

RVX-208

A Small Molecule That Increases Apolipoprotein A-I and High-Density Lipoprotein Cholesterol In Vitro and In Vivo

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Objectives

The aim of this study was to determine whether a novel small molecule RVX-208 affects apolipoprotein (apo)A-I and high-density lipoprotein cholesterol (HDL-C) levels in vitro and in vivo.

Background

Increased apoA-I and HDL-C levels are potential therapeutic targets for reducing atherosclerotic disease.

Methods

HepG2 cells were treated with 0 to 60 $\mu\text{mol/l}$ RVX-208 followed by assays for apoA-I and HDL-C production. For in vivo studies, African green monkeys (AGMs) received 15 to 60 mg/kg/day RVX-208, and the serum was analyzed for lipoprotein levels, HDL-subparticle distribution, cholesterol efflux, and activity of lipid-modifying enzymes. A phase I clinical trial was conducted in healthy volunteers (given 1 to 20 mg/kg/day of RVX-208) to assess safety, tolerability, and pharmacokinetics.

Results

The RVX-208 induced apoA-I messenger ribonucleic acid and protein synthesis in HepG2 cells, leading to increased levels of pre- β -migrating and α -lipoprotein particles containing apoA-I (LpA-I) in spent media. Similarly, in AGMs, RVX-208 treatment for 63 days increased serum apoA-I and HDL-C levels (60% and 97%, respectively). In addition, the levels of pre- β_1 -LpA-I and α_1 -LpA-I HDL-subparticles were increased as well as adenosine triphosphate binding cassette AI, adenosine triphosphate binding cassette G1, and scavenger receptor class B type I-dependent cholesterol efflux. These changes were not mediated by cholesteryl-ester-transfer protein. Treatment of humans for 1 week with oral RVX-208 increased apoA-I, pre- β -HDL, and HDL functionality.

Conclusions

RVX-208 increases apoA-I and HDL-C in vitro and in vivo. In AGMs, RVX-208 raises serum pre- β_1 -LpA-I and α -LpA-I levels and enhances cholesterol efflux. Data in humans point to beneficial features of RVX-208 that might be useful for treating atherosclerosis. (J Am Coll Cardiol 2010;55:2580–9) © 2010 by the American College of Cardiology Foundation

Raising plasma levels of high-density lipoprotein cholesterol (HDL-C) with small molecules that up-regulate apolipoprotein A-I (apoA-I) expression has been a therapeutic goal in cardiovascular medicine for more than 2 decades. Although current treatments for atherosclerotic cardiovascular disease (ACVD) are dominated by lowering low-density lipoprotein cholesterol (LDL-C), intervention trials highlight the benefits of raising HDL-C (1,2). The idea to

raise HDL stems from the consistent inverse correlation of ACVD with plasma HDL-C (3). Indeed, animal studies have shown that infusion of HDL or apoA-I or genetic overexpression of apoA-I limits progression and reduces pre-existing atherosclerosis (2). Additionally, a recent human trial demonstrated reduction of atheroma volume by direct infusion of pre- β HDL-like particles comprising apoA-I Milano and phospholipids (4). These outcomes support interest in apoA-I and HDL-C as major therapeutic targets to potentially reduce ACVD.

Additionally, evidence suggests that HDL and apoA-I possess pleiotropic properties that might contribute to their anti-atherogenic effects (5), such as anti-inflammatory (6), anti-oxidative (7), and antithrombotic activities (8). However, the best-recognized property of HDL is its proposed role in retrieving excess cholesterol from peripheral cells, including vessel wall macrophages, via specific transporters

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(e.g., the adenosine triphosphate-binding cassette proteins ABCA1 and ABCG1 and scavenger receptor BI [SR-B1]) en route to the liver for excretion into the bile. It is widely accepted that apoA-I is a critical mediator of this reverse cholesterol transport (RCT) process (1,2).

Although a variety of HDL/apoA-I–based therapies are under investigation, raising endogenous apoA-I expression is generally considered the most enviable approach to target HDL. The goal to raise HDL-C particles is tempered by the fact that increased levels do not necessarily equal better outcome. For example, the cholesteryl-ester-transfer protein (CETP) inhibitor torcetrapib raised levels of HDL particles but failed to reduce atherosclerotic burden (9). Similarly, analysis of the IDEAL (Incremental Decrease in Endpoints through Aggressive Lipid lowering) and EPIC-Norfolk (European Prospective Investigation Into Cancer in Norfolk) data showed that very high HDL-C mass and size correlated with ACVD. These findings point to the complexity of HDL biology and suggest that not only HDL-C levels but also size, composition, and functionality are important. The current study examined the potential of a novel small molecule, RVX-208, to increase apoA-I, HDL levels, and HDL function in liver cells, African Green monkeys (AGMs) and humans.

Methods

HepG2 cell and metabolic pulse-chase studies. HepG2 cells were treated with RVX-208 (0 to 60 $\mu\text{mol/l}$) for 48 h in minimum essential medium 0.5% fetal bovine serum. Total ribonucleic acid was isolated (Isogen reagent), and relative *APOA1* messenger ribonucleic acid (mRNA) expression was determined against endogenous cyclophilin (7500 RT-PCR System, Applied Biosystems, Foster City, California). To determine the effect of RVX-208 on the production of apoA-I, HepG2 were treated with or without RVX-208 (60 $\mu\text{mol/l}$) for different time periods, and the apoA-I content of the media as well as the cell lysates was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and 2-dimensional polyacrylamide non-denaturing gradient gel electrophoresis (2D-PAGGE). For pulse-chase studies, HepG2 cells treated with or without RVX-208 (60 $\mu\text{mol/l}$) were pulsed with 150 $\mu\text{Ci/ml}$ [^{35}S] methionine/cysteine in serum free-RPMI for 10 min and then chased with 5 mmol/l methionine/cysteine. Immunoprecipitation, sodium dodecyl sulfate polyacrylamide gel electrophoresis, and fluorography were performed on cell lysates and spent media in duplicate.

AGM study. Naïve adult male AGMs (*Chlorocebus aethiops sabaues*, 4.0 to 5.7 kg, housed at Barbados Primate Research Center) were fed ad libitum (Teklad global 2050 diet, Teklad, Madison, Michigan). All animals were fasted for 12 h before sample collection. All studies adhered to the Care and Use of Laboratory Animals (as per National Institutes of Health Publication No. 86-23, revised 1996) and were approved by the Institutional Animal Care and

Use Committee in accordance with National Institutes of Health (Office for Protection from Research Risks) and U.S. Department of Agriculture Animal and Plant Health Inspection Service. In single-dose studies, 6 AGMs were given 60 mg/kg RVX-208 once daily dissolved in 1N hydrochloride and carboxymethyl cellulose (pH = 2.5 to 3.0) or vehicle for 63 days followed by a 2-week washout. Serum samples were taken on days 21, 28, 42, and 63 and at 1 and 14 days after final dosing. For the dose response study, AGMs (6/group) were given vehicle or 7.5, 15, or 30 mg/kg of RVX-208 twice a day for 73 days before 11 days of washout. Samples were taken on days 14, 28, 42, 56, 70, and 73 and at 4 and 11 days after final dosing.

Cynomolgus monkey study.

Male young adult cynomolgus monkeys (n = 3) were fasted overnight before the administration of varying doses of RVX-208 via oral gavage or intravenously for pharmacokinetic studies. Food was withheld during the first 4 h after dosing but not for subsequent blood sampling (femoral or jugular venipuncture) up to 48 h. All studies were conducted in accordance with MPI Research (Mattawan, Michigan) standard operating procedures, and to ensure compliance the protocol was reviewed and approved by the Institutional Animal Care and Use Committee.

RVX-208 administration in humans.

To study the effects of RVX-208, healthy volunteers were confined to a study center (PPD, Austin, Texas) 1 day before and released shortly after treatment. All subjects consented to the study as approved by the IntegReview Ethical Review Board (Austin, Texas), which complied with the Declaration of Helsinki 2000. The RVX-208 was suspended in Ora-Blend SF (Paddock Laboratories, Minneapolis, Minnesota); Ora-Blend SF served as placebo. Six subjects received 1 mg/kg, and an equal number were given 4 mg/kg RVX-208 twice a day for 6 days, followed by a single dose on day 7. Six other subjects were given 3 mg/kg RVX-208 once daily for 7 days. In total the subjects received 2 to 8 mg/kg/day of RVX-208. There were 2 control

Abbreviations and Acronyms

2D-PAGGE = 2-dimensional polyacrylamide non-denaturing gradient gel electrophoresis

ABCA1 = adenosine triphosphate binding cassette AI

ABCG1 = adenosine triphosphate binding cassette GI

ACVD = atherosclerotic cardiovascular disease

AGM = African green monkey

apo = apolipoprotein

BHK = baby hamster kidney

CETP = cholesteryl-ester-transfer protein

FER = fractional cholesterol esterification rate

HDL = high-density lipoprotein

HDL-C = high-density lipoprotein cholesterol

LCAT = lecithin:cholesterol acyl transferase

LDL-C = low-density lipoprotein cholesterol

LpA-I = lipoprotein particles containing apoA-I

mRNA = messenger ribonucleic acid

ND-PAGGE = non-denaturing gradient gel electrophoresis

PLTP = phospholipid transfer protein

RCT = reverse cholesterol transport

SR-BI = scavenger receptor class B type I

subjects in each of the 3 groups who received placebo. Sampling of clinical parameters was collected before dosing on day 1 and after dose administration on days 1, 2, 4, 7, and 10. Pharmacodynamic data were expressed with the following formula:

$$\left(\frac{[\text{Day7}_{\text{RVX-208}} - \text{Day1,pre-treatment}_{\text{RVX-208}}]}{\text{Day1,pre-treatment}_{\text{RVX-208}}} - \left(\frac{[\text{Day7}_{\text{Placebo}} - \text{Day1,pre-treatment}_{\text{Placebo}}]}{\text{Day1,pre-treatment}_{\text{Placebo}}} \right) \right) \times 100$$

Analysis of HDL species. Nondenaturing gradient gel electrophoresis (ND-PAGE) 5% to 35% was performed as previously described (9) on serum (25 μl) along with radiolabeled molecular weight standards. apoA-I-containing particles were detected with an anti-human-apoA-I antibody (Biodesign, Bar Harbor, Maine) or an anti-monkey-apoA-I antibody from Dr. J. Parks (Wake Forest University, Winston-Salem, North Carolina) and quantitated with densitometry. Abundance of apoA-I in each HDL subfraction was expressed as a percentage of the total. Differences in HDL subclass distribution between vehicle and RVX-208-treated monkeys were determined by 2-tailed *t* test.

ABCA1, ABCG1, and SR-BI-cholesterol efflux. Cells (J774, Fu5AH, or baby hamster kidney [BHK] overexpressing ABCG1 under mifepristone-induction) were labeled with 3 $\mu\text{Ci/ml}$ ^3H -free cholesterol (Perkin Elmer, Norwalk, Connecticut) for 24 h. Simultaneously, J774 cells were incubated with 2 $\mu\text{g/ml}$ ACAT inhibitor (Pfizer, New York, New York). Afterward, J774 and BHK-ABCG1 were stimulated with 0.5 mmol/l 8-Br-cyclic adenosine monophosphate or 10 nmol/l mifepristone for 18 h, respectively (10). Cells were incubated with 2% serum for 4 h at 37°C. The %cholesterol efflux = $\frac{^3\text{H-cpm}_{\text{medium}}}{(^3\text{H-cpm}_{\text{medium}} + ^3\text{H-cpm}_{\text{cells}})} \times 100\%$. Human apoA-I and standard human serum were analyzed in parallel with test samples. The J774-efflux was normalized by the lipid-free apoA-I-efflux, whereas Fu5AH- and BHK-ABCG1-efflux was normalized by the serum standard-efflux. Cholesterol efflux from nonstimulated cells was used to control for induction efficiency.

Lecithin:cholesterol acyl transferase (LCAT), CETP, and phospholipid transfer protein activity. The fractional cholesterol esterification rate (FER) and LCAT activity were assayed with standard methodology (11,12). The CETP and phospholipid transfer protein (PLTP) activity was determined as described previously (12).

Lipid and lipoprotein assays. The 2D-PAGE was performed as described previously (13). Plasma lipoproteins were separated by high-performance liquid chromatography on a Superose-6HR column (Amersham Pharmacia Biotech, Piscataway, New Jersey), and cholesterol content was determined as described previously (14). Serum apoA-I, apoB, HDL-C, LDL-C, total cholesterol, and triglycerides were measured on an Olympus AU640 or AU2700 (Olympus, Tokyo, Japan) immunoautoanalyzer at MPI Research, Inc.

Statistical analysis. IN VITRO AND AGM STUDIES. Results were compared by paired 2-tailed *t* test.

HUMAN STUDIES. Results were compared by paired 2-tailed *t* test when compared with baseline (day 1, pre-treatment) and by 1-way analysis of variance when compared with placebo.

Results

Origins of RVX-208. Expression of the *APOA1* gene is regulated by many components, including nuclear transcription factors. However, it is difficult to manipulate the activity of such factors. Instead, we sought to identify novel compounds capable of directly raising apoA-I production. The lead compound, RVX-208, was found with a HepG2 cell-based assay to screen agents with the potential to raise apoA-I production. The RVX-208 is a small novel synthetic molecule belonging to the Quinazoline family (Fig. 1A), a compound class that has been used across a variety of therapeutic areas.

The RVX-208 compound has a molecular weight below 400 Da and obeys the Lipinski et al. (15) and Veber et al. (16) guidelines for drug-like characteristics. It is soluble in acidic (pH 1), aqueous media (3.0 to 3.5 mg/ml) and has good metabolic stability in the monkey, on the basis of incubation with liver microsomes (>90% remaining after 30 min incubation). The pharmacokinetics and oral bioavailability of RVX-208 were studied in a number of species, including the monkey, indicating that the compound was generally well-absorbed orally. In cynomolgus monkeys, it possessed low systemic clearance (5 ml/min/kg), moderate volume of distribution at steady state (V_{ss}) of 0.8 l/kg, a relatively short half-life (1.5 h), and an oral bioavailability of 44%. A single oral dose of 10 mg/kg produced a plasma exposure of approximately 14,500 h \times ng/ml. Mouse studies showed that most RVX-208 was localized in tissues that express apoA-I, the small intestine, and liver, at concentrations several-fold higher than those in plasma (data not shown). Further characterization of the apoA-I-elevating capacity of RVX-208 was pursued, given its promising pharmacological and drug-like properties.

RVX-208 induces apoA-I mRNA and de novo synthesis of apoA-I in cultured HepG2. To determine whether RVX-208 affected HDL biogenesis, we measured apoA-I mRNA expression, de novo apoA-I synthesis, and nascent HDL formation in HepG2 cells. Results (Fig. 1B) showed that RVX-208 significantly increased apoA-I mRNA and protein mass in a dose-dependent manner (0 to 60 $\mu\text{mol/l}$). Additionally, treatment of HepG2 with RVX-208 (60 $\mu\text{mol/l}$) for varying periods of time showed a significant increase in apoA-I released into the medium or associated with cell lysates for 24- and 48-h time points compared with untreated cells (Fig. 1C). As expected, RVX-208 had no significant effect on α_2 -macroglobulin ($\alpha_2\text{M}$) levels used as a control for protein loading. The increase in apoA-I released into media from

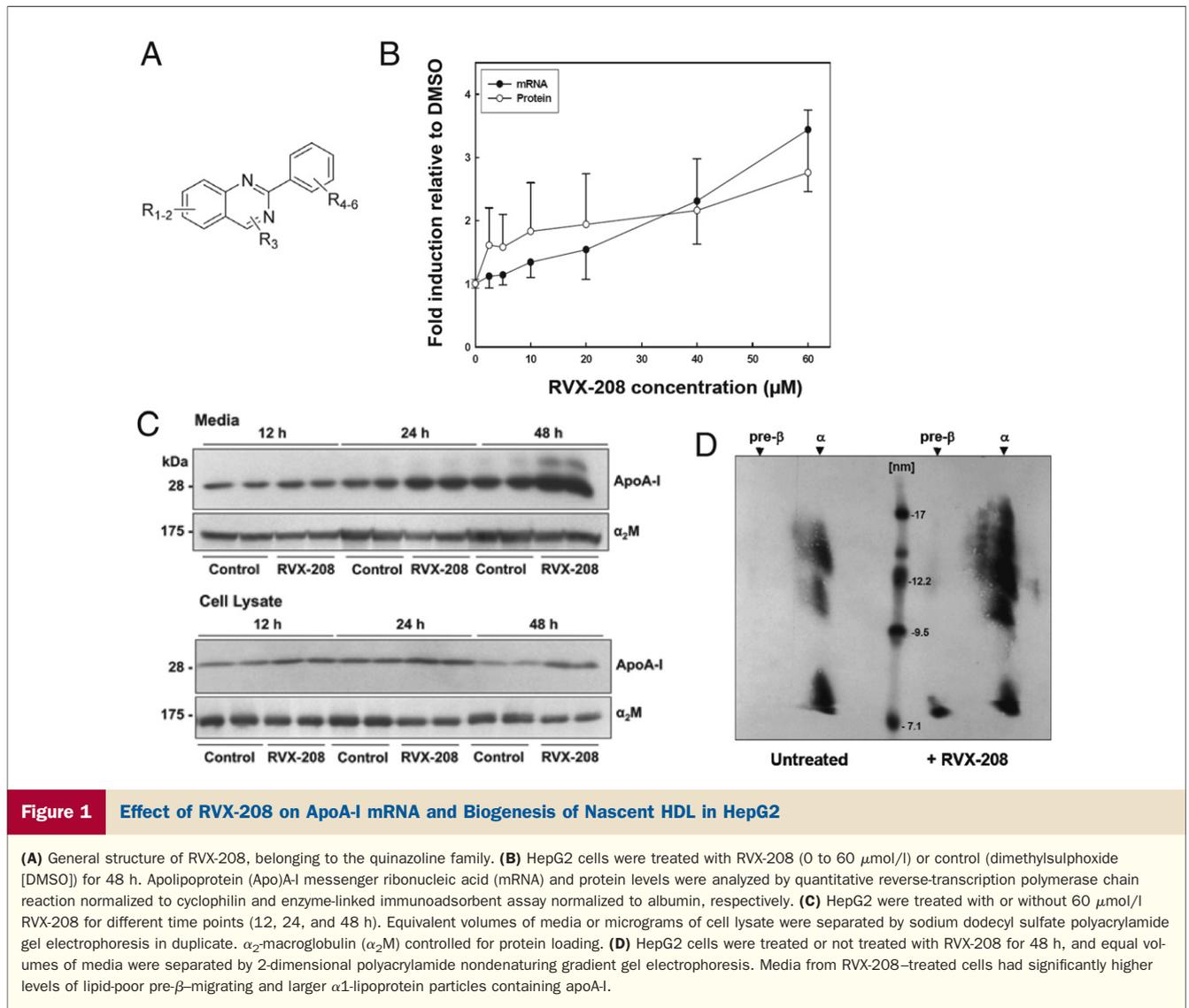


Figure 1 Effect of RVX-208 on ApoA-I mRNA and Biogenesis of Nascent HDL in HepG2

(A) General structure of RVX-208, belonging to the quinazoline family. (B) HepG2 cells were treated with RVX-208 (0 to 60 μmol/l) or control (dimethylsulphoxide [DMSO]) for 48 h. Apolipoprotein (Apo)A-I messenger ribonucleic acid (mRNA) and protein levels were analyzed by quantitative reverse-transcription polymerase chain reaction normalized to cyclophilin and enzyme-linked immunosorbent assay normalized to albumin, respectively. (C) HepG2 were treated with or without 60 μmol/l RVX-208 for different time points (12, 24, and 48 h). Equivalent volumes of media or micrograms of cell lysate were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis in duplicate. α₂-macroglobulin (α₂M) controlled for protein loading. (D) HepG2 cells were treated or not treated with RVX-208 for 48 h, and equal volumes of media were separated by 2-dimensional polyacrylamide nondenaturing gradient gel electrophoresis. Media from RVX-208-treated cells had significantly higher levels of lipid-poor pre-β-migrating and larger α₁-lipoprotein particles containing apoA-I.

cells treated with RVX-208 (60 μmol/l) for 48 h was measured by Western-blot (Fig. 1C) or enzyme-linked immunosorbent assay (Fig. 1B) were similar at 2.5-fold. The 2D-PAGE analysis of the medium from cells treated with RVX-208 (60 μmol/l, 48 h) revealed increased abundance of both lipid-poor pre-β-migrating and larger α-LpA-I particles (Fig. 1D, right), compared with untreated cells (Fig. 1D, left).

The fate of nascent apoA-I was assessed with a pulse-chase protocol. In cells treated with RVX-208, there was increased incorporation of the radiolabel into apoA-I that appeared intracellularly at 20 min (peak incorporation) (Fig. 2A, left) and extracellularly at 180 min chase time (peak secretion) (Fig. 2A, right). Densitometric quantification of [³⁵S]-apoA-I from these studies is shown in Fig. 2B. Cellular toxicity after RVX-208 treatment was assessed by [³H]-adenine leakage. No significant increase of [³H]-adenine release was observed under the concentrations of RVX-208 used in the experiments described in the preced-

ing text (data not shown). These findings show that RVX-208 increases endogenous apoA-I production.

RVX-208 increases serum apoA-I and HDL-C in AGMs. Next, RVX-208 was tested in male AGMs, because this model shows many similarities to human subjects in the effects of dietary cholesterol and fatty acids on plasma lipoproteins and cholesterol metabolism. Furthermore, AGMs shared sufficient preclinical pharmacokinetic characteristics with cynomolgus species, which were used for pharmacological testing as described in the preceding text (17). The AGMs received RVX-208 (60 mg/kg) or vehicle once daily for 63 days. The RVX-208 treatment produced a marked and significant increase in both apoA-I and HDL-C levels of 133 ± 4 mg/dl versus 203 ± 5 mg/dl (+53%) and 57 ± 2 mg/dl versus 111 ± 2 mg/dl (+97%), respectively, by 28 days when baseline was compared with RVX-208 treatment (Table 1). These increases were sustained throughout the duration of treatment, yielding average apoA-I and HDL-C values on day 63 of treatment of

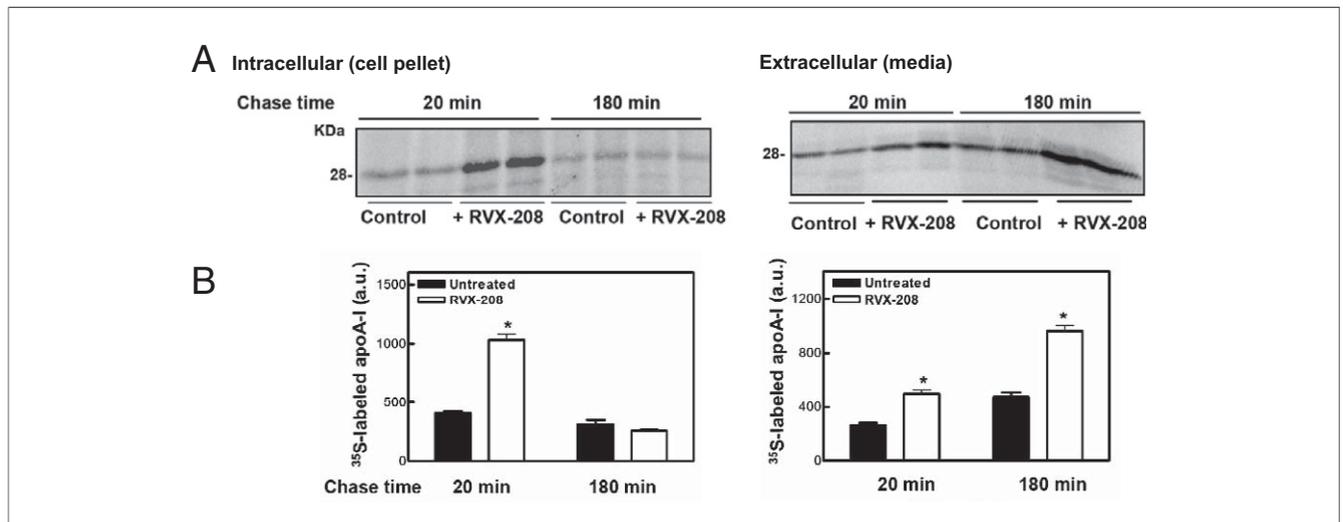


Figure 2 Effect of RVX-208 on De Novo Synthesis of ApoA-I in HepG2

(A) De novo synthesis of apolipoprotein (apo)A-I was assessed with [³⁵S]methionine/cysteine as described in the Methods section. HepG2 cells were treated with 60 μmol/l RVX-208 for 48 h before exposure to the radiolabeled amino acid. Autoradiography was used to detect radiolabeled apoA-I immunoprecipitated from labeled cells (intracellular fraction, left) and media (extracellular fraction, right) separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis. (B) Representative densitometry of radiolabeled apoA-I in intracellular and extracellular fractions are shown. Experiments were performed twice in duplicate, and results were pooled. The values are mean ± SD. *p < 0.001.

133 ± 4 mg/dl versus 213 ± 9 mg/dl (+60%) and 57 ± 2 mg/dl versus 112 ± 4 mg/dl (+97%), respectively (baseline vs. RVX-208-treated). Similarly, apoA-I and HDL-C levels increased significantly and in a dose-dependent manner in the escalating dose study (Online Table 1). The RVX-208 treatment did not affect serum LDL-C or apoB concentrations. Although RVX-208 treatment given at 60 mg/kg once daily increased total serum triglyceride levels (Table 1), the same dose given at 30 mg/kg twice daily had no effect on triglyceride levels (Online Table 1). These findings show that RVX-208 given orally to AGMs increases serum levels of both apoA-I and HDL-C.

Effect of RVX-208 on serum levels of HDL species. To further explore the effects of the compound, we examined whether RVX-208 affected HDL populations. There were 2

parts to this study. The first was the use of 2D-PAGGE and high-performance liquid chromatography analysis of plasma to assess qualitative changes; the second required use of ND-PAGGE and densitometric scanning for quantitative analysis. The 2D-PAGGE analysis of apoA-I-containing particles revealed that both pre-β₁-LpA-I and larger α₁-LpA-I subpopulations were significantly increased in RVX-208 treated serum (Fig. 3, top). Lipoprotein separation by high-performance liquid chromatography showed, consistent with this finding, increased total cholesterol in the HDL fraction and a left-shifted HDL-C peak, indicating a larger HDL-particle size after treatment (Fig. 3, bottom).

To further examine the HDL size distribution, serum was separated by ND-PAGGE, and apoA-I-containing particles were detected with an iodinated anti-apoA-I an-

Table 1 Serum Levels of ApoA-I, HDL-C, ApoB And Lipids of Vehicle and RVX-Treated Monkeys

	ApoA-I	HDL-C	Total Cholesterol	LDL-C	Triglycerides	Total Phospholipids	ApoB
Vehicle (n = 6)							
Baseline	134 ± 10	58 ± 5	108 ± 7	36 ± 3	22 ± 4	155 ± 13	28 ± 2
Day 28	153 ± 14	70 ± 6	123 ± 10	41 ± 5	32 ± 6	174 ± 19	32 ± 4
Day 42	145 ± 10	68 ± 6	115 ± 8	36 ± 3	39 ± 6	145 ± 10	30 ± 3
Day 63	154 ± 12	71 ± 5	121 ± 9	46 ± 2	31 ± 2	176 ± 17	34 ± 1
Day 64 (washout)	142 ± 12	63 ± 6	116 ± 12	33 ± 2	19 ± 1	152 ± 13	28 ± 2
Day 77 (washout)	149 ± 12	66 ± 6	115 ± 7	41 ± 4	26 ± 3	152 ± 10	33 ± 3
RVX-208 (n = 6), 60 mg/kg/day							
Baseline	133 ± 4	57 ± 2	104 ± 3	36 ± 2	28 ± 3	157 ± 13	31 ± 2
Day 28	203 ± 5*	111 ± 2*	160 ± 4*	41 ± 3	57 ± 8*	256 ± 10*	36 ± 2
Day 42	209 ± 5*	106 ± 3*	160 ± 5*	43 ± 3	69 ± 5*	211 ± 10*	37 ± 2
Day 63	213 ± 9*	112 ± 4*	170 ± 9*	46 ± 3	64 ± 10*	285 ± 8*	37 ± 1
Day 64 (washout)	191 ± 8*	105 ± 3*	135 ± 17	37 ± 3	39 ± 3	273 ± 9*	32 ± 1
Day 77 (washout)	170 ± 6	77 ± 3	125 ± 11	44 ± 3	30 ± 3	178 ± 8	36 ± 1

Values are mg/dl. *p < 0.05 compared with vehicle samples taken at the same treatment day.

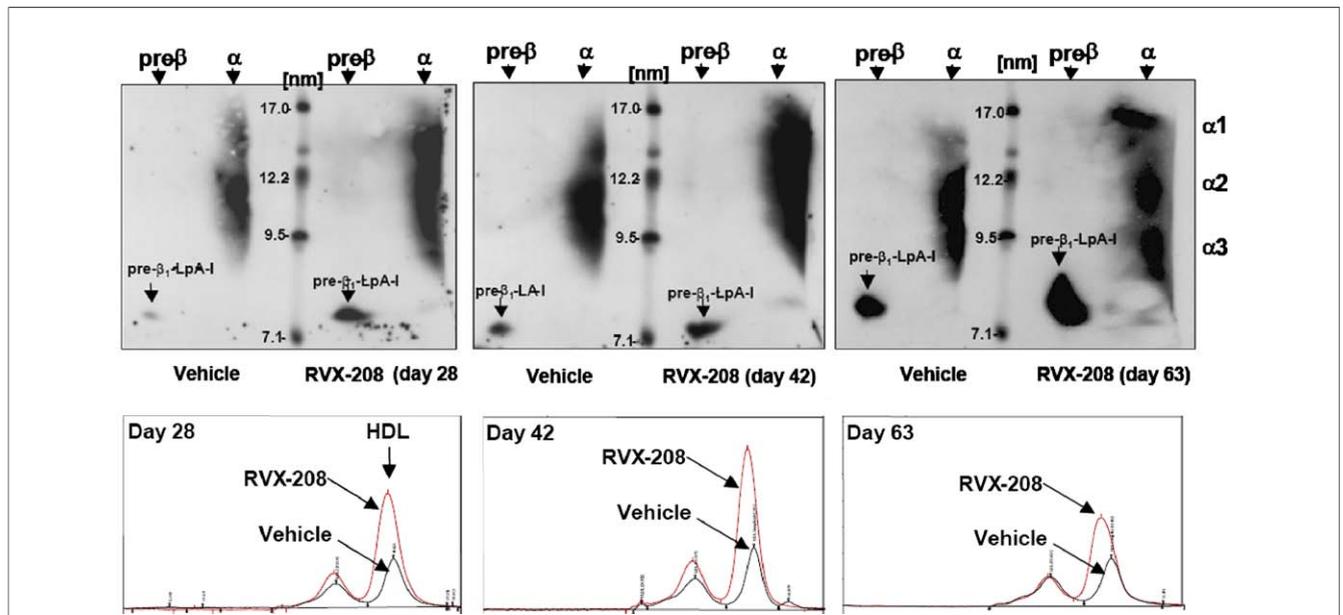


Figure 3 2D-PAGE and HPLC Analysis of RVX-208-Modified HDL

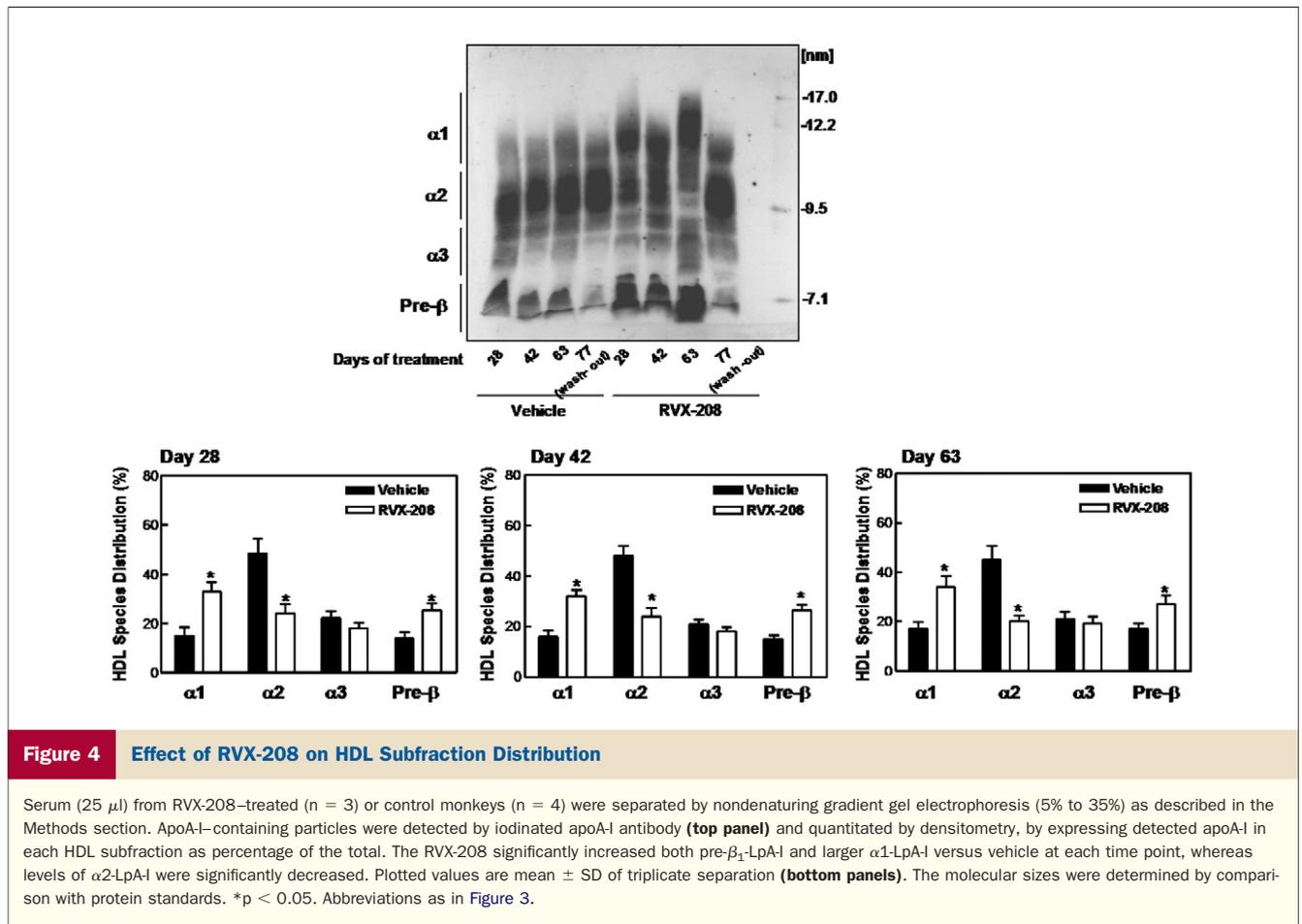
Serum (25 μ l) from monkeys treated for 63 days with vehicle or RVX-208 (60 mg/kg once daily) were separated along with radiolabeled molecular weight markers by 2-dimensional polyacrylamide nondenaturing gradient gel electrophoresis (2D-PAGE). Apolipoprotein (Apo)A-I-containing particles were detected by iodinated apoA-I antibody (top panels). Both pre- β -lipoprotein particles containing apoA-I (LpA-I) and larger α 1-LpA-I subpopulations were significantly increased in RVX-208-treated serum. Serum (250 μ l) from vehicle- or RVX-208-treated monkeys was separated by high-performance liquid chromatography (HPLC), and the total cholesterol was measured as described in the Methods section (bottom panels). A representative 2D-PAGE and HPLC (n = 4) from 1 RVX-208-treated and 1 control monkey are shown. HDL = high-density lipoprotein.

tibody (Fig. 4, top). The HDL subpopulations were quantified by densitometry. The RVX-208 (60 mg/kg; once daily) significantly increased both pre- β -LpA-I and larger α 1-LpA-I as compared with vehicle, whereas levels of α 2-LpA-I were significantly decreased (Fig. 4, bottom). No significant change in α 3-LpA-I particles was observed. As expected, the HDL size distribution of RVX-208-treated monkeys normalized after the washout period (Fig. 4, top) (day 77). Importantly, there was no difference between the human apoA-I antibody (Biodesign) and the monkey apoA-I antibody (from Dr. John S. Parks) in detecting monkey HDL species (data not shown). Together these data show that the RVX-208-stimulated increase in HDL is accompanied by changes in the profile of this class of particles.

RVX-208 stimulates cellular cholesterol efflux via different pathways. The RVX-208-induced rise in apoA-I and pre- β -HDL should enhance cholesterol efflux. Therefore, we measured ABCA1, ABCG1, and SR-BI-mediated cholesterol efflux with serum from RVX-208 and vehicle-treated AGMs. Cyclic adenosine monophosphate-stimulated J774 macrophages, BHK-cells stably over-expressing ABCG1, and Fu5AH were used as cellular models of ABCA1, ABCG1, and SR-BI-mediated cholesterol efflux, respectively. Data (Fig. 5) showed a significant increase in cholesterol efflux mediated by any 1 of the pathways (Figs. 5A to 5C) across all treatment days with levels returning to baseline upon washout. Because RVX-208-enhanced cholesterol efflux

activity had reached near plateau levels by 28 days of treatment, we pooled data from all RVX-208 treatment periods (28, 42, and 63 days) versus control subjects (Fig. 5D) for each transporter (ABCA1: $7.6 \pm 1.2\%$ vs. $15.4 \pm 1.7\%$; ABCG1: $19.9 \pm 3.3\%$ vs. $30.3 \pm 4.5\%$; and SR-BI: $8.7 \pm 1.3\%$ vs. $14.7 \pm 1.0\%$; vehicle vs. RVX-208-treated; $p < 0.001$ for ABCA1 and SR-BI, $p < 0.01$ for ABCG1, n = 4/group).

RVX-208 decreases serum LCAT activity but has no effect on CETP or PLTP activities. The RVX-208 was shown to increase apoA-I and HDL-C levels and to cause profound changes in the HDL-size distribution. Because these alterations might be due to changes in the activities of HDL-remodeling enzymes induced by RVX-208, we examined the activities of LCAT, CETP, and PLTP. Both the FER and exogenous LCAT activity were significantly decreased in the serum of RVX-208-treated monkeys as compared with control subjects (FER: 33.3 ± 9.9 vs. 59.0 ± 1.5 , $p < 0.01$, day 63; exogenous LCAT: 49.0 ± 15.8 vs. 83.8 ± 11.3 , $p < 0.05$, day 63). Previous studies have documented that subjects treated with CETP inhibitors possess larger HDL particles (18). Therefore, the increase in larger α 1-LpA-I in RVX-208-treated monkeys might arise from CETP inhibition. However, no significant difference in CETP activity was observed between RVX-208- and vehicle-treated monkeys (data not shown). Similarly, no significant difference in PLTP activity was observed (data not shown).



RVX-208 increased apoA-I, pre- β HDL, and cholesterol efflux in man. The favorable features of RVX-208 action in the AGMs prompted us to test the compound in humans. Sera from the 18 subjects receiving multiple and varying doses (2 to 8 mg/kg/day) of RVX-208 for 7 days were assayed for 4 parameters: apoA-I, HDL-C, pre- β 1-LpA-I and ABCA1 cholesterol mediated efflux as described. The results showed that all doses of RVX-208 tested induced a rise in the levels of the 4 parameters. The use of analysis of variance to compare these rises above the baseline versus placebo was significant in some groups but not all. Given the limited number of treated subjects, the value of these parameters was pooled regardless of dose administered. Results (Fig. 6) showed that plasma apoA-I was 10% higher (p < 0.05) in RVX-208–treated subjects compared with placebo-treated subjects. Similarly, there was a marked increase in pre- β 1-HDL levels and ABCA1-mediated cholesterol efflux (Fig. 6) of 42% and 11%, respectively (p < 0.05). Both HDL-C and larger α -HDL particles showed trends toward higher levels in the treated patients of 10% and 21%, respectively, but these increases were not statistically significant. The ApoB, LDL-C, and triglyceride levels were within the reference range (data not shown). Together the preceding findings show that RVX-208 increases apoA-I, pre- β 1-HDL, and cholesterol efflux in humans.

Discussion

In this report, we show that a novel small molecule, RVX-208, increases plasma apoA-I and HDL-C and modulates the biochemical properties, metabolism, and function of HDL. The RVX-208 tested in vitro on HepG2 cells increased apoA-I mRNA and protein levels in a dose-responsive manner (Fig. 1B). This finding is significant, because the liver is proposed to be the major source of plasma HDL-C (19) and hepatic ABCA1 is a significant contributor to plasma HDL-C concentrations (20).

To confirm that RVX-208 increased de novo apoA-I-protein synthesis, we used [35 S]methionine/cysteine pulse-chase studies (Fig. 2). The RVX-208 stimulated intracellular synthesis of apoA-I (Figs. 2A and 2B) that was secreted into the medium. The increased production of apoA-I was associated with a rise in the abundance of the larger nascent-LpA-I and lipid-poor pre- β -migrating particles (Fig. 1D). These in vitro studies show that RVX-208 increases apoA-I synthesis in and HDL secretion from HepG2 cells.

Next, we proceeded to in vivo studies using AGMs where RVX-208 (60 mg/kg once daily or 30 mg/kg twice a day) treatment had no effect on non-HDL-C levels but led to robust increases in serum apoA-I and HDL-C levels of 60%

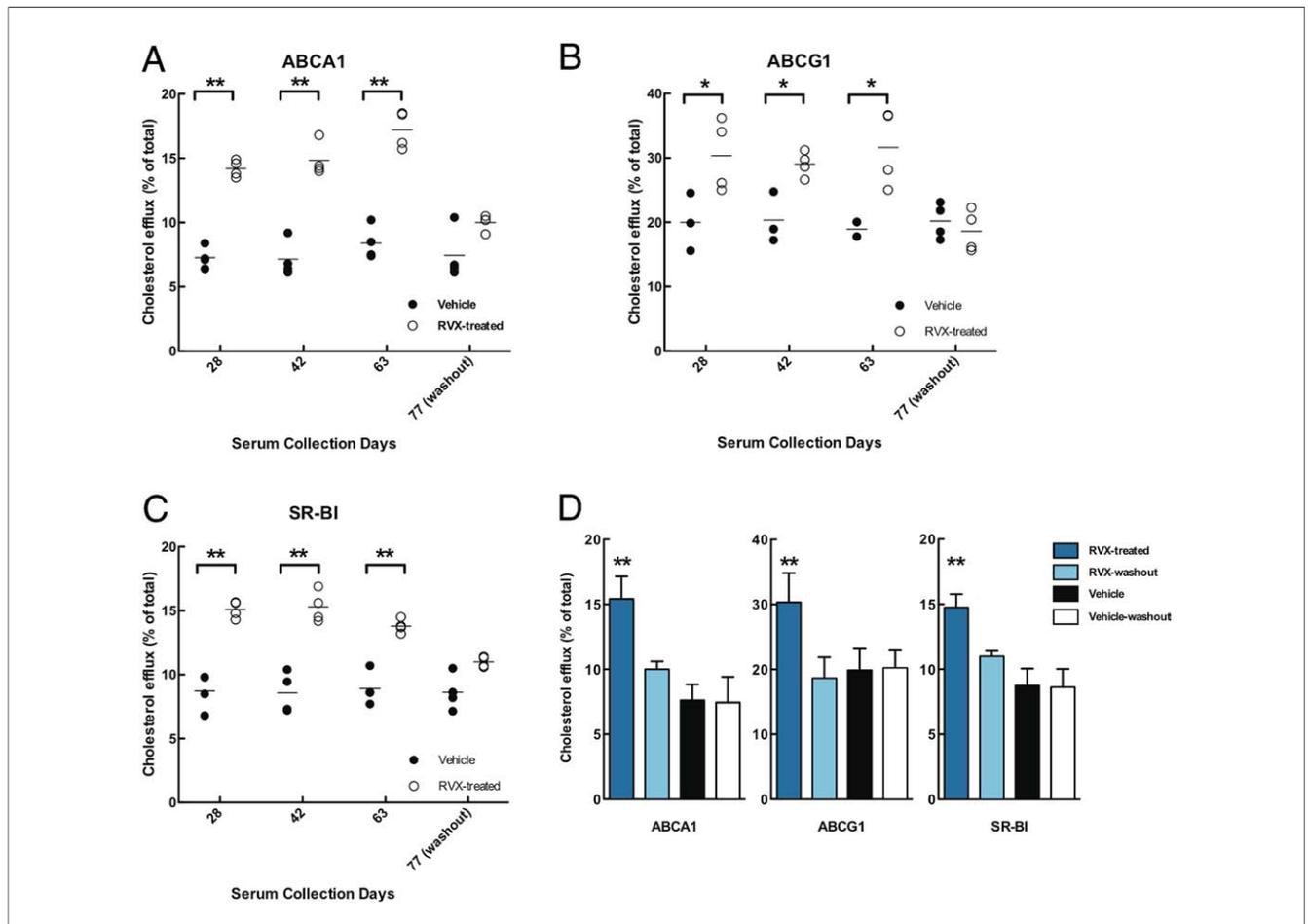


Figure 5 Serum From Monkeys Treated With RVX-208 Stimulates Cholesterol Efflux

Sera from RVX-208–treated (n = 4) or control (n = 4) monkeys were analyzed for their ability to mediate cholesterol efflux. Cyclic adenosine monophosphate-stimulated J774 (A), baby hamster kidney over-expressing ABCG1 (B), and Fu5HA (C) were radiolabeled for 24 h with [³H]free cholesterol as described in the Methods section. Cells were exposed to serum (2%) from vehicle- and RVX-208–treated monkeys for 4 h at 37°C. Each data point represents the mean efflux for an individual animal from triplicate analysis. The line illustrates the average efflux/group. The combined data from all treatment periods (28, 42, and 63 days) versus day 77 wash-out or controls are presented in D. *p < 0.05; **p < 0.01. SR-BI = scavenger receptor class B type I.

and 97%, respectively, by day 63 of treatment (Table 1, Online Table 1). A 24-h washout failed to reduce pharmacodynamic effects of RVX-208, as reflected by the sustained increase in HDL-C (Table 1). This finding fits with the prolonged plasma residence time of mature HDL (4 to 5 days) in humans and monkeys (21,22). The RVX-208–induced elevation of serum apoA-I and HDL-C was accompanied by changes in the HDL size distribution (Figs. 3 and 4). This observation in vivo mirrored the findings in HepG2 cells (Fig. 1D).

Sera from RVX-208–treated AGMs had more pre- β_1 -LpA-I and the larger α_1 -LpA-I particles but less α_2 -LpA-I (Fig. 4). This shift in the HDL distribution pattern might be due to the direct effect of RVX-208 on the biogenesis of nascent apoA-I-containing particles in the liver and intestine. Alternatively, these changes might arise from an indirect effect of RVX-208 on plasma factors—including LCAT, CETP, and PLTP—that are known to affect HDL concentration, composition, and subpopulation distribution

(23). Data in the current study support both possibilities. Specifically, exogenous LCAT activity and FER were significantly decreased in serum of RVX-208–treated monkeys. However, the cause of this reduction is unclear. Decreased FER might arise from inability of LCAT to use larger α_1 -LpA-I particles generated by RVX-208 as a substrate or to the reduction of α_2 -LpA-I species after treatment (Fig. 3). This idea is consistent with a previous study showing the inverse relationship between FER and high plasma levels of the large HDL2b particles (24). Whether decreased LCAT activity impacts on atherosclerosis is controversial (25). In studies of a large family with LCAT deficiency, there was no observed increase in ACVD over 25 years (26).

The RVX-208–induced shift to larger HDL particles could have important therapeutic implications for ACVD. Indeed, previous studies showed ACVD patients to have lower levels of the larger α_1 -HDL than control subjects, independently of HDL-C, suggesting that these larger

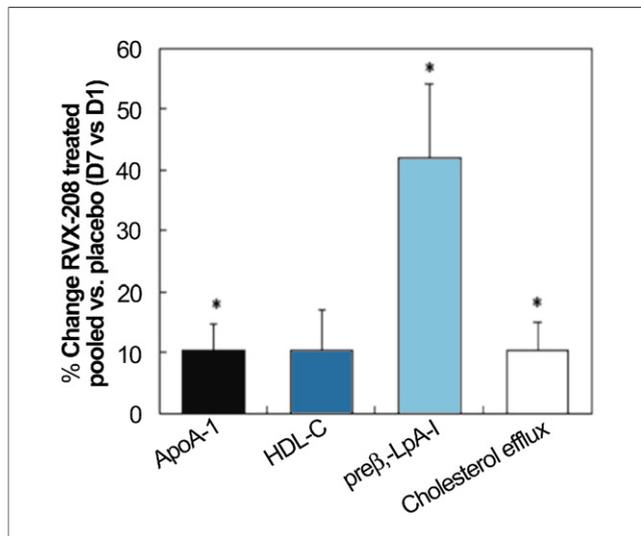


Figure 6 Effects of RVX-208 in Humans

To determine whether RVX-208 had pharmacodynamic effects, serum samples from the 18 subjects receiving multiple and varying doses of RVX-208 were examined. Depicted are the increases in serum apolipoprotein (apo)A-I, high-density lipoprotein cholesterol (HDL-C), pre- β 1-lipoprotein particles containing apoA-I, and ABCA1-mediated cholesterol efflux from pooled data regardless of dose. *Significant elevation ($p < 0.05$) in a parameter from RVX-208-treated subjects compared with placebo. Although HDL-C levels trended higher, $p = 0.09$.

particles might have an atheroprotective role (27,28). Additionally, progression of coronary atherosclerosis in younger patients who survive myocardial infarcts was inversely associated with the largest HDL particles (29). Although CETP-deficient individuals or treatment with CETP-inhibitors leads to more and larger HDL particles (18), whether they reduce ACVD remains controversial (30), because CETP might participate in RCT. The increase in larger α 1-LpA-I species in RVX-208-treated monkeys was not attributed to CETP inhibition but likely due to de novo synthesis.

The protective role of HDL against ACVD is believed to arise from the flux of cholesterol via RCT pathway (2,3,5). The RVX-208-induced rise in serum apoA-I and HDL-C correlated with enhanced cholesterol efflux activity mediated by ABCA1, ABCG1, and SR-BI-dependent pathways (Fig. 4). The ability of RVX-208 to enhance efflux likely arises from the increased total mass of HDL comprising more pre- β 1-LpA-I (Figs. 1D and 3). This change in HDL profile is expected to promote RCT, especially from macrophages, which are particularly reliant on the ABCA1 pathway (31). Indeed, pre- β 1-LpA-I has been shown to be the initial acceptor of cell-derived cholesterol and lipid-free apoA-I and pre- β 1-LpA-I, the preferred substrates for ABCA1 (32).

The RVX-208 treatment also enhanced ABCG1 and SR-BI-mediated cholesterol efflux (Fig. 5). Recently, ABCG1 was shown to mediate macrophage cholesterol efflux to mature and nascent LpA-I in vitro. Furthermore,

mice lacking ABCG1 display lipid accumulation in macrophages (33). The SR-BI facilitates bidirectional flux of cholesterol between cells and HDL (34), and it is likely that this receptor promotes selective uptake of free cholesterol and cholesteryl ester from larger HDL particles generated by RVX-208 in the liver. Our finding that cholesterol efflux mediated by all 3 pathways is enhanced by RVX-208 suggests it impacts the physicochemical properties and cholesterol efflux function of HDL.

The promising features of RVX-208 action in vitro and in AGMs prompted progression to a study in humans. Assessment of RVX-208-induced effects (Fig. 6) after 7 days of treatment showed promising results. Despite the limited number of subjects, short duration of treatment, and lack of knowledge regarding optimal dosing, the treated subjects had statistically significant increases in apoA-I protein, pre- β HDL, ex vivo ABCA1-mediated cholesterol efflux, and a trend toward increases in HDL-C and large α -HDL. This early dataset in humans supports the notion that RVX-208 treatment increases apoA-I synthesis and HDL functionality with the potential to reduce atherosclerosis.

Improving the functional capacity of HDL to remove cholesterol from macrophage foam cells in order to stabilize and possibly regress atherosclerotic plaques is a highly desirable goal in treating ACVD. A small molecule with the capacity to increase apoA-I production and plasma levels of apoA-I and HDL-C is conceptually an ideal way to achieve functional HDL. In vivo treatment with RVX-208 resulted in a significant increase in serum apoA-I and HDL-C levels. As expected, these changes led to improved HDL-mediated cholesterol efflux function. However, we wish to make clear that no attempt was made to claim that RVX-208 has atheroprotective effects. Ongoing studies that examine the effects of RVX-208 on the HDL metabolic pathway in humans and animals will define the therapeutic potential of this compound in treating atherosclerotic cardiovascular disease.

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Key Words: apoA-I ■ atherosclerosis ■ HDL-based therapy ■ reverse cholesterol transport.

▶ APPENDIX

For a supplementary table, please see the online version of this article.