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RESEARCH PAPER

The thienotriazolodiazepine Ro 11-1464 increases plasma apoA-I and promotes reverse cholesterol transport in human apoA-I transgenic mice

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BACKGROUND AND PURPOSE

Ro 11-1464 is a thienotriazolodiazepine previously described to selectively stimulate apolipoprotein A-I (apoA-I) production and mRNA level in human liver cells. Here, we studied its effects upon oral administration to human apoA-I transgenic (hapoA-I) mice.

EXPERIMENTAL APPROACH

HapoA-I mice were treated for 5 days with increasing doses of Ro 11-1464. Macrophage reverse cholesterol transport (mph-RCT) was assessed by following [^{3}H]-cholesterol mobilization from pre-labelled i.p. injected J774 macrophages to plasma, liver and faeces. Effects on plasma lipids, apoproteins, lecithin-cholesterol : acyltransferase (LCAT) and liver enzymes, as well as on faecal excretion of cholesterol and bile salts, and on liver lipids and mRNA contents were determined.

KEY RESULTS

Treatment with Ro 11-1464 300 mg·kg^{-1}·day^{-1} resulted in a nearly 2-fold increase in plasma apoA-I, a 2- to 3-fold increase in the level of large sized-pre-β high-density lipoprotein and a 3-fold selective up-regulation of hepatic apoA-I mRNA, but a marked decrease in all plasma lipids and LCAT activity. Mpm-RCT was decreased in blood but markedly increased in faecal sterols (4-fold) and bile acids (1.7-fold). However, liver weight and liver enzymes in plasma were also increased, in parallel with an increase in liver cholesterol ester content (all these effect being significant).

CONCLUSION AND IMPLICATIONS

In this model Ro 11-1464 causes increased hepatic expression and plasma levels of apoA-I and a suppression of LCAT, and a marked enhancement of reverse cholesterol transport, but also some symptoms of liver toxicity. The compound may therefore be a prototype for a next generation of anti-atherosclerotic medicines.

Abbreviations

ABCA1, ATP binding cassette A1; ABCG1, ATP binding cassette G1; AcLDL, acetylated LDL; apoA-I, apolipoprotein A-I; cpt-cAMP, 8-(4-chlorophenylthio) cAMP; HDL, high density lipoproteins; hapoA-I mice, human apoA-I transgenic mice; LCAT, lecithin-cholesterol : acyltransferase; mph-RCT, macrophage reverse cholesterol transport; SR-BI, scavenger receptor class B type I

Introduction

There is wide consensus that increasing the production of apolipoprotein A-I (apoA-I) by the liver and intestinal cells has an anti-atherosclerotic effect. This is based on the observations that increasing expression of apoA-I in the liver by inserting the gene for human apoA-I (hapoA-I) or by administration of an adenovirus coding for hapoA-1 induces atherosclerosis regression in animal models (Rubin *et al.*, 1991; Plump *et al.*, 1994; Duverger *et al.*, 1996; Tangirala *et al.*, 1999). Also, administration of isolated or bio-manufactured apoA-I directly reduces the amount and fat content of atherosclerotic plaques in rabbits (Badimon *et al.*, 1990) and humans (Nissen *et al.*, 2003). One of the reasons why high-density lipoproteins (HDL) and apoA-I may be protective against atherosclerosis is that HDL plays a key role in the process of reverse cholesterol transport (RCT), as it induces the efflux of excess cholesterol from peripheral tissues and arterial wall macrophages, and returns it to the liver for biliary excretion as free cholesterol or bile acids (Rader *et al.*, 2009). HDL is a mixture of various particles differing in size, shape, lipid and apolipoprotein composition, the only property shared by them being the possession of apoA-I (Rye *et al.*, 1999). Although cholesterol in macrophages represents only a tiny fraction of overall cholesterol in the body, the RCT process that depletes this pool is the most important with regard to atherosclerosis (Lewis and Rader, 2005; Cuchel and Rader, 2006; Duffy and Rader, 2006). Recently, a method has been developed to measure this macrophage RCT (mph-RCT) by following the appearance of radioactivity in plasma, liver and faeces after i.p. injection of ^{3}H-labelled cholesterol-loaded macrophages (Zhang *et al.*, 2003). This method captures the overall effect of a drug or gene manipulation on mph-RCT, starting with the cholesterol release from the macrophage and ending with excretion of labelled cholesterol and bile salts in the faeces. Using this method, over-expression of apoA-I in the liver was found to clearly enhance mph-RCT (Zhang *et al.*, 2003).

Ro 11-1464 is a thienotriazolodiazepine previously shown to increase apoA-I expression in HepG2 cells (Kempen, 1997a) and in cultured monkey and human hepatocytes (Princen and Kooistra, 1998), and to elevate serum apoA-I levels in hamsters (Kempen, 1997b). The aim of the current study was to establish that the apoA-I-increasing activity of Ro 11-1464 can be reproduced in the hapoA-I mouse model, and to assess whether it is associated with the promotion of mph-RCT and faecal sterol and bile salt excretion in hapoA-1 transgenic mice. The drug activity *in vivo* was further characterized by determining its effect on plasma lipids, preβ-HDL and lecithin-cholesterol : acyltransferase (LCAT) activity and on the expression of genes possibly involved in apoA-I and HDL levels. We demonstrated that Ro 11-1464 strongly increases the hepatic production of apoA-I and resulted in the enhancement of mph-RCT with increase in both labelled faecal sterols and faecal bile acids, at least in part attributable to marked increases in the circulating apoA-I and preβ-HDL and suppression of LCAT activity.

Methods

Animals and drug administration in vivo

Animal care and experimental procedures complied with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and were performed with the approval of the Ethical Committees overseeing animal experiments at Hoffmann-La Roche and at the University of Parma.

Fourteen-week-old male C57BL/6-Tg(APOA-I)1Rub/J mice from Jackson Laboratories (Harbor, ME, USA) were housed in a controlled environment at 25 ± 2°C with alternating 12 h light and dark cycles. These animals were created by injecting a transgenic construct containing the entire human apolipoprotein A-I gene, including its natural promoter, into fertilized C57BL/6 mouse eggs (Rubin *et al.*, 1991). Mice carrying this transgene show a greater than 4-fold decrease in the levels of mouse apoA-I and a human-like HDL phenotype (Rubin *et al.*, 1991). Mice were treated for 5 days by oral gavage with Ro 11-1464 suspended in 0.5% methyl cellulose at a dose of 150 mg·kg^{-1} twice a day (08 h and 17 h), or 10, 30 or 100 mg·kg^{-1}·day^{-1} once a day at 08 h. The control group received the vehicle only. Mice were individually caged and received standard diet and water *ad libitum*. On day 5, 4 h after the last drug administration, mice were killed by excess anaesthesia with ethyl ether. Blood was collected by cardiac puncture and recovered in plastic tubes containing sodium citrate 3.8% (1 part citrate: 9 part blood). Plasma was isolated by low speed centrifugation and stored at –80°C until used as described below. Livers were collected at the end of the treatment period and immediately frozen in liquid nitrogen. Faeces were collected on day 4 and day 5 of drug treatment. Samples of liver and faeces were extracted by the Bligh and Dyer method (Iverson *et al.*, 2001; Zanotti *et al.*, 2008); radioactivity in the lipid extracts was measured by liquid scintillation counting, mass content of lipids and bile salts in liver and faeces was determined as described below.

ApoA-I secretion by hepatocytes of hapoA-I transgenic mice

Hepatocytes were isolated from untreated hapoA-I mice using the three-step collagenase perfusion method, as previously described (Luttringer *et al.*, 2002). Cells were plated in 24-well plates for a 24-h recovery period in L-15 medium supplemented with 10% new born calf serum. Cells were then incubated with increasing concentrations of Ro 11-1464 for 72 h with dimethyl sulphoxide final concentration of 0.1%, with media refreshments after each 24 h, and apoA-I was quantified in the medium by a commercial ELISA-kit (Alercheck, Portland, ME, USA). At the end of the incubation, the cell layers were washed twice with phosphate buffered saline (PBS) and then dissolved in bichincholinic acid reagent for protein determination.

Hepatic gene expression

Total RNA from the livers of vehicle and Ro 11-1464-treated mice was isolated using the RNeasy mini kit (Qiagen AG, Hombrechtikon Switzerland) without DNase treatment. Taqman® polymerase chain reaction (PCR) was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland). The following

cycling parameters were used for combined reverse transcriptase PCR: 50°C for 30 min for reverse transcription, then 95°C for 15 min to activate the HotStar Taq DNA polymerase (Qiagen) and inactivate the RT enzymes, followed by detection for 40 cycles of denaturation at 94°C for 15 s, and annealing and extension at 60°C for 1 min. The relative threshold cycle (CT) method (delta CT; DCT) was used for quantification of expression of each gene. The average CT values for each condition were calculated and the DCT values were transformed to absolute values (fold change) using the equation: $2^{(DCT)}$ for each pair-wise comparison. All values represent the mean ± SD of three replicates from each liver.

Measurements of plasma lipids, apoA-I and enzymes

Plasma total cholesterol and free was measured with a colorimetric assay using a commercially available kit (Sclavo, Sovicille, Italy). HDL-cholesterol was determined using the dextran sulphate-$MgCl_2$ precipitation method. Plasma levels of triglycerides and phospholipids were measured by enzymatic kits as described previously (Calabresi *et al.*, 2004). Plasma apoA-I levels were measured by immunoturbidimetry using commercially available polyclonal antibody. HDL subclasses were separated by two-dimensional electrophoresis as described before (Zanotti *et al.*, 2008), in which agarose gel electrophoresis was followed by non-denaturing polyacrylamide gradient gel electrophoresis and subsequent immunoblotting. Plasma cholesterol esterification rate (CER) and LCAT activity were measured as previously described (Calabresi *et al.*, 2005).

Activities of alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in plasma were determined on a Roche Cobas Integra automatic analyser (Roche S.p.A., Milano, Italy) with the IFCC method.

Liver lipid measurement

Liver lipids were extracted according to the Folch method (Folch *et al.*, 1957): a sample of liver (200 mg) was mixed with 4 mL chloroform : methanol (2:1) and heated at 60°C for 30 min followed by continuous shaking overnight at 4°C. The upper phase was collected. An aliquot of 2 mL was mixed with 0.4 mL of 2% CaCl2 and 0.4 mL chloroform and shaken overnight at 4°C. After centrifugation for 5 min at 800× *g* the lower phase was collected and 50 µL were dried under N_2 and dissolved in 0.4 mL of chloroform, 2% Thesit. Chloroform was evaporated under N_2. A volume of 0.8 mL solubilization buffer (PBS 0.1 M, 50 mM NaCl, 5 mM cholate) was added and heated at 90°C for 20 min, followed by an ultrasound bath for 20 min. An aliquot of 20 µL was used for the determination of total cholesterol, unesterified cholesterol (Amplex red cholesterol assay kit, Molecular Probes kit A12216, Invitrogen Ltd., Paisley, UK) and triglyceride (colorimetric test from Sigma TR0100-1kt, Sigma-Aldrich, St. Louis, MO, USA).

Measurement of mph-RCT

Measurement of RCT was performed according to the method published previously (Zhang *et al.*, 2003). In brief, on day 1 of pharmacological treatment with Ro 11-1464 300 $mg \cdot kg^{-1} \cdot day^{-1}$, murine macrophages J774 were cholesterol-enriched with 100 $\mu g \cdot mL^{-1}$ acetylated LDL (AcLDL) and radiolabelled with 5 $\mu Ci \cdot mL^{-1}$ [^{3}H]-cholesterol. On day 3, cells were injected i.p. into recipient mice that had received Ro 11-1464 (n = 5) or vehicle (n = 5). Animals were then caged individually. On day 5, mice were killed and blood was collected, recovered in plastic tubes and anticoagulated with sodium citrate 3.8% (1 part citrate: 9 part blood). Plasma was isolated by low-speed centrifugation and stored at –80°C until use. Livers were extracted by the Bligh and Dyer method, as described in detail before (Iverson *et al.*, 2001; Zanotti *et al.*, 2008), and radioactivity in the lipid extracts measured by liquid scintillation counting. Radioactivity in faecal sterols and bile acids was determined as described in the next section.

Faecal neutral sterol and bile acid measurements (radioactivity and mass)

Extraction of faecal neutral sterols and faecal bile acids was done as follows: a portion of 100 mg of ground dried faeces were soaked in 1 mL water overnight at 4°C. The next day, 3 mL of ethanol was added and vortexed. The samples were saponified by the addition of 400 µL 1 M NaOH at 95°C for 2 h. Neutral sterols were extracted with three times 5 mL of hexane with overnight shaking at 4°C. Each upper phase was collected, dried, dissolved in 800 µL of hexane. An aliquot of 100 uL hexane extract was mixed with liquid scintillation cocktail and radioactivity measured in a beta counter. An aliquot of 50 uL hexane extract was dried under N_2, dissolved in 1 mL chloroform, dried again under N_2 and heated for 20 min at 105°C to eliminate any trace of solvent. The dried neutral sterols were dissolved in 0.2 mL solubilization buffer (PBS 0.1 M 50 mM NaCl, 5 mM cholate) and allowed to stand for 1 h at room temperature. Neutral sterols were quantified by an enzymatic method using Amplex Red reagents (Molecular Probes, Kit A11216). The aqueous phase remaining after removal of neutral sterols (lower phase) was acidified to pH 1 with 0.4 mL 1 N HCl. Acidic sterols were extracted with five times 5 mL ethyl acetate, with overnight shaking at 4°C. Each upper phase was collected, dried and dissolved in 1 mL of 20% methanol plus 1.25% Triton X-100, and placed in an ultrasound bath for 1 h. The solution was filtered through paper. An aliquot of 200 uL was mixed with scintillation cocktail and radioactivity was measured in a beta-counter. The bile acids mass was determined by an enzymatic method (Kit Diazyme, DZ042A, Diazyme Labs, Poway, CA, USA).

Efflux potential of plasma

Plasma capacity to promote *in vitro* cellular cholesterol efflux was evaluated with a standard lipid efflux assay on different cellular cultures to estimate the specific mechanism. Briefly, cells were radiolabelled with 2 $\mu Ci \cdot mL^{-1}$ [^{3}H]-cholesterol in the presence of the ACAT inhibitor Sandoz 58–035 for 24 h, equilibrated in an albumin-containing medium for 18 h and exposed to 2% whole plasma for 4 h. Quantification of the cholesterol efflux was performed using a time zero (T = 0) set of cells to calculate total [^{3}H]-cholesterol content in the monolayer. Fractional efflux is calculated as (cpm ^{3}H in the medium/^{3}H at T = 0)*100. J774 macrophages treated or not with cpt-cAMP were used to measure passive diffusion and ATP binding cassette A1 (ABCA1)-mediated cholesterol efflux, respectively (Bortnick *et al.*, 2000): Fu5AH rat hepatoma cells were used to detect scavenger receptor class B type I (SR-BI)-

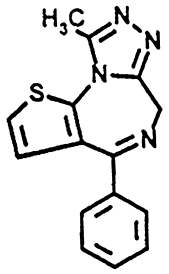

Figure 1
Structure of Ro 11-1464.

mediated cholesterol efflux (de la Llera-Moya *et al.*, 1999). The contribution of ATP binding cassette G1 (ABCG1) to cholesterol efflux in macrophages was assessed according to Rothblat and collaborators (Adorni *et al.*, 2007): J774 macrophages were radiolabelled, and were enriched with cholesterol by addition of AcLDL 50 μg·mL^{-1}; after the equilibration period, cells were treated with probucol 10 μM and BLT-1 10 μM for 2 h, in order to specifically block ABCA1- (Favari *et al.*, 2004) and SR-BI-mediated activities (Nieland *et al.*, 2002) before being incubated with the plasmas.

Materials

Ro 11-1464 (9-methyl-4-phenyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepine) (Figure 1) was synthesized at Hoffmann-La Roche according to Hellerbach *et al.* (1977).

Bovine serum albumin, cpt-cAMP, 8-(4-chlorophenylthio)cAMP, the ACAT inhibitor Sandoz 58–035, fenofibric acid and probucol were purchased from Sigma-Aldrich (Milano, Italy). Organic solvents were purchased from Merck (Darmstadt, Germany). 1,2-[^{3}H]-cholesterol was from Perkin Elmer (Monza, Italy). Tissue culture flasks and plates were from Corning (Corning, NY, USA) and Falcon (Lincoln, NY, USA). Cell culture media, foetal calf serum and PBS were purchased from Lonza Sales LTD (Milano, Italy). BLT-1 (2-hexyl-1-cyclopentanone thiosemicarbazone) was a kind gift of Prof George Rothblat (Children's Hospital of Philadelphia, PA, USA).

Results

In vitro *study: apoA-I production by cultured hepatocytes from hApoA-I mice*

Incubation of cultured hepatocytes isolated from hapoA-I mice with increasing concentrations of Ro 11-1464 resulted in a dose- and time-dependent stimulation of apoA-I secretion into the culture media; this increased secretion was five-fold at 50 μM Ro 11-1464 on the third day. In comparison, the PPAR-α agonist fenofibric acid was significantly less potent and efficacious (Figure 2). Neither compound had an effect on cell protein at the end of the 72-h incubation period (not shown).

In vivo *studies: hepatic expression of genes involved in HDL metabolism*

Treatment with Ro 11-1464 at 300 mg·kg^{-1}·day^{-1} for 5 days led to a 3.5-fold increase in the hapoA-1 mRNA content of the liver; treatment with 100 mg·kg^{-1}·day^{-1} fenofibrate for 5 days had a significantly smaller effect (Figure 3A). Ro 11-1464 had no significant effects on hepatic mRNA contents of the mouse apoA-I, ABCA1, phospholipid transfer protein (PLTP), acyl-CoA oxidase or SR-BI genes (Figure 3B), whereas treatment with fenofibrate markedly increased acyl-CoA-oxidase (not shown).

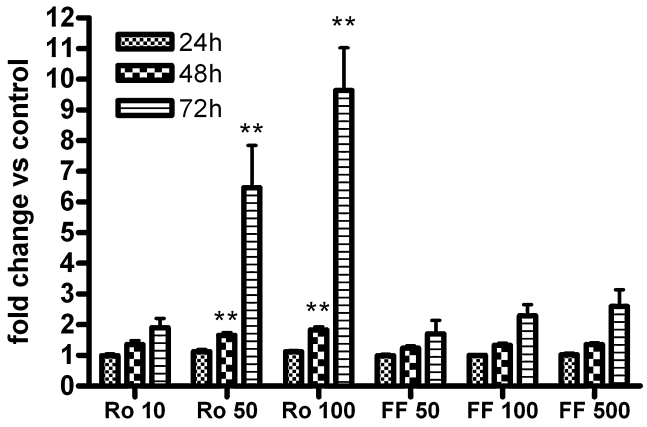

Figure 2
Effect of Ro 11-1464 on apolipoprotein A-I (apoA-I) secretion from human apoA-I transgenic mice hepatocytes. Hepatocytes were incubated in 96 well plates with dimethyl sulphoxide (DMSO) 0.1%, Ro 11-1464 (Ro) at 10, 50 and 100 uM or fenofibric acid (FF) at 50, 100 and 500 uM. Medium was collected at 24 h, 48 h and 72 h to quantify human apoA-I by ELISA. Data are mean ± SD of 4 separate wells (for Ro 50 and 100 uM 8 separate wells). Statistically significant vs. DMSO control using ANOVA followed by Dunnett's post-hoc test: *P < 0.05; **P < 0.01. Cells secreted 1.9 μg of apoA-I over the 72h-culture period.

In vivo *studies: changes in plasma lipids, apo A-I, preβ-HDL and LCAT levels, in liver lipids and in faecal sterol excretion*

Administration of Ro 11-1464 by oral gavage for 5 days caused consistent and dose-dependent decreases in plasma levels of total cholesterol, HDL cholesterol, non-HDL-cholesterol, triglycerides and phospholipids, as well as of the LCAT activity and CER (Table 1). At the dose of 300 mg·kg^{-1}·day^{-1}, the compound caused a significant nearly 2-fold increase in plasma apoA-I and a 2- to 3-fold increase in the percentage of apoA-I located in preβ-HDL, accompanied by an increase in the free/total cholesterol ratio (Table 1), and by a marked shift in the preβ-HDL particle distribution towards large sizes (Figure 4), without significantly affecting the mass of faecal neutral sterols and bile acids (Table 1). At the dose of 100 mg·kg^{-1}·day^{-1}, plasma apoA-I and preβ-HDL were not increased, although LCAT and CER were significantly reduced (Table 1). Lower doses tested (10 and 30 mg·kg^{-1}·day^{-1}) had no effect on any of these parameters (not shown).

In vivo *studies: changes in body weight, liver weight, liver lipids and liver enzymes*

The handling of the mice during the 5-day period (twice daily oral gavage) resulted in a 10% decrease in body weight, which

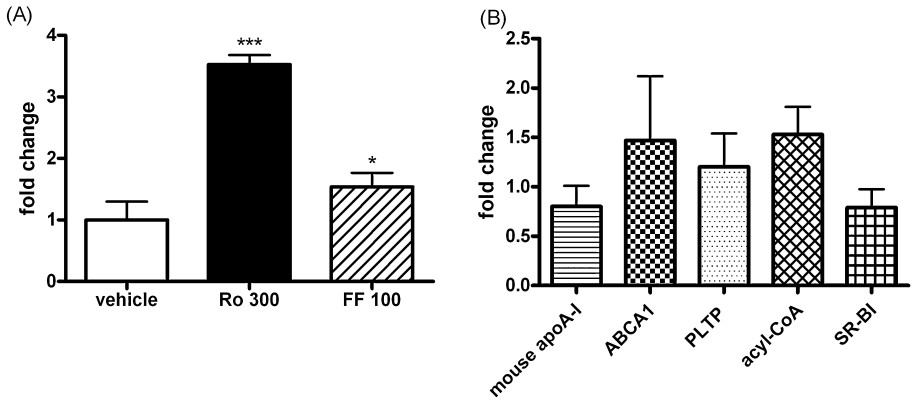

Figure 3

Effect of Ro 11-1464 on hepatic expression of selected genes that regulate HDL levels. Human apolipoprotein A-I (apoA-I) transgenic mice were treated with Ro 11-1464 300 mg·kg^{-1}·day^{-1} (Ro) or fenofibrate 100 mg·kg^{-1} (FF) for 5 days and liver was collected to isolate RNA and quantify mRNA of (A) human apoA-I or (B) mouse apoA-I, mouse ABCA-1, mouse phospholipid transfer protein, mouse acyl-CoA oxidase and mouse SR-BI levels by Q-PCR. Data are expressed as fold increase of Δ cycle time vs vehicle-treated mice and are mean ± SD (each replicate represents one animal, values are mean of 3 animals). *$P < 0.05$, ***$P < 0.001$ vs. vehicle. HDL, high-density lipoproteins; SR-BI, scavenger receptor class B type I.

was equal in the vehicle and drug-treated mice (Table 2). However, drug treatment at a dose of 300 mg·kg^{-1}·day^{-1} caused a significant increase in liver weight, and in the liver cholesteryl ester and triglyceride contents (the latter being non-significant) as compared with the control mice (Table 2), as well as in plasma levels of ALP and ALT, but not of AST (Table 2).

mph-RCT in vivo

Administration of Ro 11-1464 300 mg·kg^{-1}·day^{-1} to hapoA-1 transgenic mice produced significant changes in the distribution of labelled cholesterol effluxed from prelabelled macrophages: the amount of radioactivity mobilized from [^{3}H]-cholesterol preloaded macrophages in blood was reduced by 50% (Figure 5A), while the amount of label in the liver was not affected (Figure 5B). Strikingly, the excretion of radioactivity into faecal neutral sterols was enhanced by more than 4-fold and that into faecal bile acids by 1.7-fold during the 48 h after administration of prelabelled macrophages (Figure 5C).

Plasma efflux potential ex vivo

Plasma from mice treated with Ro 11-1464 300 mg·kg^{-1}·day^{-1} for 5 days was somewhat more efficient than plasma from vehicle-treated mice as an acceptor through ABCA1, although the effect was not statistically significant (Figure 6A). Conversely, the efflux via passive diffusion (Figure 6B) and SR-BI-mediated transport (Figure 6C) were lower in plasma from drug-treated mice than in from plasma control mice. Finally, the ABCG1-mediated efflux was not different between plasma from control and drug-treated mice (Figure 6D).

Discussion

It is widely accepted that a small molecule that is able to safely and selectively up-regulate apoA-I expression in the liver and intestine would merit being tested as a possible anti-atherosclerotic drug. In spite of the considerable efforts by various pharmaceutical companies to identify such compounds, only few molecules have actually been identified. Previous studies have shown that the compound Ro 11-1464 is able to increase apoA-I expression and secretion in HepG2 and primary liver cells, and to increase serum apoA-I levels in hamsters. Here, we investigated the effect of this compound on apoA-I expression in hapoA-I mice. We first confirmed the ability of the compound to strongly increase hapoA-1 secretion in hepatocytes of these animals *in vitro* (Figure 2), and then demonstrated that it raised human (but not mouse) apoA-I mRNA content in the liver when given by oral gavage, without affecting other mRNA levels (Figure 3).

Since fenofibrate, a well-known PPAR-α agonist, is known to increase hapoA-1 plasma level and hepatic mRNA content in the same animal model (Berthou *et al.*, 1996), this compound was tested in parallel, and was clearly less effective than Ro 11-1464 (Figure 2). Also, Ro 11-1464 did not significantly increase acyl CoA oxidase and PLTP mRNA contents in the liver (Figure 3B), whereas fenofibrate is a strong inducer of Acyl CoA oxidase (Berthou *et al.*, 1996) and PLTP (Lie *et al.*, 2005). These observations strongly suggest that Ro 11-1464 does not work as a PPAR-α agonist. However, the exact molecular mechanism by which Ro 11-1464 promotes apoA-I expression is still unclear.

We found that while the compound given in doses below 100 mg·kg^{-1}·day^{-1} had no effects, Ro 11-1464 given at 300 mg·kg^{-1}·day^{-1} markedly increased plasma apoA-I and preβ-HDL levels (Table 1, Figure 4), but also strongly lowered plasma total and HDL-cholesterol as well as phospholipid and triglyceride levels (Table 1). Although the increase in plasma apoA-I is explained by the elevation of hapoA-1 mRNA in the liver (Figure 3A), the drop in plasma lipids was unexpected as the introduction of the hapoA-1 transgene in the mouse in itself was found to be associated with an increase in plasma total and HDL-cholesterol (Rubin *et al.*, 1991; Zhang *et al.*, 2003). Several possible explanations can be considered for

Table 1

Effect of 5 days of oral administration of Ro11-1464 to hApoA-I mice on plasma lipids, apoA-I, preβ-HDL and LCAT, CER, faecal neutral sterols and bile salts

Dose ($mg \cdot kg^{-1} \cdot d^{-1}$)	0(n = 5)	100(n = 5)	% of control	0 (n = 10)	300(n = 10)	% of control
Total-Cholesterol ($g \cdot L^{-1}$)	1.12	0.83**	74.6	1.86	0.52***	28.1
	0.19	0.09		0.21	0.11	
HDL-Cholesterol ($g \cdot L^{-1}$)	0.97	.75**	77.7	1.48	0.27***	18.1
	15.1	0.07		0.24	0.09	
Non-HDL-Cholesterol ($g \cdot L^{-1}$)	0.15	0.08**	54.6	0.38	0.25	66.9
	0.04	0.02		0.17	0.07	
Free Cholesterol ($g \cdot L^{-1}$)	0.32	0.25*	77.6	0.48	0.20***	41.5
	0.06	0.02		0.05	0.04	
FC/TC (%)	28.8	30.3	104.5	26	41***	157.6
	1.9	1.2		1	5	
PL ($g \cdot L^{-1}$)	221	177**	80.1	3.33	1.38***	41.4
	33	15		0.35	0.37	
TG ($g \cdot L^{-1}$)	0.81	0.51**	63.1	0.69	0.33	47.1
	0.19	0.09		0.36	0.12	
apoA-I ($g \cdot L^{-1}$)	2.75	2.89	105.1	3.60	6.94**	192.7
	0.48	0.87		0.36	2.04	
preβ-HDL (%)[a]	20.9	26.2	125	29.6	61.0***	206
				7.3	3.4	
LCAT ($nmol \cdot mL^{-1} \cdot h^{-1}$)	20.4	16.5**	80.9	28.1	18.2**	64.7
	2.3	1.0		3.6	4.5	
CER ($nmol \cdot mL^{-1} \cdot h^{-1}$)	48.3	30.0**	62.1	51.6	34.0**	65.8
	1.1	2.7		6.3	9.7	
Faecal neutral sterols[b] (mg $2day^{-1}$)				8.04	8.54	106.2
				1.60	1.93	
Faecal bile acids[b] (μmol $2day^{-1}$)				3.55	5.08	143.1
				1.26	2.40	

Values are expressed as means ± SD for (SD below mean in each cell).
[a]Expressed as % of total apoA-I surface area on the immunoblot.
[b]Pooled collection of last 2 days of treatment.
*P < 0.05, **P < 0.01, ***P < 0.001 vs. animals treated with vehicle (unpaired two-tailed t-test).
apoA-I, apolipoprotein A-I; hapoA-1, human apoA-1; HDL, high-density lipoproteins; LCAT, lecithin-cholesterol : acyltransferase.

this unexpected drug effect. First, the drug might have stimulated SR-BI expression in the liver, which in transgenic mice was described to cause a drop in HDL-cholesterol (Kozarsky *et al.*, 1997). This possibility seems unlikely in view of the lack of effect on the hepatic SR-BI mRNA content (Figure 3B), although a post-transcriptional drug effect cannot yet be excluded. Furthermore, Ro 11-1464 given *in vitro* at 10 μM did not affect SR-BI function, as demonstrated by the lack of effect on HDL-cholesterol uptake by Fu5AH hepatoma cells, a cell line highly expressing this receptor (Jian *et al.*, 1998) (data not shown). Secondly, the drug might have had an inhibitory effect on ABCA1 expression, which in genetic studies is associated with a drop in HDL-cholesterol (Frikke-Schmidt *et al.*, 2004) as exemplified in patients with Tangier disease. However, this possibility also seems unlikely as we observed no change (if anything an increase) in the hepatic ABCA1 mRNA content (Figure 3B). It is more likely that the drop in lipids is the consequence of the observed decreases in LCAT and CER activity (Table 1): indeed patients with familial LCAT deficiency have markedly lower HDL-cholesterol levels (Calabresi *et al.*, 2005), a finding that has been reproduced in mouse models (Furbee *et al.*, 2002; Tanigawa *et al.*, 2009). These decreases in LCAT and CER activities were accompanied by an increased free cholesterol/total cholesterol ratio in plasma, which in turn, may have caused an increased flux of free cholesterol into the liver. The increase in hepatic cholesteryl ester content is consistent with this scenario. The mechanism by which Ro 11-1464 exerts inhibitory effects on LCAT and CER is currently unknown and requires further investigation.

The reduction in HDL-cholesterol observed here does not necessarily detract from the potential anti-atherosclerotic

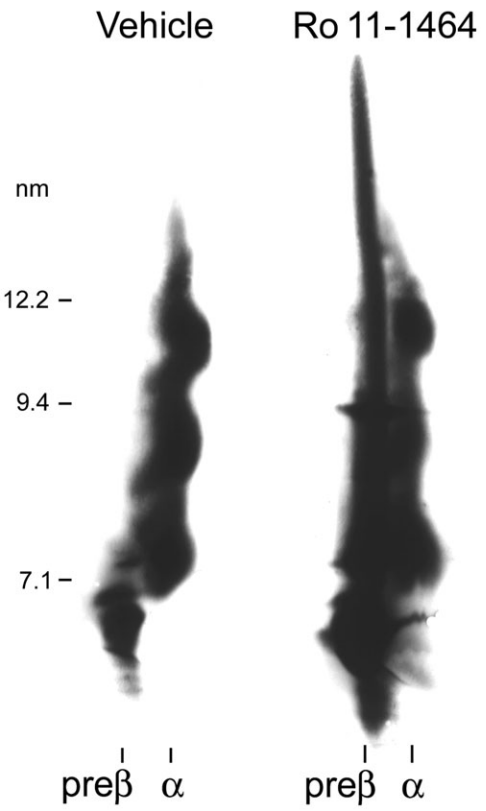

Figure 4

Effect of Ro 11-1464 on high-density lipoprotein (HDL) subclass distribution. HDL subclasses were separated by two-dimensional electrophoresis and transferred onto a nitrocellulose membrane, on which lipoproteins were detected with a sheep anti-human apoA-I, apolipoprotein A-I antibody. Left: serum from control mice, right: serum from a mouse treated with 300 mg·kg^{-1}·day^{-1} Ro 11-1464. This picture is from a single mouse, but is representative of 8 control or drug-treated animals.

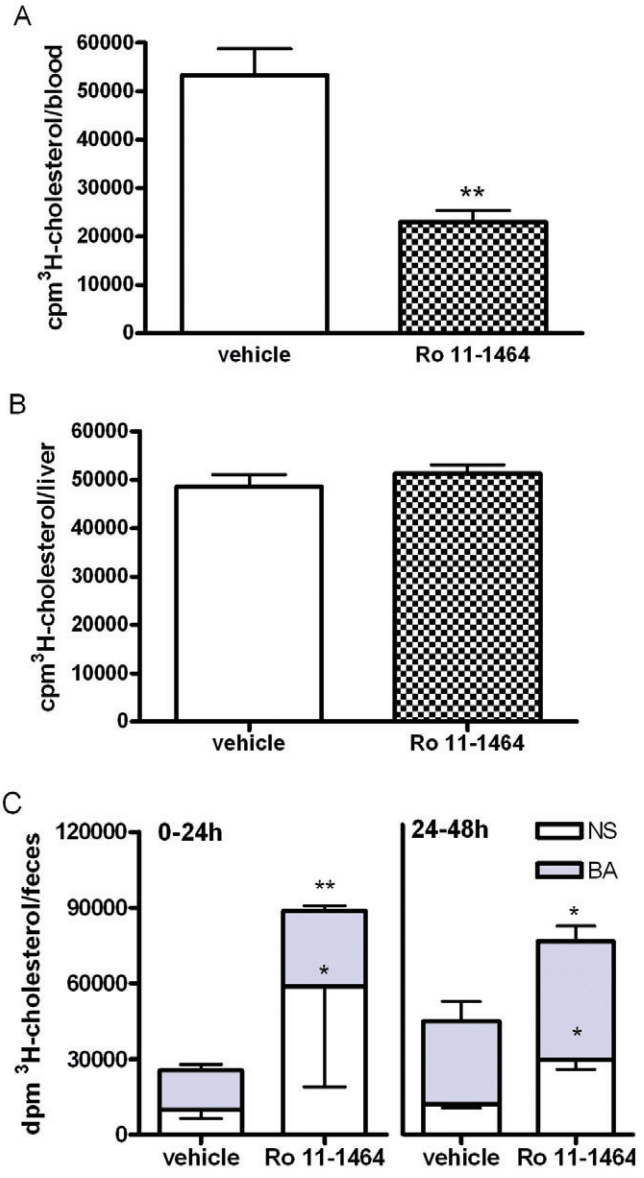

Figure 5

Effect of Ro 11-1464 on macrophage reverse cholesterol transport (mph-RCT) *in vivo*. Human apolipoprotein A-I (apoA-1) transgenic mice were treated with 300 mg·kg^{-1}·day^{-1} of Ro 11-1464 for 5 days and i.p. injected with [^{3}H]-cholesterol loaded macrophages on day 3 of drug treatment. After the mice had been killed on day 5, [^{3}H]-cholesterol distribution was quantified in blood, liver and faeces, as described in the Methods section. (A) [^{3}H]-cholesterol in blood; (B) [^{3}H]-sterols in the liver; (C) ^{3}H-labelled neutral sterols (NS) and bile acids (BA) in faeces collected 0–24 h and 24–48 h after the injection of [^{3}H]-cholesterol loaded macrophages. Data are means ± SEM (n = 5). *P < 0.05, **P < 0.01,***P < 0.001 vs. vehicle.

efficacy of Ro 11-1464. Indeed, several studies have shown that HDL levels do not always inversely correlate with atherosclerosis: mice in which the gene for SR-BI is deleted (Trigatti *et al.*, 1999), or the gene for LCAT is over-expressed (Berard *et al.*, 1997), or the gene for ABCA1 is over-expressed (Joyce *et al.*, 2006), all have a higher HDL level but nevertheless display increased atherosclerosis. Conversely, patients with LCAT deficiency seem to be protected against atherosclerosis in spite of low HDL levels (Calabresi *et al.*, 2009).

Recent studies provide evidence that mph-RCT is a better predictor of atherosclerosis than HDL or apoA-I levels (Rader *et al.*, 2009) and therefore, we tested the effect of Ro 11-1464 on this process as well. In mice treated with Ro 11-1464, the mph-RCT assay revealed that the drug led to a decrease in labelled cholesterol in the plasma compartment, accompanied by a strong increase of label excretion in the faecal sterols and bile salts, and no change in the amount of label in the liver (Figure 5). This result differs from earlier findings, in which wild-type mice, after injection of recombinant adenoviral vector coding for apoA-I, showed an increased appearance of label into plasma, liver and faecal sterols but not into faecal bile salts, as compared with untreated mice (Zhang *et al.*, 2003). It was recently reported that partial LCAT knock-out does not change the rate of appearance of labelled sterols in hApoA-I mice and that a complete knock-out reduces this rate by about half (Tanigawa *et al.*, 2009). Since we observed a clear increase in labelled neutral and acid sterols excretion,

Table 2

Effect of 5 days of oral administration of 300 mg·kg^{-1}·day^{-1} Ro11-1464 on body and liver weight, liver lipids and plasma levels of liver enzymes

	Vehicle (n = 10)	Ro 11-1464 (n = 10)	% of control
BW day 0 (g)	30.7 ± 3.1	30.7 ± 2.1	
BW day 5 (g)	27.7 ± 2.7	27.0 ± 1.9	
Δ BW (g)	–3.6 ± 2.2	–4.0 ± 1.2	
Liver wt day 5 (g)	1.16 ± 0.18	1.55 ± 0.24*	133.6
Liver TG (mg g^{-1} ww)	6.7 ± 4.0	10.8 ± 5.7	161
Liver FC (mg g^{-1} ww)	3.4 ± 0.5	3.3 ± 1.0	97.1
Liver CE[a] (mg g^{-1} ww)	0.18 ± 0.09	0.48 ± 0.31*	266
Plasma ALP (U L^{-1})	73.4 ± 14.6	217 ± 21***	296
Plasma AST (U L^{-1})	227 ± 108	219 ± 56	96.5
Plasma ALT (U L^{-1})	23.0 ± 4.2	51.0 ± 15.4**	222

Values are expressed as means ± SD.
[a]Difference between total and free cholesterol.
*P < 0.05, **P < 0.01, ***P < 0.001 vs. animals treated with vehicle (unpaired t-test).
ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; BW, body weight; CE, cholesterol esters; FC, free cholesterol; TG, triglycerides; ww, wet weight.

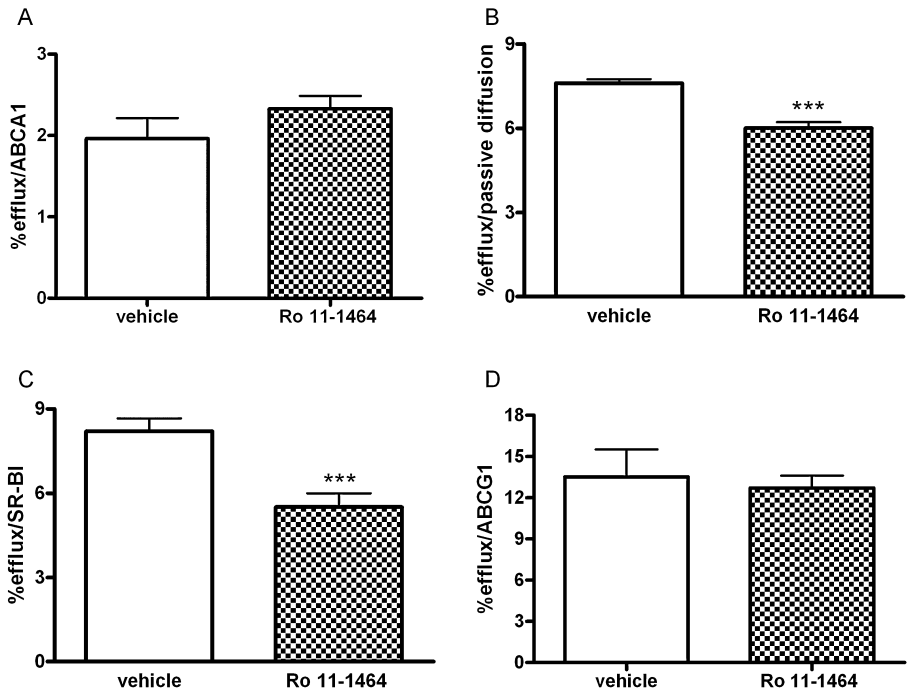

Figure 6

Effect of Ro 11-1464 on plasma efflux potential *ex vivo*; 2% plasma from control or RO 11-1464-treated mice was used as a lipid acceptor in cholesterol efflux experiments from different pre-labelled cell types. (A) ABCA1-mediated mechanism in J774 macrophages treated with cpt-cMP 0.3 mM. ABCA1-mediated efflux was calculated as the difference between efflux percentage in cpt-cAMP-treated and untreated cells; (B) passive diffusion mechanism in J774 macrophages; (C) SR-BI-mediated mechanism in Fu5AH rat hepatoma cells; and (D) ABCG1-mediated mechanism in J774 macrophages incubated with 50μg mL^{-1} AcLDL, probucol 10 μM and BLT-1 10 μM. ABCG1 contribution to total efflux was calculated as the difference with efflux shown as a percentage of that in untreated J774. The experiment was carried out in triplicate; 5 plasmas/group were tested. Data are expressed as mean ± SD. ***P < 0.001 vs control. ABCA1, ATP binding cassette A1; ABCG1, ATP binding cassette G1; AcLDL, acetylated LDL; cpt-cAMP, 8-(4-chlorophenylthio) cAMP; SR-BI, scavenger receptor class B type I.

we suggest that the increase in plasma apoA-I and preβ-HDL – and not the partial inhibition of LCAT and CER – are the main cause of the increased faecal sterol excretion in our experiments.

Free cholesterol can be effluxed from macrophages via different cholesterol transporters to various acceptor particles in plasma. Small preβ-HDL particles are the preferred acceptors when ABCA1 is the transporter (Oram, 2003; Yancey *et al.*, 2003), while mature spherical HDL acceptor preferably binds to ABCG1 or SR-BI (Tall, 2008). In our experiments, plasma from Ro 11-1464-treated mice showed only a slightly higher ABCA1-mediated efflux than seen with control plasma, while cholesterol efflux via passive diffusion and via SR-BI was even somewhat lower with plasma from Ro 11-1464-treated mice (Figure 6). Although the concentration of pre-β migrating HDL was clearly increased, the size of these particles was also much bigger (Figure 4), indicating an excess of nascent HDL particles unexposed to the remodelling by LCAT and/or PLTP. It was recently shown that such big minimally lipidated pre-β migrating particles can be generated by incubation of lipid-free apoA-I with cultured cells *in vitro*, and that they have little or no capacity to stimulate cholesterol efflux (Mulya *et al.*, 2007).

In conclusion, in the present study, we have shown that short-term administration of the thienotriazolodiazepine Ro 11-1464 to hapoA-I TG mice results in the promotion of mph-RCT and faecal excretion of neutral and acid sterols. These effects seem to be primarily driven by the up-regulation of apoA-I expression and increased plasma levels of apoA-I and preβ-HDL, whereas the drops in plasma lipids are probably mainly due to a reduction of LCAT activity and cholesterol esterification rate, although additional mechanisms cannot be excluded. Further studies are needed to characterize the compound in other animal models, and to elucidate its molecular mechanisms of action.

At the dose of 300 $mg \cdot kg^{-1} \cdot day^{-1}$, Ro 11-1464 caused a significant increase in liver weight and in plasma levels of the liver enzymes ALP and ALT. No formal toxicity study has yet been undertaken, but these findings detract from the further development of Ro 11-1464 itself as a candidate for therapeutic use. However, the findings reported here in our view justify the search for related thienotriazolodiazepines with higher potency, and devoid of the effect on liver size and liver enzymes, as potential novel anti-atherosclerotic drugs.

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Conflicts of interest

C M and P S are employees of Hoffmann-La Roche Ltd.

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