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Avasimibe and atorvastatin synergistically reduce cholesteryl ester content in THP-1 macrophages

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Abstract

Evidence suggests that the inhibition of both acyl-CoA:cholesterol acyltransferase and hydroxymethyl glutaryl-CoA reductase causes a synergistic direct antiatherosclerotic effect on the vessel wall. To investigate this synergism in a single cell type and to avoid the confounding effect of plasma cholesterol lowering by these drugs, we have used an in vitro model of human macrophages (phorbol ester-treated THP-1 cells). In macrophages incubated simultaneously with acetyl low-density lipoproteins, the novel acyl-CoA:cholesterol acyltransferase inhibitor avasimibe (0.01–0.5 μM) caused a concentration-dependent reduction in cell cholesteryl ester content that was not accompanied by an increase in intracellular free cholesterol. A 5 μM concentration of atorvastatin enhanced by approximately twofold the ability of 0.5 μM avasimibe to reduce the mass of esterified cholesterol, and this was reversed by co-incubation with 200 μM mevalonate or 10 μM geranylgeraniol. Based on these data, we propose that the synergism between acyl-CoA:cholesterol acyltransferase and hydroxymethyl glutaryl-CoA reductase inhibitors found in several in vivo studies may be explained by a direct additive effect of both agents reducing the lipid content of the macrophages present in the lesion area.

Keywords: Avasimibe; Atorvastatin; Acyl-CoA cholesterol acyltransferase; Hydroxymethyl glutaryl-CoA reductase; Macrophage; Cholesteryl ester

1. Introduction

Atherosclerosis continues to be the leading cause of death in industrialized nations despite the availability of drugs such as statins, which are able to modify plasma lipid levels beneficially and to reduce cardiovascular mortality significantly (Roth, 1998; Kusunoki et al., 2001; Doshi et al., 2001). Thus, there is a need to develop novel therapeutic strategies, such as inhibition of acyl-CoA:cholesterol acyltransferase, an intracellular enzyme localized in the endoplasmatic reticulum, which catalyzes the esterification of cholesterol by long-chain fatty acyl-CoA derivatives (Chang et al., 1997). There are two isoforms of acyl-CoA:cholesterol acyltransferase: type 1, which is ubiquitously distributed (liver, pancreas, macrophages and steroidogenic tissues), and type 2, expressed only in the liver and intestine (Buhman et al., 2000). As acyl-CoA:cholesterol acyltransferase is the primary enzyme responsible for the esterification of cholesterol in intestinal cells and hepatocytes, its inhibition would lower plasma cholesterol levels by reducing dietary cholesterol absorption and liver lipoprotein assembly.

Acyl-CoA:cholesterol acyltransferase type 1 is also implicated in the synthesis and storage of cholesteryl esters in arterial monocyte-derived macrophages (Miyazaki et al., 1998). The building up of large amounts of cholesteryl esters in the cytosol converts macrophages into foam cells, which accumulate and leads to the formation of fatty streaks, the hallmark of atherosclerosis. As the increase in lipid deposition in foam cells is due to the excessive cholesterol esterification, the inhibition of this enzyme activity may also help to prevent this process (Ishii et al., 1998; Ohgami et al., 2000). Thus, in addition to their hypolipidemic action, acyl-CoA:cholesterol acyltransferase

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inhibitors may be considered antiatherosclerotic agents acting directly on the arterial wall (Sliskovic and White, 1991; Roth, 1998; Kusunoki et al., 2001).

The combination of acyl-CoA:cholesterol acyltransferase inhibitors with statins has been proposed as a more adequate strategy than using an acyl-CoA:cholesterol acyltransferase inhibitor alone (Bocan et al., 2001). Statins are the most commonly prescribed agents for the treatment of hypercholesterolemia due to their efficacy, tolerability and safety (Maron et al., 2000). In addition to their cholesterol-lowering effects, statins can exert a multiplicity of effects in different cellular systems, since mevalonic acid, the product of the hydroxymethyl glutaryl-CoA reductase reaction, is the precursor of numerous metabolites (Davignon and Laaksonen, 1999). Some of these effects can directly interfere with the atherosclerotic process, in a fashion that is independent of the hypocholesterolemic properties of statins (Corsini et al., 1996).

The inhibition of both acyl-CoA:cholesterol acyltransferase and hydroxymethyl glutaryl-CoA reductase would not only potentiate the lipid-regulating properties of the former, but also the direct antiatherosclerotic effects of both agents on the arterial wall; thus, several authors (Bocan et al., 1998, 2001, Junquero et al., 2001) have studied the effect of an acyl-CoA:cholesterol acyltransferase inhibitor plus a statin on several in vivo animal models, and clinical studies are currently underway to assess the safety and efficacy of this combination therapy (Doshi et al., 2001). However, as acyl-CoA:cholesterol acyltransferase inhibition could affect several targets in the in vivo studies (liver, intestine and arterial cells), it is difficult to assess the contribution of each cellular system to the final effects produced.

The present study was designed to determine the direct antiatherosclerotic action of the novel acyl-CoA:cholesterol acyltransferase inhibitor avasimibe, alone and in combination with atorvastatin, by assessing their effects on cholesterol accumulation in an in vitro model of human macrophages. This cell system was chosen due to its critical role in the initiation and progression of the atherosclerotic process. The use of an in vitro model avoids the confounding effect of the cholesterol lowering involved in most of the in vivo studies, and allows the pharmacological effects of these agents on an isolated cell system to be assessed.

2. Materials and methods

2.1. Materials

Avasimibe ([[2,4,6-tris(1-methylethyl)phenyl]acetyl]sulfamic acid, 2,6-bis(1-methylethyl)phenyl ester), depicted in Fig. 1, and atorvastatin were kindly provided by Parke-Davis (Ann Arbor, MI; now Pfizer). All the reagents used for cell culture were from Gibco, Life Technologies (Paisley, Scotland), with the exception of 4-β phorbol 12-β myristate 13 alpha acetate (PMA), which was purchased

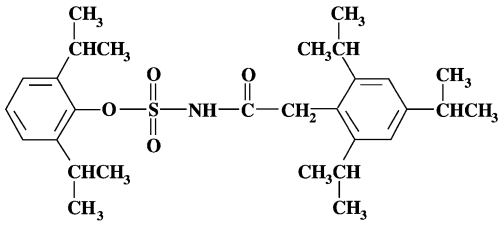

Fig. 1. Chemical structure of the ACAT inhibitor avasimibe ([[2,4,6-tris(1-methylethyl)phenyl]acetyl]sulfamic acid, 2,6-bis(1-methylethyl)phenyl ester).

from Sigma (Madrid, Spain). Apolipoprotein A-I, cholestanol and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were also from Sigma. Pyridine was from Merck (Barcelona, Spain), and Sylon BTZ was purchased from Supelco (Bellefonte, USA). Other general chemicals were obtained from commercial sources and were of analytical grade.

2.2. Lipoproteins

Low-density lipoproteins (LDL, d 1.006–1.063) and high-density lipoproteins (HDL, d 1.063–1.21) were prepared from the plasma of healthy donors by sequential ultracentrifugation (Mills et al., 1984). Lipoproteins were dialyzed against phosphate saline buffer, and their protein concentration was assessed by the method of Bradford (1976), using bovine serum albumin as a standard. LDL were acetylated as described by Basu et al. (1976), dialyzed and concentrated by using Millipore Centriplus filters (Colli et al., 1997). The degree of acetylation of LDL was confirmed by an increase of at least 2.5-fold in their electrophoretic mobility using a 1% agarose gel in barbital buffer (McCrohon et al., 1999).

2.3. Cell culture

THP-1 cells were obtained from American Type Culture Collection and maintained in RPMI 1640 with L-glutamine supplemented with 10% fetal bovine serum (Myoclone super plus FBS USA), 50 μM 2-mercaptoethanol, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin at 37 °C in 5% CO_2. Differentiation of monocytes to macrophages was achieved by exposing the cells to 50 ng ml^{-1} PMA during 72 h (Auwerx, 1991). Later, 48 h after differentiation, macrophages were incubated by using two systems (Hakamata et al., 1995): (i) sequential incubation, in which cells were initially converted to foam cells by exposure to 150 μg ml^{-1} of acetyl-LDL during 48 h. After three washings, cells were incubated for an additional 48 h with avasimibe, atorvastatin or a combination of both, in the presence of 200 μg ml^{-1} HDL; (ii) simultaneous incubation, in which cells were exposed simultaneously to 150 μg ml^{-1} of acetyl-LDL and the drug(s), in the presence or absence of HDL or apolipoprotein A-I (50 μg ml^{-1}), for 48 h. In some

experiments, cells were pre-incubated with atorvastatin for 24 h, and subsequently treated with atorvastatin, acetyl-LDL±HDL, for a further 48 h. Drug solutions were prepared in dimethylsulfoxide (DMSO), and the final DMSO concentration was 0.1%. Cell viability was determined by measuring the ability of THP-1 cells to reduce MTT (Mossman, 1983).

2.4. Mass determination of cellular cholesterol contents

After treatments, cells were scraped in phosphate-buffered saline (PBS) and sonicated with a Branson sonifier 450, set at 25 W, three times during 10 s. An aliquot of the lysate was used to determine cell protein by the method of Bradford (1976). An internal cholestanol standard was added to the rest of the cell lysate, and lipids were extracted with chloroform/methanol (2:1 v/v) (Ravandi et al., 1999). Aliquots of the lipid extract were saponified according to the method described by Klansek et al., (1995), dried under nitrogen flush and stored at −20 °C until used to determine total cellular cholesterol. The non-saponified extracts were used to determine cellular free cholesterol content. In both cases, dry extracts were dissolved in dry pyridine, an equal volume of Sylon BTZ was added and this solution was incubated at room temperature for 30 min to complete the sylanization reaction (Guardiola et al., 1994). Samples were analysed on a Hewlett-Packard gas chromatograph model 5890, equipped with a flame ionization detector and a 100% dimethylpolysiloxane fused silica capillary column (25 m×0.25 mm internal diameter) (Chrompack, Middelburg, The Netherlands). Peak areas were determined by the computer programme Hewlett-Packard Chemstation, and the ratio cholesterol/cholestanol area was used to calculate cholesterol mass from a standard curve. The results were expressed in micrograms of cholesterol per milligram of protein, and cholesteryl ester mass was calculated by subtracting the content of free cholesterol from that of total cholesterol.

2.5. Statistical analysis

Experiments were performed in duplicate wells and repeated at least three times. Data are presented as mean±S.D. An analysis of variance (ANOVA), combined with the Student–Newman–Keuls test, was used to evaluate the statistical significance of the differences. The computer programme GraphPad Instat was used for the calculations.

3. Results

3.1. Effect of avasimibe on intracellular cholesterol content

PMA-treated THP-1 cells were cholesterol loaded by incubation with 150 μg ml^{-1} acetyl-LDL for 48 h. This treatment caused a marked increase in the cellular cholesterol content, particularly in the cholesteryl ester fraction (from 2.08±1.38 to 23.40±5.00 μg mg^{-1} protein). This was accompanied by changes in cell morphology typical of the formation of foam cells, with multiple lipid droplets in the cytosol, as assessed by Oil Red O staining (data not shown).

When these lipid-loaded cells were incubated with avasimibe (0–0.2 μM) for an additional 48 h, in the presence of 200 μg ml^{-1} HDL in the culture medium, cholesteryl ester mass did not change significantly (Table 1). On the contrary, in macrophages incubated simultaneously with 150 μg ml^{-1} acetyl-LDL and avasimibe for 48 h, there was a concentration-dependent reduction in cellular cholesteryl ester content, 62% and 70% ($P<0.05$) at 0.1 and 0.2 μM, respectively. Total cholesterol was reduced as a consequence of the decrease in cholesteryl ester content; however, despite the absence of HDL in the culture medium, cellular free cholesterol was not increased (Fig. 2). When the same experiment was performed in the presence of 200 μg ml^{-1} HDL, higher concentrations of avasimibe were needed to achieve the same level of cholesteryl ester reduction as in the absence of HDL; thus, at the concentration of 0.1 μM, avasimibe reduced by 62% cellular cholesteryl ester content, while in the presence of HDL, the reduction was only of 23% ($P<0.05$), achieving a 54% reduction at 0.75 μM ($P<0.01$) (Fig. 3). As occurred in the absence of HDL, total cholesterol was reduced, but free cholesterol did not accumulate inside the cell. To exclude the interference of lipids contained in the HDL particle, the same experiment was performed using apolipoprotein A-I instead of whole HDL as a cholesterol acceptor. Under these conditions, 0.75 μM avasimibe significantly reduced intracellular cholesteryl ester from 60.92±8.54 to 21.59±3.62 μg mg^{-1} protein (64% reduction, $P<0.001$) in the absence of acceptors, and to 5.83±0.17 in the presence of apolipoprotein A-I (90% reduction).

Table 1
Effect of avasimibe on intracellular sterol content in the sequential incubation system

	CE	FC	TC
Control	19.40 ± 1.91	36.17 ± 1.97	55.57 ± 3.39
Avasimibe 0.02 μM	19.69 ± 2.50	35.18 ± 3.35	54.47 ± 4.60
Avasimibe 0.1 μM	18.40 ± 0.71	34.97 ± 1.65	53.37 ± 1.48
Avasimibe 0.2 μM	19.28 ± 0.97	35.55 ± 1.65	54.82 ± 2.53

PMA-treated THP-1 cells were converted to foam cells by 48 h of incubation with 150 μg ml^{-1} acetyl-LDL. After washing, cells were incubated for an additional 48 h with avasimibe in the presence of 200 μg ml^{-1} HDL. Cellular lipid was extracted and cholesterol mass was determined as described in Materials and methods. Results are expressed as μg sterol mg^{-1} protein and are the mean ± S.D. of three independent experiments performed in duplicate wells.

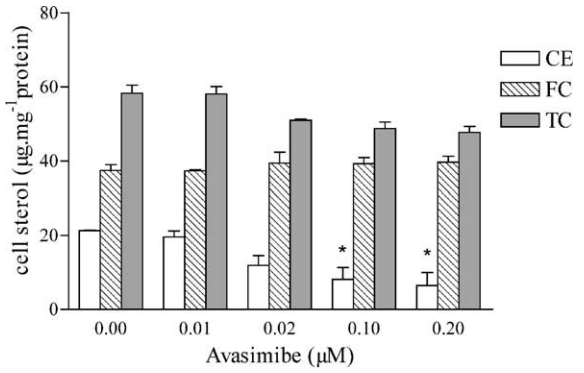

Fig. 2. Effect of avasimibe on cell sterol content in the simultaneous incubation system in the absence of HDL. PMA-treated THP-1 cells were incubated simultaneously with avasimibe and 150 μg ml^{-1} acetyl-LDL for 48 h. After washing, cellular lipid was extracted and total cholesterol (TC) and free cholesterol (FC) mass was determined as described in Materials and methods. Cholesteryl ester (CE) content was calculated by subtracting FC from TC. Results are expressed as μg sterol mg^{-1} protein, and are the mean ± S.D. of three independent experiments performed in duplicate wells. *P<0.05.

3.2. Effect of atorvastatin on intracellular cholesterol content

Atorvastatin did not reduce intracellular cholesteryl ester or total cholesterol in macrophages treated during the loading phase, at least in the concentration range assayed (0–5 μM). However, when the drug was included in the incubation medium 24 h before cholesterol loading, intracellular cholesteryl ester content was reduced between 30–40%, (P<0.05), both in the absence and in the presence of HDL (Table 2).

Table 2
Effect of atorvastatin on intracellular sterol content in the simultaneous incubation system

	CE	FC	TC
−HDL			
Control	22.54±6.44	41.77±0.81	64.31±6.27
Atorvastatin (2.5 μM)	14.00±2.24[a]	37.86±0.98	51.85±1.82
Atorvastatin (5 μM)	12.56±2.01[a]	41.13±2.24	53.69±4.22
+HDL			
Control	29.10±3.81	45.71±4.87	74.81±8.59
Atorvastatin (2.5 μM)	22.10±1.81[a]	51.12±1.79	73.22±0.26
Atorvastatin (5 μM)	20.00±2.66[a]	48.54±6.16	68.55±8.64

PMA-treated THP-1 cells were incubated with atorvastatin for 24 h, and subsequently treated with atorvastatin, 150 μg ml^{-1} acetyl-LDL±200 μg ml^{-1} HDL for further 48 h. After washing, cellular lipid was extracted and cholesterol mass was determined as described in Materials and methods. Results are expressed as μg sterol mg^{-1} protein and are the mean±S.D. of three independent experiments performed in duplicate wells.

[a] P<0.05.

3.3. Combination of avasimibe and atorvastatin

The effect of a 48-h incubation with avasimibe alone (0.2 and 0.5 μM) on cell cholesterol mass, using a simultaneous incubation system with acetyl-LDL and HDL, was compared to the effect of a combined treatment with this drug plus 5 μM atorvastatin. The reduction of cholesteryl ester mass increased from 16% to 26% at 0.2 μM, and from 20% to 39% at 0.5 μM avasimibe when atorvastatin was added, compared to avasimibe alone (Fig. 4). The inclusion of 200 μM mevalonate or 10 μM geranyl-geraniol reversed the effect of atorvastatin, returning to percentages of intracellular

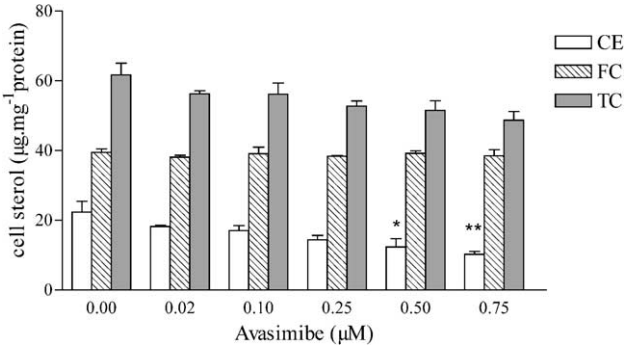

Fig. 3. Effect of avasimibe on cell sterol content in the simultaneous incubation system in the presence of 200 μg ml^{-1} HDL. PMA-treated THP-1 cells were incubated simultaneously with avasimibe and 150 μg ml^{-1} acetyl-LDL for 48 h. After washing, cellular lipid was extracted and total cholesterol (TC) and free cholesterol (FC) mass was determined as described in Materials and methods. Cholesteryl ester (CE) content was calculated by subtracting FC from TC. Results are expressed as μg sterol mg^{-1} protein, and are the mean ± S.D. of three independent experiments performed in duplicate wells. *P<0.05 and **P<0.01.

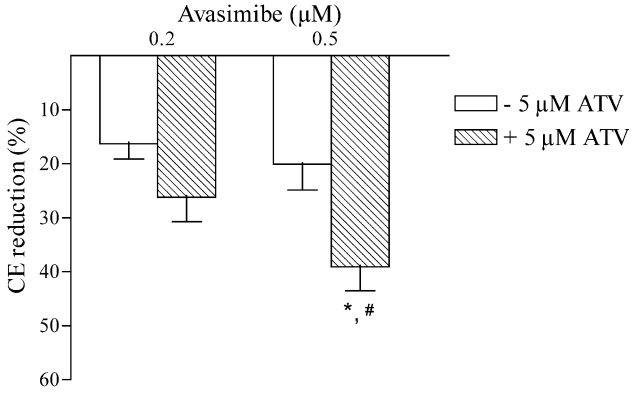

Fig. 4. Reduction of intracellular cholesteryl ester content (CE) in PMA-treated THP-1 cells incubated with 150 μg ml^{-1} acetyl-LDL and avasimibe (AVAS), alone or in combination with 5 μM atorvastatin (ATV), for 48 h in the presence of 200 μg ml^{-1} HDL. After treatment, cells were washed, cellular lipid was extracted and CE mass was determined as described in Materials and methods. Results are expressed as percentage of reduction of intracellular CE content referred to macrophages incubated with acetyl-LDL and HDL (32.05 ± 14.66 μg mg^{-1} protein, n=4). *P<0.05 vs. AVAS 0.5 μM. #P<0.05 vs. AVAS 0.2 μM.

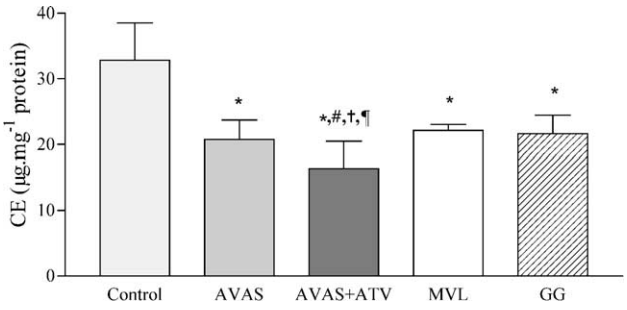

Fig. 5. Effect of mevalonate (MVL) and geranyl-geraniol (GG) on intracellular cholesteryl ester (CE) content in THP-1 cells. PMA-treated THP-1 cells were incubated with 150 μg ml^{-1} acetyl-LDL and 0.5 μM avasimibe (AVAS), ±5 μM atorvastatin (ATV) for 48 h in the presence of 200 μg ml^{-1} HDL. After treatment, cells were washed, cellular lipid was extracted and CE mass was determined as described in Materials and methods. Results are the mean ± S.D. of four experiments performed in duplicate wells. *$P<0.001$ vs. control; #$P<0.05$ vs. AVAS 0.5 μM; †$P<0.05$ vs. MVL; ¶$P<0.05$ vs. GG.

cholesteryl ester reduction similar to those obtained with avasimibe alone (Fig. 5).

4. Discussion

Accumulation of cholesteryl esters in human macrophages leads to the formation of foam cells and fatty streaks, the hallmark of atherosclerosis (Ross, 1986). We show here that the novel acyl-CoA:cholesterol acyltransferase inhibitor avasimibe, formerly known as CI-1011 (Lee et al., 1996), reduces cholesteryl ester content in human macrophages without increasing intracellular free cholesterol, and that this effect is enhanced by the hydroxymethyl glutaryl-CoA reductase inhibitor atorvastatin.

Our results show that the effect of the acyl-CoA:cholesterol acyltransferase inhibitor avasimibe is different depending on the incubation system used. Thus, avasimibe did not reduce intracellular cholesteryl ester content in the sequential incubation system (Table 1), an effect that has been also reported for other acyl-CoA:cholesterol acyltransferase inhibitors (Hakamata et al., 1995; Azuma et al., 1999). From these results, it is tempting to speculate that this class of drugs would not be efficient in the regression phase of atherosclerosis (Hakamata et al., 1995); however, in vivo studies (Bocan et al., 1991, 1993, 2001) have shown that acyl-CoA:cholesterol acyltransferase inhibitors indeed promote lesion regression. This discrepancy could mean that the mechanism by which avasimibe induces regression is related to processes other than reducing cholesteryl ester content in foam cells, as Bocan et al. (2001) suggested.

When using the simultaneous incubation system in the absence of HDL, avasimibe prevented the accumulation of cholesteryl ester (Fig. 2). Although the experiment was performed in the absence of HDL as an exogenous cholesterol acceptor, the reduction of cholesteryl ester was not accompanied by an increase in intracellular free cholesterol. In contrast with our results, incubation of murine (Hakamata et al., 1995; Warner et al., 1995; Kellner-Weibel et al., 1998), THP-1 (Azuma et al., 1999) or human monocyte-derived macrophages (Rodriguez et al., 1999) with other acyl-CoA:cholesterol acyltransferase inhibitors, together with acetyl-LDL in the absence of extracellular acceptors, significantly increased intracellular free cholesterol levels. Accumulation of free cholesterol in macrophages exposed to acyl-CoA:cholesterol acyltransferase inhibitors has been shown to be highly cytotoxic in vitro (Warner et al., 1995; Kellner-Weibel et al., 1998, 1999) and in vivo (Perrey et al., 2001). Thus, the lack of free cholesterol accumulation found in our study, despite a high degree of acyl-CoA:cholesterol acyltransferase inhibition, could indicate a better safety profile for avasimibe; this is in accordance with the excellent tolerability showed by this compound in a recent clinical study (Insull et al., 2001).

When HDL was added as an external cholesterol acceptor in the simultaneous incubation model, the effect of avasimibe on cholesteryl ester content was reduced. It has been observed that the IC_{50} for avasimibe decreases when the microsome concentration used in the assay of acyl-CoA:cholesterol acyltransferase activity was reduced, and that adding reconstituted lipids back to the media restores the IC_{50} value (Lee et al., 1996). To exclude the interference of lipids contained in the HDL particle, an experiment was performed where apolipoprotein A-I was used as an extracellular cholesterol acceptor instead of whole HDL. It was found that the presence of apolipoprotein A-I in the medium did not reduce the effect of avasimibe; on the contrary, the ability of avasimibe to reduce intracellular cholesteryl ester content was even enhanced (from 64 to 90%) when apolipoprotein A-I was included as an extracellular cholesterol acceptor. Thus, it seems that in the presence of HDL, there was an increase in the nonspecific binding of avasimibe to the lipid that reduced the number of drug molecules interacting with the acyl-CoA:cholesterol acyltransferase enzyme.

Several authors (Bocan et al., 1998, 2001; Junquero et al., 2001) have studied the effects of co-administration of an acyl-CoA:cholesterol acyltransferase inhibitor plus an hydroxymethyl glutaryl-CoA reductase inhibitor in animal models, and clinical studies are ongoing to evaluate the efficacy and safety of this combination therapy (Doshi et al., 2001). Thus, we were also interested in assessing the effects of a combination of avasimibe and atorvastatin on our in vitro model of cultured human macrophages.

The combination of both substances was more effective than avasimibe alone to inhibit intracellular cholesteryl ester accumulation; thus, the addition of 5 μM atorvastatin enhanced by approximately twofold ($P<0.05$) the effect of 0.5 μM avasimibe (Fig. 4).

The additive effects of hydroxymethyl glutaryl-CoA reductase and acyl-CoA:cholesterol acyltransferase inhibitors have been attributed to their action on different cell types of the arterial wall (Bocan et al., 2001). However, our results support the notion that both agents act synergistically at the macrophage level. Thus, the more marked reduction in the cholesteryl ester content of the thoracic aorta (Bocan et al., 1998, 2001) obtained with the combination therapy could be attributed, in light of our results, to a more profound reduction in the cholesteryl ester content of the macrophages of these areas.

The mechanism by which atorvastatin potentiates the effect of avasimibe seems to be related to the inhibition of hydroxymethyl glutaryl-CoA reductase and the depletion of some mevalonate derivatives, as the addition of mevalonate or geranyl-geraniol abolishes this effect (Fig. 5). According to Cignarella et al. (1998), statins inhibit cholesterol esterification in macrophages by blocking the delivery of cholesterol to the plasma membrane, a process which seems to depend on some proteins that require coupling to isoprene units to function correctly, probably a geranylgeranylated protein (Bernini et al., 1993, 1995).

On the other hand, although treatment with statins did not modify acyl-CoA:cholesterol acyltransferase activity directly in animal models (Alegret et al., 1998, Verd et al., 1999), it is well known that they may affect acyl-CoA:cholesterol acyltransferase activity indirectly, by limiting the availability of substrate for the esterification reaction (Bocan et al., 1998); this could explain the more profound effect of avasimibe on an enzyme that is already inhibited by the previous addition of atorvastatin.

In summary, we demonstrate that the novel acyl-CoA:cholesterol acyltransferase inhibitor avasimibe reduces cholesteryl ester accumulation in human THP-1 macrophages exposed simultaneously to acetyl-LDL, but not in macrophages that are already lipid loaded. The reduction of cholesteryl ester was not paralleled by an increase in free cholesterol levels, which points to a better safety profile for avasimibe. Moreover, we show here that avasimibe and atorvastatin act synergistically to reduce cholesteryl ester content in THP-1 cells, which could explain the in vivo results of Bocan et al. by a direct effect of both agents on the lipid content of the macrophages of the lesion area.

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