Atherosclerosis 247 (2016) 48–57

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

RVX-208, a BET-inhibitor for treating atherosclerotic cardiovascular disease, raises ApoA-I/HDL and represses pathways that contribute to cardiovascular disease

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ARTICLE INFO

Article history:
Received 21 May 2015
Received in revised form
20 January 2016
Accepted 21 January 2016
Available online 22 January 2016

Keywords:
HDL
ApolipoproteinA-I
RVX-208
BET bromomodomain
Atherosclerosis

ABSTRACT

High density lipoproteins (HDL), through activity of the main protein component apolipoprotein A-I (ApoA-I), can reduce the risk of cardiovascular disease (CVD) by removing excess cholesterol from atherosclerotic plaque. In this study, we demonstrate that the bromodomain and extraterminal domain (BET) inhibitor RVX-208 increases ApoA-I gene transcription and protein production in human and primate primary hepatocytes. Accordingly, RVX-208 also significantly increases levels of ApoA-I, HDL-associated cholesterol, and HDL particle number in patients who received the compound in recently completed phase 2b trials SUSTAIN and ASSURE. Moreover, a post-hoc analysis showed lower instances of major adverse cardiac events in patients receiving RVX-208. To understand the effects of RVX-208 on biological processes underlying cardiovascular risk, we performed microarray analyses of human primary hepatocytes and whole blood treated ex vivo. Overall, data showed that RVX-208 raises ApoA-I/HDL and represses pro-inflammatory, pro-atherosclerotic and pro-thrombotic pathways that can contribute to CVD risk.

1. Introduction

Cardiovascular disease (CVD) is the leading cause of death within industrialized nations and accounts for approximately one out of three deaths in the United States [1]. Epidemiological studies show an inverse correlation between high density lipoprotein associated cholesterol (HDL-c) and incidence of CVD [2,3], suggesting a cardioprotective role for HDL. HDL is a heterogeneous collection of lipoprotein particles with roles in inflammation, oxidation and reverse cholesterol transport (RCT). The most common underlying cause of CVD is atherosclerosis, which leads to development of cholesterol-rich plaques in the artery wall that may rupture to induce acute events [6]. HDL mediates cholesterol efflux from atherosclerotic plaque via RCT, potentially slowing down the atherogenesis process and stabilizing the plaque. The concept that increased production of HDL can decrease atherosclerosis is supported by studies in which ApoA-I, the major protein component of HDL, was infused into subjects, resulting in regression of coronary plaque [7,8]. Currently, strategies that increase HDL-c are being evaluated in the clinic with the objective to reduce cardiovascular events [9,10]. However, findings from most of these studies suggest that raising concentrations of HDL-c is not sufficient to reduce CVD risk [11]. Conversely, increasing HDL functionality to augment RCT may potentially decrease cardiovascular events [11,12].

RVX-208 is an orally active small molecule being developed for the treatment of CVD [13,14]. Recent evidence indicates that serum cholesterol efflux capacity, which represents a critical step in RCT, provides a more accurate prediction of cardiovascular events than levels of HDL-c [11,12]. In preclinical studies of African green monkeys, RVX-208 increased plasma ApoA-I, HDL-c (pre-beta and alpha HDL), and enhanced the ability of serum to efflux cholesterol via the ABCA1, ABCG1, and SR-BI-dependent pathways [15]. The phase 1a trial showed that serum from healthy volunteers taking RVX-208 had increased cholesterol efflux capacity [15], consistent

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http://dx.doi.org/10.1016/j.atherosclerosis.2016.01.036

with enhanced production of HDL that is functional in RCT. In the phase 2a ASSERT trial of statin-treated patients with stable coronary artery disease, those receiving RVX-208 for 12 weeks had significant increases in HDL-c and large HDL, indicating elevated production of ApoA-I and efflux of cholesterol to nascent HDL particles [14]. The same trial showed that ApoA-I and HDL-c levels increased rapidly from week 8 to week 12, suggesting peak pharmacological effect had not been reached. Subsequently, SUSTAIN and ASSURE, 24 and 26 week phase 2b trials were undertaken to assess the long term efficacy of RVX-208 [13].

RVX-208 increases ApoA-I transcription through an epigenetic mechanism that is mediated by bromodomain and extra-terminal domain (BET) protein 4 (BRD4) [16,17]. BRD4 can recruit and tether the positive transcription elongation factor (P-TEFb) to gene promoters, thereby promoting transcription [18]. BET proteins contain two bromodomains that interact with acetylated lysines on histone tails [19]. RVX-208 disrupts this interaction by selectively binding to the second bromodomain (BD2). This leads to differential effects on gene expression as compared to pan BET inhibitors (BETi) [17]. RVX-208 is currently the only BD2 selective BETi in clinical development for CVD.

In vitro, increased ApoA-I transcription is a recognized property of BETi in hepatocarcinoma cells [15,16,20–22]. In this study, human and primate primary hepatocyte systems that maintain many characteristics of the liver were used to confirm induction of ApoA-I gene expression and protein production in response to BETi, including RVX-208. Further, we explored the effects of RVX-208 on known markers of CVD risk in the SUSTAIN and ASSURE trials and in cultured primary hepatocytes and whole blood using microarrays. Results indicate that, in addition to expected effects on lipoprotein profile, RVX-208 represses pro-inflammatory, pro-atherosclerotic and pro-thrombotic pathways that can contribute to CVD risk.

2. Methods

2.1. SUSTAIN and ASSURE human trials

Clinical data from two randomized, double-blind, placebo-controlled, similarly designed phase 2b clinical trials of RVX-208 treatment over 6 months in patients with coronary artery disease (CAD) were retrospectively analyzed to evaluate the effects of RVX-208 on various lipid, inflammatory and metabolic biomarkers as well as incidence of major adverse cardiac events (MACE) defined as death, nonfatal myocardial infarction, coronary revascularization and hospitalization for unstable angina or heart failure. The full design and rationale of the two studies, SUSTAIN (NCT01423188) and ASSURE (NCT01067820) has been published previously [13]. The similarities of the clinical trial designs include the inclusion criteria, identical dosing regimen of RVX-208, comparable treatment durations and the replicate placebo groups, providing rationale for the data to be pooled and analyzed. The major difference in the patient groups was the severity of the CAD. In the SUSTAIN study, patients were required to have documented stable coronary artery disease whereas in the ASSURE study patients were scheduled to undergo coronary angiography for a clinical indication. This difference provided a broader and more integrated analysis of the effects of RVX-208 across the CAD spectrum over 6 months of treatment. A total of 55 matching biomarkers were collected at various time points throughout the course of both studies in addition to several screening parameters. Secondary outcome measures included HDL-c, ApoA-I and HDL-subclasses. The proApoA-I clinical data were collected in the SUSTAIN study.

2.2. Statistical analysis

The change from baseline to 24/26 weeks (SUSTAIN/ASSURE) in the efficacy parameters was analyzed using a 2-sided Van Elteren test of RVX-208 vs placebo, stratified by study in the modified-intent-to-treat patient population. For each analysis, the median change from baseline, median percentage change from baseline, median difference between treatment groups and p-value for the difference between RVX-208 and placebo are presented. Shapiro–Wilk tests on each parameter illustrated non-normal distribution ($p < 0.001$) and thus non-parametric statistical tests were used. Baseline was defined as the last non-missing value prior to randomization. The censoring date for the quantification of MACE was taken to be 30 days after the last dose, and only the first event was used.

2.3. Chemical synthesis

RVX-208 (2-(4-(2-hydroxyethoxy)-3,5-dimethylphenyl)-5,7-dimethoxyquinazolin-4(3H)-one) and JQ1 were synthesized as described previously by NAEJA Pharmaceuticals (Edmonton, Canada) or IRIX Pharmaceuticals (Florence, SC) [23–25]. GSK1210151A (IBET-151) was purchased from ShangHai Biochempartner Co. Ltd. (Wuhan, China) while GSK525762 (IBET-762) was from Chemscene (Monmouth Junction, NJ).

2.4. Primary hepatocyte culture systems

Human hepatocytes in the HepatoPac® microliver system prepared by Hepregen (Medford, MA) and cryopreserved human hepatocytes (CellzDirect/Life Technologies) were plated as recommended by the suppliers. RVX-208 treatments were performed in media recommended by the supplier containing 0.1 or 0.2% DMSO.

2.5. mRNA abundance

mRNA analysis was performed by TaqMan® assay based real-time PCR as described previously [16].

2.6. ELISA

A rabbit monoclonal anti-proApoA-I antibody was generated using a synthetic peptide RHFWQQ_DEPP. The antibody was used to coat EIA/RIA high binding surface microplates (Corning) overnight. Plates were washed, and then blocked with 5% skim milk. Recombinant poly-histidine tagged human proApoA-I (Genscript, Piscataway, NJ) was used as a standard. Recombinant protein or samples were introduced onto plates, incubated with anti-human ApoA-I (Calbiochem # 178470), and then with HRP conjugated anti-mouse IgG (Calbiochem # 401253). Color was developed by treatment with tetramethylbenzidine, followed by sulfuric acid. Plates were read on a Thermo Scientific Multiskan GO Spectrophotometer at 450 nm. ApoA-I ELISA was performed in a similar fashion as proApoA-I, except using the mouse anti-human ApoA-I antibody (Calbiochem # 178470). The standard was purified ApoA-I (Calbiochem # 178452) and it was detected using a polyclonal rabbit anti-human ApoA-I antibody (Calbiochem # 178422), followed by HRP conjugated anti-rabbit IgG (Calbiochem # 401353). Media was collected from Huh-7 cells after 48 h, then incubated with recombinant bone morphogenetic protein-1 (R&D Systems) followed by ELISA to measure remaining proApoA-I.

2.7. Western blots

Proteins in media from hepatocyte cultures maintained for 72 h were separated in 4–12% NuPAGE Bis-Tris gels (Invitrogen/Life Technologies) under reducing conditions. Proteins were transferred to a nitrocellulose membrane (Bio-Rad). Immunoblots were performed with rabbit anti-proApoA-I followed by peroxidase conjugated anti-rabbit IgG (Calbiochem # 401353). Immunoreactive proteins were visualized by chemiluminscence (Pierce Biotechnology). The primary antibody used to detect total ApoA-I was from Calbiochem (# 178422).

2.8. Microarrays

Primary human hepatocytes (CellzDirect/Life Technologies) were plated in 24 well format at 500,000 cells/well, then overlaid with Matrigel™ as recommended by the supplier. Cells were treated with RVX-208 at 30μM for 48 h. Total RNA was extracted with the mirVana™ kit (Ambion) and sent to Asuragen Inc. (Austin, TX) for microarray analysis using Affymetrix Human Genome U133 Plus 2.0 Array. After obtaining informed consent, whole blood was collected from three healthy volunteers into BD Vacutainer Sodium Heparin tubes (# 367874) and samples were inverted 10 times. Blood samples (1 mL) were combined with 1 mL of RPMI containing 2 mM glutamine, 1% penicillin/streptomycin, 20% FBS and 20μM RVX-208 or vehicle (0.1% DMSO), followed by a 3 h or 24 h incubation at 37 °C in an tissue culture incubator (CO_2 concentration 5.5%). Treated samples were transferred to a PAXgene RNA tube (PreAnalytix/Qiagen), inverted 5 times and frozen. RNA was isolated with the PAXgene RNA kit according to manufacturer's instructions. Microarrays were performed by Asuragen using the Affymetrix human U133 plus 2.4 Array. Data were analyzed using Ingenuity Pathway Analysis Software (Qiagen).

2.9. Multi-analyte profiling (MAP)

Plasma samples were collected from 20 RVX-208 treated subjects and 10 placebo treated subjects at baseline and after final dose in the ASSURE trial. Levels of plasma proteins were quantitated using microsphere-based immuno-multiplexing analysis. Changes in amounts of each protein were calculated versus baseline. Shapiro–Wilk tests were used to determine data distribution. For normally distributed parameters, paired student t-tests were used to calculate statistical significance, while for non-normally distributed parameters, Wilcoxon signed-rank tests were applied.

3. Results

3.1. RVX-208 increases ApoA-I mRNA in primary human hepatocyte systems

RVX-208 and other BET inhibitors enhance ApoA-I gene activity in hepatocarcinoma cell lines [15,16,20–22]. Primary hepatocytes mimic in vivo activity more accurately than cell lines, and so the effects of RVX-208 on ApoA-I expression were examined in two different primary liver systems including HepatoPac® microliver cultures (Hepregen Corp., Medford, MA) and cryopreserved human hepatocytes (Cellz Direct, Life Technologies). The HepatoPac® microliver system co-cultures stromal cells with human hepatocytes to provide an environment that enhances hepatocyte function in vitro [26,27]. In such co-cultures, hepatocytes retain liver-specific characteristics including albumin secretion, synthesis of urea and formation of bile canaliculi [28]. The cryopreserved system utilizes human hepatocytes plated on collagen to form monolayers. In both models, exposure to RVX-208 increased ApoA-I mRNA in a time and dose dependent manner (Fig. 1A and B). Similar observations (data not shown) were made using RegeneMed 3-D liver cell cultures (RegeneMed Inc., San Diego, CA). Together the results show that RVX-208 induces ApoA-I expression in a variety of human hepatocyte systems. A similar finding was evident in 3-D cultures of primary hepatocytes from African green monkeys.

Increased levels of ApoA-I mRNA were also measured in HepatoPac® microliver and cryopreserved hepatocytes treated with the pan BET inhibitors JQ1, I-BET762 and I-BET151 (Fig. 1C). The chemical scaffold for these compounds is distinct from RVX-208, indicating enhanced ApoA-I transcription is a property of these BET inhibitors. I-BET762 is being tested in humans for treating NUT midline carcinoma and other cancers (ClinicalTrials.gov NCT01587703), while the related compound I-BET151 induces ApoA-I transcription in vitro [21].

Next, actinomycin D, an inhibitor of transcription, was used to block induction of ApoA-I mRNA by RVX-208 in cryopreserved primary human hepatocytes (Fig. 1D), supporting the transcriptional mechanism demonstrated in carcinoma cells [16]. Cycloheximide, an inhibitor of protein synthesis, also abrogated ApoA-I mRNA induction (Fig. 1D). These findings suggest that RVX-208 stimulates ApoA-I gene transcription, and although this process is BET protein-dependent, it involves synthesis of a protein intermediate in human liver cells.

3.2. RVX-208 increases proApoA-I protein secretion

To determine if RVX-208 induced transcription of the ApoA-I gene leads to synthesis of the protein in vitro and in vivo, an antibody targeting proApoA-I was developed. ApoA-I is secreted from the liver and intestine as proApoA-I which carries an N-terminal hexapeptide [29,30] that is cleaved by bone morphogenetic protein-1 (BMP-1) [31,32]. Thus, levels of circulating proApoA-I reflect recently synthesized and secreted protein that has not yet been processed. An antibody directed against the cleavage motif plus 4 amino acids (RHFWQQ_DEPP) was created, and it recognized a single band in immunoblots from primary human hepatocytes, illustrating specificity (Fig. 2A). A modest increase in both proApoA-I and total ApoA-I was detected by Western blots in spent media from primary hepatocytes exposed to RVX-208. The abundance of mature and proApoA-I in spent media was dose-dependent (Fig. 2B). The ELISA signal was eliminated by digestion with recombinant BMP-1, consistent with recognition of proApoA-I by the antibody (Fig. 2C). JQ1 also induced proApoA-I mRNA and protein levels, confirming that BET inhibition increases ApoA-I production as expected [33]. In the SUSTAIN trial, statistically significant increases in both serum proApoA-I (+2.37%; $p < 0.05$) and total ApoA-I (+4.38%; $p < 0.01$) proteins were observed following treatment with RVX-208 versus placebo. Importantly, these protein changes were accompanied by an increase in HDL-c ($p < 0.001$) and HDL particle number ($p < 0.05$), suggesting that, in humans, RVX-208 increases production of ApoA-I protein that matures to HDL.

3.3. RVX-208 impacts CVD risk in the SUSTAIN and ASSURE trials

Phase 2b SUSTAIN and ASSURE trials were designed to evaluate the effects of RVX-208 on CVD [34,35]. Both trials enrolled patients with established CVD receiving standard of care therapy, including statins and then randomized to receive either RVX-208 or placebo for a period of 6 months. After combining data from the two trials, there was a total of 499 subjects that received either 100 mg b.i.d. of RVX-208 ($n = 331$) or placebo ($n = 168$) (Fig. 3). Pooled data showed that RVX-208 treatment led to a statistically significant increase in HDL-c, ApoA-I, large HDL particles and average HDL particle size of 7.69% ($p < 0.001$), 10.3% ($p < 0.01$), 30.7% ($p < 0.01$)

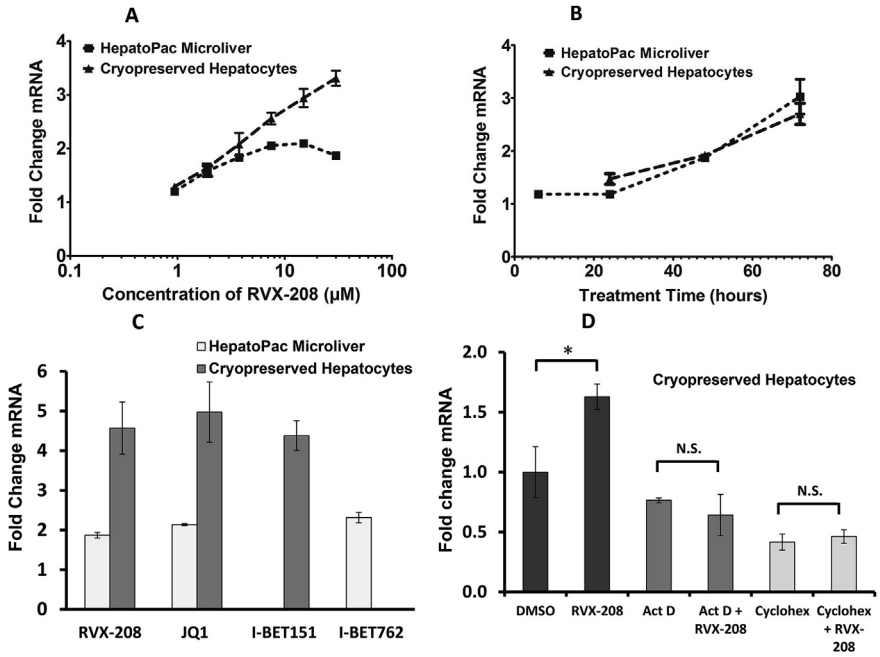

Fig. 1. RVX-208 increases ApoA-I expression in primary hepatocytes in a time and dose dependent manner. Primary human hepatocytes were cultured in the two systems indicated. (A) Hepatocytes were treated with increasing concentrations of RVX-208 for 48 h, then ApoA-I mRNA abundance determined by TaqMan® real-time PCR. (B) The time course for ApoA-I induction was performed using RVX-208 at 30μM (HepatoPac® microliver or cryopreserved hepatocytes). (C) Hepatocytes in the indicated culture systems were treated for 48 h with BETi with chemical scaffolds different from RVX-208, then ApoA-I mRNA levels were determined. In the HepatoPac® microliver system, RVX-208 was used at 30μM, JQ1 at 1μM, and I-BET762 at 1μM. In cryopreserved hepatocytes, RVX-208 was used at 30μM, JQ1 at 0.5μM and I-BET151 at 0.9μM. For all data in Fig. 1A through C, $p < 0.05$ versus DMSO treated samples at the same time point using two-tailed student's t-tests. D) ApoA-I mRNA induction by RVX-208 is dependent on transcription and production of new protein. Cryopreserved primary human hepatocytes were treated with the indicated compounds for 48 h. ApoA-I mRNA induction by 30μM RVX-208 is inhibited by 1μM actinomycin D (Act D) and by 1μM cycloheximide (Cyclohex). All data are the mean from independent triplicate samples, while error bars represent standard deviation. *$p < 0.05$ N S. = not significant.

and 1.16% ($p < 0.05$), respectively, versus placebo (Table 1). Additional CVD risk factors and metabolic parameters (blood pressure, high-sensitivity C-reactive protein [hsCRP] and glucose) were analyzed, but there were no differences between treatment groups. Quantification of MACE showed 17/168 (10.1%) and 18/331 (5.4%) in the placebo and RVX-208 treatment groups, respectively (Table 2). The post-hoc analysis of pooled data from our phase 2b trials shows that RVX-208 improves the HDL profile and appears to lower MACE in CVD patients.

3.4. RVX-208 regulates pathways that underlie CVD

The observed reduction in MACE (Table 2) was more pronounced than that predicted from changes in ApoA-I/HDL alone. This implies RVX-208 had other effects that could impact CVD risk. To explore this, a microarray analysis of cryopreserved hepatocytes treated with RVX-208 (30μM) was performed. Pathway analysis of these data showed that RVX-208 downregulates the complement cascade, fibrin clotting, acute phase response, cholesterol synthesis, fatty acid synthesis, and diabetes pathways (Table 3). These pathways, which are predominantly expressed in the liver, are linked to processes that are known to affect CVD risk, including atherosclerosis, thrombosis and inflammation. Gene sets contributing to these pathways and the effect of RVX-208 on expression are shown in Fig. 4.

In addition to the pathways above, it is known that vascular inflammation and atherosclerotic plaque development contribute to CVD. These processes can be mediated by circulating cells in blood, such as monocytes, lymphocytes and neutrophils. To assess the effects of RVX-208 on these cell types, human whole blood from healthy donors was treated ex vivo for 3 or 24 h with 20μM RVX-208. This dose maximally suppresses inflammation markers in both U937 and peripheral blood mononuclear cells (data not shown). The gene expression profile in whole blood was examined by microarrays. Mining of the scientific literature shows that almost 600 genes expressed in multiple cell types have documented roles in inflammation, atherogenesis and angiogenesis [36 and references therein]. This list of genes was cross-referenced with our microarray data showing that, in human whole blood, RVX-208 affected expression of 64 genes with functional relevance in atherosclerosis (Fig. 5). Specifically, RVX-208 downregulated the expression of chemokines, cytokines, integrins and their cognate receptors, which control immune cell migration and activation in atherosclerotic plaque. These genes are implicated in atherosclerotic plaque development and instability. Overall, out of 46 affected pro-atherogenic genes, 37 were downregulated in response to RVX-208, whereas 8 out of 18 anti-atherogenic genes were upregulated by RVX-208 (Fig. 5). Lastly, the top canonical pathways downregulated by RVX-208 treatment are involved in cytokine signaling, cell–cell adhesion, extracellular matrix organization, G protein signaling and Th1/Th2 responses (Table 4). Downregulation of these pathways may translate into anti-atherogenic effects in human vasculature.

To confirm the microarray findings, select proteins were measured versus baseline in plasma samples collected in the ASSURE trial using multi-analyte profiling to determine effects of RVX-208 on inflammatory cytokines, adhesion molecules and markers of APR (Table 5). In patients receiving RVX-208, protein levels of pulmonary and activation-regulated cytokine (CCL18), IL-18, interleukin-12 subunit p40 and the adhesion molecule ICAM-1 were significantly reduced by 22%, 10%, 6% and 7%, respectively. Moreover, APR proteins serum amyloid P-component, ceruloplasmin and complement C3 were reduced by 7–15% (Table 5). These data show an effect of RVX-208 on inflammatory mediators

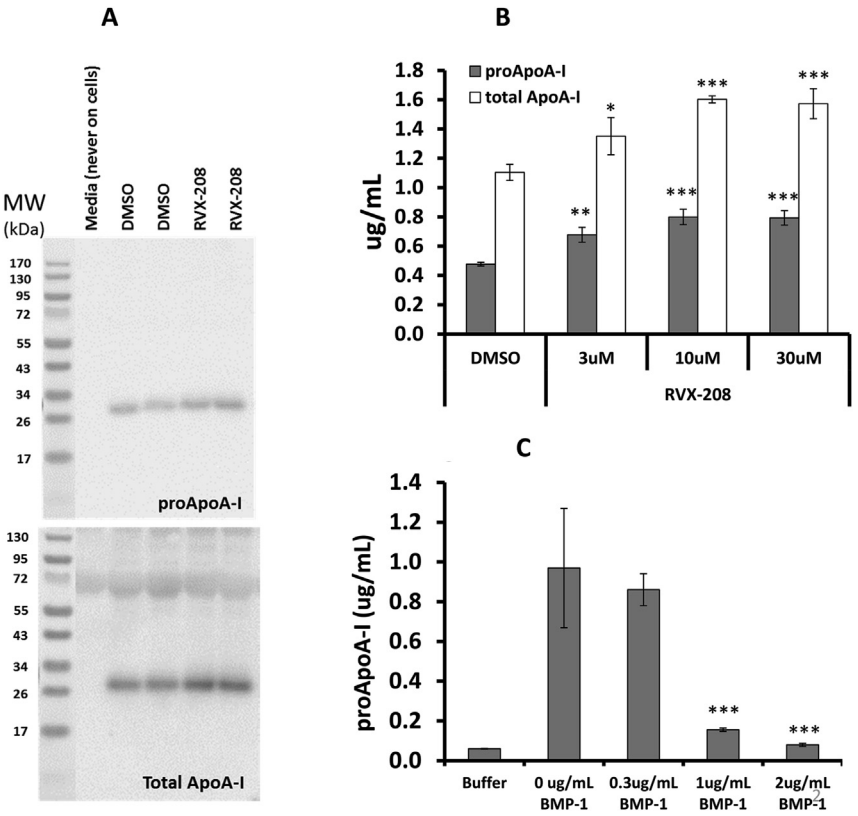

Fig. 2. RVX-208 increases secretion of proApoA-I and total ApoA-I protein from cryopreserved human primary hepatocytes. A) Cryopreserved human hepatocytes plated in duplicate wells were treated with 30μM RVX-208 or DMSO for 72 h. Immunoblots were used to probe for proApoA-I and total ApoA-I in the media. B) Cryopreserved human hepatocytes were treated for 72 h with RVX-208 or DMSO. ApoA-I and proApoA-I protein in the media were quantified using ELISA assays. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus DMSO treated cells. C) Media from Huh-7 cells was collected and incubated with recombinant human BMP-1. ProApoA-I remaining in the media was determined by ELISA. Data are the mean from independent triplicate samples, while error bars represent standard deviation. $^{***}p < 0.001$ versus samples lacking BMP-1 in a two-tailed student's t-test.

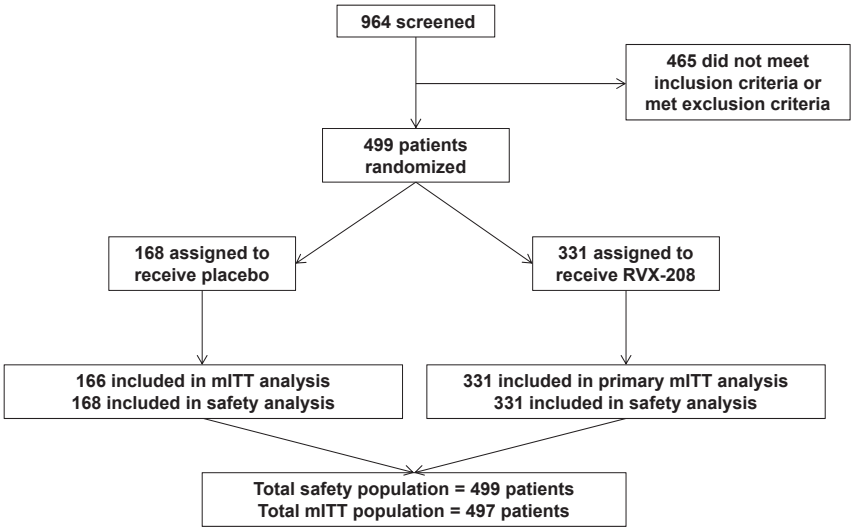

Fig. 3. Flow chart of patients through the SUSTAIN and ASSURE clinical trials.

in patients with CVD, and extend microarray findings in response to RVX-208 treatment.

4. Discussion

ApoA-I is the precursor of new HDL particles, and provides the vehicle to promote RCT. Enhancement of ApoA-I production and formation of efflux-competent HDL can oppose plaque progression, resulting in increased stability and reduced cardiovascular risk. In addition to RCT, HDL counters atherosclerosis through antioxidant, anti-inflammatory and antithrombotic mechanisms [37]. Some pharmacological strategies that increase HDL-c have been

Table 1
Combined analysis of the ASSURE and SUSTAIN phase II trials.

Biomarker	RVX-208 (n = 331)		Placebo (n = 166)		Between treatment difference (%▲)	p value vs placebo
	Baseline	Change from baseline (%▲)	Baseline	Change from baseline (%▲)		
HDL-cholesterol (mg/dL)	39.0	+3.0 (+7.69)	38.0	0.0 (0.0)	+3.0 (7.69)	0.0003
ApoA-I (mg/dL)	119.2	+12.3 (+10.3)	118.1	+4.8 (+3.8)	+7.5 (6.5)	0.005
Large HDL particles (μmol/L)	2.4	+0.8 (+30.7)	2.1	+0.1 (+4.11)	+0.7 (26.6)	0.03
HDL particle size (nm)	8.7	+0.1 (+1.16)	8.7	0.0 (0.0)	+0.1 (1.16)	0.049
Total HDL particles (μmol/L)	27.2	+1.9 (+6.51)	26.9	+0.1 (+0.40)	+1.8 (6.11)	0.07
LDL-cholesterol (mg/dL)	2.4	−0.2 (−10.8)	2.3	−0.2 (−8.05)	0.0 (−2.75)	NS
Glucose (mmol/L)	5.8	+0.1 (+2.08)	5.7	+0.2 (+3.57)	+0.1 (+1.49)	NS
hsCRP (mg/L)	2.3	−0.36 (−28.4)	2.5	−0.33 (−22.4)	−0.03 (−6.03)	NS

Table 2
Incidence of MACE in the ASSURE and SUSTAIN phase II trials.

Cardiovascular events	RVX-208 (n = 331)	Placebo (n = 168)
MACE n (%)	18 (5.4)	17 (10.1)
Death n (%)	1 (0.3)	3 (1.8)
Myocardial infarction n (%)	4 (1.2)	0 (0.0)
Coronary revascularization n (%)	9 (2.7)	7 (4.2)
Hospitalization for unstable angina or heart failure n (%)	4 (1.2)	7 (4.2)

Table 3
Pathways that can impact MACE were identified by microarrays from cryopreserved primary human hepatocytes treated with 30μM RVX-208 for 48 h. The normalized enrichment score (NES) and the p-value are indicated.

Pathway	NES	p value
REACTOME_COMPLEMENT_CASCADE	−2.30	<0.001
REACTOME_FORMATION_OF_FIBRIN_CLOT_CLOTTING_CASCADE	−1.98	<0.001
REACTOME_CHOLESTEROL_BIOSYNTHESIS	−1.88	<0.001
INGENUITY_ACUTE_PHASE_RESPONSE_SIGNALING	−1.84	<0.001
REACTOME_DIABETES_PATHWAYS	−1.67	<0.001
REACTOME_FATTY_ACYL_COA_BIOSYNTHESIS	−1.65	0.01

evaluated in pre-clinical and clinical studies. Current investigation of RVX-208 through analysis of clinical trials and in vitro studies show that this novel BETi affects mechanisms that enhances production of the ApoA-I protein and HDL particles which are efflux-competent. In addition, microarray-based gene expression analyses of human primary liver cells and whole blood identify additional pathways regulated by RVX-208 that contribute to CVD risk.

Studies from laboratories including our own have shown that BETi with various chemical scaffolds induce ApoA-I gene transcription in hepatocarcinoma cell lines [15,16,20–22]. This report confirms that BETi induces ApoA-I gene expression in multiple primary human and primate hepatocyte systems (Figs. 1–2). Moreover, RVX-208 induces production of new ApoA-I protein that is detected with an anti-proApoA-I antibody in primary hepatocyte culture and in patients' serum from the SUSTAIN trial (Fig. 2). Mature ApoA-I, HDL-c, and HDL particle numbers are also significantly elevated in patients receiving RVX-208 in the SUSTAIN/ASSURE trials (Table 1). We have previously demonstrated increased efflux potential in serum from human trials [15]. In addition, RVX-208 enhanced cholesterol efflux three-fold from J774 macrophages to lipid poor recombinant ApoA-I (data not shown). Together, these findings suggest that RVX-208 promotes cholesterol efflux through increased production of ApoA-I by hepatocytes and maturation into HDL particles, as well as increased efflux potential of macrophages.

The data collected in the recently completed phase 2b trials SUSTAIN and ASSURE provided an opportunity to look at potential impact of RVX-208 on cardiovascular events. While SUSTAIN did not pre-specify MACE as an endpoint, in ASSURE, in addition to the primary endpoint of arterial plaque regression ($p = 0.08$), MACE was an exploratory endpoint that showed trending towards a reduction in patients receiving RVX-208 (data not shown). To expand on this observation, we performed a post-hoc analysis of the combined MACE data from the two trials. Treatment with RVX-208 was associated with lower incidence of MACE (Table 2), but the magnitude was greater than predicted by changes in ApoA-I/HDL (Table 2) [38], and thus prompted microarray-based gene expression analysis to identify properties of RVX-208 beyond lipid and lipoprotein profile.

Microarray analysis of primary human hepatocytes revealed a significant RVX-208 mediated repression of cholesterol and fatty acid synthesis, glucose processing and acute phase response (APR) pathways (Table 3 and Fig. 4). The contribution of cholesterol and fatty acyl-CoA biosynthesis as well as diabetes pathways on progression of atherosclerosis is well established. Plasma triglycerides, LDL-c and diabetes are independent risk factors for CVD [39,40]. However, biologically significant changes in LDL-c, triglycerides or fasting glucose were not observed in the ASSURE/SUSTAIN trials (Table 1). Components of the APR are pro-inflammatory, pro-atherogenic and markers of CVD risk [41]. Chronic inflammation leads to formation of dysfunctional and pro-inflammatory HDL particles that no longer promote cholesterol efflux or prevent LDL oxidation (reviewed in [42,43]). RVX-208 downregulates expression of 14 genes that encode APR proteins associated with HDL. Three of these proteins are also reduced in patients' plasma, including ceruloplasmin, amyloid P component and complement C3 (Table 5). Thus, downregulation of APR by RVX-208 may reduce formation of pro-inflammatory HDL and counter the

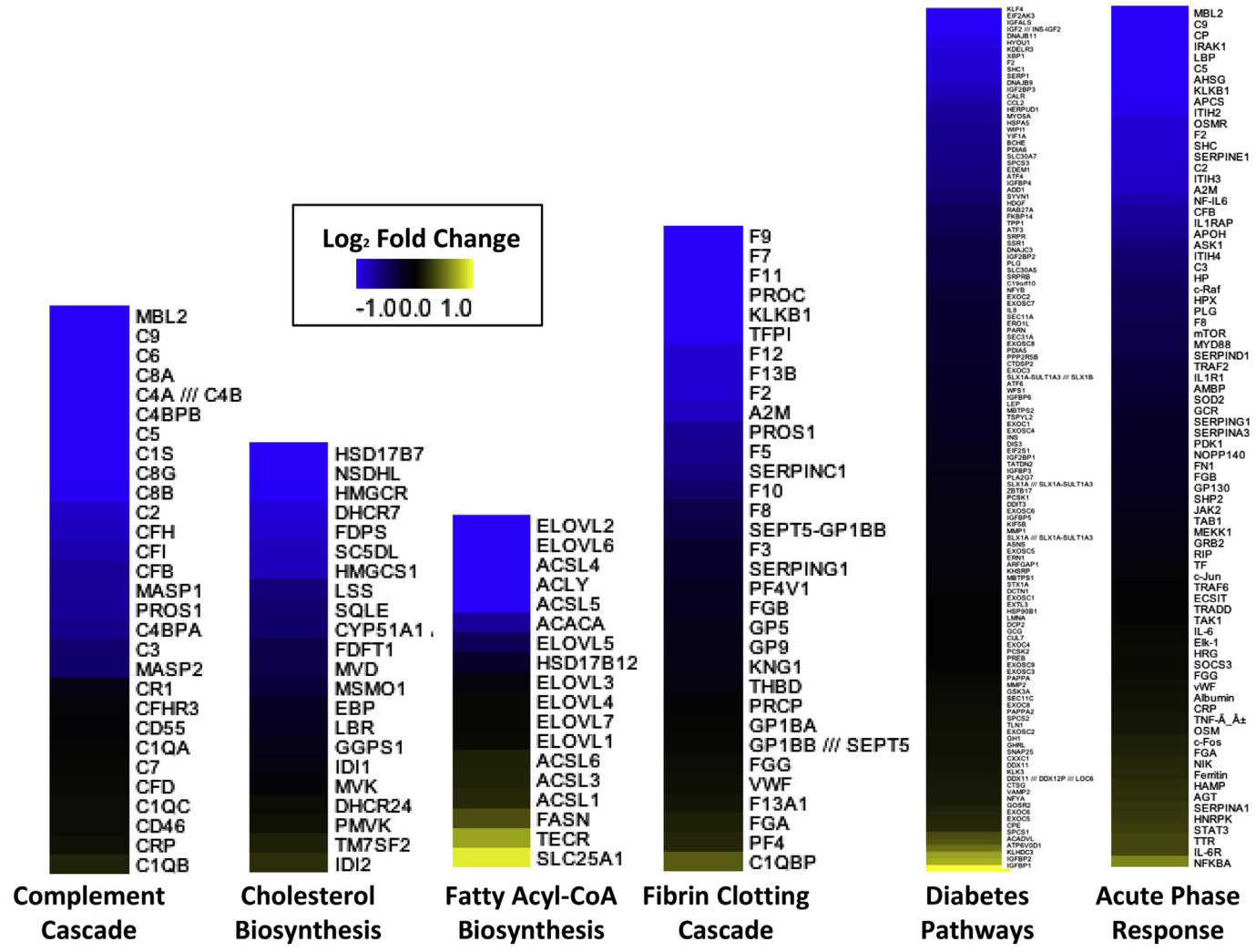

Fig. 4. Microarray analysis of RVX-208-induced gene expression changes in primary human hepatocytes. Cryopreserved human hepatocytes were treated with 30μM RVX-208 for 48 h. Heatmaps represent log2 fold changes in gene expression in pathways known to impact MACE.

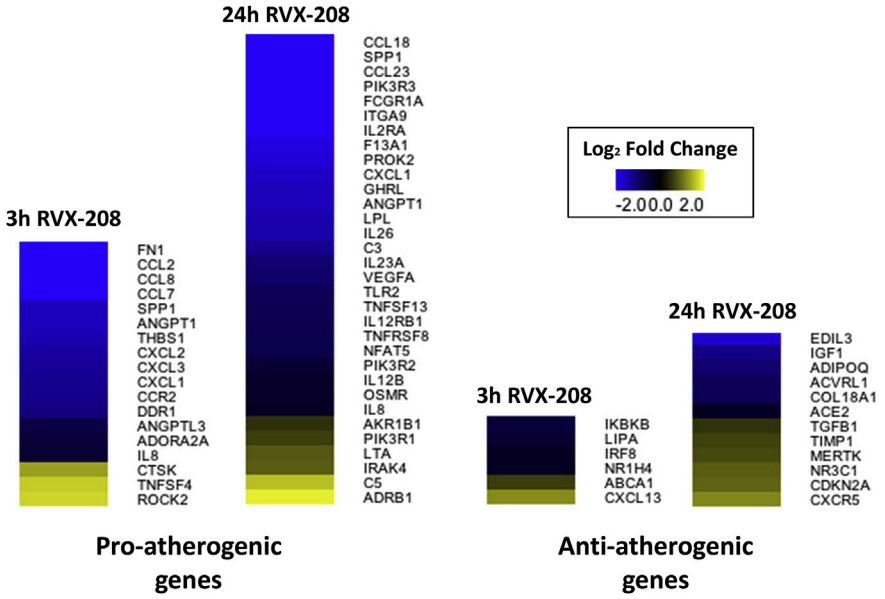

Fig. 5. Microarray analysis of RVX-208-induced gene expression changes in human whole blood. Samples were treated ex-vivo with 20μM RVX-208 for 3 or 24 h. Heatmaps represent log2-fold changes in expression of genes with known pro- and anti-atherosclerotic functions.

Table 4
Top pathways downregulated by 20μM RVX-208 identified in microarrays from human whole blood treated ex vivo for 3 h or 24 h. The normalized enrichment score (NES) and the p-value are indicated.

Pathway	NES	p value
REACTOME_COLLAGEN_FORMATION	−2.04	<0.001
PID_AVB3_INTEGRIN_PATHWAY	−1.97	<0.001
REACTOME_BETA_DEFENSINS	−1.78	0.002
REACTOME_DEFENSINS	−1.77	0.004
KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION	−1.73	<0.001
REACTOME_EXTRACELLULAR_MATRIX_ORGANIZATION	−1.71	0.005
REACTOME_G_ALPHA_Q_SIGNALLING_EVENTS	−1.66	<0.001
BIOCARTA_DC_PATHWAY	−1.65	0.005
PID_INTEGRIN_A9B1_PATHWAY	−1.63	0.02
BIOCARTA_TH1TH2_PATHWAY	−1.57	0.02

inflammatory signaling axis. This is of importance as CCR2 is the target of developing anti-atherosclerotic therapies [59]. Furthermore, FN1, CCL2, CCL18 and SPP1 were upregulated in blood taken from sites of occlusion during acute myocardial infarction, indicating they either play a direct role in the acute event or they constitute a tissue response to the occlusion [60]. Importantly, these four are amongst the top 10 downregulated genes in RVX-208-treated whole blood, suggesting that treatment with RVX-208 may disrupt pathogenic pathways underlying CVD and/or an acute event.

Based on findings in microarrays, we analyzed plasma samples from our clinical trials and found decreased level of circulating cytokines vs. baseline in patients receiving RVX-208. Specifically

Table 5
RVX-208 reduces circulating levels of cytokines and APR proteins in clinical samples. Multi-Analyte Profiling was performed with plasma samples from the ASSURE trial (N = number of samples) collected at baseline and after the final dose of RVX-208.

Multi-analyte profiling of plasma samples from clinical trails.			
Analyte/Gene symbol	N	Percent change from baseline	p-value vs baseline
Pulmonary And Activation-Regulated (CCL18)	20	−22.2	0.01
Serum Amyloid P-Component (APCS)	20	−15.0	0.001
Interleukin-18 (IL18)	20	−10.3	0.02
Complement C3 (APR and Inflammation)	20	−9.28	0.002
Ceruloplasmin (CP) (APR)	17	−7.35	0.02
Intercellular Adhesion Molecule 1 (ICAM1)	10	−6.87	0.01
Interleukin-12 Subunit p40 (IL12B)	19	−5.65	0.04

downregulation of HDL associated with activation of the innate immune system (reviewed in [43]), resulting in improved HDL profile as observed in the ASSURE/SUSTAIN trials.

RVX-208 had profound effects on the complement and fibrin clotting pathways (Table 3 and Fig. 4). Overactivation of the complement pathway has been implicated in plaque development and destabilization [44,45]. Complement activation also influences thrombosis through activation of platelets, promotion of fibrin formation, and impairment of fibrinolysis. Fibrin clotting is fundamental in the formation of thrombi and emboli. Fibrin and fibrinogen degradation products have been associated with CVD development and severity [46,47], as well as cardiac events and death [48,49]. Bleeding disorders were not found in clinical trials with RVX-208, however, downregulation of the fibrin clotting pathway may avoid cardiac events similar to other pharmacological agents that target coagulation [50].

BETi have well established anti-inflammatory properties [20,22,51]. JQ1 was demonstrated to downregulate inflammatory signaling in vascular endothelial cells and to decrease leukocyte chemoattraction to endothelium in ApoE−/− mice, potentially leading to reduced atherosclerosis [51]. RVX-208 was shown to lower inflammatory markers in human vascular and monocytic cell lines and to reduce circulating cytokines in ApoE−/− mice [52]. Microarray data summarized here identify pro-inflammatory and pro-atherosclerotic genes that are downregulated by RVX-208 in primary human hepatocytes and whole blood (Figs. 4 and 5). Many of the targets repressed by RVX-208 are expressed at high levels in patients with atherosclerosis and CVD, and the corresponding protein levels are predictive of the disease. For example, plasma levels of secreted phosphoprotein 1 (SPP1) predict MACE in patients with coronary artery disease [53–55] or type 1 diabetes [56], while CCL23 and Toll-like receptor 2 are independent risk factors for atherogenesis [57,58]. It was previously shown that RVX-208 downregulates aortic CCL2 in mice and in human aortic endothelial cells [52]. Downregulation of CCL2 and its receptor CCR2 in human blood confirms that RVX-208 can interfere with this pro-

CCL18 (PARC) and IL-18 were reduced by −22% and −10%, respectively (Table 5). These findings matched microarray data from RVX-208 treated whole blood showing a 7-fold repression of CCL18 and from primary hepatocytes, where a 3-fold repression of IL-18 was observed. Both cytokines play a role in CVD and are associated with MACE [54,61]. Furthermore, IL-18 neutralizing antibodies are being tested for clinical benefit [62], thus highlighting the potential of RVX-208 in reducing CVD.

Elevation of serum inflammatory markers is consistently associated with risk of MACE. In the ASSURE trial, MACE rate was reduced particularly in RVX-208-treated patients with elevated levels of the inflammatory marker CRP (≥2 mg/L) [63]. Thus, RVX-208 may have more favorable effects in the context of systemic inflammation, which is consistent with effects on inflammatory gene expression in liver and whole blood.

In summary, RVX-208 targets multiple processes that underlie CVD, including reverse cholesterol transport, atherogenesis, thrombosis and vascular inflammation. Transcriptional changes in any of these pathways, independently or cumulatively, may impact CVD and lower incidence of MACE observed in the ASSURE and SUSTAIN trials. These effects of RVX-208 are consistent with an epigenetic mechanism of action of BETi that modulate transcription of genes and pathways which can drive disease [51]. Downregulation of multiple pathogenic processes by inhibiting a single target is an attractive property of RVX-208 that can be used to treat a complex multifactorial disease such as CVD.

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