

Colesevelam HCl Decreases Atherosclerosis and May Activate Reverse Cholesterol Transport in Cholesterol-Fed Rabbits

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ABSTRACT

Objective: This study evaluated the effects of colesevelam on plasma lipids, aortic plaque area, and reverse cholesterol transport enzymes and genes in a rabbit model of atherosclerosis.

Methods: Thirty New Zealand white rabbits were fed standard chow for 1 week, placed on a 0.5% cholesterol diet for 2 weeks, then randomized equally to receive either the 0.5% cholesterol diet (control) or the 0.5% cholesterol diet with 1.5 g of colesevelam for 11 weeks. Serum total cholesterol and high-density

lipoprotein cholesterol concentrations and lecithin-cholesterol acyltransferase (LCAT) activity were measured before randomization and at treatment endpoint. Rabbits were killed and plaque area was measured in one aorta segment. Gene expression was measured by reverse transcription-polymerase chain reaction in liver RNA extracts.

Results: Colesevelam markedly reduced the circulating cholesterol concentration and decreased plaque area by 81% compared with controls ($P < 0.001$). LCAT fractional rate was markedly increased versus controls (12.6% and 0.2%, respectively; $P < 0.001$). Colesevelam enhanced fecal cholesterol concentrations more than two-fold. Colesevelam significantly increased hepatic gene expression for scavenger receptor type BI, apolipopro-

tein A1, LCAT, cholesterol ester transfer protein, cholesterol 7- α -hydroxylase (CYP7A1), low-density lipoprotein receptor, and liver X receptor (LXR).

Conclusions: In cholesterol-fed rabbits, colessevelam reduced atherosclerosis through cholesterol lowering and potentially by enhancing reverse cholesterol transport, mediated by changes in LXR-regulated proteins.

INTRODUCTION

Bile acid sequestrants (BAS) interrupt enterohepatic bile acid recirculation, thereby decreasing hepatic bile acid stores and promoting low-density lipoprotein (LDL) receptor upregulation to provide the cholesterol necessary for bile acid synthesis.¹ Whereas much attention has been focused on the effects of BAS on upregulation of LDL receptors and the compensatory increase in hepatic cholesterol synthesis, little information has been published on the effects of BAS on high-density lipoprotein (HDL) metabolism, particularly the various components of the reverse cholesterol transport pathway. HDL cholesterol (HDL-C) is presumably a major source of biliary cholesterol,² but a regulatory response to promote reverse cholesterol transport due to reduced hepatic bile acid stores has not been fully documented. Although both statins and BAS effectively lower LDL cholesterol (LDL-C) concentrations, these classes of drugs work through different mechanisms and may differ in their effects on HDL metabolism.

Although controversial, there is evidence that cholesterol returning to the liver in HDL via the scavenger receptor type B1 (SRB1) is preferentially used in the formation of biliary cholesterol that is excreted into the intestines.³ In the intestines, most bile acids are reabsorbed into the enterohepatic circulation by the

ileal bile acid transporter. Consequently, the majority of the HDL-C returned to the liver is reabsorbed into the circulation. Therefore, a BAS such as colessevelam HCl, by inhibiting bile acid reabsorption, may enhance reverse cholesterol transport through enhanced uptake of HDL via SRB1 and/or increased apolipoprotein (apo) A1 synthesis.

Cholesterol homeostasis is maintained through cholesterol synthesis, absorption of intestinal cholesterol and bile acids, and excretion of these compounds into bile.⁴ The oxysterol liver X receptor (LXR) plays a major role in the regulation of cholesterol homeostasis, stimulating hepatic bile acid synthesis, modulating intestinal cholesterol absorption, and enhancing the expression of many of the genes involved in reverse cholesterol transport.⁵ In contrast, Farnesoid X receptors (FXR) protect hepatic cells from bile acid toxicity by repressing bile acid import into hepatocytes and by stimulating their biliary excretion. FXR activation also induces the expression of small heterodimer partner (SHP), which appears to inhibit LXR-mediated gene activation.⁶⁻⁸ The LXR-mediated genes include apo A1, lecithin-cholesterol acyltransferase (LCAT), cholesterol ester transfer protein (CETP), and SRB1, which are all involved in reverse cholesterol transport. LXR also activates cholesterol 7- α -hydroxylase, the rate-limiting enzyme in bile acid synthesis, as well as the gene for production of the LDL receptor.^{9,10} Therefore, the interruption of enterohepatic bile acid reabsorption by BAS is likely to have some effect on FXR and LXR activation.

The purpose of this study was to evaluate the effects of colessevelam HCl, a potent, specifically engineered BAS, on atherosclerotic plaque development and hepatic expression of genes for proteins involved in HDL metabolism. The

cholesterol-fed rabbit was the animal model chosen because its bile acid physiology and components of the reverse cholesterol pathway are similar to those in humans.

METHODS

Animals and Diet

Thirty New Zealand white rabbits (Charles River Laboratories, Inc, Wilmington, MA) received standard rabbit chow for 1 week (starting at week 0) and then were placed on a 0.5% cholesterol diet (Research Diets, Inc, New Brunswick, NJ). After 2 weeks on the high-cholesterol diet (week 3), the rabbits, weighing approximately 3 kg each, were randomly assigned to 2 groups (n=15 per group). One group received 1.5 g/day of colestevlam HCl premixed into the 0.5% cholesterol diet, whereas the other group (controls) continued on the 0.5% cholesterol diet. Colestevlam HCl was provided by Sankyo Pharma Inc, Parsippany, NJ.

Blood was collected in EDTA tubes via the central auricular artery at weeks 1, 2, 6, 10, and 14. Plasma cholesterol and HDL-C concentrations and LCAT and CETP activity were measured by the methods described below.

Analysis of Atherosclerosis and Lesion Composition

The rabbits were killed at the end of the treatment period; the aortas were removed for analysis of atherosclerosis and lesion composition, and a portion of each liver was removed for evaluating gene expression by reverse transcription polymerase chain reaction (RT-PCR). One segment of blood vessel from each rabbit from the aortic arch to the iliac arteries was placed in formalin and sent to Pathology Associates Inc, Boston, MA. The vessels were cut axially and pinned open on a piece of dental wax, which was placed in a tissue cassette containing the animal identification. The

vessels were stained with Sudan IV for at least 24 hours. Each vessel segment was photographed with 1-cm metric calibration marks using a digital camera.

Morphometry was performed using a Bioquant TCW98 image analysis system (R&M Biometrics, Nashville, TN) that was calibrated using rulers present in each image. The following measurements were made: intimal area (total area of the opened vessel) and plaque area (the area stained dark red). The intimal area was measured by tracing the perimeter of the opened vessel's endothelial surface. The thresholding feature was used on the dark red staining to calculate plaque area. Intimal and plaque areas were measured in square millimeters and percent plaque area was then calculated by the formula $100 \times \text{plaque area/intimal area}$.

Lipid and Lipoprotein Analysis

Total and free cholesterol were measured in the rabbit plasma using Wako kits (Wako Chemicals USA, Richmond, VA). HDL-C was measured after precipitation of apo B-containing lipoproteins with heparin-Mn²⁺.¹¹

LCAT Activity

LCAT was assayed by measuring the rate of esterification using cholesterol labeled with radioactive carbon (¹⁴C).¹² A [¹⁴C] cholesterol-albumin emulsion was prepared as described by Stokke and Norum.¹² The plasma samples were preincubated for 30 minutes at 37°C with 2 mmol/L freshly made 5,5'-dithio-bis [2-nitrobenzoic acid (DTNB)] in 0.2 mol/L phosphate-buffered saline, pH 7.0, followed by incubation with labeled albumin-cholesterol complex for 4 hours to equilibrate with endogenous cholesterol. The inhibition by DTNB was released by adding 10 mmol/L mercaptoethanol, and the sample incubated for 30 minutes. The reaction was stopped by adding 1 mL of ethanol and 50 µL of

Table 1. Total Cholesterol, High-Density Lipoprotein Cholesterol (HDL-C), and Total/HDL-C Ratio Levels by Treatment Group and Week of Study

| Parameter | Study Week | Control (n=15) | Colesevelam HCl (n=15) | P Value |
|--------------------------------------|------------|----------------|------------------------|---------|
| Mean total cholesterol, mmol/L (SD)* | 2 | 11.68 (3.65) | 12.57 (4.03) | 0.530 |
| | 6 | 24.42 (9.85) | 1.23 (0.86) | <0.001 |
| | 10 | 25.80 (9.00) | 1.14 (0.29) | <0.001 |
| | 14 | 64.77 (20.23) | 3.74 (3.23) | <0.001 |
| Mean HDL cholesterol, mmol/L (SD)* | 2 | 0.64 (0.13) | 0.66 (0.28) | 0.723 |
| | 6 | 0.83 (0.38) | 0.42 (0.28) | 0.003 |
| | 10 | 1.24 (1.29) | 0.44 (0.24) | 0.047 |
| | 14 | 0.94 (1.09) | 0.60 (0.26) | 0.654 |

*To convert mmol/L to mg/dL, divide values by 0.02586.
SD = standard deviation.

5% trichloroacetic acid. Lipids were extracted with 2 mL hexane (twice), and free cholesterol and cholesterol ester were separated by thin-layer chromatography using hexane:ethyl ether (85:15 v/v) as the solvent system. The percent conversion of cholesterol to cholesterol-ester (fractional esterification rate) and molar esterification rate (nmol of cholesterol esterified per hour per mL of plasma) were calculated.

CETP Activity

CETP activity was determined as the percent transfer of cholesterol ester from HDL-C to apo B-containing lipoproteins.¹³ LCAT activity in the plasma samples was inhibited by the addition of 2 mmol/L DTNB. HDL-C was separated from apo B-containing lipoproteins at 0 and 3 hours by heparin-Mn²⁺ precipitation and collected by centrifugation at 4000g for 20 minutes. In the case of severely hyperlipidemic plasma, the centrifugation was repeated. Total and free cholesterol were meas-

ured in the supernatant. The decrease in cholesterol ester (total cholesterol minus free cholesterol) from 0 to 3 hours was calculated, and CETP activity was expressed as the percent HDL-C ester decrease during 3 hours of incubation.

Gene Expression

RNA was extracted from 250 mg of rabbit liver with TRI Reagent (Molecular Research Center, Inc, Cincinnati, OH). Samples were homogenized in liquid nitrogen and then suspended in 2.5 mL of TRI Reagent. The aqueous phase was separated with bromochloropropane and precipitated with 0.8 volume of isopropanol. To get pure RNA, the precipitate of total RNA was reextracted using an RNeasy Mini Kit (Qiagen Inc, Valencia, CA). RT-PCR was carried out in a GeneAmp PCR System 9600 (Applied Biosystems, Foster City, CA) thermocycler. Reverse transcription (RT) was carried out using the ImProm II Reverse Transcription System (Promega, Madison, WI). Polymerase

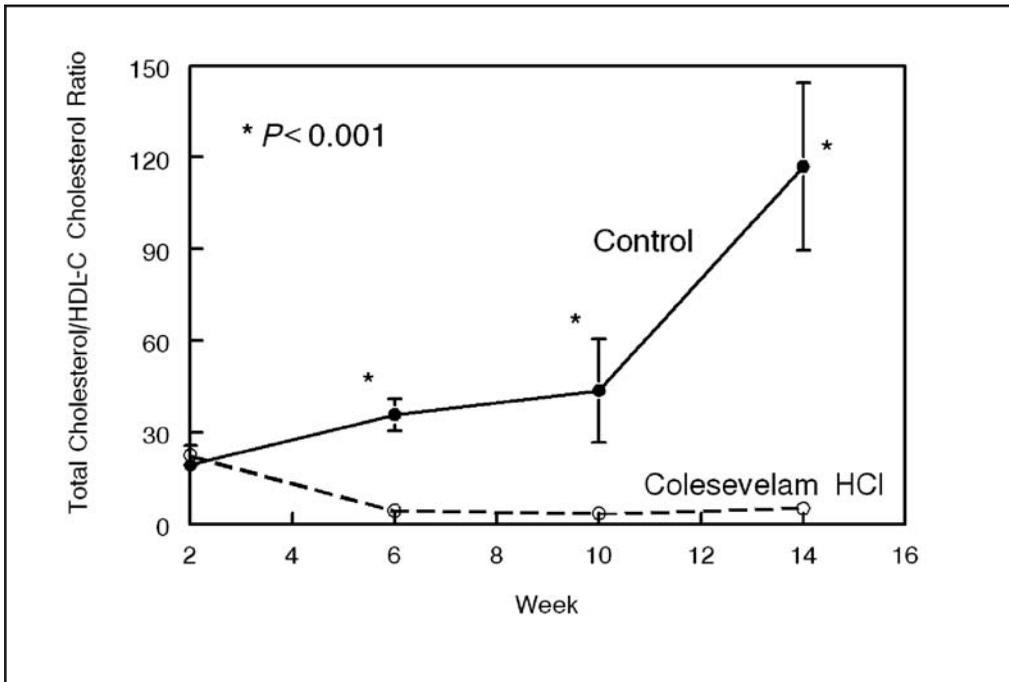


Figure 1. Total cholesterol/high-density lipoprotein cholesterol (HDL-C) ratios by treatment group and week of study.

chain reaction (PCR) was completed using Master Taq Kit from Eppendorf Inc (Westbury, NY). Both RT and PCR were performed using the conditions recommended by the manufacturers. The primer sequences were as described by Rea et al¹⁴ and Wu et al.¹⁵

Statistical Analyses

Statistical modeling was performed using SAS Version 8.2 (SAS Institute, Cary, NC). All tests for significance were performed at $\alpha=0.05$, 2-tailed. Analysis of variance models were used to assess between-group differences at each time point for each variable. Normality assumptions were tested using the Shapiro-Wilk test. If the data were found not to approximate a normal distribution, values were ranked prior to generating the models.

RESULTS

Lipid values for each of the groups are

shown in Table 1. The colesevelam HCl-treated rabbits had markedly lower total cholesterol concentrations than the control group during the treatment period. The mean total cholesterol concentration for the control animals at week 14 was 64.77 mmol/L (2504.6 mg/dL) compared with 3.74 mmol/L (144.6 mg/dL) in the colesevelam HCl group ($P<0.001$). HDL-C concentrations were lower in the colesevelam HCl group during treatment and the differences between groups were statistically significant at weeks 6 and 10. The total cholesterol/HDL-C ratios were significantly lower in the colesevelam HCl group throughout the treatment period ($P<0.001$) (Figure 1).

LCAT activity (fractional esterification rate) was markedly increased compared with controls in the colesevelam HCl group at all time points during treatment ($P<0.001$) (Figure 2A). When expressed as molar esterification rates,

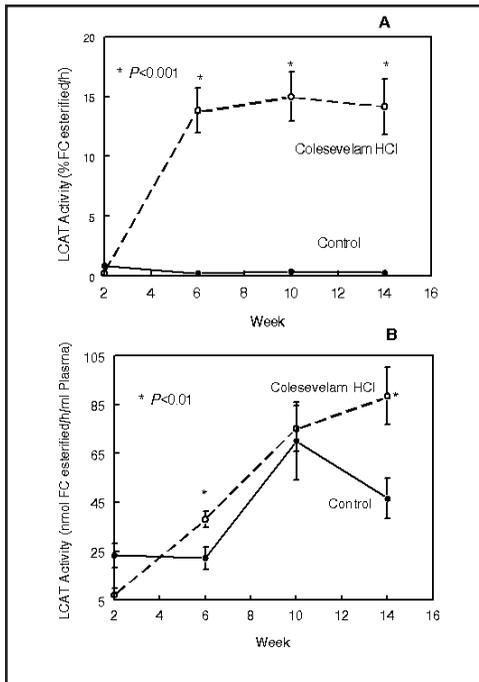


Figure 2. Lecithin-cholesterol acyltransferase (LCAT) fractional esterification rates (A) and molar esterification rates (B) by treatment group and week of study. LCAT = lecithin-cholesterol acyltransferase; FC = cholesterol.

the differences were less marked because of the reduction in the unesterified cholesterol concentration following treatment ($P < 0.01$) (Figure 2B). CETP activity was not significantly different between the colesevelam HCl and control groups at week 14 (results not shown). Plaque area was 81% lower in the colesevelam HCl group than in controls ($P < 0.001$) (Figure 3). Fecal cholesterol concentrations were higher in the colesevelam HCl group than in controls (1.09 g/day vs. 0.53 g/day, respectively; $P = 0.037$) (Figure 4).

Hepatic gene expression for LXR and the expression of genes regulated by LXR, including SRB1, apo A1, LCAT, CETP, CYP7A1, and the LDL receptor, were all significantly higher in the colesevelam HCl group than in controls (Figure 5).

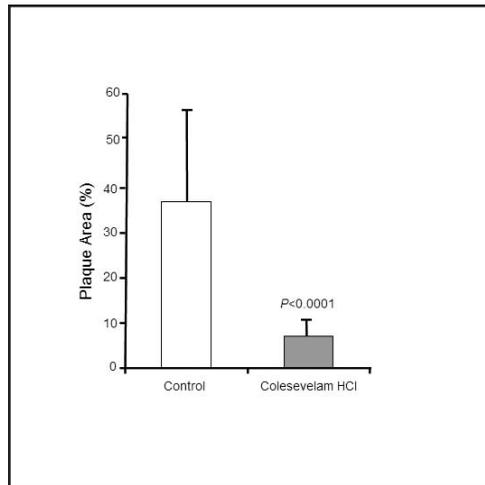


Figure 3. Plaque area by treatment group at week 14.

DISCUSSION

The results of this study show colesevelam HCl, a specifically engineered BAS, markedly reduces plasma cholesterol concentrations and aortic atherosclerosis in cholesterol-fed rabbits. Although cholesterol lowering alone may explain the anti-atherosclerotic effect of colesevelam HCl, the hepatic gene expression data support the hypothesis that inhibition of bile acid reabsorption enhances the expression of genes involved with reverse cholesterol transport, which may also have played a role in the anti-atherosclerotic effect of the drug. There were significant increases in the gene expression for apo A1, LCAT, and CETP in the colesevelam HCl-treated animals compared with controls, probably mediated by an enhanced expression of LXR. LXR agonists have been documented to enhance expression of apo A1, CETP, and ABCA1 and ABCG1, which are involved in promoting cellular efflux of cholesterol from the periphery, which is carried by HDL particles to the liver for conversion into bile salts.

Cholesterol feeding in rabbits has been documented to increase cellular levels of oxysterols, which rapidly

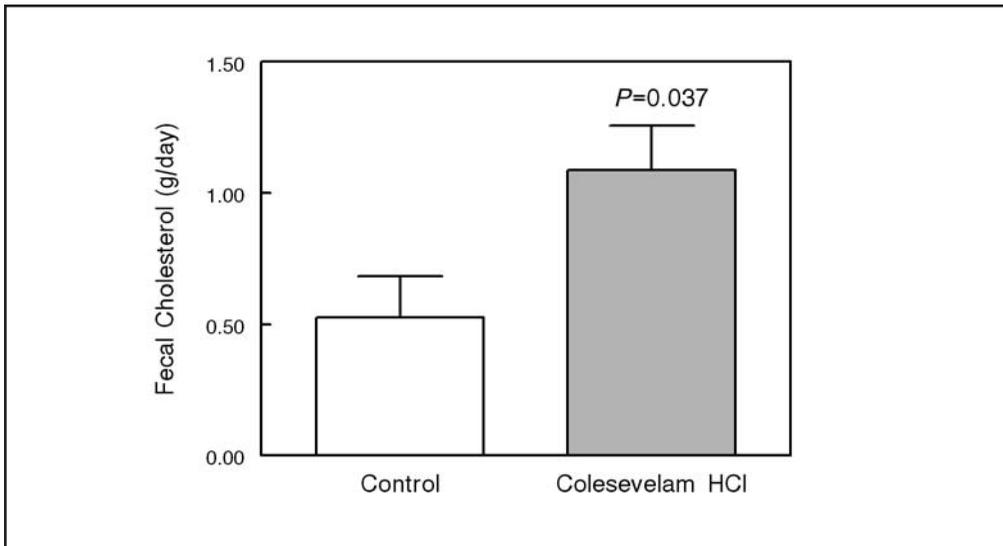


Figure 4. Fecal cholesterol concentration by group at week 14.

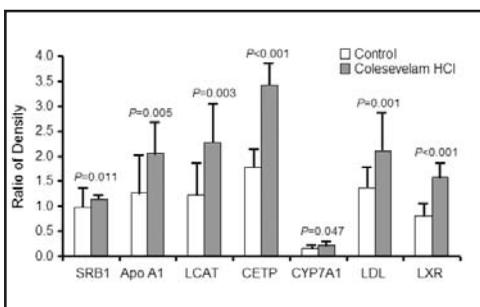


Figure 5. Hepatic gene expression by treatment group at week 14. SRB1 = scavenger receptor type B1; apo A1 = apolipoprotein A1; LCAT = lecithin-cholesterol acyltransferase; CETP = cholesteryl ester transfer protein; CYP7A1 = cholesterol 7- α -hydroxylase; LDL = low-density lipoprotein; LXR = liver X receptor.

increase expression of hepatic LXR and its target genes, such as ABCA1 and CETP.¹⁶ After long-term cholesterol feeding, the bile-acid pool size increases, resulting in activation of FXR.¹⁶

Although cholesterol feeding alone appears to increase expression of LXR and FXR in rabbits, colesevelam HCl treatment, while significantly lowering the serum cholesterol concentration,

also appears to further upregulate LXR.

A significant increase in fecal cholesterol concentrations in the rabbits treated with colesevelam HCl also supports the hypothesis that BAS may enhance reverse cholesterol transport. The LXR induction of ABCG5 and ABCG8 results in enhanced transport of hepatic cholesterol into bile, as well as increased efflux of intestinal cholesterol from the enterocyte back into the gut, thereby decreasing net cholesterol absorption.¹⁷ However, the possibility that colesevelam HCl directly inhibits intestinal cholesterol absorption cannot be ruled out as a cause of increased fecal cholesterol concentrations. Lastly, a marked increase in LCAT activity in the colesevelam HCl-treated rabbits further supports the conclusion that inhibiting bile acid reabsorption and depleting hepatic bile acid stores results in stimulation of reverse cholesterol transport.

The mechanism by which colesevelam HCl may increase LXR is unclear. Since oxysterols are the ligands for LXR activation, BAS, by interrupting bile acid reabsorption, should decrease hepatic

cholesterol stores and reduce LXR activation. However, in the cholesterol-fed rabbit, which has a rich supply of plasma cholesterol, BAS, by suppressing FXR activity, may reduce SHP, which would, in turn, inhibit LXR. Therefore, BAS, by reducing SHP activity, may increase activation of LXR in a cholesterol-rich state. This potential mechanism is supported by a previous study in cholesterol-fed rabbits treated with a competitive inhibitor of the ileal apical sodium-dependent bile acid cotransporter.¹⁸ In this study, rabbits were treated with a competitive inhibitor of apical sodium-dependent bile cotransporter SC-435, and the expression of SHP was markedly reduced.¹⁸

LXR activation also increases expression of sterol response element binding protein and fatty acid synthase, which results in enhanced triglyceride production.¹⁹ This may explain the mechanism by which BAS treatment increases plasma triglyceride levels. Bile acids, by activating FXR, induce the expression of SHP. SHP interferes with SREBP-1c expression by inhibiting the activity of LXR and eventually other transcription factors that stimulate SREBP-1c expression.⁸ In this study, colessevelam HCl did not increase the HDL-C concentration, but the enhancement of the expression of apo A1, LCAT, and, possibly, ABCA1 may have been offset by an increase in CETP gene expression, which would tend to lower the HDL-C concentration. Therefore, although reverse cholesterol transport may be enhanced with BAS administration, the net effect on the plasma HDL-C concentration may have been a reduction from baseline. In an apo E knockout mouse model of atherosclerosis, enhanced expression of LXR did not increase HDL-C concentration if CETP was also present.²⁰

Statins appear to inhibit LXR, at least in macrophages, probably by lower-

ing cellular oxysterol levels.²¹ Although statins have proven cardiovascular outcome benefits, the anti-atherosclerotic efficacy may be counteracted to some extent by reduced LXR ligand formation. LXR agonists have been shown to decrease atherosclerosis in mice, possibly through their putative effects on HDL-C metabolism.²² However, upregulation of the lipogenesis target genes SREBP-1c and FAS by LXR, as well as the genes involved in reverse cholesterol transport, results in hypertriglyceridemia and hepatic steatosis.²³ Therefore, the potential clinical use of LXR agonists to reduce atherosclerosis in humans requires a dose that allows enhancement of reverse cholesterol transport, while not significantly inducing fatty acid synthesis. The results of this study suggest the intriguing hypothesis that BAS, by enhancing reverse cholesterol transport, might not only further enhance LDL-C reduction when used in combination with a statin, but also offset the effects of statins to reduce LXR activity in macrophages. Although limited data are available from clinical trials that included both a BAS and a statin or another lipid-lowering agent, the results to date have suggested larger declines in clinical events than those from statin monotherapy trials in which similar degrees of LDL-C reduction have been achieved.²⁴⁻²⁷

Additional research is warranted to evaluate the hypothesis that the combination of a BAS with a statin might enhance reverse cholesterol transport and produce additive or synergistic anti-atherosclerotic effects.

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