B4 and presented relative to controls. $n = 3$. Significant differences between control and treatment groups are indicated as $^*P < 0.05$. LXR, liver X receptor; RT-PCR, real-time polymerase chain reaction.

appeared to be more effective in suppressing expression than the same concentration of ATI-111.

ATI-111 selectively activated LXR target genes in vivo

Pharmacokinetics of ATI-111 revealed a similar bioavailability as ATI-829 in mice (data not shown), suggesting that a dose of around 3 $mg \cdot kg^{-1} \cdot day^{-1}$ of ATI-111 should activate LXR target gene expression in mice because the structurally similar but less potent ATI-829 (30% potency of T0901317 *in vitro*) induced LXR target gene expression when administered to mice at 10 $mg \cdot kg^{-1} \cdot day^{-1}$ (Peng *et al.*, 2008). Agonist-induced changes in LXR target gene expression in various tissues were determined by using quantitative real-time PCR. In the small intestine, doses of 3 and 5 $mg \cdot kg^{-1} \cdot day^{-1}$ of ATI-111 moderately induced ABCA1, ABCG5 and ABCG8 mRNA expression, more than twofold and greatly increased SREBP-1c mRNA up to sevenfold (Figure 3A). Furthermore, a slight but significant increase in PLTP mRNA was found in mice treated with the higher dose, while only a slight elevation of LPL mRNA was detected in the small intestine in these mice (Figure 3A). Intestinal expression of FAS, apoE and NPC1L1 mRNA was not increased in ATI-111-treated mice (Figure 3A). In peritoneal macrophages isolated from the treated mice, the higher dose of ATI-111 slightly but significantly increased ABCA1 and ABCG1 mRNA expression, while the expression of apoE mRNA was not altered in these mice (Figure 3B). In the liver, the expression of the mRNA for the lipid transporters ABCA1 and ABCG8 was

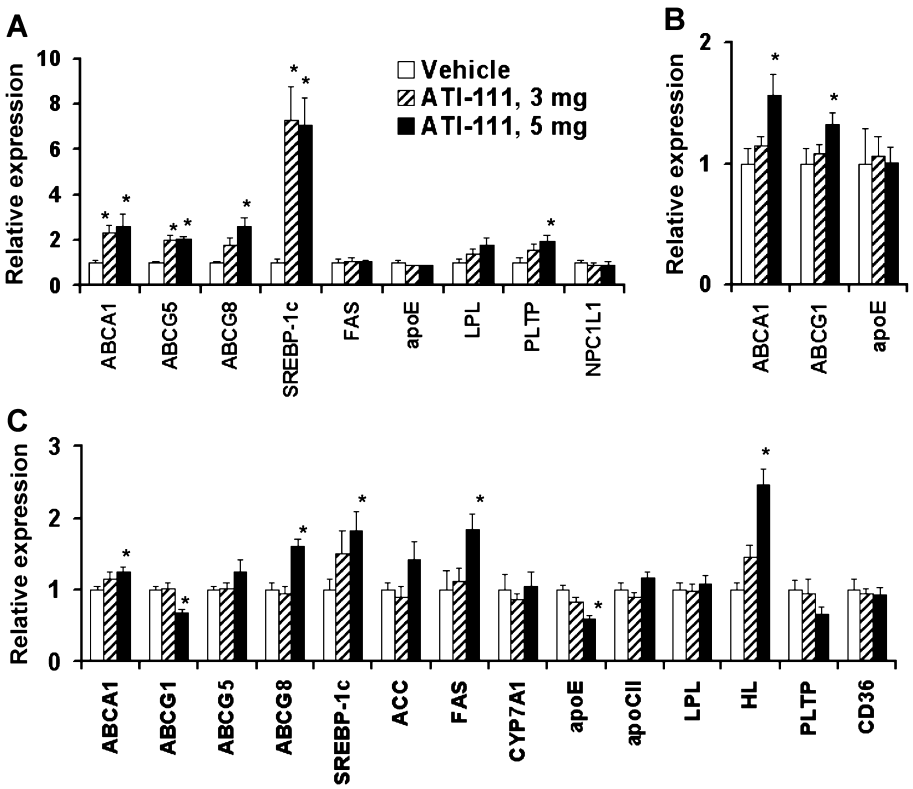

Figure 3

Effect of ATI-111 on gene expression in mouse intestine (A), peritoneal macrophages (B) and liver (C). Male LDLR$^{-/-}$ mice on an atherogenic diet were gavaged daily for 8 weeks with vehicle (control, $n = 8$) and 3 ($n = 9$) and 5 ($n = 9$) mg·kg^{-1} ATI-111. Specific mRNA levels were measured by quantitative RT-PCR, normalized with 36B4 and presented relative to controls. Significant differences between control and treatment groups are indicated as *$P < 0.05$. RT-PCR, real-time polymerase chain reaction.

slightly but significantly increased, while ABCG1 and apoE mRNA expression was clearly inhibited at the higher dose of ATI-111 (Figure 3C). The mRNA level of genes involved in liver lipogenesis, such as SREBP-1c, ACC and FAS, was slightly elevated at the higher dose of ATI-111 (Figure 3C). The higher dose of ATI-111 also slightly increased ABCG5 and inhibited PLTP mRNA expression, although these changes were not statistically significant (Figure 3C). Surprisingly, the higher dose of ATI-111 moderately but significantly increased the expression of mRNA for hepatic lipase (HL), a non-LXR target gene (Figure 3C). Overall, ATI-111 treatment had no effect on the expression of CYP7A1, apoCII, LPL and CD36 mRNA in mouse liver (Figure 3C).

Liver lipid and plasma lipase analysis

Treatment with 3 and 5 mg·kg^{-1}·day^{-1} of ATI-111 did not induce liver accumulation of cholesterol or triglycerides compared with vehicle-treated mice (Figure 4A,B). At 5 mg·kg^{-1}·day^{-1}, ATI-111 slightly but significantly increased the activity of plasma post-heparin hepatic lipase but not total lipase (Figure 4C). Because both plasma post-heparin hepatic and lipoprotein lipase contribute to plasma post-heparin total lipase activity, the activity of plasma post-heparin lipoprotein lipase did not appear to be increased in ATI-111-treated mice.

Analysis of SREBP-1 processing

Oxysterols, such as 25-HC, have been found to inhibit cholesterol synthesis by blocking SREBP processing (Adams *et al.*, 2004). ATI-111 induced SREBP-1c mRNA expression in mouse intestine without increasing the expression of FAS mRNA, a gene regulated by SREBP-1c (Figure 3A), suggesting that ATI-111 may also inhibit SREBP processing. To address this question, we analysed SREBP-1 processing in Caco-2 (Figure 4D) and HepG2 (Figure 4E) cells treated with vehicle (DMSO), 25-HC and ATI-111. As agonists of LXR, 25-HC and ATI-111 dramatically increased the amount of the precursor form of SREBP-1 in both Caco-2 and HepG2 cells compared with vehicle-treated cells (Figure 4D,E). However, the cleaved nuclear forms of SREBP-1 did not accumulate in either 25-HC- or ATI-111-treated Caco-2 and HepG2 cells compared with vehicle-treated cells (Figure 4D,E). In addition, the higher dose of ATI-111 (10 μM) clearly suppressed SREBP-1 processing in Caco-2 cells (Figure 4D). Based on these data, ATI-111 may inhibit SREBP-1c processing.

Plasma lipid and lipoprotein analysis

In our previous studies, the nonsteroidal LXR agonist T0901317 induced hypertrigylceridaemia (Peng *et al.*, 2008). To determine if ATI-111 also induces hypertriglyceridaemia, plasma lipids and lipoproteins were measured in mice fed the

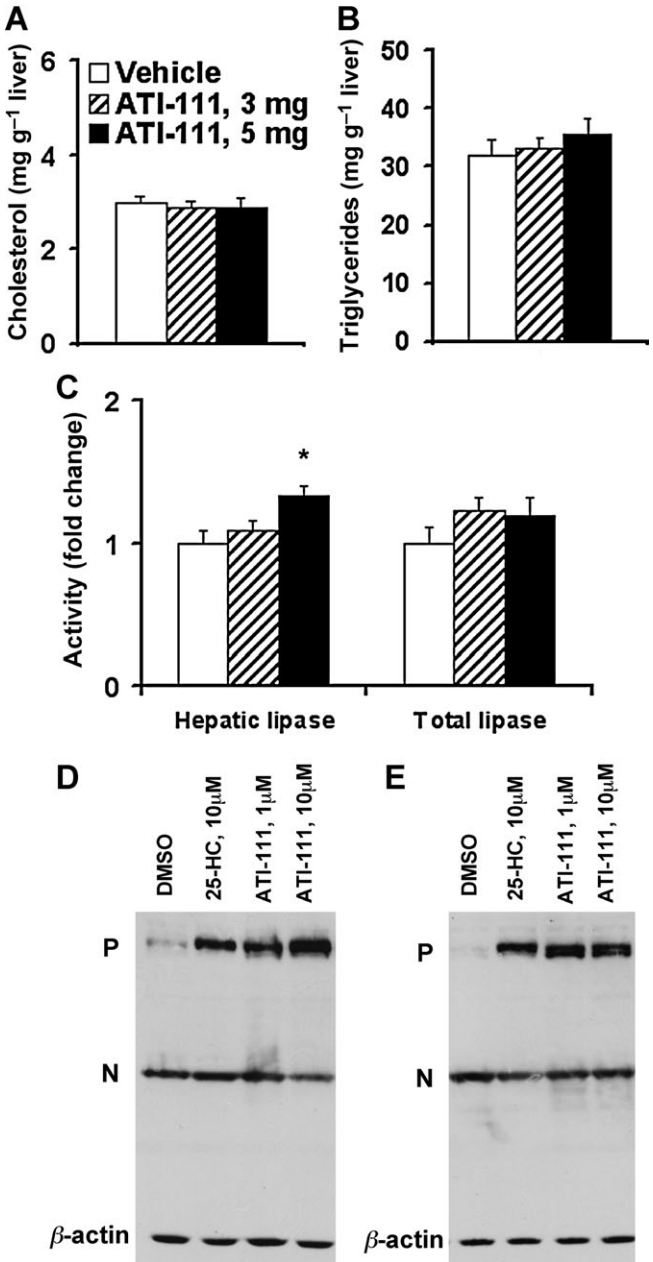

Figure 4

Effect of ATI-111 on hepatic lipid levels (A and B) and plasma lipase activity (C) in atherogenic diet-fed male LDLR$^{-/-}$ mice after 8 weeks of treatment with vehicle (control, $n = 8$) and 3 ($n = 9$) and 5 ($n = 9$) mg·kg^{-1}·day^{-1} of ATI-111. Significant differences between vehicle and treatment groups are indicated as *$P < 0.05$. Effect of LXR agonist 25-HC and ATI-111 on SREBP-1 processing in Caco-2 cells (D) and HepG2 cells (E). P and N denote the uncleaved membrane precursor and processed forms of SREBP-1 respectively. Total cell extracts (50 μg protein) were analysed by immunoblot with antibody against SREBP-1. LXR, liver X receptor; SREBP-1, sterol regulatory element binding protein 1.

atherogenic diet and treated with vehicle or 3 and 5 mg·kg^{-1}·day^{-1} of ATI-111, a steroidal LXR agonist, for 8 weeks. There was a marked decrease in total and VLDL cholesterol with either dose of ATI-111, but IDL/LDL and HDL

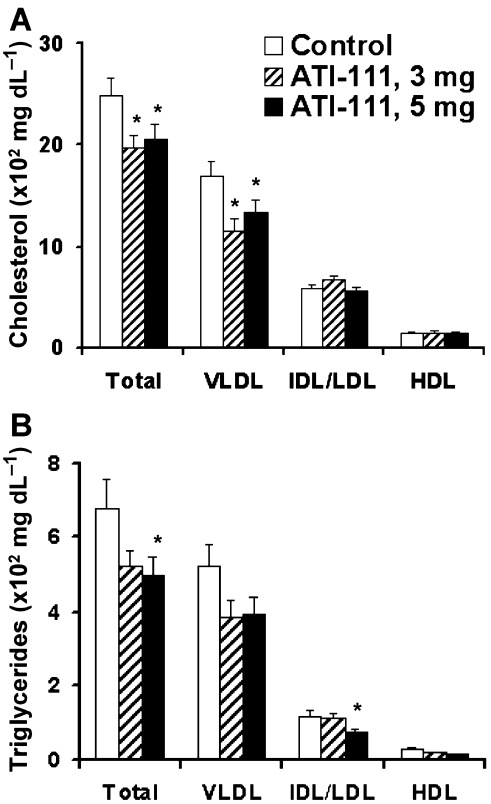

Figure 5

Effect of ATI-111 on plasma lipoprotein cholesterol (A) and triglyceride (B) levels in atherogenic diet-fed male LDLR$^{-/-}$ mice after 8 weeks of treatment with vehicle (control, $n = 15$) and 3 ($n = 15$) and 5 ($n = 13$) mg·kg^{-1}·day^{-1} of ATI-111. Plasma samples were fractionated by FPLC for lipoprotein lipid analysis. Significant differences between vehicle and treatment groups are indicated as *$P < 0.05$. FPLC, fast protein liquid chromatography

cholesterol levels were not altered with either dose of ATI-111 (Figure 5A). Both doses of ATI-111 also reduced plasma triglyceride levels, resulting in a decrease in VLDL triglyceride. IDL/LDL triglyceride was decreased only at the higher dose (Figure 5B).

Treatment of LDLR$^{-/-}$ mice with T0901317 induced the accumulation of small VLDL particles (Peng *et al.*, 2008); thus, we examined the impact of ATI-111 on the size of plasma VLDL and IDL/LDL particles. Pooled VLDL (FPLC fractions 7–14) and IDL/LDL (FPLC fractions 15–35) particles were analysed by EM. Large VLDL particles (>100 nm in diameter) were observed in both vehicle- and ATI-111-treated mice (Figure 6A to C). The average diameter of the plasma VLDL particles in ATI-111-treated mice was similar to vehicle-treated mice (Figure 6D). The distribution of different sizes of VLDL particles was also similar between vehicle- and ATI-111-treated mice (Figure 6E). ATI-111 treatment slightly increased the number of smaller IDL/LDL particles compared with vehicle treatment (Figure 7A to C), with a reduction in the number of larger IDL/LDL particles (>40 nm in diameter) and an increase in the number of 20 to 30 nm IDL/LDL particles (Figure 7E), although these changes were not statistically significant. The average size of IDL/LDL was not altered after

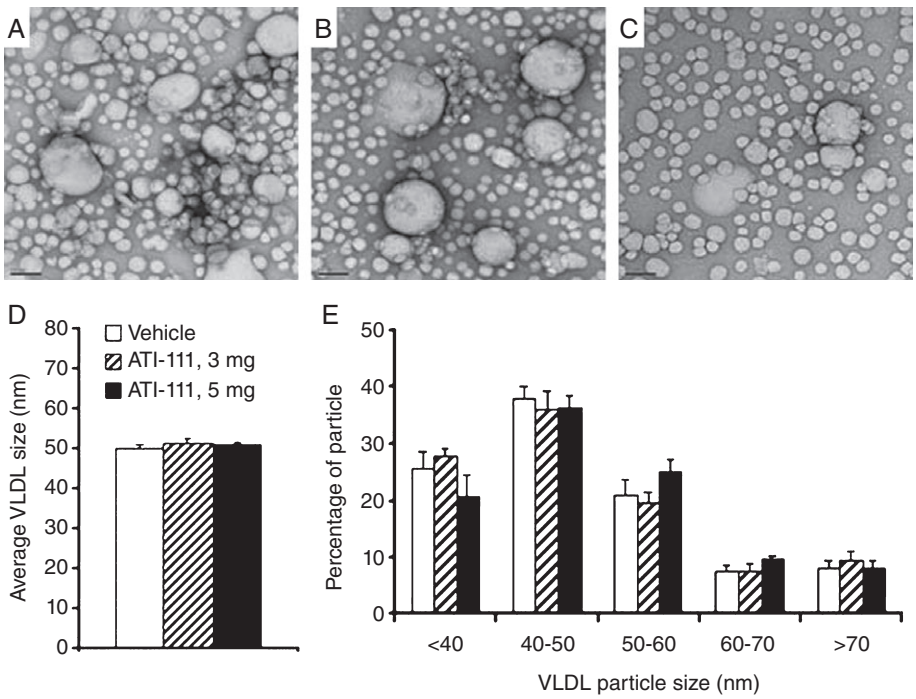

Figure 6

Effect of ATI-111 on plasma VLDL size. Male LDLR-/- mice were fed an atherogenic diet and gavaged with vehicle (A) and 3 (B) and 5 (C) mg·kg^{-1}·day^{-1} of ATI-111 for 8 weeks. FPLC fractions 7 to 14 were pooled for EM analysis. Size bar is 100 nm. The average size (D) and size distributions (E) of VLDL particles from mice treated with vehicle (n = 4) and 3 (n = 4) and 5 (n = 5) mg·kg^{-1}·day^{-1} of ATI-111 were assessed. A total of 250 to 500 particles from each mouse were measured. No significant differences were observed. FPLC, fast protein liquid chromatography; VLDL, very low density lipoprotein.

ATI-111 treatment compared with treatment with vehicle (Figure 7D).

Nascent lipoprotein analysis

The reduction of plasma lipoprotein levels after ATI-111 treatment may be due to differences in liver lipoprotein secretion, post-secretion remodelling of plasma lipoproteins and/or intestinal absorption of dietary lipids between the ATI-111- and vehicle-treated mice. To address this question, mice fed the atherogenic diet and treated with vehicle or ATI-111 for 8 weeks were injected with Triton WR1339 to prevent lipolysis and clearance of triglyceride-rich lipoproteins. Mice were deprived of food for 16 h to exclude exogenous sources of fat before the Triton WR1339 injection. Under these conditions, the main source of plasma lipoproteins would be from endogenous hepatic synthesis and/or secretion. Three hours after injection of Triton, mice were bled, and the plasma samples were fractionated by FPLC for lipoprotein lipid analysis. Plasma total, VLDL, IDL/LDL and HDL cholesterol and triglyceride levels were not different between ATI-111- and vehicle-treated mice (Figure 8A,B). Therefore, ATI-111 treatment did not alter the secretion of newly synthesized lipoproteins from the liver.

Anti-atherosclerotic effects of ATI-111

The impact of ATI-111 on the development of atherosclerosis was examined in mice fed an atherogenic diet. After 8 weeks of treatment with ATI-111, atherogenesis was quantified in the innominate artery, ascending aorta and aortic root. In the innominate artery, treatment with 5 mg·kg^{-1}·day^{-1} of ATI-111 significantly decreased atherosclerotic plaque size by 78% compared with vehicle-treated mice (Figure 9A). Treatment with 3 mg·kg^{-1}·day^{-1} of ATI-111 had no effect on the size of atherosclerotic plaques at this site (Figure 9A). In the ascending aorta, a 63% decrease in plaque size was observed with the higher dose of ATI-111, while the lower dose of ATI-111 produced only a 10% reduction of plaque size (Figure 9B), which was not significantly different from vehicle-treated mice. In the aortic root, the mean atherosclerotic lesion area was significantly reduced by 24% and 54% in mice treated with 3 and 5 mg·kg^{-1}·day^{-1} of ATI-111 respectively (Figure 9C). Overall, ATI-111 had a greater efficacy in blocking development of atherosclerosis in the innominate artery and ascending aorta than in the aortic root, perhaps due to regional differences in sensitivity of atherosclerotic plaque development.

Discussion

In this work, we describe the properties of a new steroidal LXR agonist on lipid metabolism and atherosclerosis. We have previously reported on a related steroidal compound ATI-829, which differs from the current agonist ATI-111 on the basis of the saturation of its side chain (Peng *et al.*, 2008).

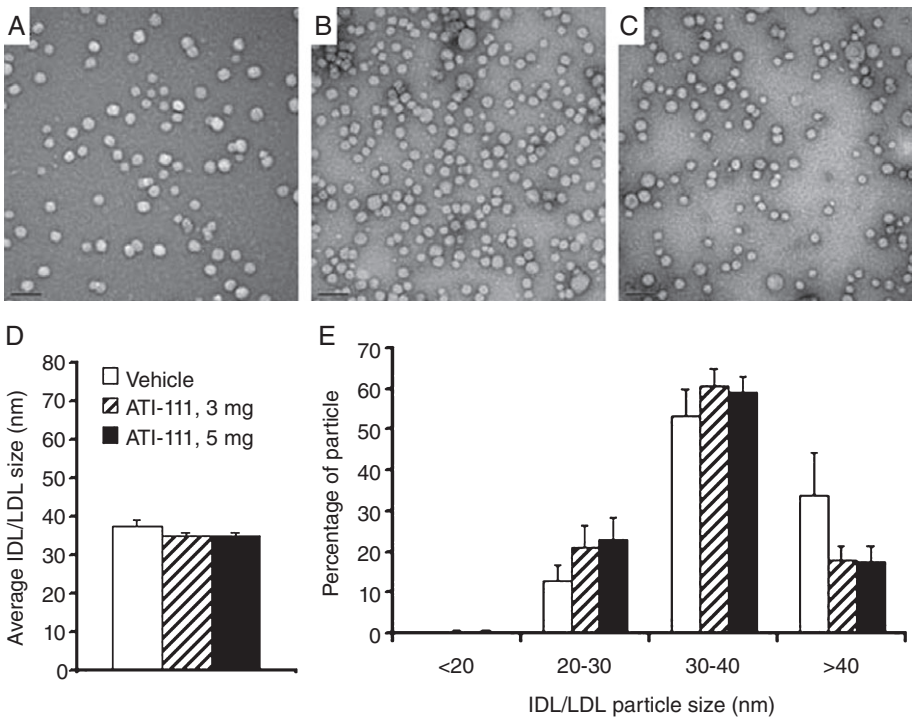

Figure 7

Effect of ATI-111 on plasma IDL/LDL size. Male LDLR$^{-/-}$ mice were fed an atherogenic diet and gavaged with vehicle (control) (A) and 3 (B) and 5 (C) mg·kg^{-1}·day^{-1} of ATI-111 for 8 weeks. FPLC fractions 15 to 35 were pooled for EM analysis. Size bar is 100 nm. The average size (D) and size distributions (E) of IDL/LDL particles from mice treated with vehicle ($n = 4$) and 3 ($n = 4$) and 5 ($n = 5$) mg·kg^{-1}·day^{-1} of ATI-111 were assessed. A total of 250 to 500 particles from each mouse were measured. No significant differences were observed. IDL, intermediate density lipoprotein; LDL, low density lipoprotein; FPLC, fast protein liquid chromatography.

ATI-111 is predominantly an agonist for LXRα with modest activation of LXRβ. The potency of ATI-111 is much higher than its relative ATI-829 and even higher than the nonsteroidal LXR agonist T0901317. This makes it possible to use a lower dose *in vivo* and avoid potential cytotoxicity associated with high concentrations of an oxysterol.

Various LXR agonists have been studied by different groups, seeking agents that would be atheroprotective without the side effects of hepatic steatosis and hypertriglyceridaemia that are contingent upon an up-regulation of SREBP-1c in the liver. Several of these agents appeared to have the desired properties (Albers *et al.*, 2006; Phelan *et al.*, 2008). However, many of these agents are weak LXR ligands. Here we show that the highly potent LXR agonist ATI-111 induced PLTP mRNA expression in intestine and ABCG1 mRNA expression in peritoneal macrophages, while it inhibited their expression in liver, suggesting tissue selectivity. In contrast, ATI-111 induced ABCA1, ABCG8 and SREBP-1c mRNA expression in both intestine and liver, indicating that gene selectivity is probably limited with this highly potent synthetic steroidal LXR agonist. Despite notable up-regulation of SREBP-1c mRNA in the intestine and modest up-regulation in the liver, there was no increase in either hepatic lipids or plasma lipids and lipoproteins. It is known that some oxysterols, and perhaps also for ATI-111, inhibit the dissociation of SCAP from Insig in the endoplasmic reticulum, thus precluding the proteolysis and activation of the SREBP-1c precursor in the Golgi apparatus (Adams *et al.*, 2004; Radhakrishnan *et al.*, 2007). Indeed, when 25-HC and ATI-111 were incubated with Caco-2 and HepG2 cells, there was a very notable up-regulation of SREBP precursor protein, which was not accompanied by an increase in the nuclear (proteolysed) derivative (Figure 4). ATI-111-mediated inhibition of SREBP-1c processing might be responsible for the absence or minimal increase in FAS mRNA expression in intestine and liver. In addition, mice with FAS knockout in liver developed hypoglycemia and fatty liver when fed a diet without fat, indicating that 'new' but not 'old' hepatic fat is required to activate PPARα for the maintenance of glucose and lipid homeostasis (Chakravarthy *et al.*, 2005). In primates, a LXRβ-selective agonist markedly induced hepatic lipid accumulation, while the expression of SREBP1 and FAS in liver was dramatically inhibited (Quinet *et al.*, 2009). Therefore, suitable induction of fatty acid biosynthesis by LXR agonists might be beneficial for hepatic fatty acid metabolism. ATI-111 is highly active, being at least as effective at doses of 3–5 mg·kg^{-1} administered by oral gavage as ATI-829 used at 10 mg·kg^{-1} (Peng *et al.*, 2008), GW3965 used at 20 mg·kg^{-1} (Bradley *et al.*, 2007), DMHCA at 8 mg·kg^{-1} (Kratzer *et al.*, 2009) and WAY 252623 at 15 to 50 mg·kg^{-1} (Quinet *et al.*, 2009). While each of these agents is approximately as effective in modulating murine atherosclerosis in either apoE or LDL receptor deficient mice, they do differ in subtle ways in their metabolic phenotypes.

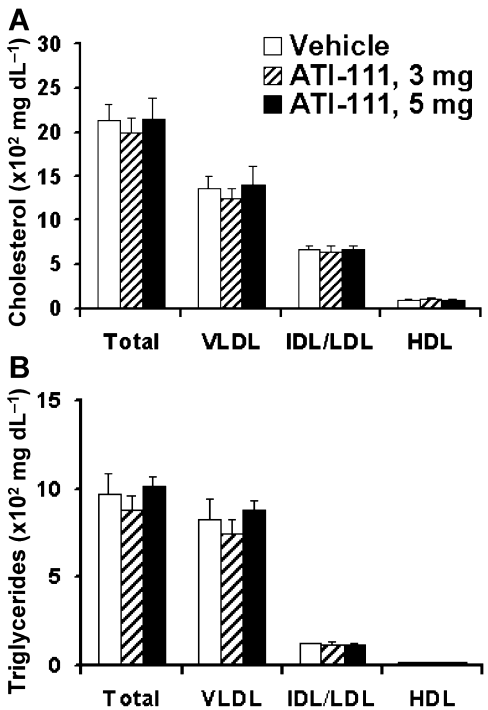

Figure 8

Effect of ATI-111 on nascent plasma lipoprotein cholesterol (A) and triglyceride (B) levels in the atherogenic diet-fed male LDLR$^{-/-}$ mice after 8 weeks of treatment with vehicle (control, $n = 8$) and 3 ($n = 9$) and 5 ($n = 8$) mg·kg^{-1}·day^{-1} of ATI-111. Mice were deprived of food for 16 h followed by Triton WR1339 injection. Three hours after injection of Triton, mice were bled, and the plasma samples were fractionated by FPLC for lipoprotein lipid analysis. No significant differences were observed. FPLC, fast protein liquid chromatography.

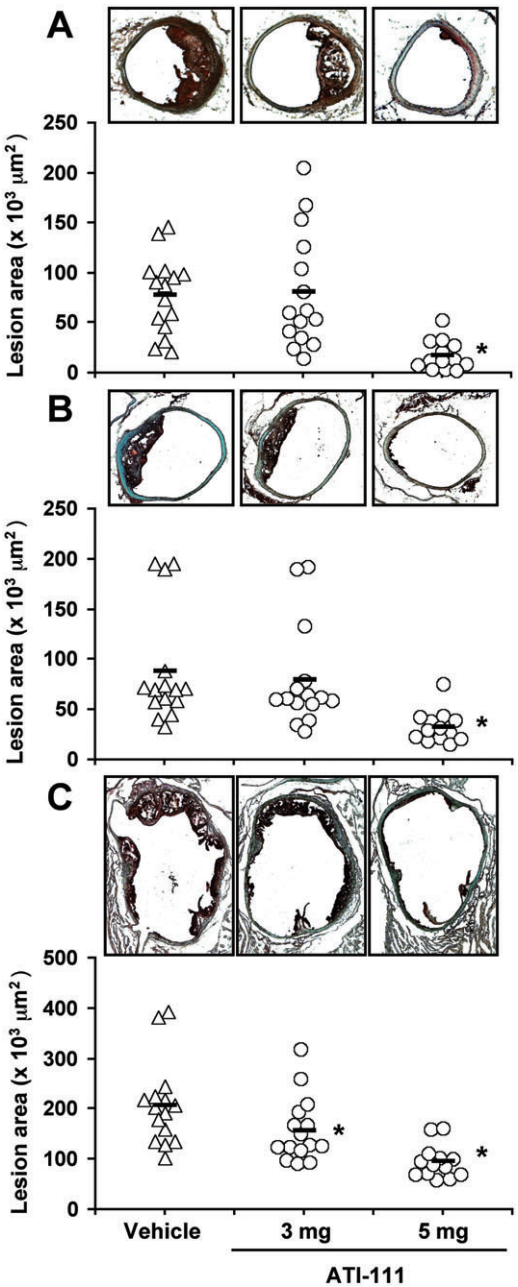

Figure 9

Effect of ATI-111 on atherosclerosis lesion area in the innominate artery (A), ascending aorta (B) and aortic root (C). Male LDLR$^{-/-}$ mice on an atherogenic diet were gavaged daily for 8 weeks with vehicle (control, $n = 15$) and 3 ($n = 15$) and 5 ($n = 13$) mg·kg^{-1}·day^{-1} of ATI-111. Atherosclerotic lesions were measured as described in Methods. Significant differences between vehicle- and ATI-111-treated groups are indicated as $^*P < 0.05$. Original magnification: 100× (A) and 40× (B and C).

In this study, we note that 3 and 5 mg·kg^{-1} doses of ATI-111 administered by oral gavage to high-fat high cholesterol diet-fed LDL receptor deficient mice for 8 weeks, exhibited reductions in total plasma cholesterol and triglycerides, and VLDL cholesterol and triglycerides. There was no increase in hepatic triglycerides despite a modest increase in SREBP-1c mRNA accompanied by a modest increase in its target genes fatty acid synthase and acetyl CoA carboxylase particularly at the higher dose. To understand the physiological significance of these changes, we examined the hepatic secretion of triglycerides and cholesterol but noted no significant change with LXR agonist treatment. Thus, the reduction in plasma triglyceride does not appear to be attributable to a reduction in hepatic output of VLDL. Either the Triton method employed to measure hepatic output of VLDL is not sensitive enough to assess a decline in output sufficient to account for the decreased plasma lipid levels, or the changes are attributable to intraplasma remodelling of VLDL or an increase in lipid export from the intestine during the experiment. With respect to remodelling of VLDL, there was no change in LPL mRNA levels in the liver in contrast to what we observed with mice treated with T0901317 (Peng *et al.*, 2008; 2010). In both liver and intestine, ATI-111 induced changes in the ABC transporters mRNA levels. In the intestine, there was a dose-independent up-regulation of ABCA1 and ABCG5 transcripts by ATI-111 and ABCG8 at the higher dose of agonist. In the liver, there was a more modest transcript increase of ABCA1 and ABCG8 mRNA. These changes, if translated into increased level of protein, could give rise to a modest export of cholesterol especially in the intestine. However, ATI-111

down-regulated apoE expression in liver, which could attenuate ABC transporter-related production of HDL from liver, a major resource of plasma HDL. An unexpected observation among the liver transcripts was the up-regulation of hepatic lipase by the higher dose of ATI-111. This was accompanied by a modest increase in hepatic lipase activity in the plasma (Figure 4), which could possibly account for the remodelling of IDL/LDL described in Figure 7. Hepatic lipase is not a known target of LXR activation, and this change may not be attributable to a direct gene transactivation. A recent study revealed up-regulation of hepatic lipase expression in the liver of $LDLR^{-/-}$ mice after inhibition of lymphotoxin (LT) and LIGHT signalling with a soluble lymphotoxin β receptor decoy protein (Lo *et al.*, 2007). LXR agonists have well-established anti-inflammatory properties, and the effects of ATI-111 on the immune system may indirectly regulate hepatic lipase expression. It has been shown that the activation of LXR can lead to downstream interruption of Toll-like receptor 4, IL-1β and TNFα signalling with a reduction in the expression of several NFκB-dependent genes, including iNOS, IL-6, COX1, MMP9, CCL2, CCL7 and IL-1 (Bensinger and Tontonoz, 2008). In this study, ATI-111 was also observed to suppress the expression of LPS-induced inflammatory genes, such as iNOS, COX2, TNFα, IL-1β, IL-6, IL-12 and MCP-1, in mouse peritoneal macrophages. For most of these genes, GW3965 was at least as effective as ATI-111 in suppressing gene expression especially at the higher concentration (but not TNFα). Additionally, uptake of apoptotic cells by macrophages leads to the activation of LXR and up-regulation of Mertk, responsible for the clearance of apoptotic cells, which may also lead to the up-regulation of other cholesterol-regulated genes, as well as a suppression of pro-inflammatory cytokines (A-Gonzalez *et al.*, 2009).

In these experiments, the reduction in atherosclerosis could be the result of at least three mechanisms: the reduction in plasma lipids, the promotion of reverse cholesterol transport and the LXR-mediated anti-inflammatory processes. The reduction in plasma cholesterol and triglycerides was equivalent at each dose of ATI-111, yet it was only at the higher dose that there was a reduction in innominate artery and aortic arch atherosclerosis. In the aortic roots, there was a dose-dependent reduction in lesion area despite the fact that the plasma lipid reduction was not dose-dependent. Taken together, the reduction in atherosclerosis at any of the three vascular sites we examined does not appear to be simply attributable to the change in plasma lipids. The anti-inflammatory effect of ATI-111 on mouse peritoneal macrophages suggests that an effect of ATI-111 on the immune system may also play a role in its anti-atherosclerotic effect.

The activation of reverse cholesterol transport is a mechanism that probably makes a major contribution to the changes in atherosclerosis. Macrophages constitute critical cell components of the atherosclerotic plaque. In peritoneal macrophages, there was a dose-dependent up-regulation of both ABCA1 and ABCG1, which if applicable to lesional macrophages, could lead to an increased efflux of cholesterol from these cells and a reduction in atherosclerosis. Furthermore, a recent study has suggested that the activation of LXR in the intestine, as has been shown in this present study, is associated with the promotion of reverse cholesterol transport *in vivo* (Yasuda *et al.*, 2010).

Here we have shown an atheroprotective action at three separate vascular sites by cross-sectional analysis. This has not previously been shown. We know that not all vascular sites respond similarly to various interventions (VanderLaan *et al.*, 2004) including LXR-dependent treatment (Peng *et al.*, 2008, 2009). Taken together, it is clear that a number of potentially valuable therapeutic LXR agonists are available for further development. These agents do not cause the undesirable side effect of hypertriglyceridaemia. However, much work is required to ascertain precisely how the LXR agonists provide atheroprotection at various athero-susceptible sites.

Acknowledgements

We thank Yimei Chen from the Electron Microscopy Facility of the University of Chicago for her invaluable technical support. This research was partly supported by National Institutes of Health grants AT00850 and CA58073 and funding from Anagen Therapeutics Inc.

Conflicts of interest

Conflict of interest statement: DP, RAH, JTX, Q D, J M K, CAR and GSG have no conflicts to disclose. SL has a financial interest in Anagen Therapeutics Inc., which has licensed patents dealing with ATI-111 (U.S. patent US7012069B2, 14March 2006).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Procedure of synthesis of 3α, 6α-24-trihydroxy-22-en-24-di-trifluoromethyl-5β-cholane (ATI-111).
Figure S2 Information regarding compound T0901317, GW3965, ATI-829 is available from the following References:
Figure S3 Effect of T0901317, GW3965, ATI-829, and ATI-111 on luciferase reporter gene expression in HEK293 cells without expressing LXRα or LXRβ.
Figure S4 Nuclear receptors were activated by their own agonists.

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