Acyl-CoA:Cholesterol Acyltransferase Inhibitor Avasimibe Reduces Atherosclerosis in Addition to Its Cholesterol-Lowering Effect in ApoE*3-Leiden Mice

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Background—The present study investigated whether the ACAT inhibitor avasimibe can reduce atherogenesis independently of its cholesterol-lowering effect in ApoE*3-Leiden mice.

Methods and Results—Two groups of 15 female ApoE*3-Leiden mice were put on a high-cholesterol (HC) diet; 1 group received 0.01% (wt/wt) avasimibe mixed into the diet. The HC diet resulted in a plasma cholesterol concentration of 18.7±2.6 mmol/L. Addition of avasimibe lowered plasma cholesterol by 56% to 8.1±1.2 mmol/L, caused mainly by a reduction of and composition change in VLDL and LDL. In a separate low-cholesterol (LC) control group, plasma cholesterol was titrated to a level comparable to that of the avasimibe group (10.3±1.4 mmol/L) by lowering the amount of dietary cholesterol. After 22 weeks of intervention, atherosclerosis in the aortic root area was quantified. Treatment with avasimibe resulted in a 92% reduction of lesion area compared with the HC control group. Compared with the LC control, avasimibe reduced lesion area by 78%. After correction for the slight difference in cholesterol exposure between the LC control and avasimibe groups, the effect of avasimibe on lesion area (73% reduction) remained highly significant. In addition, monocyte adherence to the endothelium, free cholesterol accumulation, and lesion severity were reduced by avasimibe treatment.

Conclusions—Treatment with avasimibe potently lowered plasma cholesterol levels in ApoE*3-Leiden mice and considerably reduced atherosclerotic lesion area in addition to its cholesterol-lowering effect. Because monocyte adherence to the endothelium and lesion severity were also reduced by avasimibe, treatment with avasimibe may result in higher plaque stability and therefore a reduced risk of plaque rupture. (Circulation. 2001;103:1778-1786.)

Key Words atherosclerosis n inhibitors n lipoproteins n cell adhesion molecules

The enzyme acyl-CoA:cholesterol-O-acyltransferase (ACAT) catalyzes the intracellular formation of cholesterol esters. It is involved in the absorption of cholesterol in the intestine and the secretion of VLDL from the liver. The enzyme has also been implicated in esterification and thereby storage of cholesterol ester in monocyte-derived macrophages in the arterial wall.3 Because the accumulation of cholesterol esters in the latter cells is one of the earliest steps in the development of atherosclerosis,2 it has been suggested that inhibition of the enzyme ACAT has a direct antiatherogenic potential.3,4 Moreover, because the stability of the atherosclerotic plaque is increased when fewer lipid-laden macrophages are present,5 a decrease of the number of lipid-laden macrophages in the arterial wall on ACAT inhibition could lead to a higher plaque stability and consequently a reduced risk of plaque rupture.

Several animal studies with various ACAT inhibitors have shown cholesterol-lowering effects of these drugs.6–8 Data on direct antiatherosclerotic effects of ACAT inhibitors, however, remain disputed. ACAT inhibition has been shown to decrease atherosclerotic lesion areas or cholesterol ester enrichment in the arterial wall of animal models for atherosclerosis, but in most of these studies, substantial lipid-lowering was involved.9–12 Others found less atherosclerosis at a dose that showed some systemic availability but did not lower plasma total cholesterol levels.13,14 Thus, a number of models have been used to test the direct antiatherosclerotic activity of ACAT inhibitors, but so far, conclusions drawn from these experiments are based on circumstantial evidence.15

The present study was designed to determine the direct antiatherosclerotic effects of the ACAT inhibitor avasimibe (CI-1011: [2,4,6-tris-(1-ethylethyl)phenyl]acetetyl)sulfamic acid, 2,6-bis(1-methyl-ethyl)phenyl ester) in ApoE*3-Leiden transgenic mice by ruling out the confounding effects of

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cholesterol lowering. ApoE*3-Leiden mice were used because they exhibit elevated plasma cholesterol and triglyceride levels, mainly confined to the VLDL/LDL-size lipoprotein fraction.\textsuperscript{16,18} Because they are highly responsive in their plasma lipid levels to dietary treatment,\textsuperscript{17} their plasma cholesterol levels can easily be titrated to a constant level by the amount of cholesterol in the diet. In addition, on high-fat, high-cholesterol feeding, these mice develop atherosclerotic lesions resembling those found in humans, depending on their plasma cholesterol levels.\textsuperscript{18,19} Because ApoE*3-Leiden mice have been shown to be responsive to a number of cholesterol-lowering therapies, such as lovastatin, gemfibrozil, and fish oil,\textsuperscript{20,21} these mice were considered a suitable model for the testing of the antiatherosclerotic properties of the ACAT inhibitor avasimibe.

**Methods**

**Mice**

Heterozygous female ApoE*3-Leiden mice of F14 generations, 31 to 41 weeks of age at the start, were used. Identification of transgenic mice was performed by an ELISA for human apoE, as previously described.\textsuperscript{17} All animal experiments were approved by the institutional committees on animal welfare of TNO Prevention and Health.

**Diets**

Before the start of the study, animals were kept on a standard rat/mouse chow diet (Hope Farms). During a 3-week run-in period, all animals received a semisynthetic high-fat/high-cholesterol (HFC) diet as used before.\textsuperscript{17} After this period, the animals were divided into 3 groups of 15 mice each on the basis of age and cholesterol level. The high-cholesterol (HC) control group received an HFC diet with addition of 0.5\% wt/wt cholesterol and 0.1\% wt/wt cholate, the latter added to facilitate intestinal uptake of fat and cholesterol, thereby increasing plasma cholesterol levels. The avasimibe group received the same diet as the HC control group, with an extra addition of 0.01\% wt/wt avasimibe (kindly provided by Dr Krause, Parke Davis, Ann Arbor, Mich), approximately equaling a daily dose of 10 mg/kg body wt. The diet of the low-cholesterol (LC) control group was chosen to reach the same plasma cholesterol level in these mice as in the avasimibe group and contained 0.5\% wt/wt cholesterol and no cholate. Animals had free access to water and food.

**Lipid and Lipoprotein Analysis**

After a 4-hour fasting period from 9 AM to 1 PM, blood samples were obtained from each individual mouse by tail incision. Total plasma cholesterol and triglyceride levels were measured enzymatically, and lipoprotein profiles were obtained by size fractioning, as described.\textsuperscript{17,22} For the determination of the composition of the apoB-containing lipoproteins, ultracentrifugation of total plasma was performed. VLDL and IDL/LDL fractions were pooled, and lipids were analyzed as described previously.\textsuperscript{22} Total plasma apoB and apoE were measured by gel electrophoresis followed by Coomassie blue staining and calculation of the contribution of apoB and apoE to total protein.

**Histological Assessment of Atherosclerosis**

After 24 weeks of diet feeding, mice were killed after anesthetization and blood collection as described.\textsuperscript{17} The hearts were dissected, stored overnight in phosphate-buffered 3.8\% formalin fixation, and embedded in paraffin. Serial cross sections (5 \(\mu\)m thick) throughout the entire aortic valve area were used for histological analysis. Sections were routinely stained with hematoxylin-phloxine-saffron (HPS). Per mouse, 4 sections with intervals of 30 \(\mu\)m were used for quantification and qualification of atherosclerotic lesions. For determination of severity of atherosclerosis, the lesions were classified into 5 categories (types I through V) as described before.\textsuperscript{17,24}
control (443 and 436 μg/mL, respectively). Plasma apoE in these latter groups was 39% lower than in the HC control (720 μg/mL). These data indicate that in the HC control group, the number of VLDL/LDL particles and their total cholesteryl ester content are largest. Treatment with avasimibe causes a reduction of these particles, which become relatively enriched in triglycerides. Compared with the LC control, the avasimibe-treated animals have ≈2-fold more VLDL/LDL particles in their plasma, which contain less cholesteryl ester.

Figure 1. Effect of avasimibe on lipoprotein profiles and composition. A, Lipoprotein profiles for cholesterol. B, Effect of avasimibe on lipid composition of VLDL and IDL/LDL. FC indicates free cholesterol; CE, cholesteryl ester; and TG, triglycerides.
Effect of Avasimibe on Macrophage ACAT Activity

Plasma concentrations of avasimibe were 50±24 nmol/L. To investigate whether esterification of cholesterol is inhibited at these concentrations, the effect of avasimibe on ACAT activity was measured in the mouse macrophage cell line J774 in the presence of an additional source of cholesterol to increase the intracellular cholesterol pool. Incubation with avasimibe resulted in a dose-dependent reduction in ACAT activity, showing a significant 25% reduction at 50 nmol/L (Figure 2, P<0.005).

Effect of Avasimibe on Atherosclerosis Development

Atherosclerotic lesions were quantified in cross sections of the aortic valve area in the heart of each individual mouse. The average lesion area per section for the individual groups is shown in Figure 3A. For the HC control group, the average lesion area per section was 95.5±35.2 μm²±1000. Treatment with avasimibe resulted in a 92% reduction of lesion area (7.6±7.0 μm²±1000), related to the total effect of avasimibe. Comparison of the avasimibe group and the LC control (34.1±24.5 μm²±1000) showed a 78% reduction of atherosclerotic lesion area by avasimibe, suggesting an effect independent of its cholesterol-lowering effect.

Although the avasimibe group and the LC control were almost matched for their plasma total cholesterol levels, there was a slight difference in cholesterol level between those groups. Because it has been shown previously that there is a linear relationship between atherosclerotic lesion area and exposure of the arterial wall to plasma cholesterol in ApoE*3-Leiden mice, total cholesterol burden over time for the latter groups was calculated to correct the lesion areas for differences in these cholesterol exposures. Mean cholesterol exposure in mice treated with avasimibe was 184±26 mmol·L⁻¹·wk⁻¹ and in mice receiving the LC control diet, 236±34 mmol·L⁻¹·wk⁻¹ (P<0.005). Correction of the lesion areas for cholesterol exposure by dividing the lesion area per mouse by its individual cholesterol exposure showed that the lesion area in the avasimabe group was 73% smaller than in the LC control group (P=0.012 by Mann-Whitney test, Figure 3B). These data indicate that avasimabe reduces the development of atherosclerosis independently of its cholesterol-lowering effect.

We also evaluated the effect of avasimabe treatment on the severity of the lesions. Figure 4 shows representative pictures. The percentages of lesions classified in the respective lesion categories were calculated (Figure 5). In the HC control group, the largest percentage of all lesions is constituted of severe lesions. Lesions in the LC control group are almost equally distributed over the fatty streaks and severe lesions. In the avasimabe group, however, most lesions are mere small fatty streaks, and only a few lesions belong to the severe types of plaques. These results, in combination with the stainings for macrophages (Figure 4B, 4E, and 4H) and smooth muscle cells (Figure 4C, 4F, and 4I) indicate that treatment with avasimabe resulted in the development of only small numbers of foam cells in the intima of the arterial wall containing small lipid pools compared with the LC control group, in which foam cell accumulation with larger lipid pools also occurred in the media. In the HC control group, in contrast, large lipid pools were found in the intima and media, the presence of smooth muscle cells in the media was diminished, and severe necrosis and calcification were found.

Effect of Avasimibe on Free Cholesterol Content of Atherosclerotic Lesions

Because avasimabe reduced cholesteryl ester accumulation in the cell, it is conceivable that treatment with avasimabe would result in the accumulation of free cholesterol in the atherosclerotic lesions. Because free cholesterol above a certain threshold concentration is toxic to the cell, accumulations of free cholesterol can be found only in the form of cholesterol clefts. Therefore, we determined whether treatment with avasimabe increased the number of sections containing cholesterol clefts. However, the opposite was found (Figure 6). Treatment with avasimabe significantly reduced the number of atherosclerotic lesions containing cholesterol clefts, indicating that avasimabe does not increase free cholesterol accumulation.
Effect of Avasimibe on Monocyte Adhesion to the Endothelium

The number of monocytes adhering to the endothelium, as shown in Figure 7A through 7D, was counted in the same slides as used for quantification of atherosclerosis. Figure 7E shows that monocyte attachment to the endothelium was not different between the HC and LC control groups. Avasimibe, however, showed a significant 80% reduction of the number of monocytes adhering to the endothelium (P<0.001), suggesting that avasimibe reduces endothelial activation independently of its cholesterol-lowering effect.

Discussion

In the present study, the ACAT inhibitor avasimibe decreases plasma cholesterol levels by >50% in cholesterol-fed ApoE*3-Leiden mice by reduction of VLDL/LDL cholesterol and induces a relative enrichment of triglycerides and decrease of cholesteryl esters in these lipoproteins, making them potentially less atherogenic. In addition, treatment with avasimibe results in reduced progression and severity of atherosclerosis, in addition to its cholesterol-lowering effect.

The present study was designed to demonstrate a direct effect of avasimibe on the development of atherosclerosis, in addition to its cholesterol-lowering effect. Therefore, an HC control group, an HC group receiving avasimibe, and a matched LC group were used to compare the development of atherosclerosis in the avasimibe group with that in the groups without ACAT inhibitor at high or at equally low cholesterol levels.

Our results on the lipid-lowering effects of the ACAT inhibitor avasimibe are in agreement with previous studies showing that avasimibe can potently reduce plasma lipid concentrations in rats, rabbits, and hamsters. At the dosage used (10 mg·kg body wt⁻¹·d⁻¹), however, the plasma cholesterol reduction observed in the present study (−56%) exceeds that in previous studies in rats (−32%) and hamsters (−37%). The reduction of plasma cholesterol levels by ACAT inhibitors can be attributed primarily to a decreased intestinal cholesterol absorption. For inhibitors with increased bioavailability compared with the early nonabsorbable hypocholesterolemic agents, however, a reduction of the secretion of VLDL apoB from the liver and the subsequent proper disposal of cholesterol into the bile acid synthetic pathway may also contribute to the cholesterol-lowering effect. The present study shows that treatment with avasimibe also changed lipoprotein...
composition. Compared with the HC and LC controls, avasimibe treatment resulted in a relative enrichment of VLDL/LDL with triglycerides, probably as a consequence of the decrease in cholesterol esterification in the liver. Compared with the HC control group, the avasimibe group showed 44% lower total plasma apoB levels, in agreement with data obtained in miniature pigs.\textsuperscript{30} Compared with the LC control, however, apoB levels were more than twice as high in the avasimibe group. This indicates that plasma from the avasimibe group contains VLDL/LDL particles that are less atherogenic than those in the LC control, but also that more of these particles are present in plasma, which again adversely contributes to atherogenicity in the avasimibe group.

**Figure 4.** Representative photomicrographs of atherosclerotic lesions in different groups. A, Severe plaque from HC control group (type V). D, Mild plaque from LC control group (type III). G, Early fatty streak from avasimibe group (type I). B, E, and H, Immunohistochemistry of macrophages (M\textsubscript{6}); staining with AlA31240. C, F, and I, Immunohistochemistry of smooth muscle cells (SMC); staining with \textalpha-actin.

**Figure 5.** Effect of avasimibe on severity of atherosclerosis. Data are presented as mean±SEM. Fatty streaks indicate type I to III lesions; Plaques, type IV or V lesions. Statistically significant differences in distributions of atherosclerotic lesions over fatty streaks and plaques between groups are indicated: **P<0.01, ***P<0.005.
Because the enzyme ACAT is also responsible for the esterification and storage of cholesterol in the macrophage, it has been hypothesized that ACAT inhibition may prevent the accumulation of lipid-laden macrophages in the arterial wall.2,4 Bocan et al.22 recently showed that avasimibe reduced the intracellular cholesteryl ester concentration in human monocyte–derived macrophages in a dose-dependent manner. To investigate whether esterification of cholesterol is inhibited in mouse macrophages at concentrations found in plasma of the avasimibe group (50±24 mmol/L), the effect of avasimibe on ACAT activity was measured in the mouse macrophage cell line J774. At 50 mmol/L avasimibe, cholesterol esterification was significantly inhibited by 25%, suggesting that in our animal study, macrophage ACAT in the vessel wall was also inhibited by avasimibe.

Comparison of atherosclerotic lesion areas in the HC control and the avasimibe group shows that avasimibe reduced atherosclerosis by 92% in the ApoE*3-Leiden mice. This finding is in line with previous observations with avasimibe in hamsters2 and other ACAT inhibitors in rabbits5–7,11 showing an antiatherosclerotic activity of ACAT inhibitors concomitant with reductions in plasma cholesterol concentrations. After correction of the extent of atherosclerosis for the slight difference in cholesterol exposure between the LC control and the avasimibe groups, comparison of these corrected lesion areas showed that avasimibe significantly reduced the development of atherosclerosis by 73%. It has been suggested that an atherogenic diet containing cholate, such as that used in the avasimibe group, may induce an inflammatory response in the liver, resulting in adverse effects on atherogenesis.3,4 Others, however, have found no such effect in C57BL/6J mice and LDLr−/− mice, indicating that the effect of cholate on the liver has not been unequivocally established. The fact that we found that avasimibe in a cholate-containing diet significantly reduced atherosclerosis compared with the LC control group without cholate clearly favors the direct antiatherosclerotic effect of avasimibe.

We also demonstrate that avasimibe reduced lesion severity compared with the LC and the HC controls. In the HC control group, atherosclerotic lesions were characterized by large lipid depositions in the intima and media, with severe fibrosis, necrosis, calcification, and reduction of smooth muscle cells. Lesions found in the avasimibe group contained only a small number of foam cells and a small lipid pool. The media was hardly ever affected, and the number of smooth muscle cells was not reduced. This is in contrast to the mild plaques found in the LC control group, in which the lipid pools were larger and foam cells were also found among the smooth muscle cells in the media. It is currently understood that clinical manifestations of atherosclerosis are characterized by disruption of the plaque13 and that the most important features of a vulnerable and potentially unstable plaque are an abundant presence of T lymphocytes and macrophages, a large lipid pool in the plaque, a low density of smooth muscle cells, and a thin, collagen-poor fibrous cap.3 Therefore, our results indicate that treatment with avasimibe can contribute to a higher plaque stability and consequently reduce the risk of plaque rupture, as has recently also been suggested in studies with hypercholesterolemic rabbits.25 Also, the finding that treatment with avasimibe significantly reduced the number of atherosclerotic lesions containing cholesterol clefts indicates that avasimibe does not increase free cholesterol accumulation in the lesions. Thus, the additional effect of avasimibe is an attenuated accumulation of lipids in the arterial wall and inhibition of the infiltration of macrophages into the media. In addition, our results show that avasimibe significantly reduced the number of monocytes adhering to the endothelium, which is considered to be one of the first steps in the development of atherosclerosis.5 This is in agreement with in vitro studies that have shown that ACAT inhibitors can decrease monocyte adhesion to endothelial cells.35 It suggests that treatment with avasimibe reduces endothelial activation. Whether avasimibe indeed decreases the expression of adhesion and/or chemoattractant molecules in the endothelium remains to be investigated.

In conclusion, this study demonstrates that the ACAT inhibitor avasimibe potently lowers plasma cholesterol levels in transgenic ApoE*3-Leiden mice. Although it cannot be fully excluded that the change in lipoprotein composition in the avasimibe-treated animals contributes to its antiatherogenic effect, the dramatic reduction in atherosclerotic lesion area in the avasimibe group clearly
appears to be attributable to an effect additional to that of cholesterol lowering. Because lesion severity is also reduced by avasimibe, treatment with avasimibe may result in higher plaque stability and therefore a reduced risk of plaque rupture.

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**References**


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