Inhibition of ileal bile acid transport and reduced atherosclerosis in apoE$^{-/-}$ mice by SC-435

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Abstract Blocking intestinal bile acid absorption by inhibiting the apical sodium codependent bile acid transporter (ASBT) is a target for increasing hepatic bile acid synthesis and reducing plasma LDL cholesterol. SC-435 was identified as a potent inhibitor of ASBT (IC$_{50}$ = 1.5 nM) in cells transfected with the human ASBT gene. Dietary administration of 3 mg/kg to 30 mg/kg SC-435 to apolipoprotein E$^{-/-}$ (apoE$^{-/-}$) mice increased fecal bile acid excretion by >2.5-fold. In vivo inhibition of ASBT also resulted in significant increases of hepatic mRNA levels for cholesterol 7α-hydroxylase and HMG-CoA reductase. Administration of 10 mg/kg SC-435 for 12 weeks to apoE$^{-/-}$ mice lowered serum total cholesterol by 35% and reduced aortic root lesion area by 65%. Treatment of apoE$^{-/-}$ mice also resulted in decreased expression of ileal bile acid binding protein and hepatic nuclear hormone receptor small heterodimer partner, direct target genes of the farnesoid X receptor (FXR), suggesting a possible role of FXR in SC-435 modulation of cholesterol homeostasis. In dogs, SC-435 treatment reduced serum total cholesterol levels by ≤12% and, in combination with atorvastatin treatment, caused an additional reduction of 25%.⬛ These results suggest that specific inhibition of ASBT is a novel therapeutic approach for treatment of hypercholesterolemia resulting in a decreased risk for atherosclerosis.—Bhat, B. G., S. R. Rapp, J. A. Beaudry, N. Napawan, D. N. Butteiger, K. A. Hall, C. L. Null, Y. Luo, and B. T. Keller. **Inhibition of ileal bile acid transport and reduced atherosclerosis in apoE$^{-/-}$ mice by SC-435.** *J. Lipid Res.* 2003. **44:** 1614–1621.

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Many epidemiological and prospective studies have identified hypercholesterolemia and elevated LDL as risk factors for coronary artery disease (CAD). Several randomized clinical trials with cholesterol-lowering drugs in a variety of populations, including patients with or without established cardiovascular disease and patients with severe or moderate hypercholesterolemia, have demonstrated that reduction of plasma LDL cholesterol levels leads to decreased mortality due to cardiovascular disease (1). Although statins are the most widely used agents to lower LDL cholesterol levels and demonstrate significant clinical benefits, alternate and/or additional treatment strategies to further reduce LDL cholesterol and CAD risk are also being studied (2). One alternative mechanism to lower LDL cholesterol is to therapeutically modulate fecal bile salt wasting and compensatory hepatic up-regulation of bile acid biosynthesis from cholesterol (3).

As an important component of the enterohepatic circulation, the apical sodium codependent bile acid transporter (ASBT) mediates the active reabsorption of conjugated bile acids in the terminal ileum (4). A specific inhibitor of the ASBT protein would block the reabsorption of bile acids in the ileum and promote their excretion in the feces, thereby reducing the amount of bile acids returning to the liver. The reduction in the bile acid pool due to increased fecal loss following administration of an ASBT inhibitor is expected to result in increased hepatic oxidation of cholesterol to bile acids, eventually depleting the liver pool of esterified cholesterol (3). In order to maintain liver cholesterol levels to support bile acid synthesis, hepatocytes increase both de novo synthesis of cholesterol and expression of cell surface LDL receptors (LDLRs). Increased expression of LDLRs leads to increased hepatic uptake of LDL cholesterol, thereby reducing serum LDL levels, a primary therapeutic effect. Validation for the concept of blocking enterohepatic circulation of bile acids comes from the Program on the Surgical Control of Hyperlipidemias (POSCH) trial, in which partial ileal bypass surgery to reduce ileal bile acid absorption resulted in significant reductions in both plasma LDL levels and CAD (5). Hence, specific ASBT inhibition is an attractive therapeutic target to lower LDL cholesterol. Moreover, with the

Abbreviations: ASBT, apical sodium codependent bile acid transporter; CAD, coronary artery disease; Cyp7α1, cholesterol 7α-hydroxylase; FXR, farnesoid X receptor; HMGR, HMG-CoA reductase; IBABP, intestinal bile acid binding protein; SHP, small heterodimer partner.

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target protein expressed on the luminal surface of the enterocytes, an inhibitor of ASBT would not have to gain access to the systemic circulation, thereby affording an additional safety factor for this approach. The cloning and expression of human ASBT (6, 7) have provided us a method to identify selective inhibitors of this transporter as potential drug candidates for treating hypercholesterolemia. Here we report on SC-435, a potent and selective small-molecule ASBT inhibitor. We demonstrate mechanism-based hypocholesterolemic properties of SC-435 in apolipoprotein $E^{-/-}$ (apoE$^{-/-}$) mice and dogs and the antiatherosclerotic properties in the apoE$^{-/-}$ mouse model of atherosclerosis. In addition, inhibition of ASBT causes a further reduction in serum LDL cholesterol when coadministered in combination with a statin. Thus, ASBT inhibition is a novel therapeutic approach for reduction of LDL cholesterol and CAD.

MATERIALS AND METHODS

Baby hamster kidney cells transfected with human ASBT and bile acid uptake assay

Culture conditions for baby hamster kidney (BHK) cells expressing functional human ASBT and the 96-well cellular bile acid uptake assay using 5 μM [^{14}C]taurocholic acid were performed as described previously (8). Specificity of the ASBT inhibitor was tested against sodium-dependent [^{3}H]alanine uptake in these cells under identical conditions.

Bile acid transport assays

CaCo-2 cells were cultured on Transwell filters (0.4 μm pore, 6.5 mm) at confluence for 3 weeks. Radiolabled taurocholic acid (^{14}C, 6 μM, 0.3 μCi/ml) in 100 μl HBSS was added to apical chamber in the absence or presence of 0 to 100 nM SC-435, and 600 μl HBSS was added to the basolateral chamber. After a 2 h incubation, radioactivity in an aliquot of basolateral chamber buffer was quantitated. Taurocholate (TC) transport was determined as a percentage of the total apical chamber [^{14}C]TC transported to the basolateral chamber.

Animals

The Institutional Animal Care Committee approved all animal procedures and experiments. Male apoE$^{-/-}$ mice on C57BL/6J genetic background, ∼8 weeks old, were obtained from the Jackson Laboratory (Bar Harbor, ME). Purpose-bred adult male beagle dogs were obtained from Marshall Farms (North Rose, NY). Animals were single-housed in a constant-temperature environment with alternating 12 h light and dark cycles and were given free access to food and water.

Diets, dosing, and sample collection

ApoE$^{-/-}$ mice were fed a pelletted Western high-fat diet containing 0.15% cholesterol (Research Diets, New Brunswick, NJ; Diet #D12079B) without or with 0.002, 0.006, or 0.018 g % SC-435 (equivalent to 3, 10, and 30 mg/kg/day) for 6 weeks. For the atherosclerosis study, apoE$^{-/-}$ mice were fed a Western high-fat diet without or with 0.006 g % SC-435 for 12 weeks. There was no adverse effect on visible health during the 12 week feeding of SC-435 in these mice. Following a 4 h fasting, mice were sacrificed, blood was collected, and heart tissue was processed for atherosclerosis evaluation as described below.

Dogs were fed a standard pelletted chow diet (Ralston Purina, St. Louis, MO) and were grouped based on pretreatment serum total cholesterol values and body weights. Animals were dosed daily for 4 weeks with 2.0 mg/kg atorvastatin, 0.33, 1.0, 3.0, or 9.0 mg/kg SC-435 alone, or combination doses of 2.0 mg/kg atorvastatin + 1 mg/kg SC-435 or 2.0 mg/kg atorvastatin + 9.0 mg/kg SC-435 or vehicle. All doses were administered per os in gelatin capsules to each dog between 9:00 and 9:30 AM prior to feeding. Blood samples were taken at the end of each week after an overnight fast for comparison with pretreatment serum total cholesterol levels. Three consecutive 24 h fecal samples were collected from individual dogs in all treatment groups during the last 72 h period of each week of treatment and used to determine the fecal bile acid concentration.

Evaluation of aortic root atherosclerosis

The heart was separated from the aorta and was then cut between the tips of the atria for aortic root sectioning. The aortic root was embedded into paraffin and sectioned as described (9). Serial 5 micron thick sections were cut with a Leica RM2035 or RM2155 rotary microtome, processed, and stained. The first slide taken for hematoxylin and eosin staining was recognized by the presence of valves from the aortic semilunar valve at the base of the aortic root. Then the following five slides posterior to the valve cusps, each 50 microns apart, were also stained. Image analysis of six aortic root stained slides from each animal were done using Optimus Image Analysis software V 6.5 (Media Cybernetics, Silver Spring, MD). The lesion area was quantitated using a Sony DKC ST5 Digital Camera (Sony Corporation, Japan) connected to an Olympus BX50 microscope.

Taqman gene analysis

ApoE$^{-/-}$ mouse ileal and liver RNA was isolated using the Qiagen RNA purification Kit according to manufacturer's instructions and was used in real-time PCR using Applied Biosystems one-step Taqman reagents and ABI 7700 Taqman machine. Primer probe sets for cholesterol 7α-hydroxylase (Cyp7α1), HMG-CoA reductase (HMGR), ASBT, intestinal bile acid binding protein (IBABP), small heterodimer partner (SHP), and LDLR were designed based on published mouse sequences. The results were analyzed by SDS calculator and expressed as fold changes after correcting for cyclophilin levels.

Serum and liver lipids

Liver lipids were extracted as described previously (10) and resuspended in PBS containing 3% Triton X-100 for lipid analysis. Lipoproteins were fractionated using equal volumes of pooled serum samples on Superose 6 HR 10/30 column fast protein liquid chromatography (FPLC) column in 1 mM EDTA, 0.15 M NaCl, and 0.02% sodium azide. Serum, FPLC fraction, and liver total cholesterol and triglycerides were measured colorimetrically using commercial kits from Wako Chemicals USA, Inc., Richmond, VA (276-64909) and Sigma Diagnostics, St. Louis, MO (337-B), respectively.

Fecal bile acid and neutral sterol measurements

For bile acid measurements, fecal materials were collected over a 72 h period, weighed, and extracted with 50% v/v t-butanol in water for 45 min at 37°C and centrifuged at 2,000 g. The concentration of bile acids (μmol/g) in the supernatant was determined by a spectrophotometric method described using 3α-hydroxysteroid dehydrogenase and using sodium TC standard (11). For fecal neutral sterol measurements, the fecal samples collected over a 72 h period were dried, weighed, and ground to a fine powder. An aliquot of 0.1 g of feces was saponified with 0.3 N KOH at 70 C for 1 h followed by extraction with petroleum ether to determine fecal neutral sterols by gas-liquid chromatog-

raphy (Hewlett Packard HP6890 GC system using Ultra2 5% Phenyl column and flame ionization detector).

Statistical analysis

A two-tailed, paired Student's *t*-test (12) without an assumption of equal variance was used to determine the statistical significance of changes in serum lipids in treated animals compared with pretreatment values. A two-tailed, two-sample, Student's *t*-test without an assumption of equal variance was used to determine the statistical significance of changes in serum total cholesterol and fecal bile acids in SC-435-treated animals compared with controls. A *P* value <0.05 was considered significant.

RESULTS

The potency and selectivity of ASBT inhibitors was assessed with transfected BHK cells that constitutively express human ASBT (8). Using a [^{14}C]TC uptake assay in these cells, we identified SC-435 as a potent inhibitor of human ASBT. Selectivity was measured in the same assay system, by replacing [^{14}C]TC with [^{3}H]alanine to determine the effect of the compound on amino acid uptake, another cellular sodium-dependent cotransporter. As shown in **Fig. 1A**, SC-435 [(1-[4-[4-[(4*R*, 5*R*)-3,3-dibutyl-7-(dimethyl-amino)-2,3,4,5-tetrahydro-4-hydroxy-1,1-dioxido-1-ben-zothiepin-5-yl]phenoxy]butyl]4-aza-1-azoniabicyclo[2.2.2] octane methanesulfonate (salt)] is a specific ASBT inhibitor with an IC$_{50}$ = 1.5 nM having more than 66,000-fold selectivity, as determined by sodium-dependent alanine uptake.

To determine the effects of SC-435 on bile acid transport by endogenous ASBT in human cells, we used the CaCo2 human colon carcinoma cell line grown on permeable transwell membranes. As shown in Fig. 1B, when differentiated CaCo2 cells were incubated with [^{14}C]TC and SC-435 in the apical chamber, a dose-dependent decrease in transcellular transport of bile acid into the basal chamber was observed, with almost complete inhibition at 100 nM. Thus, results shown in Fig. 1 demonstrate that SC-435 is a potent and selective inhibitor of ASBT and blocks both bile acid uptake and transport in two in vitro systems.

The apoE$^{-/-}$ mouse is a spontaneous atherosclerosis model and has been extensively used to study the role of cholesterol and various gene products on the development of atherosclerosis (13, 14). In this report, we attempted to characterize the effects of SC-435 on the enterohepatic metabolism of sterols and their relationship to atherosclerosis in these animals. As shown in **Fig. 2**, we treated apoE$^{-/-}$ mice with 0.002%, 0.006%, or 0.018% SC-435 (~3, 10, and 30 mg/kg/day) in a Western high-fat diet for 3 weeks and measured their fecal bile acid excretion. Treatment of apoE$^{-/-}$ mice with SC-435 caused a significant increase in fecal bile acids at all doses studied (2.2- to 2.5-fold), although the effects were not observed to be dose dependent. These data suggest that we may have reached the optimal efficacious dose with as little as 3 mg/kg/day treatment.

In the same study, we also measured fecal neutral sterol excretion in these mice to determine whether inhibiting SC-
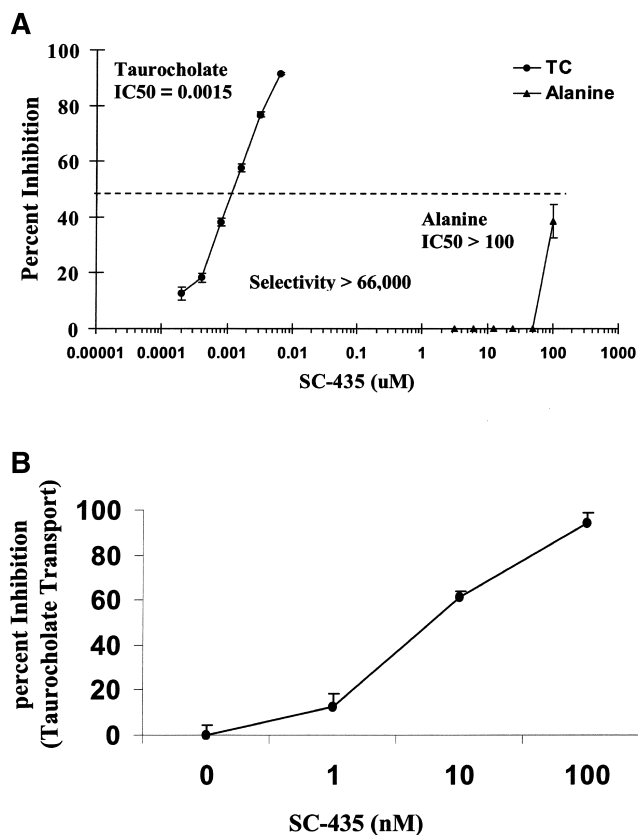

A

B

Fig. 1. In vitro inhibition of apical sodium codependent bile acid transporter (ASBT) by SC-435. A: In vitro potency and selectivity of SC-435 using baby hamster kidney cells transfected with human ASBT. Cell uptake of [^{14}C]taurocholate (TC) and [^{3}H]alanine were measured as described in Materials and Methods. Results are mean ± SD. Inset, chemical structure of SC-435: 1-[4-[4-[(4*R*,5*R*)-3,3-dibutyl-7-(dimethylamino)-2,3,4,5-tetrahydro-4-hydroxy-1,1-dioxido-1-ben-zothiepin-5-yl]phenoxy]butyl]4-aza-1-azoniabicyclo[2.2.2]octane methanesulfonate (salt). B: Effect of SC435 on TC transport in CaCo-2 cells. CaCo2 cells were cultured on Costar filter plates (0.4 μm pore, 6.5 mm) at confluence for 3 weeks. Cells were incubated with 6 μM [^{14}C]taurocholic acid (0.3 uCi/ml) in 100 μl HBSS in the absence or presence of the indicated concentrations of SC-435 in the apical chamber. After 2 h incubation, basolateral chamber medium was collected and an aliquot was counted. TC transport was presented as percentage of total radioactivity transported to the basolateral chamber. Values are mean ± SEM.

435-mediated bile acid absorption had any influence on cholesterol absorption. The results indicated that SC-435 treatment for 3 weeks had no significant effect on fecal neutral sterol excretion in apoE$^{-/-}$ mice (Control, 13.91 ± 1.13; 10 mg/kg/day SC-435, 16.76 ± 1.35; and 30 mg/kg/day SC-435, 16.03 ± 1.17 mg neutral sterols/g feces).

To determine the effects of chronic treatment of SC-435 on serum lipids and on the development of atherosclerosis, apoE$^{-/-}$ mice were fed a Western high-fat and high-cholesterol diet without or with 0.006% (10 mg/kg/day) SC-435 for 12 weeks. No significant differences in food intake, measured at week 3 of treatment, or in body weight, measured weekly, were observed (data not shown). Serum lipids were monitored at weeks 3, 6, and 12 of treatment. As shown in **Fig. 3A**, serum total cholesterol levels were not
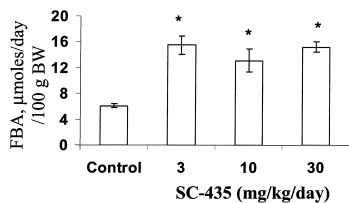
Fig. 2. Effect of SC-435 on fecal bile acid excretion in apolipoprotein E$^{-/-}$ (apoE$^{-/-}$) mice. Animals were treated with SC-435 at indicated levels in Western high-fat diet for 3 weeks. Fecal materials were collected over 72 h and fecal bile acids were extracted and quantitated as described in Materials and Methods. Values are mean $\pm$ SEM. * $P < 0.005$.

significantly affected after 3 weeks of SC-435 treatment. However, after 6 and 12 weeks of treatment, there were significant decreases of 16% and 35% in serum total cholesterol compared with control animals (Fig. 3A). As shown in Fig. 3B, serum triglycerides were more variable throughout the 12 week study. There was a transient increase at week 3 of treatment, no change at week 6, and at week 12, serum triglyceride was significantly reduced by 28%. Pooled serum samples from control and 10 mg/kg/day SC-435-treated mice (12 weeks of treatment) were analyzed for serum lipoproteins using FPLC (Fig. 3C, D). Typical of apoE$^{-/-}$ mice on Western high-fat diet, the majority of the cholesterol resided in VLDL and VLDL remnant particles. As shown in Fig. 3C, serum VLDL and LDL cholesterol were lower in the SC-435-treated mice than in the control group without a detectable change in HDL particles. Almost all of the triglycerides were recovered in the VLDL fractions, and SC-435 treatment resulted in a de-

crease in VLDL triglycerides (Fig. 3D). We also measured hepatic lipids after 6 weeks of treatment with 10 mg/kg/day SC-435. A significant reduction in hepatic total cholesterol levels from 7.13 $\pm$ 0.3 mg/g to 5.79 $\pm$ 0.19 mg/g (19%, $P < 0.001$) was observed without any significant change in hepatic triglycerides (data not shown).

Following the 12 week administration of SC-435, the mice were sacrificed and the atherosclerotic lesion area in aortic root sections was quantitated by image analysis. **Figure 4A** shows representative cross-sections of the aortic root from mice fed the Western high-fat and high-cholesterol diet with or without SC-435. Quantification of the lesions area revealed a statistically significant 65% reduction in the animals administered 10 mg/kg/day SC-435 (Fig. 4B). These results show that ASBT inhibition by SC-435 is effective in reducing serum cholesterol levels and in inhibiting the progression of aortic root lesions in apoE$^{-/-}$ mice.

To understand the mechanism of reduction in serum cholesterol by ASBT inhibition in apoE$^{-/-}$ mice, we examined the effect of 10 mg/kg/day SC-435 on mRNA levels of farnesoid X receptor (FXR) target genes involved in hepatic LDL cholesterol metabolism (SHP, Cyp7α1, and ileal IBABP) by quantitative real-time PCR. As shown in **Fig. 5A**, hepatic mRNA levels of Cyp7α1, the rate-limiting enzyme involved in oxidative conversion of cholesterol to bile acid, was significantly increased (13-fold) in mice treated with SC-435 when compared with the control group. In agreement with the proposed mechanism, hepatic expression of HMGR, the rate-limiting enzyme for cholesterol biosynthesis, was also significantly increased by 51%. Although not statistically significant, hepatic LDLR expression was increased by 29% in SC-435-treated
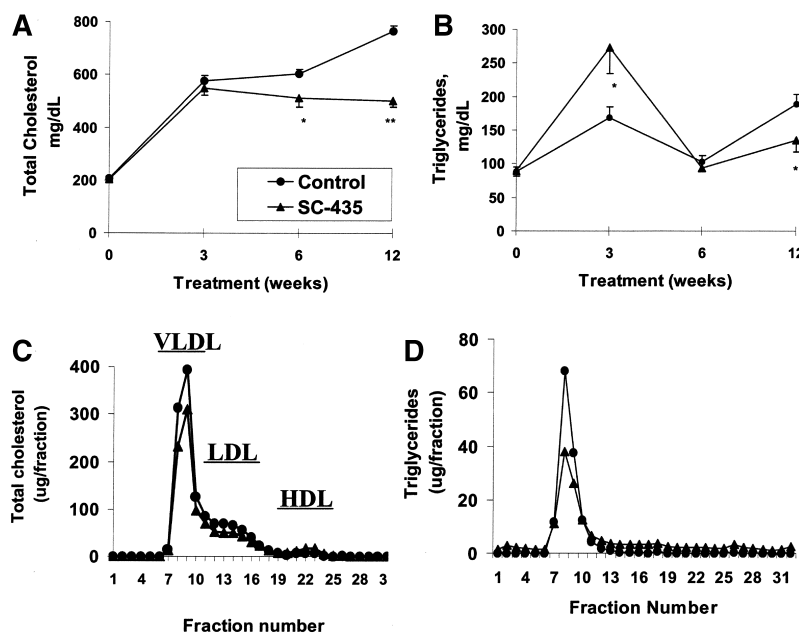

Fig. 3. Effect of SC-435 on serum lipids. A and B: ApoE$^{-/-}$ mice were treated with 10 mg/kg/day SC-435 in Western high-fat diet for 3–12 weeks. Serum total cholesterol (A) and triglycerides (B) were measured at 3, 6, and 12 weeks following treatment. C and D: Following 12 weeks of treatment, pooled serum samples from control and SC-435-treated mice were analyzed by fast protein liquid chromatography, and fractions were measured for total cholesterol (C) and triglycerides (D). Values are mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.005$.
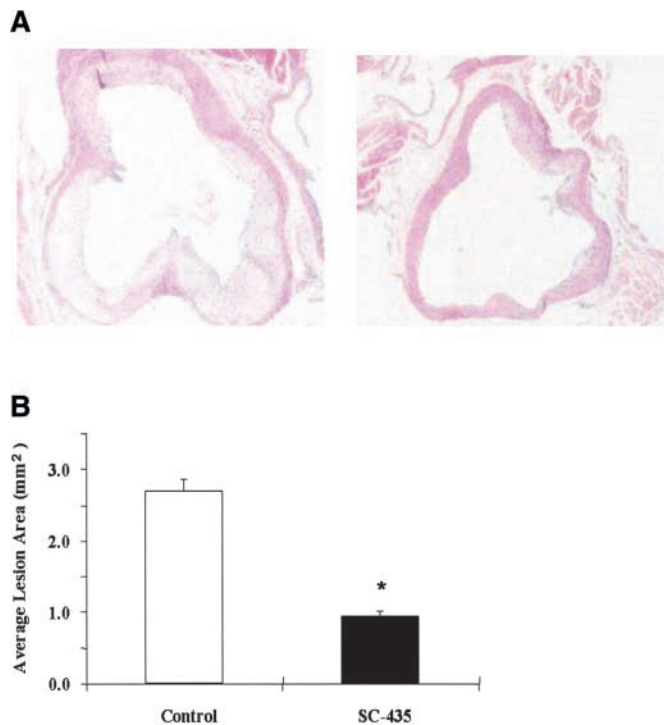
Fig. 4. Effect of 12 weeks dietary feeding of SC-435 on development of atherosclerosis in apoE$^{-/-}$ mice. Animals (n = 15/group) were fed a Western high-fat and high-cholesterol diet with or without 10 mg/kg/day SC-435 for 12 weeks. Aortic root sections (A) are representative samples from control and SC-435-treated mice. B: Quantitative aortic root lesion area in control and SC-435-treated mice. Values are mean ± SEM from 15 animals. * $P < 0.001$.

apoE$^{-/-}$ mice, suggesting that both HMGR and LDLR provide cholesterol to support increased bile acid synthesis in the liver. In vivo inhibition of ASBT with SC-435 increased the expression of ileal ASBT (Fig. 5B). We also tested whether inhibition of bile acid absorption has any effect on the expression of the FXR target genes, IBABP and SHP. As shown in Fig. 5A and B, SC-435 treatment resulted in significant decreases in expression of both ileal IBABP and hepatic SHP.

The statin class of cholesterol-lowering drugs (e.g., atorvastatin) decreases plasma LDL cholesterol levels by inhibiting HMGR, which results in a secondary elevation of LDLRs. Because ASBT inhibition results in a significant increase in HMGR expression (Fig. 5), we determined the effect of SC-435 and atorvastatin, alone or in combination, on cholesterol and bile acid metabolism in chow-fed dogs. Dogs have previously been shown to be responsive to statins (15), and we have observed that they are also responsive to ASBT inhibitors. We chose 2 mg/kg/day atorvastatin, a dose that has minimal effect on serum total cholesterol in dogs, to test the concept of combination therapy. As shown in **Fig. 6A**, treatment of dogs with SC-435 caused a significant dose-dependent increase in fecal bile acid excretion, yielding a maximum increase of 287% in dogs treated with a dose of 9 mg/kg/day. Coadministration of 2 mg/kg/day atorvastatin in combination with various doses of SC-435 caused no additional significant effect on fecal bile acid excretion. As shown in Fig. 6B, following 4 weeks
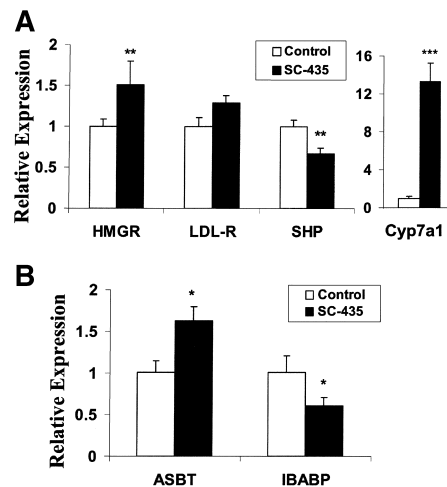

Fig. 5. Effect of SC-435 treatment on the expression of genes involved in cholesterol homeostasis in apoE$^{-/-}$ mice. A: Hepatic expression of HMG-CoA reductase (HMGR), and low density lipoprotein receptor (LDLR), small heterodimer partner (SHP), and cholesterol 7α-hydroxylase (Cyp7α1) genes. B: Ileal expression of apical sodium codependent bile acid transporter (ASBT) and intestinal bile acid binding protein (IBABP). Animals were treated with 10 mg/kg/day SC-435 in a Western high-fat diet for 6 weeks as described in Materials and Methods. Liver and ileal RNA were isolated and gene expression of ileal ASBT and IBABP and hepatic HMGR, LDLR, SHP, and Cyp7α1 were quantitated by real-time PCR. Values are relative to cyclophyllin mRNA and are mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

of treatment in dogs, there was little, if any, change in the concentration of serum total cholesterol in either the vehicle group (−0.2%) or the 2 mg/kg/day atorvastatin monotherapy group (−1.2%) compared with pretreatment values. However, 4 weeks of monotherapy treatment with 0.33, 1, 3, and 9 mg/kg/day SC-435 resulted in statistically significant ($P < 0.05$) reductions in serum total cholesterol of 7%, 7%, 8%, and 12%, respectively, compared with pretreatment values. Four weeks of combination treatment with 2 mg/kg/day atorvastatin plus 1.0 mg/kg/day SC-435 or 2 mg/kg/day atorvastatin plus 9 mg/kg/day SC-435 resulted in additional reductions in serum total cholesterol of 16% and 23%, respectively, compared with pretreatment values. The reductions seen in the SC-435 monotherapy or coadministration groups were rapid, with most of the effect observed during the first week of treatment and sustained for the duration of the 4 week study (data not shown). These data demonstrate that inhibition of bile acid uptake by SC-435 results in increased fecal bile acid excretion and decreased serum total cholesterol levels in dogs. They also indicate that reduction of LDL cholesterol with pharmacological agents of different mechanisms can be more effective when used in combination treatment regimens.

DISCUSSION

Bile acids are synthesized from cholesterol in the liver and secreted into the small intestine to facilitate lipid absorption. More than 95% of intestinal bile acids are reab-
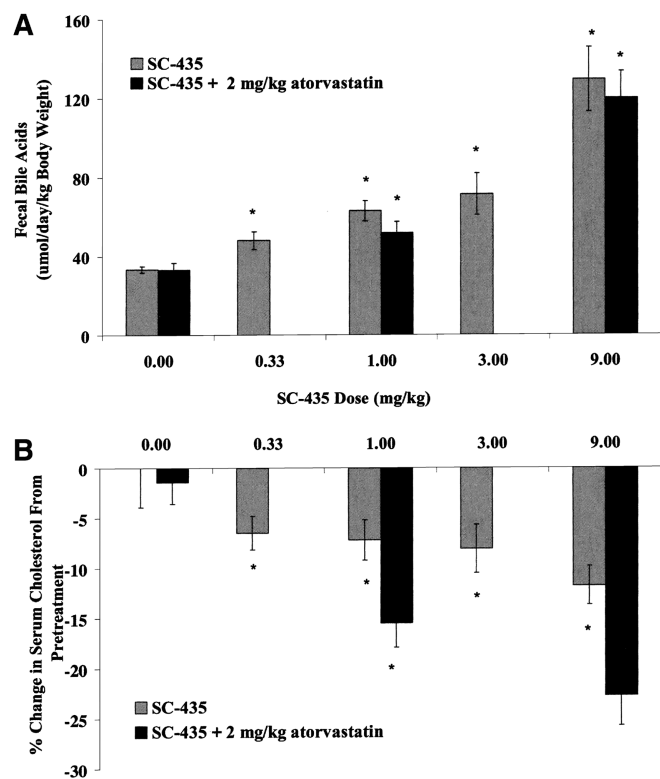
Fig. 6. Effect of SC-435 and atorvastatin and their combination treatment on fecal bile acids (A) and serum total cholesterol (B) in male beagle dogs. Dogs were randomly assigned to five groups such that each group had mean pretreatment serum total cholesterol values and body weights within 5% of each other. Animals (n = 6/group) were dosed once daily with SC-435 and/or atorvastatin in gelatin capsules at indicated doses for 4 weeks. Fecal bile acids were measured from feces collected during last 2 day period in the last week of treatment. Control-dog serum total cholesterol (mg/dl) values were 158 ± 19 and 148 ± 18 at weeks 0 and 4, respectively. Values are mean ± SEM. * $P < 0.05$ versus respective pretreatment values.

sorbed either by passive diffusion along the entire intestine or by active transport at the terminal ileum (16). Because the total bile acid pool is tightly regulated, interruption of enterohepatic circulation results in increased bile acid biosynthesis from cholesterol to compensate for the reduced flux through the liver (3). Partial depletion of hepatic cholesterol by the enhancement of cholesterol catabolism induces HMGR activity (increased cholesterol synthesis) and increased uptake of plasma LDL cholesterol via the hepatic LDLR. The bile acid binding resins, cholestyramine and colestipol, are drugs currently in clinical use that interrupt enterohepatic bile acid recirculation (17), and their use in combination with other hypocholesterolemic agents, such as HMGR inhibitors, results in further reduction in LDL cholesterol compared with monotherapy (18, 19). However, the limitations of bile acid sequestrant therapy include discomfort owing to their bulkiness (gram quantities), unpalatable texture, and the gastrointestinal side effect of constipation, which contributes to poor patient compliance (3). Therefore, it is desirable to develop a hypocholesterolemic agent that interrupts the reabsorption of bile acids,

resulting in increased cholesterol catabolism at more reasonable therapeutic doses. Inhibitors of ASBT have been reported with IC_{50} values in the micromolar range, and some of these have been shown to reduce total cholesterol in various animal models (20–25). Development of potent agents that specifically inhibit intestinal uptake of bile acids by ASBT are likely to be more efficacious than the bile acid binding resins (26). Moreover, the cardiovascular benefits of LDL cholesterol lowering by reducing enterohepatic circulation of bile acids has been well established by the POSCH trial (5, 27).

The aim of our study was to characterize a potent and specific ASBT inhibitor SC-435, which represents a novel class of pharmaceutical agents, to lower LDL cholesterol levels by altering cholesterol and bile acid metabolism through a mechanism that is distinct from inhibition of HMGR (i.e., statins). Because bile acids are synthesized from cholesterol in the liver and are the major pathway for cholesterol excretion in vivo, a specific inhibitor of ASBT would lower plasma cholesterol by reducing the intestinal reabsorption of bile acids and consequently increasing the conversion of cholesterol to bile acids to maintain the bile acid pool (28).

A key advantage of this approach is the localization of ASBT on the luminal surface of the ileal enterocyte and thus, inhibitors of ASBT do not have to be absorbed into the systemic circulation to exert their therapeutic effect. This has distinct safety advantages for an ASBT inhibitor as a therapeutic agent. In this report, using a cell line expressing the human ASBT protein, we have described SC-435 as a potent and selective ASBT inhibitor with an in vitro $IC_{50} = 1.5$ nM. Treatment of apoE$^{-/-}$ mice and dogs with SC-435 results in increased fecal excretion of bile acids with a concomitant decrease in serum total cholesterol. The SC-435-mediated reduction in bile acid absorption did not appear to affect cholesterol absorption as measured by fecal neutral sterols. Following administration of SC-435 to Western high-fat-fed apoE$^{-/-}$ mice, an increase in hepatic expression of HMGR, Cyp7α1, and LDLR was observed, demonstrating that the compensatory mechanism to replenish the bile acid pool is an increased biosynthesis of cholesterol and bile acids, and a receptor-mediated LDL uptake from plasma. Coadministration of SC-435 with atorvastatin to chow-fed dogs provided additional reduction of serum total cholesterol over statin monotherapy. This is anticipated, because the statin would negate the increase in HMGR activity in response to ASBT inhibition, and supports an added benefit of combination treatment with an ASBT inhibitor over statin monotherapy. In a genetic model of atherosclerosis, changes in serum lipid levels in the apoE$^{-/-}$ mouse by dietary treatment of SC-435 not only resulted in a 35% reduction of serum total cholesterol, but also caused a 65% reduction in the aortic root lesion area after 12 weeks of treatment. Thus, inhibition of ileal bile acid absorption using a specific ASBT inhibitor should be useful in the treatment of hypercholesterolemia, leading to a decreased risk for CAD in humans. The present report describes a new approach to lower LDL cholesterol and atherosclerosis either as monother-

apy or in combination with existing effective treatments (26, 29). Combination treatments involving the use of ASBT inhibitors together with statins, fibrates, or niacin in humans remain to be tested, and may constitute attractive strategies for treatment of hyperlipidemia and atherosclerosis.

Bile acids have recently been identified as the natural ligands for the nuclear receptor FXR (30, 31). FXR regulates key proteins and enzymes involved in bile acid and cholesterol homeostasis. Bile acid activation of FXR results in the inhibition of Cyp7α1 expression via increased expression of SHP, resulting in decreased catabolism of cholesterol to bile acid. In addition, FXR also appears to modulate the expression of a number of apolipoproteins, including positive regulation of apoC-II (32) and negative regulation of apoA-I (33). Thus, bile acids not only aid absorption of dietary fat from the body, but also regulate cholesterol homeostasis through FXR (30, 31). Recently, Xu et. al. have shown that removal of the bile acid pool using an in vivo whole rabbit bile cannulation model resulted in decreased expression of the FXR target genes I-BABP and bile salt export pump, and increased expression of Cyp7a, suggesting that FXR activity was inhibited or diminished under these conditions (34). Similarly, interruption of ileal bile acid absorption by SC-435 resulted in a decrease in FXR target genes I-BABP and SHP and an increase in Cyp7α1 mRNA expression. These results suggest that an ASBT inhibitor will decrease hepatic bile acid flux, deactivate FXR, and result in regulation of cholesterol and bile acid metabolism through an FXR-mediated mechanism.

Although we have not seen any adverse effects of SC-435 in the various animal models tested, the potential for adverse side effects in humans needs to be examined. Because ASBT inhibitors would increase the luminal concentration of free bile acids, it is possible that ASBT inhibition could lead to a variety of intestinal pathologies, including increased rates of colon cancer and diarrhea (3). It is noteworthy that no increased incidence of colon cancer has been reported after several years of follow-up in patients from the POSCH study (35). Another potential adverse effect of ASBT inhibition is increased plasma triglyceride levels. This has been brought to light in a study with a few hypertriglyceridemic patients with diminished gene expression of ileal ASBT, although, as the authors indicated, this does not establish a cause-and-effect relationship (36). In transgenic mice or in cultured rat hepatoma cells overexpressing Cyp7α1, it has been shown that Cyp7α1 plays a homeostatic role in balancing the SREBP-regulated anabolic lipoprotein assembly/secretion pathway and the cholesterol catabolic bile acid synthetic pathway (37, 38). However, we have recently shown in miniature pigs that a low dose of the ASBT inhibitor SC-435 significantly reduces plasma LDL cholesterol through enhanced LDLR-mediated LDL apoB clearance, secondary to increased expression of Cyp7α1, while the plasma triglyceride or VLDL pool size was not significantly altered by the treatment (39). Nevertheless, the effect of ASBT inhibitors on plasma triglycerides in humans will need to be established.

In conclusion, ASBT inhibitors provide a new therapeutic approach to lowering LDL cholesterol and slowing the progression of atherosclerosis. Based on their nonsystemic mechanism of action, they can be used as monotherapy or in combination with existing effective treatments, and appear to be particularly effective in combination with statins in negating the increase in HMGR activity in response to ASBT inhibition. The safety and efficacy of this novel mechanism are currently under investigation in humans.

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