Increased HDL Cholesterol and ApoA-I in Humans and Mice Treated With a Novel SR-BI Inhibitor

David Masson, Masahiro Koseki, Minako Ishibashi, Christopher J. Larson, Stephen G. Miller, Bernard D. King, Alan R. Tall

Objectives—Increasing HDL levels is a potential strategy for the treatment of atherosclerosis.

Methods and Results—ITX5061, a molecule initially characterized as a p38 MAPK inhibitor, increased HDL-C levels by 20% in a human population of hypertriglyceridemic subjects with low HDL levels. ITX5061 also moderately increased apoA-I but did not affect VLDL/LDL cholesterol or plasma triglyceride concentrations. ITX5061 increased HDL-C in WT and human apoA-I transgenic mice, and kinetic experiments showed that ITX5061 decreased the fractional catabolic rate of HDL-CE and reduced its hepatic uptake. In transfected cells, ITX5061 inhibited SR-BI–dependent uptake of HDL-CE. Moreover, ITX5061 failed to increase HDL-C levels in SR-BI−/− mice. To assess effects on atherosclerosis, ITX5061 was given to atherogenic diet–fed Ldlr+/− mice with or without CETP expression for 18 weeks. In both the control and CETP-expressing groups, ITX5061-treated mice displayed reductions of early atherosclerotic lesions in the aortic arch −40%, P<0.05, and a nonsignificant trend to reduced lesion area in the proximal aorta.

Conclusions—Our data indicate that ITX5061 increases HDL-C levels by inhibition of SR-BI activity. This suggests that pharmacological inhibition of SR-BI has the potential to raise HDL-C and apoA-I levels without adverse effects on VLDL/LDL cholesterol levels in humans. (Arterioscler Thromb Vasc Biol. 2009;29:2054-2060.)

Key Words: scavenger receptor B-I ■ high-density lipoproteins ■ inhibitors ■ p38 MAPK ■ atherosclerosis

Plasma high-density lipoprotein (HDL) levels are inversely correlated with atherosclerotic cardiovascular disease, and raising HDL levels by lifestyle changes or pharmacological interventions is an emerging strategy that might help to reduce the residual burden of disease in patients treated with low-density lipoprotein (LDL)–lowering approaches.1,2 Whereas increasing synthesis or infusion of apolipoprotein A-I (apoA-I) or HDL reduces atherosclerosis in animals and humans, it is not clear that all approaches to raising HDL will reduce atherosclerosis.

The scavenger receptor B-I (SR-BI) is a major factor regulating HDL catabolism. SR-BI binds HDL and mediates the selective uptake of HDL-cholesteryl ester (CE) in the liver and steroidogenic tissues.3 Although the role of SR-BI in HDL metabolism in mice and rats has been clearly shown, much less information is available on the function of SR-BI in humans. Primary human hepatocytes do demonstrate selective uptake of HDL-CE,4 but the quantitative importance of this pathway has not been shown. In humans, cholesteryl ester transfer protein (CETP), a factor lacking in mice and rats, plays a major role in HDL-CE catabolism. In the present study, we demonstrate that ITX5061, a molecule initially characterized as a p38 mitogen-activated protein kinase (MAPK) inhibitor, caused an increase in HDL cholesterol (HDL-C) and apoA-I levels in humans. Studies in mice and cultured cells indicated that ITX5061 is an inhibitor of the SR-BI activity. Interestingly, ITX5061 did not cause increases in very-low-density lipoprotein (VLDL) and LDL cholesterol and reduced atherosclerosis in the aortic arch in LDL receptor (Ldlr) heterozygous mice fed an atherogenic diet.

Materials and Methods
A complete Materials and Methods section is available in the supplemental materials (available online at http://atvb.ahajournals.org).

Molecule
Structure of ITX5061 is shown in supplemental Figure I. The empirical formula of ITX5061 is C30H38ClN3O7S. The molecular weight of ITX5061 (as an HCl salt) is 620.2.

Human Data
KC706-C06 study was conducted from November 6, 2006 to May 8, 2007. It was a multicenter, randomized, double-blind, placebo-controlled study. Its primary objective was to evaluate the ability of ITX5061 to increase HDL-C in patients with low HDL-C and elevated triglycerides (TG) at baseline.
Animals
C57Bl/6 wild-type (WT) mice, C57Bl/6 SR-BI–deficient mice (SR-BI−/−),5 and C57Bl/6 transgenic mice expressing human apoA-I under the control of its natural flanking regions (HuAITg) 6 were used in the present study. Atherosclerosis studies were conducted in F1 hybrid C57BL/6 DBA/1 Ldlr−/− mice fed the Paigen diet, which is high-fat/cholesterol/bile salt diet containing 1.25% cholesterol, 7.5% cocoa butter, and 0.5% cholic acid (TD 88051; Harlan Teklad). Animal protocols were approved by the Institutional Animal Care and Use Committee of Columbia University.

Results
ITX5061 Increases HDL Levels in Humans
ITX5061 was initially characterized as a type II (non competitive) inhibitor of p38 MAPK (supplemental Figure II) and was noted to cause increases in HDL-C levels in humans. To systematically investigate effects on plasma lipoprotein levels, ITX5061 was administered at 2 different doses in a population of subjects with low HDL-C (men <45 mg/dL and women <55 mg/dL) and increased TG concentrations (TG ranging from 150 to 400 mg/dL). Baseline characteristics are given in supplemental Table I. Subjects received either placebo or ITX5061 at 150 mg or 300 mg daily. ITX5061 treatment resulted in an increase in HDL-C concentration that was similar in the 2 ITX5061-treated groups (∼20% increases). The rise in HDL-C was observed throughout the treatment period and was reversed after cessation of the drug (Figure 1). The mean HDL particle size was significantly increased in ITX5061-treated subjects (P<0.05; Figure 2A). No significant changes in other lipid parameters (LDL-C and TG) were observed, although a transient nonsignificant increase in TG was observed after 2 weeks of treatment (supplemental Figure III). ApoA-I protein levels were moderately increased in the 150 mg group (+10%, P<0.05) but were not statistically different in the 300 mg group (Figure 2B). Adverse events were similar in all groups except for one patient in the 150 mg group and 4 patients in the 300 mg group who had reversible increases in transaminase levels. These findings indicate a selective increase in HDL-C and apoA-I in subjects treated with the 150 mg dose of ITX5061. Further increases in these parameters were not seen at the 300 mg dose.

ITX5061 Increases HDL-C and Size in HuAITg Mice
To investigate the mechanism of HDL-C raising by ITX5061, further studies were conducted in mice. HuAITg mice were treated with ITX5061 (30 mg/kg/d) or vehicle for 1 week. This resulted in a 50% increase in HDL-C levels compared to baseline, but no change in non-HDL-C levels (Figure 3A). As determined by scanning SDS-PAGE gels, apoA-I levels were moderately (+15%) but significantly increased in ITX5061-treated HuAITg mice, compared to mice received vehicle (Figure 3B). Consistent with the more prominent increase in HDL-C than apoA-I, ITX5061 treatment induced a shift toward larger sized HDL as shown by native-PAGE (Figure 3C). To assess whether ITX5061 could affect expression of genes involved in HDL biosynthesis or catabolism in the liver, relative mRNA levels of apoA-I, ABCA1, and SR-BI were...
determined in ITX5061 and vehicle-treated animals. No changes in mRNA levels were found for any of these transcripts (supplemental Figure IV), suggesting that ITX5061 raised HDL independent of these transcript levels.

**ITX5061 Decreases HDL-CE Catabolism and Hepatic Uptake**

To gain more insight into the mechanisms by which ITX5061 increases HDL-C, a kinetic study was performed. HDL labeled with [3H]CE was injected via the tail vein into HuAITg mice pretreated or not with ITX5061. As shown in Figure 3D, ITX5061 significantly decreased HDL-CE catabolism with an FCR of 1.86 ± 0.26 pools/d versus 2.47 ± 0.26 pools/d in the control group (P < 0.05), whereas calculated production rates were identical in both groups (129 ± 24 µg/dl versus 129 ± 16 µg/dl). Moreover, accumulation of [3H] CE in the liver was significantly lower in ITX5061-treated mice, indicating that increased HDL-CE levels were attributable to reduced uptake by the liver (Figure 3E).

**ITX5061 Does Not Increase HDL Levels in SR-BI−/− Mice and Reduces Selective Uptake via SR-BI in Cell Culture**

Because SR-BI has a major role in the clearance of HDL-C and CE in the liver, we determined whether ITX5061 could still raise HDL levels in SR-BI−/− mice. WT and SR-BI−/− mice were treated for 1 week by daily administration of ITX5061 at 30 mg/kg/d. Although ITX5061 increased HDL-C in WT mice its effects were abolished in SR-BI−/− mice, suggesting that the mechanism of action was dependent on SR-BI (Figure 4B). A significant increase in non-HDL cholesterol levels was also observed in ITX5061-treated WT mice. Analysis of apolipoproteins by SDS-PAGE in fractions corresponding to the LDL region of similar profiles indicated they consisted primarily of apoB lipoproteins with only trace amounts of apoA-I (data not shown). Interestingly, despite increases in HDL-C, no
increase in apoA-I was observed in ITX5061-treated WT mice (Figure 4A). We also confirmed by Western blot that ITX5061 did not affect the protein levels of apoA-I, ABCA1, and SR-BI in the liver (supplemental Figure IVB).

To test the SR-BI inhibitory potential of ITX5061 in vitro, HEK 293 cells overexpressing SR-BI were incubated with [3H]CE-labeled HDL. As shown in Figure 4C, ITX5061 significantly decreased HDL uptake at 1 μmol/L concentration, whereas a structurally related p38 MAPK inhibitor (KR-004515) had no effect at the same concentration.

**ITX5061 Increases HDL-C and Reduces Atherosclerosis in Ldlr<sup>−/−</sup> Mice Fed the Paigen Diet**

Most prior studies of the effects of SR-BI deficiency or inhibition on atherogenesis have used Ldlr<sup>−/−</sup> or apoE<sup>−/−</sup> mice. We wished to determine effects of ITX5061 on atherogenesis in a setting where the clearance of VLDL/LDL particles by apoE- or Ldlr-dependent pathways is not completely impaired and with the potential availability of a compensatory pathway of reverse cholesterol transport via CETP. Indeed, it has been shown recently that CETP expression was able to reverse the atherogenic phenotype in SR-BI<sup>−/−</sup> mice. Thus, to determine the effects of ITX5061 on atherogenesis, Ldlr<sup>−/−</sup> mice, with or without expression of CETP, were fed the Paigen diet containing ITX5061 (0.037%) for 18 weeks. CETP expression was induced by injection of AAV-CETP and was monitored 2 weeks later. Plasma CETP levels were slightly lower than obtained in a human plasma sample in mice injected with AAV-CETP but within the physiological range for humans (supplemental Figure V). Plasma lipid parameters were monitored after 2 and 18 weeks of treatment. No differences in total cholesterol or TG levels were observed between ITX5061-treated or control mice. However, a 30% increase in HDL-C concentrations was observed in ITX5061-treated groups as determined by FPLC (Figure 5A) and ultracentrifugation (supplemental Table II). At human-like levels, CETP expression has only little effects on plasma lipid levels in WT mice as originally reported. As expected, animals fed the Paigen diet developed moderately elevated transaminase levels, but there was no hyperbilirubinemia and there were no differences between the 4 groups (supplemental Table II).

Figure 5 shows representative pictures of aortic arches from male mice with or without treatment by ITX5061. Lesions were observed primarily at branch points and sometimes along the lesser curvature. A significant 40% reduction of atherosclerotic lesions was observed in ITX5061-treated mice. Similar reductions in atherosclerosis were seen in ITX5061-treated mice injected with AAV-CETP or AAV-Ctrl (Figure 5C). No difference in lesion area was observed when comparing AAV-Ctrl groups and AAV-CETP groups either in control or ITX-5061 treated mice. Analysis of the lesions was also performed in the aortic valves (Figure 5D). Although there was a trend toward reduced lesion area in both groups treated with ITX5061, the differences were not statistically significant.

**Discussion**

Our findings indicate that ITX5061, initially characterized as a p38 MAPK inhibitor, is able to increase HDL-C levels in humans and rodents and strongly suggest that this occurs at least in part through inhibition of SR-BI activity. Importantly, whereas previous studies have shown that modulation of SR-BI activity could affect apoB-containing lipoprotein levels, ITX5061 did not increase VLDL/LDL cholesterol or plasma TG concentrations in humans and Ldlr<sup>−/−</sup> mice. Finally, although many studies have documented markedly increased atherosclerosis in various mouse models deficient in SR-BI, we observed that ITX5061 had no significant effect on atherosclerotic lesions in the aortic valves and reduced early atherosclerotic lesions in the aortic arch of Ldlr<sup>−/−</sup> mice fed the Paigen diet for 18 weeks.
Several lines of evidence suggest that the primary mode of action of ITX5061 is via inhibition of SR-BI activity. First, similar to what is observed in SR-BI−/− mice, ITX5061 increased HDL-C more than apoA-I and led to formation of large sized HDL enriched in cholesterol. Second, ITX5061 raised HDL-C by decreasing HDL-CE plasma catabolism and uptake by liver. Third, ITX5061 effects on HDL-C were abolished in SR-BI−/− mice. Fourth, in vitro, ITX5061 inhibited SR-BI-mediated HDL-CE uptake whereas other structurally related compounds without HDL raising ability did not. Although ITX5061 was initially developed as a p38 MAPK inhibitor, it seems unlikely that this property contributed to increased HDL because structurally similar p38 MAPK inhibitors did not increase HDL levels. Nevertheless, the exact mechanism of SR-BI inhibition by ITX5061 remains to be determined. Other SR-BI inhibitors described to date decreased HDL-CE selective uptake and increased the binding of HDL to SR-BI suggesting the formation of inactive HDL-SR-BI complexes.14,15

The effects of SR-BI deficiency/inhibition on lipoprotein profiles are well documented in mice.5,16,17 SR-BI−/− mice display a marked increase in HDL-C concentration attributable to accumulation of very large size apoE- and cholesterol- and CE-enriched HDL. In the absence of apoE or Ldlr, SR-BI deficiency also increases VLDL/LDL concentration, indicating a role for SR-BI as a back up receptor for these lipoproteins.16,18–20 In contrast to mice, much less is known about the effects of SR-BI deficiency or inhibition on lipoprotein profiles in humans. Several nonfunctional SR-BI polymorphisms have been associated with changes in HDL and LDL cholesterol levels, perhaps suggesting a role of SR-BI in humans.21–24 No genetic deficiencies for SR-BI have been described to date.25,26 Only one preliminary report on administration of SR-BI inhibitory molecules in humans has been presented. HDL376, a molecule with SR-BI inhibitory properties was able to increase HDL-C levels in humans (+20%) and also in nonhuman primates.27,28 Our results suggest that the use of SR-BI inhibitors in humans has the potential to increase HDL-C and to a lesser extent apoA-I without detrimental effects on apoB-containing lipoprotein concentration, leading to a potentially less atherogenic lipoprotein profile. Interestingly, ITX5061 increased apoA-I as well as HDL-C in humans and in HuAITg mice, but in wild-type mice only increased HDL-C levels. This suggests that there could be a difference in effects of SR-BI deficiency on HDL particles containing human apoA-I versus mouse apoA-I. Nevertheless, the increase of apoA-I was only moderate as compared to HDL-C, consistent with the SR-BI inhibition mechanism. The lack of increase VLDL and LDL cholesterol in humans treated with ITX5061 likely reflects the effect of partial SR-BI deficiency in a setting where apoE and Ldlr are still functional.

After the discovery of dramatically increased HDL levels in SR-BI−/− mice,3 there was considerable initial interest in the potential to use inhibition of SR-BI as an antiatherogenic strategy. However, this idea was discarded in the face of compelling evidence that SR-BI deficiency increased atherosclerosis in several different mouse models, sometimes in a dramatic fashion, while SR-BI overexpression had the opposite effects.7–9,16,20–29 Besides genetic models of SR-BI overexpression or deficiency, pharmacological SR-BI inhibitors such as BLTs, HDL376, and R-138329 have been characterized.14,15,27,33,34 The effects of the latter compound on atherosclerosis have been investigated in apoE−/− mice, and moderate proatherogenic effects were observed but only at high concentration.35 The increase in atherosclerosis seen in SR-BI−/− mice has been attributed to the decrease in reverse cholesterol transport that has been clearly demonstrated in these mice.36 In favor of this hypothesis, CETP expression was able to reverse the atherogenic phenotype in SR-BI−/− mice.10 However, in many of these studies changes in HDL levels were associated with parallel changes in levels of VLDL and LDL, tending to confound the analysis of the mechanisms of atherogenesis. We did not find a proatherogenic effect of CETP on atherosclerosis in our model, either with or without ITX5061 treatment. This is possibly because of the low level of CETP expression at which we chose to work. CETP was previously shown to be moderately proatherogenic in Ldlr−/− that displayed much higher plasma CETP levels.37

In the present study we observed that ITX5061 treatment resulted in a 40% reduction in atherosclerosis in the aortic arch after 18 weeks of treatment in Ldlr−/− mice fed the Paigen diet. The antiatherogenic effect of the molecule was observed in the control and in the CETP expressing group. Our results suggest therefore that partial SR-BI inhibition could have some beneficial effects per se whether an alternative pathway for HDL cholesterol uptake or transfer is provided by CETP or not.

Several points might account for differences between our results and those from previous studies. First, we used a model of Ldlr−/− mice where the clearance of apoB lipoproteins is not completely impaired, leading only to moderate hypercholesterolemia. Secondly, SR-BI inhibition was not complete in our experimental conditions, only a 30% to 40% increase in HDL levels was observed as compared with typical +125% increases in SR-BI−/− animals. Because it has been shown that SR-BI deficiency has different effects in liver and in vascular wall, selective inhibition of SR-BI by ITX5061 in different tissues could also explain some of the differences.38 Third, whereas most studies used Western-type diet, we used a cholic acid–containing diet, which have different effects on atherogenesis. Although this diet has been extensively used in atherosclerosis studies,39 it is also recognized to have significant limitations. Indeed, cholate that is present in the Paigen diet induces liver inflammation and fibrosis and these effects contribute to the peculiar atherogenicity of the diet.40 These concerns limit the usefulness of this model in extrapolating results to other settings such as in humans. Nonetheless, in the same dietary model marked overexpression of SR-BI was antiatherogenic in conjunction with both HDL and VLDL/LDL lowering. Fourth, because ITX5061 is also a potent p38 MAPK inhibitor, it is possible that this off-target effect contributes, at least
partially, to the reduction of atherosclerotic lesions in ITX5061-treated mice. However, recent data do not support a role for p38 MAPK in regulating atherosclerotic lesion size. A p38 MAPK inhibitor decreased inflammation in atherosclerotic plaques but did not affect lesion size in apoE−/− mice infused with angiotensin II.41 Similarly, p38 MAPK deficiency in macrophages increased necrotic cores in advanced lesions in Western-type diet–fed apoE−/− mice but did not change lesion size.42 Therefore, although it seems unlikely that p38 MAPK inhibition contributed significantly to the reduction of atherosclerosis in our model, we cannot rule this out. In conclusion, inhibition of hepatic SR-BI in a setting that leads to increases in HDL without concomitant increases in VLDL and LDL cholesterol levels could potentially have an antiatherogenic effect.

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Disclosures
C.J.L., S.G.M., and B.D.K. were formerly employed by Kemia Inc.

References
20. Calvo D, Gomez-Coronado D, Lasuncion MA, Vega MA. CLA-1 is an 85-kDa plasma membrane glycoprotein that acts as a high-affinity receptor for both native (HDL, LDL), and VLDL) and modified (oxLDL, and AcLDL) lipoproteins. Arterioscler Thromb Vasc Biol. 1997;17:2341–2349.


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**Human data**

KC706-C06 study was conducted from 6 November 2006 to 8 May 2007. It was a multicenter, randomized, double-blind, placebo-controlled study. Its primary objective was to evaluate the ability of ITX5061 to increase HDL-C in patients with low HDL-C and elevated triglycerides (TG) at Baseline. The study was conducted under a United States (US) Investigational New Drug Application and in compliance with US Food and Drug Administration (FDA) Code of Federal Regulations. All principles of the Declaration of Helsinki (1964) were followed. Patients were randomized in a 1:1:1 ratio to placebo, 150 mg ITX5061, or 300 mg ITX5061 for oral administration once daily for 6 weeks. A fasting lipid panel (total cholesterol, HDL-C, LDL-C, and TG), and general laboratory parameters were assessed at the Baseline Visit, every 2 weeks during dosing, and 4 weeks after the end of dosing (Follow-up Visit). The main inclusion criteria were 18 to 70 years of age, inclusive; decreased HDL-C ($\leq 45$ mg/dL in men or $\leq 55$ mg/dL in women); elevated TG (150 to 400 mg/dL); LDL-C $\leq 250$ mg/dL; on a stable dose of all medications that might have altered lipid levels or for diabetes management for at least 1 month before screening. Treatment-emergent adverse effects (TEAEs) were reported for 56.8%, 65.8%, and 67.5% of patients in the placebo, 150-mg, and 300-mg groups, respectively. Only one patient (300 mg) experienced severe adverse effects (unstable angina, atrial fibrillation); these effects resolved while continuing on study medication and were considered to be unrelated to the study drug. All other TEAEs were reported as mild or moderate. Increased indirect bilirubin was reported in 7.5% of patients in the 300-mg group; this effect is related to inhibition of the UGT1A1 enzyme responsible for bilirubin glucuronidation. Total cholesterol, LDL-C, HDL-C and TG were assayed via reagents from Roche Diagnostics and run on a Roche Modular PPP Analyzer. ApoA-I was assayed via an immunoturbidimetric assay using reagent from Roche Diagnostics run on a Roche Modular PPP Analyzer. HDL particle size distribution was determined using NMR at Liposcience (Raleigh, NC).
Animals: C57Bl/6 Wild Type (WT) mice, C57Bl/6 SR-BI deficient mice (SR-BI−/−) 1, and C57Bl/6 transgenic mice expressing human apoA-I under the control of its natural flanking regions (HuAITg) 2 were used in the present study. Animal protocols were approved by the Institutional Animal Care and Use Committee of Columbia University. Animals had free access to both food and water and were fed regular chow diet. ITX5061 was prepared as a 6 mg/ml suspension in water containing Methyl cellulose 0.5% and Polysorbate 0.1%, and given daily by oral gavage at 30mg/ body weight kg.

Atherosclerosis studies were conducted in F1 hybrid C57BL/6 × DBA/1 Ldlr+/− mice fed the Paigen diet, which is high-fat/cholesterol/bile salt diet containing 1.25% cholesterol, 7.5% cocoa butter, and 0.5% cholic acid (TD 88051; Harlan Teklad, Madison, WI). This model has been described previously 3, and it has been shown that moderate increases in VLDL/LDL levels and early atherosclerotic lesions can be readily induced in Ldlr+/− mice fed with this diet 4. For 18 weeks, the mice were put on the Paigen diet with or without ITX5061 at 0.037 %. CETP expression was induced by injection of adeno-associated virus (AAV)8 TBG human CETP vector (AAV-CETP, 3x1010 viral particles) two weeks before starting the Paigen diet as described previously 5. AAV was a kind gift of Dr. Daniel J. Rader (University of Pennsylvania School of Medicine). Doses of virus were given by IP injection.
Quantification of atherosclerosis lesions: To quantify the extent of the atherosclerotic lesions, the aortic arch and the thoracic aorta was opened longitudinally, stained with Oil Red O, and pinned out on a black wax surface. The amount of Oil Red O staining was measured using color thresholding to delimit the area of staining with Photoshop® software and the percentage of the plaque area stained by Oil Red O to the total surface area was determined. Proximal aortas were serially paraffin sectioned and stained with Haematoxylin and eosin as previously described. Aortic lesion size of each animal was calculated as the mean of lesion areas in 5 sections from the same mouse.

Plasma and liver tissue sampling: On the day of sample collection, the mice were fasted for 4 h. No differences in body weight were observed between treated and untreated mice over the period studied. Animals were euthanized with isofurane, and blood samples were collected by intracardiac puncture in heparin-containing tubes that were centrifuged at 5,000 rpm for 10 min. Plasma was harvested and stored at –80°C. Livers were excised and immediately snap-frozen in liquid nitrogen, and stored at –80°C before mRNA isolation.

Plasma Cholesterol, Triglyceride, and Lipoprotein Measurements: Total plasma cholesterol and TG were determined enzymatically using kits from Wako and Thermo scientific respectively. Pooled plasma from fasted mice was used for fast protein liquid chromatography (FPLC) analysis using one Superose 6 column with a flow rate of 0.3 ml/min. FPLC buffer contains 0.1 mol/L Tris-HCL and 0.4% NaN3 and pH was adjusted at 7.4. HDL-C and non-HDL-C were assayed in d > 1.07 and d < 1.07 g/ml plasma fractions, respectively.

Bilirubin and transaminase determinations:
Total bilirubin and transaminase were determined by enzymatic methods on a Dimension Vista analyser (Siemens, Marburg, Germany). Reference values are those observed in the same mouse line under standard diet.

Native polyacrylamide gradient gel electrophoresis (native-PAGE): Total lipoproteins were separated by ultracentrifugation as the d < 1.21 g/ml plasma fraction with one 5.5 h, 100,000 rpm spin in a TLA100 rotor in a TLX ultracentrifuge (Beckman Instruments; Palo
Alto, CA). Lipoproteins were then applied to a 50–200 g/l polyacrylamide gradient gel and electrophoresis was conducted as recommended by the manufacturer. Gels were subsequently subjected to Coomassie brilliant blue staining. The mean apparent diameters of HDL were determined by comparison with globular protein standards subjected to electrophoresis together with the samples.

**Determination of apoA-I concentration:** Relative levels of apoA-I were determined as described previously by a nonimmunologic method. Briefly, plasma samples (0.5 µl) were subjected to electrophoresis on 50–200 g/l polyacrylamide gradient gel as recommended by the manufacturer. Gels were subsequently stained by Coomassie brilliant blue and scanned on a GS-800 densitometer (Biorad, Hercules, CA).

**RNA isolation and quantitative-PCR:** Total RNA were extracted from liver by using the RNeasy Mini kit (Qiagen, Valencia, CA.) according to the manufacturer protocol. Real-time quantitative PCR assays were performed by using the Mx4000 Quantitative PCR System (Stratagene, La Jolla, CA.). Briefly, 1 µg of total RNA was reverse-transcribed into cDNA using MuMLV reverse transcriptase and oligo-dT (Invitrogen, Carlsbad, CA). Fifty nanograms of the cDNA mixture was used for real time PCR analysis using the SYBR Green PCR Master Mix® (Applied Biosystems, Carlsbad, CA). Values were normalized to GAPDH levels. Relative mRNA levels were evaluated using the ΔΔCt method.

**Western blot analysis.** Protein levels of apoA-I, ABCA1, and SR-BI were measured in the liver by Western blot analysis. Primary antibodies for mouse apoA-I, ABCA1, and SR-BI were purchased from Santa Cruz Biotechnology Inc., Abcam, and Biodesign, respectively.

**In vivo turnover studies:** Mouse HDL was labelled with $[^3]H$CE by the following procedure: Briefly, cholesteryl-1,2-$[^3]H$hexadecylether in toluene (Perkin Elmer, Boston, MA) was dried down under a stream of nitrogen and resuspended in 100 µl of absolute ethanol. The solution was added dropwise to mouse HDL (1 mg of total protein) that were subsequently incubated for 18 h at 37°C in the presence of $d > 1.21$ infranatant from human serum as CETP source. Labelled HDL were subsequently isolated by sequential ultracentrifugation at 1.07 and 1.21 g/ml density in a TLA-100.2 rotor using a TL-100 ultracentrifuge. Control and ITX5061-treated mice were injected in the tail vein with $[^3]H$CE-labelled HDL (1.5 x 10$^6$ cpm). After injection, blood was taken from the tail vein at different time points for
determination of radioactivity. The fractional catabolic rates (FCRs) were calculated from the decay curves of $[^3H]CE$ radioactivity in plasma by fitting the data to a biexponential equation according to the method of Matthews. The production rates were calculated by multiplying the FCR by the plasma cholesterol pool and dividing by the body weight. Liver was collected, weighed, homogenized, and one aliquot was radioassayed after dissolution in NaOH 0.1 M.

**Uptake of $[^3H]CE$-labeled HDL by cells in culture**

HEK293 cells were plated on 24-well plates, when 80% confluence was reached, cells were transfected with mouse SR-BI cDNA expression vector or a mock vector using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA). One day after transfection, the cells were pre-incubated with or without molecule at the indicated concentration. After the pre-incubation, $[^3H]CE$-labelled HDL were added into the medium and the cells were incubated for 4 h either at 37°C or at 4°C. The culture medium was removed and the cells were washed with PBS. Finally, the cells were dissolved in 200 μL of NaOH 100 mM. Radioactivity of the cell lysate was directly measured by liquid scintillation spectrometry. The radioactivity levels were normalized to the protein content of the lysates and values at 4°C were subtracted.

**Statistical analysis**

Mann Whitney or Wilcoxon tests were used to determine the statistical significance between two groups.
Figure S1: Structure of ITX5061

Figure S2: Effect of ITX5061 on p38 MAPK activity

Figure S3: Changes in lipid parameters over time during ITX5061 administration. Patients received placebo (n=37), ITX5061 at 150mg (n=38) and ITX5061 at 300 mg (n=40) daily. Lipid parameters were determined at indicated time points. (A): mean percent changes in LDL-C, (B): mean percent changes in triglycerides.

Figure S4: (A) Relative changes in hepatic mRNA levels in HuAITg mice upon ITX5061 treatment. Total RNA was extracted from the liver, and real-time quantitative PCR was performed as described in Materials and Methods. Data were standardized for GAPDH. mRNA levels in mice receiving the vehicle were set at 1.00. (B) Western blot analysis of apoA-I, ABCA1, and SR-BI in the liver. Values are normalized to actin.

Figure S5: Plasma CETP activity in Ldlr⁺⁻ mice injected with AAV-CETP or AAV-Ctrl. Mice were injected IP with AAV at the indicated doses. CETP was determined 2 weeks after injection using commercially available kit as described by the manufacturer.
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<th>Characteristics at baseline</th>
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<td>21 (55%)</td>
<td>23 (58%)</td>
</tr>
<tr>
<td>Female</td>
<td>14 (38%)</td>
<td>17 (45%)</td>
<td>17 (43%)</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>38.2±7.39</td>
<td>37.84±7.08</td>
<td>39.33±8.56</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>210.73±36.45</td>
<td>209.45±41.21</td>
<td>198.95±41.45</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>229.30±83.93</td>
<td>218.55±80.89</td>
<td>221.15±105.35</td>
</tr>
</tbody>
</table>
Table SII: plasma lipid parameters after 2 and 18 weeks of Paigen diet in \( Ldlr^{+/−} \) treated or not with ITX5061. a significantly different from AAV-CETP + Vehicle, b: significantly different form AAV-Ctrl + Vehicle. Lipid parameters are in mg/dl, AST and ALT are international units/L, TBIL is in µmol/L. Normal values for ALT : 40±20 UI/L, AST: 140±67 UI/L 7).

<table>
<thead>
<tr>
<th></th>
<th>2 weeks</th>
<th></th>
<th>18 weeks</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TC</td>
<td>Non HDL-C</td>
<td>HDL-C</td>
<td>TC</td>
<td>HDL-C</td>
</tr>
<tr>
<td>AAV-CETP + ITX5061</td>
<td>456</td>
<td>359</td>
<td>97.2(^a)</td>
<td>337</td>
<td>228</td>
</tr>
<tr>
<td>(n=10)</td>
<td>±115</td>
<td>±135</td>
<td>±30.7</td>
<td>±73.8</td>
<td>±88.1</td>
</tr>
<tr>
<td>AAV-CETP + Vehicle</td>
<td>509</td>
<td>455</td>
<td>54.1</td>
<td>276</td>
<td>204</td>
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<tr>
<td>(n=10)</td>
<td>±118.</td>
<td>±125</td>
<td>±26.5</td>
<td>±85.4</td>
<td>±86.5</td>
</tr>
<tr>
<td>AAV-Ctrl + ITX5061</td>
<td>406</td>
<td>317</td>
<td>89.7(^b)</td>
<td>313</td>
<td>213</td>
</tr>
<tr>
<td>(n=11)</td>
<td>±147</td>
<td>±154</td>
<td>±17.2</td>
<td>±131</td>
<td>±156</td>
</tr>
<tr>
<td>AAV-Ctrl + Vehicle</td>
<td>497</td>
<td>438</td>
<td>58.9</td>
<td>326</td>
<td>217</td>
</tr>
<tr>
<td>(n=10)</td>
<td>±132</td>
<td>±143</td>
<td>±19.9</td>
<td>±105</td>
<td>±82.3</td>
</tr>
</tbody>
</table>


Figure S1
Table 1: Preincubation Time vs. IC50

<table>
<thead>
<tr>
<th>Preincubation Time</th>
<th>IC50 (nM)</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>302</td>
</tr>
<tr>
<td>30</td>
<td>38</td>
</tr>
<tr>
<td>60</td>
<td>14</td>
</tr>
<tr>
<td>120</td>
<td>8</td>
</tr>
</tbody>
</table>

Figure S2
Figure S3
Figure S4
Figure S5