Antisense inhibition of proprotein convertase subtilisin/kexin type 9 reduces serum LDL in hyperlipidemic mice

Mark J. Graham,[1] Kristina M. Lemonidis, Charles P. Whipple, Amuthakannan Subramaniam, Brett P. Monia, Stanley T. Crooke, and Rosanne M. Crooke

Cardiovascular Group, Department of Antisense Drug Discovery, Isis Pharmaceuticals, Inc., Carlsbad, CA 92008

Abstract Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a member of a family of proteases that is thought to promote the degradation of the low density lipoprotein receptor (LDLR) through an as yet undefined mechanism. We developed second generation antisense oligonucleotide (ASO) inhibitors targeting murine PCSK9 to determine their potential as lipid-lowering agents. Administration of a PCSK9 ASO to high fat-fed mice for 6 weeks reduced total cholesterol and LDL by 53% and 38%, respectively. Moreover, inhibition of PCSK9 expression resulted in a 2-fold increase in hepatic LDLR protein levels. This phenotype closely resembles that reported previously in *Pcsk9*-deficient mice. The absence of cholesterol lowering in *Ldlr*-deficient mice effectively demonstrated a critical role for this receptor in mediating the lipid-lowering effects of PCSK9 inhibition.■ Antisense inhibition of PCSK9 is an attractive and novel therapeutic approach for treating hypercholesterolemia in human.—Graham, M. J., K. M. Lemonidis, C. P. Whipple, A. Subramaniam, B. P. Monia, S. T. Crooke, and R. M. Crooke. Antisense inhibition of proprotein convertase subtilisin/kexin type 9 reduces serum LDL in hyperlipidemic mice. *J. Lipid Res.* 2007. **48:** 763–767.

Supplementary key words antisense oligonucleotides • low density lipoprotein receptor • hyperlipidemia

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a member of the proprotein convertase family of proteases that is thought to play a role in regulating lipid metabolism (1). Recent epidemiological studies have suggested that loss-of-function mutations in PCSK9 result in lifelong reductions in LDL that are associated with significant reductions in the incidence of coronary heart disease (1–5). Mechanistic studies in *Pcsk9*-deficient mice have shown that inactivation of this gene produces ~2-fold increases in hepatic low density lipoprotein receptors (LDLRs), resulting in significant reductions in LDL via an enhanced hepatic clearance mechanism (4). Although these studies have demonstrated an important role for PCSK9 in lipid homeostasis, the mechanism of these effects has not been elucidated, nor has the pharmacological intervention of PCSK9 been described, as no such agent currently exists.

To determine whether pharmacological inhibition of PCSK9 decreases LDL through upregulation of LDLR protein, an antisense oligonucleotide (ASO) complementary to the mouse PCSK9 gene was identified and administered to hyperlipidemic mice. Inhibition of that target resulted in significant reductions in hepatic PCSK9 mRNA levels, with concomitant reductions in total cholesterol and LDL. Consistent with the results observed in *Pcsk9*-deficient mice, hepatic LDLR protein expression was increased significantly (~2-fold). Thus, lipid lowering was dependent on a functional LDLR. These findings validate PCSK9 as a pharmacological target for LDL lowering and suggest that the specific and selective inhibition of PCSK9 mRNA using ASOs may be an effective approach for decreasing LDL in human.

MATERIALS AND METHODS

ASOs

A series of chimeric 20-mer phosphorothioate oligonucleotides containing 2′-O-methoxyethyl groups at positions 1–3 and 17–20 targeted to mouse PCSK9 were synthesized and purified as described (6) with an automated DNA synthesizer (380B; Perkin-Elmer Applied Biosystems, Foster City, CA). The most potent ASO, ISIS 394814 (5′-GGGCTCATAGCACATTATCC-3′), was used for all subsequent in vivo pharmacological assessments. ISIS 141923 (5′-CCTTCCCTGAAGGTTCCTCC-3′), which is in the same chemical and mechanistic class as the PCSK9 compound but not complementary to any known gene sequences, was used as a control ASO.

Abbreviations: apoB, apolipoprotein B; apobec1, apolipoprotein B mRNA editing enzyme catalytic polypeptide 1; ASO, antisense oligonucleotide; HF, high fat; LDLR, low density lipoprotein receptor; PCSK9, proprotein convertase subtilisin/kexin type 9; SR-BI, scavenger receptor class B type I; TG, triglyceride.

[1] To whom correspondence should be addressed.
  e-mail: mgraham@isisph.com

Quantitative RT-PCR analysis

Total RNA was extracted from whole liver tissue and primary hepatocytes with Qiagen RNeasy isolation kits, as described (7). Samples (50 ng) were subjected to quantitative RT-PCR analysis using commercial reagents (Invitrogen, Carlsbad, CA) and analyzed using a Prism 7700 Sequence Detector (Perkin-Elmer Applied Biosciences). Values were normalized to glyceraldehyde-3-phosphate dehydrogenase and/or Ribogreen levels. In all cases, the probes were labeled with 5′ FAM (a 6-carboxyfluorescein reporter) and 3′ TAMRA [a 5(6)-carboxytetra-methyl-rhodamine quencher]. After 40 amplification cycles, absolute values were obtained with SDS analysis software (Applied Biosystems).

Western blots

Cells or tissues were harvested in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40, 1% Triton, 0.25% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail 1:100, and 0.2 mM sodium orthovanadate), and protein concentrations were measured with the detergent compatible (DC) kit from Bio-Rad (Hercules, CA). Mouse plasma (10 µl, 1:100) was subjected to electrophoresis on 4–12% Tris-glycine gels and transferred to polyvinylidene fluoride membranes (Invitrogen). Mouse hepatic LDLR Western blotting was performed overnight at 4°C with a primary anti-mouse LDLR antibody (1:1,000; R&D Systems, Minneapolis, MN). Additional analyses were performed using a scavenger receptor class B type I (SR-BI) antibody (Novus Biologicals, Littleton, CO), an apolipoprotein B (apoB) antibody (kindly provided by Dr. Stephen Young), and an apoA-I antibody (Biodesign, Saco, ME). Blots were detected using horseradish peroxidase-conjugated secondary antibodies, either goat anti-rabbit (Transduction Laboratories, Lexington, KY) or bovine anti-goat (Santa Cruz Biotechnology, Santa Cruz, CA). Protein bands were visualized using the ECL plus Western blot detection kit (Amersham Biosciences) and quantified using Image-Quant analysis software (Molecular Dynamics, Santa Clara, CA).
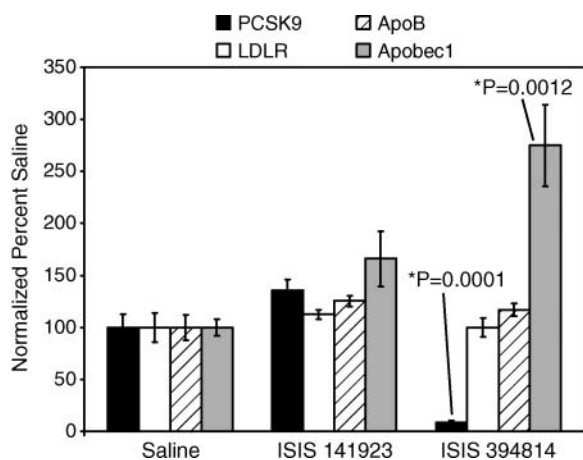
Lipoprotein analysis and metabolic measurements

Plasma concentrations of total cholesterol, LDL, HDL, triglycerides (TGs), free cholesterol, glucose, ketones, phospholipids, and transaminases were determined using an Olympus (Melville, NY) AU400e automated clinical chemistry analyzer.

Serum lipoprotein and cholesterol profiling was performed as described (8) using a Beckman System Gold 126 HPLC system, a 507e refrigerated autosampler, a 126 photodiode array detector (Beckman Instruments, Fullerton, CA), and a Superose 6 HR 10/30 column (Pfizer, Chicago, IL). HDL, LDL, and VLDL fractions were measured at a wavelength of 505 nm and validated with a cholesterol calibration kit (Sigma).

Mouse pharmacological experiments

All animal experiments were conducted in accordance with Institutional American Association for the Accreditation of Laboratory Animal Care guidelines. C57BL/6 mice were obtained from Jackson Laboratory (http://www.jax.org). *Ldlr*-deficient/apoB-100 mice were kindly provided by Dr. Larry Rudel (Wake Forest University). A majority of the mice were male, and studies were initiated when animals were 4–5 weeks of age. The mice were maintained on a 12 h light/12 h dark cycle and fed ad libitum. C57BL/6 mice were fed a Western diet consisting of 60% lard (Research Diets, New Brunswick, NJ), whereas all *Ldlr*-deficient/apoB-100 mice were fed regular chow.

Oligonucleotides were administered twice weekly (50 mg/kg) for 6 weeks by intraperitoneal injection (10 mg/ml dosing solution formulated in saline). Mice were anesthetized, and whole blood was obtained through cardiac puncture. Serum was analyzed as de-
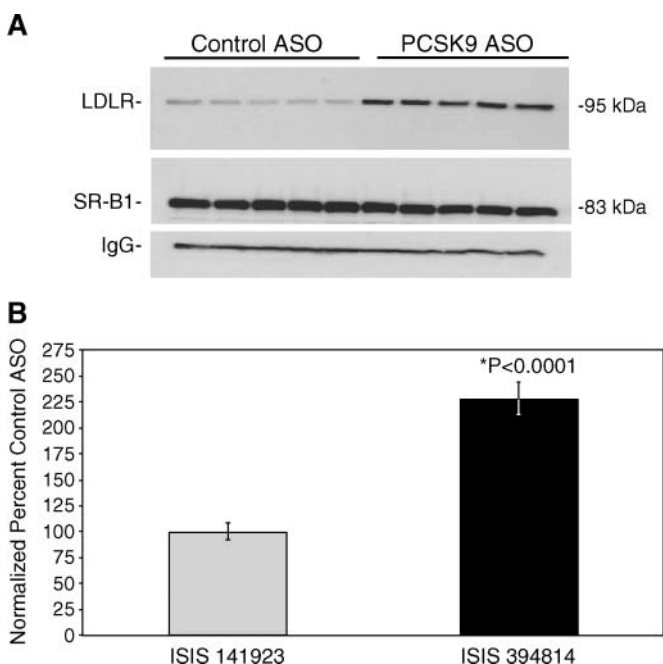

Fig. 1. Treatment with a mouse-specific proprotein convertase subtilisin/kexin type 9 (PCSK9) antisense oligonucleotide (ASO) for 6 weeks (100 mg/kg/week) reduced hepatic PCSK9 expression (black bars) by 92%, increased apolipoprotein B mRNA editing enzyme catalytic polypeptide 1 (apobec1) by 270% (gray bars), but produced no significant effect on either low density lipoprotein receptor (LDLR) mRNA (white bars) or apolipoprotein B (apoB) mRNA (hatched bars) expression in high fat (HF)-fed C57BL/6 mice. Representative groups (n = 6) were administered saline, control ASO (ISIS 141923), or PCSK9 ASO (ISIS 394814); each bar represents the mean of each treatment group with the SEM indicated.

Fig. 2. Treatment with a PCSK9 ASO increases the abundance of hepatic LDLR protein in HF-fed mice after 6 weeks of ASO administration (100 mg/kg/week). A: A total of 50 µg of liver lysates was blotted and incubated with antibodies specific for mouse LDLR and scavenger receptor class B type I (SR-BI). LDLR protein levels were increased by >2-fold after ISIS 394814 treatment, relative to control ASO treatment (ISIS 141923), whereas SR-BI protein expression was unaffected. B: Mean percentage expression of hepatic PCSK9 in control ASO treatment (ISIS 141923; gray bars) and ISIS 394814 treatment (black bars) groups, with the SEM indicated (n = 5).

TABLE 1. PCSK9 mRNA, lipid, and liver TG levels after 6 weeks of ISIS 394814 treatment (100 mg/kg/week) in HF-fed and *Ldlr*-deficient/apoB-100 mice

Phenotype	Saline PCSK9 mRNA	Total Cholesterol	Free Cholesterol	HDL	LDL	TG	Ketone	Phospholipid	Liver TG
	%	mg/dl	mg/dl	mg/dl	mg/dl	mg/dl	µmol/l	mg/dl	mg/g
HF-fed									
Saline	100 ± 13	183 ± 18	41 ± 10	133 ± 16	22 ± 4	102 ± 18	141 ± 52	354 ± 29	97 ± 10
ISIS 141923	135 ± 11	194 ± 14	58 ± 3	145 ± 10	25 ± 2	89 ± 18	146 ± 48	383 ± 21	72 ± 28
ISIS 394814	8 ± 4[a]	87 ± 19[a]	31 ± 5[b]	61 ± 16[a]	14 ± 2[b]	87 ± 12	101 ± 25	169 ± 35[a]	36 ± 18[b]
Ldlr-deficient/apoB-100									
Saline	100 ± 9	358 ± 12	159 ± 4	92 ± 4	261 ± 18	118 ± 14	265 ± 101	304 ± 10	22 ± 7
ISIS 141923	96 ± 13	375 ± 15	163 ± 3	99 ± 1	262 ± 11	155 ± 17	321 ± 187	314 ± 21	26 ± 10
ISIS 394814	9 ± 2[a]	445 ± 25[b]	186 ± 7[b]	109 ± 7[b]	329 ± 11[b]	127 ± 26	524 ± 102	354 ± 12	21 ± 4

apoB, apolipoprotein B; HF, high fat; LDLR, low density lipoprotein receptor; PCSK9, proprotein convertase subtilisin/kexin type 9; TG, triglyceride. All values indicated are group means (n = 5) and SEM.
[a] $P < 0.0001$.
[b] $P < 0.005$.

scribed above. Whole liver was processed for RNA, protein, and histological examination. Hepatic TG levels were assayed as described (9).

Statistical analysis

Pharmacological studies were performed at least twice using six mice per treatment group. A nonparametric, two-tailed *t*-test comparison was performed for all serum lipid parameters (saline or control ASO vs. PCSK9 ASO treated). Statistics for mRNA and protein changes were deemed significant at $P < 0.05$.

RESULTS

Reduction of PCSK9 hepatic mRNA expression in high fat-fed C57BL/6 mice

A series of 96 ASOs complementary to murine PCSK9 mRNA sequence were designed to hybridize throughout the PCSK9 transcript and evaluated for their ability to reduce PCSK9 mRNA expression in mouse primary hepatocytes (data not shown). The most potent ASO identified, ISIS 394814, was chosen for in vivo characterization.

ISIS 394814 (50 mg/kg) was administered intraperitoneally twice weekly to high fat (HF)-fed C57BL/6 mice. After 6 weeks of treatment, liver mRNA was isolated and quantitative RT-PCR was performed to determine the expression levels of PCSK9, LDLR, and apoB mRNA. Treatment with the PCSK9 ASO, but not the control ASO, selectively reduced hepatic PCSK9 mRNA levels by 92% after 6 weeks (**Fig. 1**). As observed previously in *Pcsk9*-deficient mice (3), suppression of PCSK9 did not affect the levels of hepatic LDLR or apoB mRNA. Additional quantitative RT-PCR analysis of key cholesterol and fatty acid biosynthetic genes, such as HMG-CoA reductase, sterol-CoA desaturase-1, fatty acid synthase, sterol response element binding factor-1, and hepatic lipase, revealed no change in expression after 6 weeks of ISIS 394814 treatment (data not shown). Interestingly, expression of hepatic apolipoprotein B mRNA editing enzyme catalytic polypeptide 1 (apobec1) mRNA was increased by 2.7-fold after 6 weeks of ASO treatment (Fig. 1).

Suppression of hepatic PCSK9 mRNA increases hepatic LDLR protein levels

To determine whether suppression of PCSK9 levels would produce an increase in hepatic LDLRs, LDLR protein levels were measured in HF-fed mice after 6 weeks of ASO treatment. Administration of ISIS 394814 resulted in a >2-fold
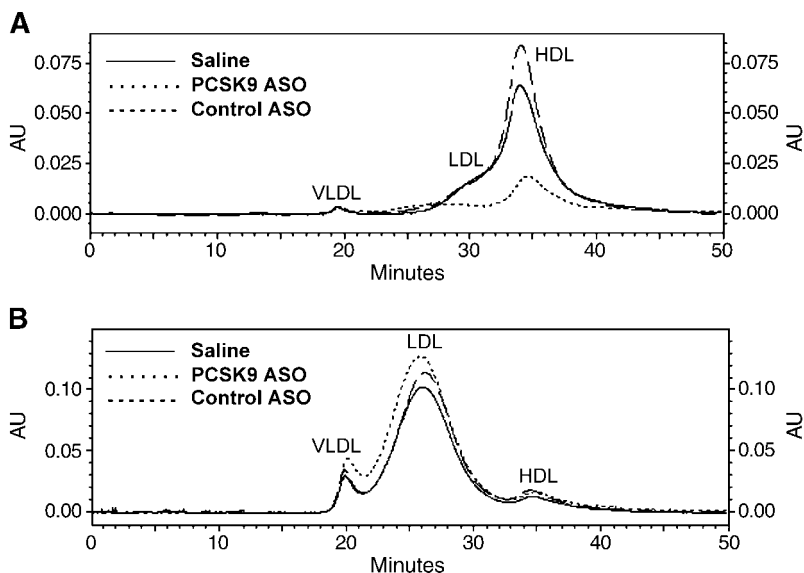
Fig. 3. HPLC lipoprotein profiles of HF-fed C57BL/6 mice (A) and *Ldlr*-deficient/apoB-100 mice (B) after 6 weeks of ASO administration at 100 mg/kg/week. For each profile, serum samples for each group (n = 5) were pooled and a composite profile was generated. Saline treatment (solid lines), control ASO (dashed lines), and PCSK9 ASO (dotted lines) are indicated. AU, arbitrary units.

increase in LDLR protein levels (**Fig. 2**), whereas control ASO (ISIS 141923)-treated animals were unaffected. ISIS 394814 effects were also specific and selective. For example, PCSK9 ASO treatment had no effect on the levels of SR-BI, a hepatic receptor involved in HDL clearance (Fig. 2A).

Effect of PCSK9 inhibition on serum lipid levels

Administration of ISIS 394814 for 6 weeks reduced total cholesterol (52%; $P < 0.0001$), LDL (32%; $P < 0.005$), and HDL (54%; $P < 0.005$) (**Table 1**). A reduction in HDL has also been observed in *Pcsk9*-deficient mice (3), and those authors suggested that this results from enhanced clearance of apoE-containing lipoproteins facilitated by increased hepatic LDLR abundance. Furthermore, Lieu et al. (10) reported similar findings in Reversa mice and suggested that HDL particle formation requires components derived from apoB-associated lipoproteins. Serum free cholesterol and phospholipids were reduced as well [25% ($P < 0.005$) and 54% ($P < 0.0001$), respectively]. HPLC analysis of pooled samples from treated mice corroborated the alterations in serum lipids measured using the clinical analyzer (**Fig. 3A**).

PCSK9 ASO treatment decreases serum apoB-100 while increasing apoB-48 levels in HF-fed C57BL/6 mice

Based upon the increased expression of hepatic apobec-1 mRNA, Western analysis was performed to determine whether serum apoB-48 and apoB-100 would be differentially affected after PCSK9 ASO administration. Consistent with those data, serum apoB-48 protein levels were increased (3-fold) but apoB-100 levels were reduced by 50% (**Fig. 4A, B**). In contrast, PCSK9 ASO treatment did not produce any significant change in apoA-I protein levels relative to those observed in ISIS 141923 (control ASO)-treated mice.

Effect of PCSK9 inhibition on liver TG content in HF-fed C57BL/6 mice

Treatment with ISIS 394814 for 6 weeks reduced liver TG content by ~65% ($P = 0.01$) relative to saline controls (Table 1). Although there was a modest reduction in hepatic TG content with the control ASO (ISIS 141923), these changes were not statistically significant. These results are consistent with liver TG reductions observed previously in *Pcsk9*-deficient mice (3), and further studies are planned to determine the underlying mechanism.

Pharmacological effects of PCSK9 suppression in *Ldlr*-deficient/apoB-100 mice

To determine whether the pharmacological effects induced by the PCSK9 ASO required the presence of a functional LDLR, the PCSK9 ASO was administered to *Ldlr*-deficient/apoB-100 mice. Consistent with the proposed mechanism of PCSK9 action on cholesterol regulation, no reduction in serum cholesterol was observed in mice lacking the LDLR (Table 1), despite a >90% reduction in hepatic mRNA levels. In fact, a 20% increase in both total cholesterol and LDL was observed in these mice ($P = 0.05$), and HPLC analysis confirmed these results (Fig. 4B). Additionally, in contrast to HF-fed mice, hepatic TG levels were unaffected by PCSK9 inhibition in *Ldlr*-deficient/
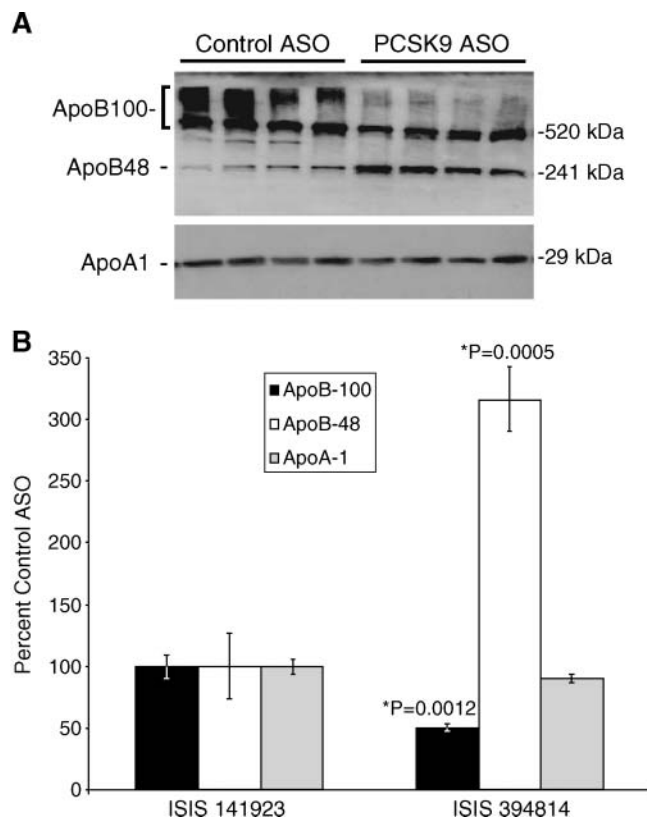
Fig. 4. Treatment with a PCSK9 ASO decreases the abundance of serum apoB-100 while increasing apoB-48 levels in HF-fed mice after 6 weeks of ASO administration (100 mg/kg/week). A: Serum samples were diluted 1:80, blotted, and incubated with antibodies specific for mouse apoB and apoA-I. ApoB-100 levels were decreased by 50%, whereas apoB-48 protein levels were increased by >3-fold after ISIS 394814 treatment, relative to control ASO treatment (ISIS 141923). In contrast, apoA-I protein expression was unaffected. B: Mean percentage expression of serum apoB-100 (black bars), apoB-48 (white bars), and apoA-I (gray bars) in control ASO (ISIS 141923) and PCSK9 ASO (ISIS 394814) treated groups, with the SEM indicated (n = 4).

apoB-100 mice (Table 1). Finally, an 80% increase in hepatic apobec1 mRNA levels was observed (data not shown).

DISCUSSION

In this article, we demonstrate for the first time that administration of a second generation PCSK9 ASO to hyperlipidemic mice reduced hepatic PCSK9 mRNA expression by 92% and resulted in significant reductions in total cholesterol and LDL. Like other second generation antisense inhibitors (8, 9), ISIS 394814 was well tolerated in mice. These results demonstrate that direct pharmacological suppression of PCSK9 can significantly reduce LDL in a manner consistent with its proposed role in the regulation of cholesterol homeostasis, as suggested by epidemiological evidence and studies in *Pcsk9*-deficient mice (1, 3, 5). Based upon these compelling initial results, studies are in progress to further evaluate the potential of PCSK9 as a targeted intervention for cholesterol lowering in animals that have more similar serum lipid profiles to humans, such as hamsters and nonhuman primates.

The expression of several key genes involved in cholesterol and fatty acid synthetic pathways were also evaluated in mice after PCSK9 ASO administration. Surprisingly, the only significant effects of PCSK9 inhibition appeared to be an increase in the expression of apobec1 mRNA (2- to 3-fold) with a concordant induction in serum apoB-48 protein levels (3-fold). Apobec1 is the enzyme responsible for the production of apoB-48-containing lipoproteins within the mammalian small intestine. It is also expressed in rodent, but not in human, liver (11). As PCSK9 is a protease of unknown function, it seems plausible that, in addition to putatively acting on LDLR, it may act upon other substrates. Thus, one possibility to explain these data is that additional substrates for PCSK9 are the proteins involved in editing apoB mRNA. Clearly, more work is required to understand this observation. It is also important to define the other substrates for PCSK9 and the potential pharmacological and toxicological effects of reducing this protein. A specific and selective antisense inhibitor of this protein makes it possible to determine its potential mechanism of action as well. Additional studies are planned to determine whether alterations in apobec1 mRNA expression in liver and intestine of treated animals correlate with any adverse physiological consequences.

To date, specific inhibition of PCSK9 via traditional pharmaceutical approaches has proven difficult. Antisense technology has been shown to target proteins not readily amenable to small molecule or antibody therapeutics (12–14). Second generation ASOs are stable, highly water-soluble drugs that can be administered conveniently in saline without special formulation via subcutaneous injection and other routes of delivery (15, 16). The pharmacokinetics of these drugs is well characterized, and after a subcutaneous dose, ~12% of the dose distributes to the liver, a principal site of PCSK9 expression. Because of their long elimination half-lives and a well-characterized safety profile, these drugs can be administered weekly or less frequently (17, 18). For example, very recently, a second generation antisense inhibitor to another lipid-lowering target, apoB-100, resulted in significant dose-dependent effects on apoB-100 and all apoB-100-containing atherogenic lipids, both as a single agent and in combination with statins with an acceptable safety profile (19). Thus, a PCSK9 drug of this class should be effective and well tolerated in the clinic and may indeed provide therapeutic benefit in patients at risk for cardiovascular disease.

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