Novel pharmaeutical interventions in experimental atherosclerosis and myocardial infarction

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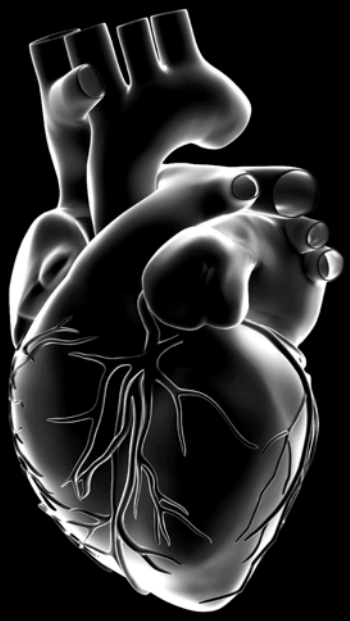

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New cholesterol absorption inhibitor AVE5530 is more effective in preventing atherosclerosis than ezetimibe in APOE*3Leiden mice

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Submitted

Abstract

Objectives The cholesterol uptake inhibitor ezetimibe is currently used as a cholesterol lowering drug mainly in combination with statin therapy. Ezetimibe is nearly 100% absorbed in the intestine and is active on cholesterol transport in macrophages. The consequence of this systemic activity is unclear. The novel cholesterol absorption inhibitor AVE5530 which is being developed for the treatment of dyslipidemia is poorly absorbed in the intestine. The aim of this study was to compare the anti-atherosclerotic activities of AVE5530 and ezetimibe.

Methods and Results APOE*3Leiden mice were fed a cholesterol-raising diet alone or either supplied with AVE5530 or ezetimibe (both 0.3 mg/kg bw/day). Effects on plasma lipids, levels of pro-inflammatory biomarkers and atherosclerosis were assessed after 20 weeks of treatment. AVE5530 and ezetimibe lowered plasma cholesterol as compared to control (-64% and -33%, respectively; $p<0.001$) and both had favorable effects on inflammation markers, as indicated by reduced plasma levels of SAA (by 70% and 69%), MCP-1 (by 38 % and 31%), E-selectin (by 30% and 29%), VCAM-1 (by 24% and 16%, all $P<0.01$, respectively). Additionally, AVE5530 also reduced fibrinogen (by 32%) levels and reduced hepatic cholesterol content (by 69%, $P<0.05$). Ezetimibe did not affect fibrinogen levels and reduced hepatic cholesterol content to a less extent. AVE5530 strongly inhibited atherosclerosis development: it decreased lesion size (by 93%), the number of lesion (by 61%), and additionally improved the quality of lesions. The percentage of severe lesions was also decreased (by 58%) and the undiseased segments were 7-fold increased (all $P<0.001$). Ezetimibe only reduced the quantity of atherosclerosis by decreasing the lesion size by 59% ($P<0.001$), but did not have significant effects on the other three parameters. AVE5530 differed significantly from ezetimibe in all atherosclerosis parameters.

Conclusions We showed that AVE5530 markedly reduced plasma cholesterol levels and therewith decreased the systemic and local vessel wall inflammation. Together these effects resulted in a more potent prevention of atherosclerosis development than ezetimibe.

Introduction

Dyslipidemia is an important risk factor for the development of cardiovascular disease (CVD). Therefore current guidelines to treat CVD emphasize targeting primarily Low-Density Lipoprotein- cholesterol (LDL-C)[1]. Since the introduction of statins, which became the gold standard of cholesterol lowering therapy, a large reduction of plasma LDL-C levels can be achieved[2]. However, there is still an important percentage of patients who do not reach their treatment goals or are statin intolerant[3]. Therefore, other lipid lowering drugs, used alone or in combination, are of great clinical significance.

Ezetimibe is the first of a new class of selective cholesterol absorption inhibitors[4]. Ezetimibe or rather its phenolic glucuronide selectively inhibit cholesterol absorption in the intestine at the brush border membranes of small intestine enterocytes, confining the cholesterol to the intestinal lumen for subsequent excretion[5,6]. The working mechanism of ezetimibe has been investigated extensively, and both Niemann–Pick 1 like 1 protein (NPC1L1) as well as aminopeptidase N (or CD13), both expressed in a.o. the intestine and macrophage, were suggested to be its molecular targets[6,7]. Ezetimibe is rapidly and completely absorbed and metabolized in the intestine and liver to its phenolic glucuronide, then it is excreted into the bile and delivered back to its site of action[8]. The compound has been shown to reduce atherosclerosis development in apoE knock-out mice[9]. Clinical trials have demonstrated the lipid-lowering properties of ezetimibe as a single agent and its additive cholesterol-lowering effects when combined with a statin[10,11]. However, unexpected and still unexplained were the results of the recently published ENHANCE trial, in which patients with familial hyperlipidemia were treated with simvastatin alone or in combination with ezetimibe. Despite lower levels of LDL-C and C reactive protein with the combination therapy, no additional protective effects in retarding on intima-media thickness were measured[12].

AVE5530 is a new cholesterol absorption inhibitor, which, in contrast to ezetimibe, is very poorly absorbed[13]. AVE5530 has similar targets as ezetimibe[7], and lowers LDL cholesterol in a similar manner, however, it is not systemically available[13].

The aim of the present study was to compare AVE5530 with ezetimibe regarding their effects on plasma lipid levels and atherosclerosis development. To this end, we used female APOE*3Leiden transgenic mice, which are a well-established mouse model for hyperlipidemia and atherosclerosis[14]. These mice have a lipoprotein profile similar to the profile of patients with familial dysbetalipoproteinemia in which the elevated plasma cholesterol and triglyceride levels are mainly confined to the VLDL/LDL-sized lipoprotein fraction. In addition, in contrast to other mouse models for dyslipidemia and/or atherosclerosis[15], these mice respond in a human-like manner to treatment of CVD (e.g. statins, cholesterol uptake inhibitors, calcium channel blockers, fibrates and, angiotensin II receptor antagonists[16-23]).

Methods

Mice and treatments

Female heterozygous APOE*3Leiden transgenic mice (11 to 16 weeks of age), characterized by ELISA for human apoE[14], were used. During a 3 week run-in period, all animals received a semi-synthetic western-type diet (WTD) containing 40.5% sucrose, 15% cacao butter and 0.75% (w/w) cholesterol. After matching into 3 groups, based on age, plasma cholesterol and triglyceride levels, the mice received WTD diet either alone (control group) or supplemented with either ezetimibe (0.3 mg/kg bw/day) or with AVE5530 (0.3 mg/kg bw/day). Both compounds were provided by Sanofi Aventis Deutschland GmbH. EDTA blood was drawn at week 2, 4, 9, 12, 16 and 20 of the study, and was assayed for lipids. After 20 weeks, mice were sacrificed and the hearts and livers were isolated to assess atherosclerosis and hepatic cholesterol content. The animals received food and water *ad libitum*. Body weight and food intake were monitored during the study. Animal experiments were approved by the Institutional Animal Care and Use Committee of The Netherlands Organization for Applied Scientific Research (TNO). Animals were bred by TNO.

Lipid and lipoprotein analysis and plasma inflammation markers

After a 4-hour fasting period from 9 a.m. to 1 p.m., EDTA plasma was collected (Sarstedt, Nümbrecht, Germany) and lipoproteins were separated by FPLC[24]. Total hepatic cholesterol content was determined after homogenization of the liver tissue[25]. Total cholesterol (TC) (No-1489437, Roche Diagnostics, USA) and triglyceride (TG) (1488872, Roche Diagnostics, USA), levels were measured. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined spectrophotometrically using a Reflotron system (Roche Diagnostics, USA). Fibrinogen (FBG) (as described before [18]), serum amyloid A (SAA) (Biosource International, Belgium), monocyte chemoattractant protein (MCP)-1, endothelial (E) -selectin, vascular cell adhesion molecule (VCAM)-1 (all R&D systems Inc, USA), were determined by ELISA.

Histological assessment of atherosclerosis

After the 20-week treatment period, the mice were sacrificed. The hearts with aortic root were dissected, formalin fixed and embedded in paraffin. Serial cross sections (5 µm thick, spaced 50 µm apart) throughout the entire aortic valve area were used for histological analysis. Sections were stained with haematoxylin-phloxine-saffron (HPS). Per mouse, 4 sections with intervals of 50 µm were used for quantification and qualification of the atherosclerotic lesions. For determination of severity of atherosclerosis, the lesions were classified into 5 categories as described before[17,18,20] I) early fatty streak, II) regular fatty streak, III) mild lesion, IV) moderate lesion, V) severe

lesion. Per mouse the percentages of all lesions found in the respective categories were calculated. The total lesion area was calculated per cross-section. In each segment used for lesion qualification the number of monocytes adhering to the endothelium was counted and the macrophage area was measured after immunostaining with AIA31240 (1:3000, Accurate Chemical and Scientific, USA). Smooth muscle cells (SMCs) were immunostained with mouse anti-human actin (DAKO, Denmark), which cross reacts with mouse actin. The collagen was stained by a Sirius Red staining. The lesion content of the different compounds was quantified morphometrically. All analyses were performed by the same operator, who was blinded for experimental group allocation.

Statistical analysis

Data are presented as means ± SD unless indicated otherwise. Statistical differences were assessed using the non-parametrical Kruskal-Wallis test followed by Mann Whitney U test. $P<0.05$ was considered significant. In tables and figures: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Results

Effect of AVE5530 and ezetimibe on plasma lipid levels

Treatment with AVE5530 or ezetimibe did not affect food intake or body weight gain. As presented in **figure 1** feeding the Western type-diet induced hyperlipidemia in the mice, giving plasma cholesterol levels of 17.5 ± 4.1 mmol/L and triglyceride levels of 3.0 ± 0.9 mmol/L at the start of treatment (week 0). Inhibiting the cholesterol uptake by AVE5530 resulted in a strong reduction of plasma cholesterol after two weeks and a 64% ($p<0.001$) reduction at the end of the study and decreased plasma triglycerides by 20% ($p<0.05$) at the later time points in the study. Ezetimibe was less potent as compared to AVE5530 treatment resulting in a 33% ($p<0.001$) reduction of plasma cholesterol levels without affecting the triglycerides. The reductions in plasma cholesterol upon AVE5530 and ezetimibe treatment were confined to apoB-containing lipoproteins as measured after plasma separation by FPLC (**figure 1C**).

		Control			AVE			reduction	EZE			reduction
FBG	mg/ml	2.9	±	1.1	2.0	±	0.6	-32%*	2.4	±	0.8	
SAA	µg/ml	9.2	±	5.3	2.8	±	2.6	-70%***	2.8	±	1.8	-69%***
MCP-1	pg/ml	162.8	±	51.8	100.8	±	36.4	-38%**	111.9	±	44.4	-31%**
E-Selectin	ng/ml	64.0	±	13.9	44.9	±	4.2	-30%***	45.1	±	13.2	-29%**
V-CAM	µg/ml	3.1	±	0.4	2.4	±	0.3	-24%***	2.6	±	0.6	-16%**

Table 1. The effect of AVE5530 and ezetimibe (EZE) on plasma inflammation markers. The parameters were measured at the end of the study after a 20 week treatment period. Values are means ± SD (n=15-16 per group). *$p<0.05$,**$p<0.01$ ***$p<0.001$ as compared to control.

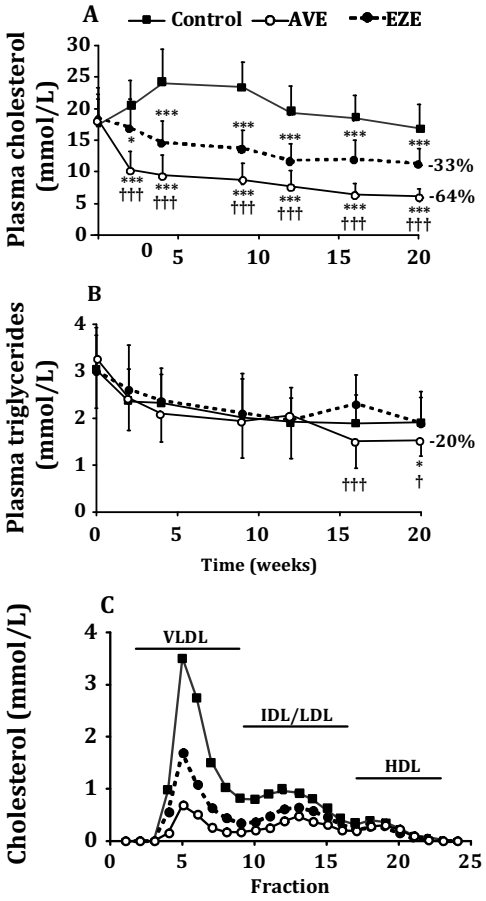

Figure 1. The effect of AVE5530 (AVE) and ezetimibe (EZE) on plasma lipid levels. The plasma cholesterol (A) and triglyceride levels (B) were measured throughout the study. After 16 weeks of treatment the lipoproteins were separated by FPLC and the cholesterol was measured in the fractions (C). *P<0.05, ***P<0.001 as compared to control and †P<0.05, †††P<0.001 as compared to ezetimibe.

Effect of AVE5530 and ezetimibe on liver and inflammatory parameters

As elevated cholesterol has an important impact on liver condition and plays a major role in enhancing inflammation[17,26], we measured hepatic cholesterol content and also plasma markers for systemic and vessel wall inflammation (**table 1)**. The relative liver weights (as percentage of body weight) tended to be reduced in the animals treated with AVE5530 (5.6 ± 1.1% vs 6.2 ± 0.9% in the control group, P=0.1 Kruskall-Wallis followed by P=0.03 Mann-Whitney), which was not observed in the ezetimibe treated group (5.9 ± 1.1%, N.S.). Concomitantly AVE5530 significantly reduced the cholesterol content of the liver by 69% (17.7 ± 5.5 vs 57.6 ± 5.5 mg/ g liver in the control group, P<0.001). Ezetimibe decreased liver cholesterol content by only 35% (to 37.2 ± 12.8 mg/ g liver, P<0.001), which was significantly less than the reduction observed after AVE5530 treatment (P<0.001). Plasma ALT and AST levels were not affected by both treatments. Additionally, treatment with AVE5530 resulted in reduced levels of liver derived acute phase proteins SAA (by 70%, P<0.001) and FBG (by 32%, P<0.05), chemokine MCP-1 (by 38%, P<0.01), and vessel wall inflammation markers E-selectin and VCAM-1 (by 30% and 24%, both P<0.01, respectively) as compared to control. Treatment with ezetimibe brought about similar reduction of inflammation markers as AVE5530, however, without affecting FBG as compared to control.

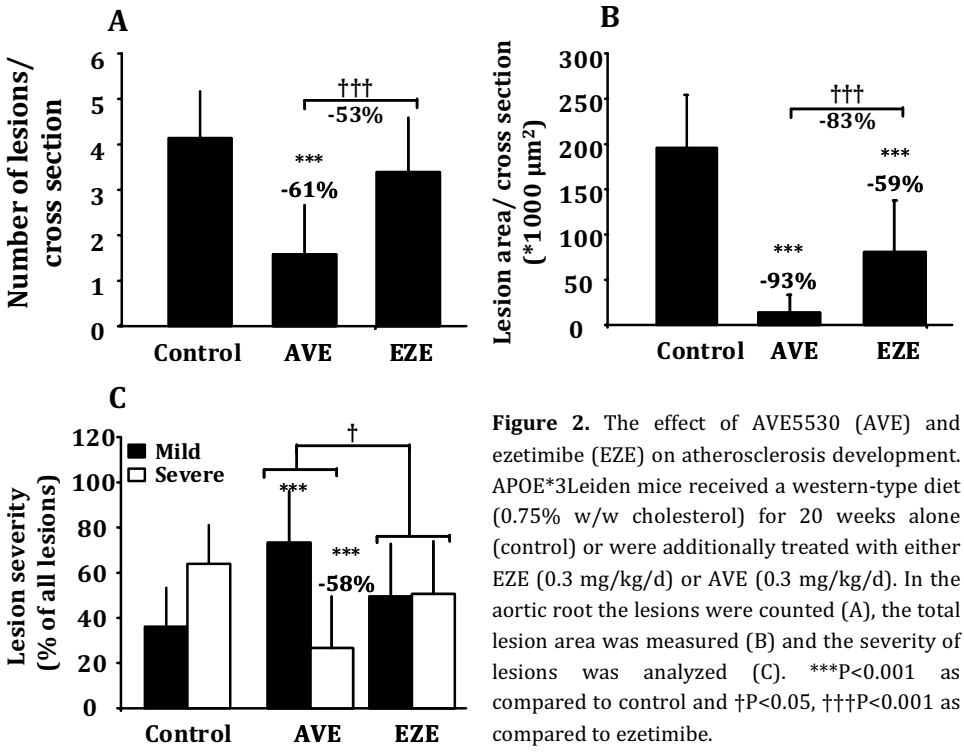

Figure 2. The effect of AVE5530 (AVE) and ezetimibe (EZE) on atherosclerosis development. APOE*3Leiden mice received a western-type diet (0.75% w/w cholesterol) for 20 weeks alone (control) or were additionally treated with either EZE (0.3 mg/kg/d) or AVE (0.3 mg/kg/d). In the aortic root the lesions were counted (A), the total lesion area was measured (B) and the severity of lesions was analyzed (C). ***P<0.001 as compared to control and †P<0.05, †††P<0.001 as compared to ezetimibe.

Effect AVE5530 and ezetimibe on atherosclerosis development

To determine the effect of AVE5530 and ezetimibe treatment on atherosclerosis development we measured the amount of lesions, the lesion area and we qualified the severity of these lesions in the aortic root (**figure 2)**. The control group had on average 4.1 ± 1.0 lesions per cross section in the aortic root with a total area of 196 ± 59 *1000 µm^2; 64 ± 17% of these lesions were severe type IV-V lesions. AVE5530 strongly reduced lesion number by 61% (1.6 ± 1.1, P<0.001), the lesion area by 93% (14 ± 19 *1000 µm^2, P<0.001) and the percentage of severe lesions (down to 27 ± 23%, P<0.001) as compared to control. AVE5530 at the same dosage was more potent in preventing atherosclerosis development than ezetimibe. This was reflected by an absence in reduction of the amount of lesions in the ezetimibe group, whereas the decrease in total lesion area was less as compared to the AVE5530 (to -59%, 80 ± 58 *1000 µm^2, P<0.001). Also the lesion severity was not affected by ezetimibe treatment. For all atherosclerosis parameters AVE5530 had significantly stronger effects than ezetimibe treatment. Representative pictures of the lesions are shown in **figure 3**. In line with these latter results we observed relatively more undiseased segments after either treatment, whereby again AVE5530 was more potent than ezetimibe (from 7 ± 15% in the control up to 54 ± 23% in the AVE5530 group and to 21 ± 25% in the ezetimibe group, P<0.001, **figure 4**).

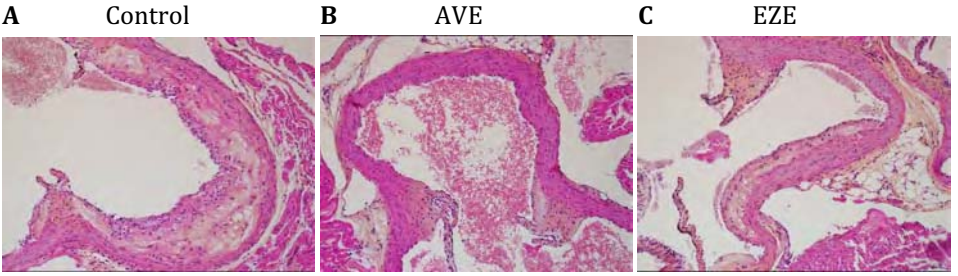

Figure 3. After 20 weeks of intervention the amount of atherosclerosis in the aortic root was measured. Representative pictures of atherosclerotic lesions of the control group (A), AVE5530 (AVE) (B) and ezetimibe (EZE) (C) are presented.

To assess the vulnerability to rupture of the lesions we measured the amount of collagen and SMCs, which can be considered to stabilize the lesions, and the amount of macrophages, known to be a destabilizing factor, in the lesions (**figure 5A**). 40 ± 13% of the lesion content in the control group was collagen. Treatment with AVE5530 or ezetimibe resulted in an equal increase in the collagen content to 65 ± 20% and 68 ± 11%, respectively ($P<0.001$). No change in the amount of SMCs was found (control 1.2 ± 1%). The macrophage content of the lesions in the control group was 30 ± 11%. Treatment with AVE5530 reduced this amount to 14 ± 14% ($P<0.01$). Ezetimibe was comparably effective and decreased it to 21 ± 9% ($P<0.05$). AVE5530 thereby tended to be more potent in reducing the macrophage content than ezetimibe ($P=0.077$).

As both the macrophage content of the lesions and the plasma markers for vessel wall inflammation were reduced by both the treatments, we also investigated the amount of monocytes adhering to the activated endothelium of the aortic root, which is considered as the first step in lesion development and a functional parameter for the extent of vessel wall inflammation. In the control group on average 7.9 ± 4.1 monocytes per cross section adhered to the activated endothelium (**figure 5B**). This was 75% reduced upon AVE5530 treatment (to 2.0 ± 1.1, $P<0.001$), while ezetimibe inhibited adhesion of monocytes less effectively, but still noteworthy by 35% (to 5.1 ± 2.8, $P<0.05$). Also for this parameter AVE5530 had significantly more effect than ezetimibe ($P<0.001$).

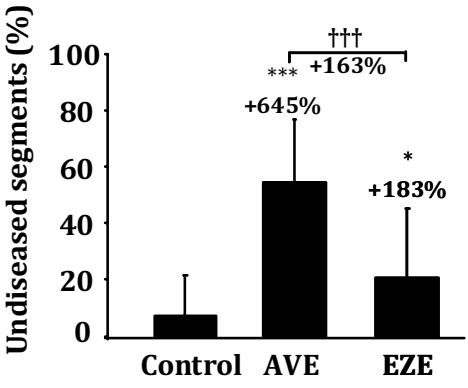

Figure 4. The effect of AVE5530 (AVE) and ezetimibe (EZE) on the percentage of undiseased (healthy) segments in the aortic root. *P<0.05, *** P<0.001 as compared to control and †††P<0.001 as compared to ezetimibe.

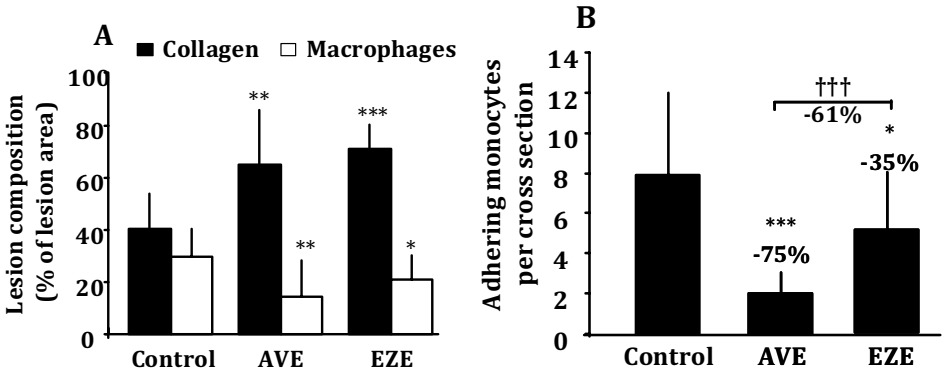

Figure 5. The effect of AVE5530 (AVE) and ezetimibe (EZE) the vessel wall. The lesion composition was analyzed by measuring the collagen content and the macrophage content (A). In the aortic root the number of adhering monocytes was counted per cross section (B). *P<0.05, ** P<0.01, *** P<0.001 as compared to control and †††P<0.001 as compared to ezetimibe.

Discussion

In this study we showed that the new low-absorbable, non-systemically acting cholesterol absorption inhibitor AVE5530 is more potent in cholesterol lowering than ezetimibe, resulting in less hepatic and vascular inflammation and in a stronger prevention of atherosclerosis development in APOE*3Leiden transgenic mice.

The present study was designed to investigate the efficacy of AVE5530 to reduce plasma lipid levels and to inhibit the development of atherosclerosis as compared to ezetimibe. To this end we used APOE*3Leiden transgenic mice, which are highly susceptible to dietary and pharmacological interventions with respect to modulating plasma lipid levels. Moreover, APOE*3Leiden mice show a human-like response to interventions aimed at treatment of CVD (*e.g.* statins, cholesterol uptake inhibitors, fibrates, calcium channel blockers and angiotensin II receptor antagonists [17-23]) with respect to alterations in the lipoprotein profile and/or atherosclerosis development at clinically relevant dosages. AVE5530 and ezetimibe both reduced plasma cholesterol; however, at an equal dose AVE5530 was markedly more effective than ezetimibe. Inhibition of cholesterol absorption with AVE5530 also resulted in reduced plasma triglycerides at the later time points, which was not observed in the ezetimibe treated animals. The reductions in cholesterol were mainly confined to the apoB-containing lipoproteins, similarly as observed previously in APOE*3Leiden mice on a low cholesterol diet and after treatment with statin, fibrates and ACAT inhibitors[17,20,21,26].

There is ample evidence that hypercholesterolemia (*i.e.* elevated plasma levels of (V)LDL) is a major causative factor in atherogenesis, but that an inflammatory component, thought to drive the initiation and progression of the disease, is also

required[27,28]. It has been shown that hypercholesterolemia and inflammation are not separate factors in diet induced hypercholesterolemia in APOE*3Leiden mice, but closely related features of the same trigger, hepatic cholesterol content[26]. We showed that inhibition of cholesterol absorption with either AVE5530 or ezetimibe reduced circulating inflammation markers mostly to the same extent. Difference was found for the liver derived acute phase protein fibrinogen, which was only reduced after AVE5530 treatment. Additionally AVE5530 strongly reduced the cholesterol content of the liver and was therewith significantly more potent than ezetimibe. Both hepatic cholesterol content and inflammation are considered as indirect measures for the amount of cholesterol absorption[26,29]. Therefore, it is likely that the strong anti-inflammatory effects of the two cholesterol absorption inhibitors are secondary to their cholesterol lowering properties. Similar anti-inflammatory and liver protective effects were observed previously in APOE*3Leiden mice treated with sphingolipids, which protect the liver from fat- and cholesterol induced steatosis[22]. Cholesterol and inflammation activate the endothelium of the vessel wall, which in turn expresses adhesion molecules facilitating the adherence and infiltration of monocytes, which is considered to be the first step in lesion development[27]. Therefore we investigated the effect of both treatments on a functional parameter of vessel wall inflammation: monocyte adherence. Whereas we did not observe differences in circulating levels of vessel wall derived inflammation markers (*i.e.* E-selectin and VCAM-1), we found less monocyte adherence to and macrophage accumulation in the arterial wall, both indicative for reduced local inflammation in the vessel wall.

Atherosclerosis development was strongly prevented by both AVE5530 and ezetimibe treatment, in line with previous reports on the anti-atherosclerotic effects of ezetimibe in apoE knock-out and apoE/eNOS double knock-out mice[9,30]. However, while AVE5530 reduced lesion area very potently (-93%, $P<0.001$) and additionally reduced the lesion number and prevented the progression of lesion severity, the effect of ezetimibe was restricted to a reduction of the lesion area (-59%, $P<0.001$) as compared to control. At an equal dose ezetimibe was markedly less efficacious as compared to AVE5530 for most atherosclerosis related read-out parameters. The reason for this marked difference in atherosclerosis development between AVE5530 and ezetimibe is likely related to the difference in potency to lower plasma cholesterol levels. However, an additional explanation may be provided by recent reports showing that NPC1L1 is not only present in the intestinal epithelial cells, but also in the liver and in monocytes and macrophages[31-33]. Herein is the protein involved in transport of cholesterol and modified lipoproteins. In macrophages in cell culture ezetimibe blocked the uptake of oxidized LDL and repressed the induction of cholesterol transporter genes ABCA1, ABCG1 and apoE[31]. Moreover, the drug has been reported to interfere with raft assembly in monocytes and to reduce the surface expression of raft-associated proteins involved in the cellular uptake of modified lipoproteins or phagocytosis[32]. What this

consequently means for the macrophage function *in vivo* remains unanswered, however, it can be hypothesized that macrophage activation, differentiation and efflux capacity are at least influenced.

Since its approval by the FDA in October 2002 many studies have confirmed the effective cholesterol lowering capacity of ezetimibe, especially when being co-administered with other lipid lowering drugs[34]. Surprisingly, the results of the ENHANCE trial[35] showed no benefit of ezetimibe/statin treatment over statin alone on intima media thickening, a surrogate endpoint for CVD, in patients with familial hypercholesterolemia, despite of a stronger decrease in LDL-cholesterol in the co-treated group. In fact, a worsening of intima media thickening in the group treated with the combination of drugs was found. Whether this also translates into differences in hard clinical endpoints, need to be investigated, *e.g.* in the currently ongoing larger IMPROVE-IT trial[36]. Also long-term safety data for ezetimibe have not been established yet. Generally ezetimibe is well tolerated, demonstrating a favorable safety profile [37]. Side effects have been infrequently reported, mainly in combination treatment with statins, concerning myopathy, liver damage and pancreatitis[38]. This might hypothetically be a consequence of the similar metabolism of the two compounds, as both statins and ezetimibe are glucuronidated by the uridine 5'-diphosphate glucuronosyltransferase isoenzymes[39,40]. However, more research is required to elucidate the consequence of the systemic availability and metabolism of ezetimibe, *e.g.* also whether this drug is active on cholesterol transport in macrophages *in vivo*[31,32]. Since AVE5530, in contrast to ezetimibe, is barely systemically available, it may have less adverse side effects in clinical use. This, however, remains to be investigated in clinical studies.

In conclusion, we showed that the new low-absorbable, non-systemically acting cholesterol absorption inhibitor AVE5530 is more effective in lowering plasma lipid levels and development of atherosclerosis than ezetimibe in APOE*3Leiden mice.

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Disclosures

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