New CETP inhibitor K-312 reduces PCSK9 expression: a potential effect on LDL cholesterol metabolism

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Miyosawa K, Watanabe Y, Murakami K, Murakami T, Shibata H, Iwashita M, Yamazaki H, Yamazaki K, Ohgiya T, Shibuya K, Mizuno K, Tanabe S, Singh SA, Aikawa M. New CETP inhibitor K-312 reduces PCSK9 expression: a potential effect on LDL cholesterol metabolism. Am J Physiol Endocrinol Metab 309: E177–E190, 2015. First published May 26, 2015; doi:10.1152/ajpendo.00528.2014.—Despite significant reduction of cardiovascular events by statin treatment, substantial residual risk persists, driving emerging needs for the development of new therapies. We identified a novel cholesteryl ester transfer protein (CETP) inhibitor, K-312, that raises HDL and lowers LDL cholesterol levels in animals. K-312 also suppresses hepatocyte expression of proprotein convertase subtilisin/kexin 9 (PCSK9), a molecule that increases LDL cholesterol. We explored the underlying mechanism for the reduction of PCSK9 expression by K-312. K-312 inhibited in vitro human plasma CETP activity (IC50; 0.06 μM). Administration of K-312 to cholesterol-fed New Zealand White rabbits for 18 wk raised HDL cholesterol, decreased LDL cholesterol, and attenuated aortic atherosclerosis. Our search for additional beneficial characteristics of this compound revealed that K-312 decreases PCSK9 expression in human primary hepatocytes and in the human hepatoma cell line HepG2. siRNA silencing of CETP in HepG2 did not compromise the suppression of PCSK9 by K-312, suggesting a mechanism independent of CETP. In HepG2 cells, K-312 treatment decreased the active forms of sterol regulatory element-binding proteins (SREBP-1 and -2) that regulate promoter activity of PCSK9. Chromatin immunoprecipitation assays demonstrated that K-312 decreased the occupancy of SREBP-1 and SREBP-2 on the sterol regulatory element of the PCSK9 promoter. PCSK9 protein levels decreased by K-312 treatment in the circulating blood of cholesterol-fed rabbits, as determined by two independent mass spectrometry approaches, including the recently developed, highly sensitive parallel reaction monitoring method. New CETP inhibitor K-312 decreases LDL cholesterol and PCSK9 levels, serving as a new therapy for dyslipidemia and cardiovascular disease.

High levels of LDL cholesterol (LDL-C) and low levels of HDL cholesterol (HDL-C) are associated with high risk of cardiovascular events (10, 16). Although HDL-C-raising therapies are expected to decrease cardiovascular events (29), their clinical benefits remain unclear. Despite a large amount of basic science and clinical evidence for the HDL-C levels, the functionality of HDL is incompletely understood, supporting the need for further investigations (47, 51). Inhibition of cholesteryl ester transfer protein (CETP) is one of the major approaches to pharmacologically raise HDL-C (5). CETP mediates transport of cholesteryl ester from HDL to apolipoprotein B-containing lipoproteins such as VLDL and LDL. Therefore, inhibition of CETP activity increases HDL-C levels. Clinical development of the first two CETP inhibitors, torcetrapib and daltepertapib, was terminated due to the increased mortality presumably caused by off-target effects of increasing blood pressure, unrelated to CETP inhibition itself, or to the lack of efficiency (2, 38). However, post hoc analysis of the ILLUSTRATE trial, which examined the effect of torcetrapib on the progression of atherosclerosis, revealed the regression of coronary atherosclerosis in the patients of the highest quartiles of achieved HDL-C levels (33). Moreover, changes in HDL-C and percent atheroma volume in torcetrapib-treated patients were inversely correlated. Thus, despite the unsuccessful clinical trials, hope remains for the development of CETP inhibitors that can achieve substantially high levels of HDL-C and prevent cardiovascular events.

In contrast to HDL-raising therapies, the clinical and preclinical evidence has established the benefits of LDL lowering for anti-inflammatory effects (28). Hydroxymethylglutaryl-CoA reductase inhibitors (statins), the most commonly used class of cholesterol-lowering drugs, potently lower LDL-C levels and prevent acute complications of atherosclerosis. However, the majority of patients on statins still suffer from cardiovascular events (27). In many patients, statin treatment fails to achieve target LDL-C levels (1, 45). Some patients with familial hypercholesterolemia respond poorly, or not at all, to statin therapy (34). Furthermore, significant numbers of individuals are intolerant to statins. These challenges have driven the development of new statin add-on therapies or statin alternatives to further reduce residual cardiovascular risk.

Proprotein convertase subtilisin/kexin 9 (PCSK9) inhibitors represent one of the most promising LDL-lowering therapeutics (46). PCSK9, which is produced mainly in the liver and the intestine (39), is secreted to the circulation and mediates degradation of LDL receptor (LDLR) in lysosomes, resulting in the reduction of LDLR numbers on the cell membrane (50). In PCSK9-deficient mice, higher expression of LDLR in the liver resulted in 48% lower plasma cholesterol levels and increased plasma LDL particle clearance compared with wild-type mice (36). In addition, individuals with loss-of-function mutations of PCSK9, which produce inactive forms of PCSK9, have lower LDL-C and lower risk of developing coronary heart disease (6, 7). These lines of evidence indicate that PCSK9 decreases clearance of LDL-C from the circulation and thus
elevates plasma LDL-C levels. Therefore, the reduction of activity or plasma concentration of PCSK9 lowers plasma LDL cholesterol levels. Clinical studies of human monoclonal antibodies for PCSK9 showed significant LDL-C lowering in patients with hypercholesterolemia or heterozygous familial hypercholesterolemia (37, 44). In addition, the phase I clinical trial of ALN-PCS, an siRNA for PCSK9, showed that delivery of the siRNA with lipid nanoparticles decreased LDL-C by up to 40% in healthy subjects (17). These antibody and RNAi biotherapeutics are delivered by injection.

Given that PCSK9 therapies would be added mainly to commonly prescribed LDL-lowering agents (e.g., statins), which are administered orally, small-molecule PCSK9 inhibitors would have substantial benefits compared with biotherapeutics. We synthesized the novel small-molecule CETP inhibitor K-312, which inhibits CETP as potently as the other CETP inhibitors anacetrapib and evacetrapib but does not indicate the adverse side effect of elevated blood pressure compared with torcetrapib. Our search for additional beneficial effects of K-312 found that it suppresses PCSK9 expression in vitro and in vivo. A novel high-resolution mass spectrometry (MS)/MS-based strategy to quantify plasma levels of PCSK9 in rabbits facilitated in vivo validation. Overall, this study supports K-312 as a potential LDL-C-lowering therapy.

MATERIALS AND METHODS

Materials. Real-time PCR primer and probe sets to the following genes were obtained from Life Technologies (Grand Island, NY): β-actin (4326315E), HNF5 encoding histone H4 transcription factor (Hs00120105_m1), hepatocyte nuclear factor (HNF)-1α (Hs00167041_m1), HNF-1β (Hs01001602_m1), PCSK9 (Hs00543599_m1), sterol regulatory element-binding protein-1 (SREBP-1: Hs01088691_m1), and SREBP-2 (Hs01081784_m1). Other primer sequences are available upon request. CETP inhibitors (K-312, anacetrapib, evacetrapib, torcetrapib, and dalceptrapib) were synthesized in the Tokyo New Drug Research Laboratories of Kowa Company (Tokyo, Japan).

Cell culture. Human hepatoma cell line HepG2 was purchased from American Type Culture Collection (ATCC; Manassas, VA) and maintained in Eagle’s minimum essential medium (ATCC) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 5% CO2. Human primary hepatocytes were obtained from Life Technologies and maintained in William’s E Medium supplemented with hepatocyte maintenance cocktail (Life Technologies) and dexamethasone (1 nM). Rat primary hepatocytes were isolated from male Sprague-Dawley rats (200–300 g). Rats were anesthetized with pentobarbital sodium, and then the livers were perfused with Hanks’ balanced salt solution, followed by perfusion with collagenase solution (Sigma-Aldrich, St. Louis, MO). Cells were seeded on plates coated with collagen type 1 (Becton Dickinson, Franklin Lakes, NJ) and grown in William’s E medium.

Cholesterol efflux assay. The mouse macrophage cell line RAW264.7 cells (ATCC) were cultured for 24 h in DMEM containing 50 μg/mL acetyl-LDL (BT-906; Biomedical Technologies, Stoughton, MA) and 2 μCi/ml [3H]cholesterol (Perkin-Elmer, Waltham, MA). After stimulation with liver X receptor agonist (T0901317; 1 μM) for 24 h, the rabbit HDL fraction (1.125 < d < 1.210 g/ml), isolated by ultracentrifugation (19), was added to the medium. The media were collected 4 h later, and radioactivity was measured in the media and cells. The cholesterol efflux was expressed as the percentage of the radioactivity in the medium relative to total radioactivity.

Promoter-reporter gene assay. The promoter region of the human PCSK9 gene was amplified from the human genome (HepG2 cells) by PCR. The amplified fragment was cloned into the pGL3-basic vector (Promega, Madison, WI). Mutation constructs for the various transcription factor binding sites were generated with a previously reported method (21). Promoter-reporter vectors were cotransfected with pGL4.74 vector (Promega) using Lipofectamine LTX reagent (Life Technologies). Luciferase activity was measured with Dual-Glo Luciferase assay system (Promega), and firefly luciferase activity was normalized to the Renilla luciferase activity.

Loss-of-function analysis. CETP, SREBP-1, and SREBP-2 were silenced on On-Target plus SMART pool siRNA (Dharmacon, Lafayette, CO). On-Target plus nontargeting control pool (Dharmacon) was used as a negative control siRNA. For transfection, Lipofectamine RNAiMAX (Life Technologies) was used according to the manufacturer’s instructions.

RNA stability assay. HepG2 cells were cultured in the presence of actinomycin D (5 μg/ml; Sigma-Aldrich) to halt transcription. K-312 was added to the culture medium and incubated for the indicated time. After RNA isolation, gene expression was measured by real-time quantitative (q)PCR to determine the decay of mRNA.

Detection of SREBP-1 and SREBP-2 by Western blot. To improve the detection of SREBP, immunoprecipitation of SREBP was carried out prior to the Western blot. Cell lysate was preclarified with Dynabeads (Life Technologies, Thermo Fisher) and normal rabbit IgG (R & D Systems) for 1 h. The lysate was then incubated for 4 h with either anti-SREBP-1 antibody (sc-8984; Santa Cruz Biotechnology) or anti-SREBP-2 antibody (10007663; Cayman Chemical), followed by the incubation with Dynabeads Protein G for 1 h. After the beads were washed, immunoprecipitated protein was subjected to Western blot with anti-SREBP-1 antibody (clone: 2A4, Active Motif) and anti-SREBP-2 antibody (clone: 1D2, MBL international).

Alternatively, the nuclear fraction was enriched using a nuclear extract kit (Active Motif), and then 20–30 μg of the lysate was analyzed by the Western blot technique with the same antibodies used above.

Chromatin immunoprecipitation assay. HepG2 cells were fixed with 1% formaldehyde for 10 min at room temperature then stopped with glycine solution. The chromatin was sheared by sonication to prepare DNA fragments with an average size of 200–500 bp. Chromatin immunoprecipitation (ChIP) was carried out with anti-SREBP-1 antibody (sc-8984; Santa Cruz Biotechnology) or anti-SREBP-2 antibody (10007663; Cayman Chemical). The immune complex was collected with Dynabeads Protein G (Life Technologies) and then purified with the QIAquick PCR purification kit (Qiagen, Germantown, MD). The enrichment of the target DNA region in the immunoprecipitated DNA was determined by real-time qPCR using a specific primer set. Reactions were performed in triplicate, and the fold enrichment was calculated against the nontarget region IGX1A.
were then measured and quantified using the PCSK9 H standard translated by the FLEXL:FLEXH ratio, which was then correlated to the measured. The absolute quantity of the PCSK9H standard was calculated with 10

immunoprecipitation and sample preparation for MS-based quantification of PCSK9. Rabbit plasma (500 μl) was incubated for 18 h with 10 μg of anti-human PCSK9 antibody (AF3888; R & D Systems) conjugated to Dynabeads Protein G (Life Technologies). After washing three times with PBS, SDS sample buffer (Boston BioProducts) was added and then boiled to elute proteins from beads. Immunoprecipitation was resolved by SDS-PAGE, and the area between 50 and 100 kDa was excised for subsequent in-gel trypsinization (40). Peptide samples were finally dissolved in 30 μl of loading buffer (5% formic acid, 5% acetonitrile).

In vitro-translated protein standard for quantification of rabbit plasma PCSK9. The full-length-expressed, stable isotope-labeled quantification (FLEXiQuant) strategy was used for relative and absolute quantification of PCSK9 in rabbit plasma (43). Rabbit PCSK9 was amplified by PCR using rabbit liver cDNA (Zyagen, San Diego, CA) as the template. The forward primer corresponded to the putative start site for the mature form of PCSK9, as judged by alignment to the human PCSK9 sequence. Several attempts to amplify rabbit PCSK9 from the predicted COOH terminus were unsuccessful. However, an amplicon was obtained with a reverse primer corresponding to 45 base pairs from the predicted COOH terminus. Rabbit PCSK9 was then cloned into the in vitro expression vector pEU-E01-His-N1-FLEX-T14K (42) (CellFree Sciences, Matsuyama, Japan). Rabbit PCSK9 was in vitro translated using WEPRO 7240H (Cambridge Isotope Laboratories, Tewksbury, MA) in the presence of [13C,15N]Arg/Lys, resulting in heavy-labeled PCSK9 (PCSK9H).

Peptide and MS/MS ion selection for quantification of PCSK9 using parallel reaction monitoring MS. The PCSK9H trypptic peptides were used to optimize instrument parameters and to establish a quantification method using parallel reaction monitoring (PRM); the advantages of this approach have been discussed previously (31, 35). The peptides used to establish the standard curves were chosen based on 1) high precursor intensities, 2) fragment quality, and 3) nonoverlapping retention times. Since the target peptides had dispersed retention times, PRM scans were dedicated to a single target peptide per duty cycle, thereby maximizing the points across the chromatographic curve for optimal quantification. Seven peptides met our criteria; however, only five peptides produced linear standard curves with a lower limit of quantification of 10–100 amol (see more below).

PRM for quantification of rabbit plasma PCSK9. To generate a standard curve, several dilutions of PCSK9H were mixed with 500 μl of plasma to achieve the same matrix effect as endogenous/light (PCSK9L), followed by immunoprecipitation, SDS-PAGE, and in-gel digestion carried out as described above. The peptide samples were analyzed by PRM (31, 35). The extracted ion chromatograms (XICs) of the monoisotopic peaks of two to three fragments per peptide were quantified and then plotted to establish the lower limit of detection and quantification (FLEXiQuant) strategy was used for relative and absolute quantification of PCSK9 in rabbit plasma (43). Rabbit PCSK9 was amplified by PCR using rabbit liver cDNA (Zyagen, San Diego, CA) as the template. The forward primer corresponded to the putative start site for the mature form of PCSK9, as judged by alignment to the human PCSK9 sequence. Several attempts to amplify rabbit PCSK9 from the predicted COOH terminus were unsuccessful. However, an amplicon was obtained with a reverse primer corresponding to 45 base pairs from the predicted COOH terminus. Rabbit PCSK9 was then cloned into the in vitro expression vector pEU-E01-His-N1-FLEX-T14K (42) (CellFree Sciences, Matsuyama, Japan). Rabbit PCSK9 was in vitro translated using WEPRO 7240H (Cambridge Isotope Laboratories, Tewksbury, MA) in the presence of [13C,15N]Arg/Lys, resulting in heavy-labeled PCSK9 (PCSK9H).

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In vitro and in vivo characteristics of K-312. K-312 is a small-molecule CETP inhibitor that is currently in a phase I clinical trial (ClinicalTrials.gov: NCT01952548). K-312 inhibits CETP activity in human plasma more potently than other CETP inhibitors, anacetrapib and evacetrapib, which are also in clinical development (Table 1). To investigate the potential effect of K-312 on blood pressure (2, 18), we measured arterial blood pressure in Sprague-Dawley rats. No difference was observed between vehicle control and K-312 groups under conditions where torcetrapib increased blood pressure (data not shown). We then examined the effect of K-312 on plasma lipoprotein cholesterol levels in normolipidemic Syrian golden hamsters. Single oral administration of K-312 or anacetrapib

<table>
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<tr>
<th>CETP Inhibitor</th>
<th>Human Plasma (IC50), μM</th>
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<tr>
<td>K-312</td>
<td>0.06</td>
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<tr>
<td>Anacetrapib</td>
<td>0.20</td>
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<tr>
<td>Evacetrapib</td>
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CETP, cholesteryl ester transfer protein.

 RESULTS

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inhibited plasma CETP activity at comparable levels (Fig. 1A). When administered for 2 wk, K-312 and anacetrapib increased HDL-C levels significantly and decreased LDL-C levels (Fig. 1B). We also examined the effects of K-312 on plasma lipid levels in cholesterol-fed New Zealand White rabbits. K-312 increased HDL-C across the 18 wk of the treatment period (Fig. 2A). Furthermore, K-312 tended to lower LDL-C, VLDL cholesterol, and triglyceride levels (Fig. 2A). Plasma CETP activity was suppressed >90% at week 18 (Fig. 2B). These results indicate that K-312 lowers CETP activity and increases HDL-C levels and also has the potential to decrease LDL-C levels, although this effect was not sustained in our animal model.

**K-312 suppresses development of atherosclerosis in hyperlipidemic rabbits.** We examined the effect of K-312 on the development of atherosclerotic plaques in cholesterol-fed New Zealand White rabbits. We measured atherosclerotic plaque area in the aorta as gauged by lipid deposition (en face Oil Red O staining; Fig. 3, A and B). K-312 administration (10 and 30 mg/kg) reduced plaque area by ≈54.8%. Further analyses linked atherosclerotic plaque area with changes of plasma lipid levels. Lesion area correlated with AUC of LDL-C levels,
whereas no significant relation was observed with AUC of HDL-C (Fig. 3C), indicating that the LDL-C-lowering effect of K-312 may have accounted for the reduction of atherosclerotic plaques.

Enhanced cholesterol efflux from cholesterol-loaded macrophages is one of the key potential benefits of HDL-raising therapies. HDL isolated from rabbits treated with K-312 had higher cholesterol efflux activity from the macrophage cell line RAW264.7 compared with that isolated from vehicle control (Fig. 3D). This result indicates that K-312 may increase the capacity of HDL to take up cholesterol from peripheral tissues.

**K-312 suppresses PCSK9 expression in HepG2 cells and primary hepatocytes.** To further explore pharmacological features of K-312 other than the CETP inhibitory activity, we conducted gene expression profiling using DNA microarray analysis of the human hepatoma cell line HepG2. We identified PCSK9 as one of the genes whose expression decreased with K-312 treatment. PCSK9, secreted mainly from the liver, accelerates degradation of LDL receptor; hence, suppression of PCSK9 expression inhibits degradation of LDL receptor, leading to LDL-C lowering. K-312 at 0.3–3 μM suppressed PCSK9 mRNA levels by > 69.2% in HepG2 cells (real-time qPCR; Fig. 4A). The other CETP inhibitors anacatreplab and torcetrapib also decreased PCSK9 mRNA, but at lower efficacies than that of K-312 (Fig. 4A). We examined the time-dependent response of PCSK9 expression to K-312 in HepG2 cells. K-312 reduced PCSK9 mRNA as early as 4 h after its addition, reaching the maximum reduction at 24 h (Fig. 4B). Treatment with K-312 decreased protein levels of PCSK9 in the culture medium of HepG2 cells in a dose-dependent man-
Fig. 4. K-312 decreases proprotein convertase subtilisin/kexin 9 (PCSK9) levels in HepG2 and primary hepatocytes. A: PCSK9 mRNA levels in HepG2 cells treated with various CETP inhibitors for 24 h (n = 4). B: time course of PCSK9 mRNA expression after the addition of K-312 (3 μM) in HepG2 cells (n = 3). Data show relative mRNA levels in K-312-treated cells compared with DMSO-treated cells at each time point. C: PCSK9 protein concentration in HepG2 culture media 24 h after K-312 or Ana treatment (n = 3). D: PCSK9 mRNA levels in human primary hepatocytes treated with K-312 or Ana for 24 h (n = 3). E and F: effects of silencing of CETP on CETP mRNA (E) or PCSK9 mRNA (F) levels in HepG2 cells. HepG2 cells were treated with siRNA for either nontarget control or CETP and then cultured for 24 h. Cells were then incubated with serum-free medium containing DMSO, K-312, or 3 μM Ana (n = 3). G: PCSK9 mRNA levels in primary rat hepatocytes treated with either K-312 or 10 μM Ana for 24 h (n = 3). Each set of mRNA data shows mRNA levels relative to DMSO control (Cont) normalized by β-actin (A–D and G) or Rppl0 (E and F). *P < 0.05, **P < 0.01, and ***P < 0.001 vs. Cont [Dunnett’s test (A, C, D, and G), unpaired t-test (E), and Tukey’s test (F)]. Tor, torcetrapib; Dal, dalcetrapib.
ner, which is in accord with PCSK9 mRNA levels (Fig. 4C). Although anacetrapib significantly decreased PCSK9 secretion, the effect was not as potent as that of K-312. Finally, we validated the inhibitory effect of K-312 on PCSK9 expression in human primary hepatocytes (Fig. 4D).

**K-312 suppresses PCSK9 in a CETP-independent manner.**

We then questioned whether the reduction in PCSK9 was dependent on CETP inhibition by K-312. We silenced CETP in HepG2 cells via siRNA (mRNA silencing efficacy >90%; Fig. 4E) and cultured the cells in serum-free medium to ensure no exogenous source of CETP. Depletion of CETP did not affect PCSK9 mRNA expression or the suppressive effect of K-312 on PCSK9 mRNA expression (Fig. 4F). Primary rat hepatocytes, which do not express CETP, also demonstrated a decrease in PCSK9 mRNA with K-312 treatment (Fig. 4G). Collectively, these data indicate that the suppression of PCSK9 by K-312 occurs in human and rat hepatocytes seemingly via a mechanism independent of CETP inhibition.

**K-312 decreases PCSK9 expression by suppressing promoter activity.** We aimed to clarify the mechanism(s) by which K-312 suppresses PCSK9 expression. First, we investigated whether the reduction in PCSK9 expression by altering the stability of PCSK9 mRNA. Measuring the decay of PCSK9 mRNA was enabled by halting transcription using actinomycin D. PCSK9 mRNA stability did not differ in the DMSO control and K-312 treatment (Fig. 5A). We then studied the effect of K-312 on the transcriptional activity of PCSK9 using the promoter-luciferase assay (Fig. 5B). Luciferase activity with PCSK9 promoter −1 to −3,150 bp, relative to translation start site, was suppressed by 60% when treated with 3 μM K-312 (Fig. 5C). Deletion analysis showed that inhibition of PCSK9 promoter activity by K-312 decreased when the promoter was

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**Fig. 5. K-312 suppresses PCSK9 promoter activity.**

A: decay of PCSK9 mRNA levels in HepG2 cells treated with 5 μg/ml actinomycin D (Act.D) with DMSO or 3 μM K-312 for the indicated time (n = 3). B: diagram of reporter constructs with various lengths of PCSK9 promoter sequence. Numbers on the diagram represent the positions relative to translation start site (ATG). C: effects of K-312 on PCSK9 promoter activity. HepG2 cells transfected with PCSK9 promoter reporter constructs were treated with DMSO or 3 μM K-312 for 24 h (n = 3). D: diagram of mutation constructs for hepatocyte nuclear factor 1 (HNF-1), HINFP, and sterol regulatory element-binding protein (SREBP) binding sites in −450-bp PCSK9 promoter reporter construct. E: effects of K-312 on PCSK9 promoter activity in mutation constructs (n = 3). Data show %inhibition of promoter activity by K-312 compared with promoter activity in DMSO treatment.
shortened to less than −450 bp, suggesting the existence of key responsive elements around −450 bp (Fig. 5C). Previous reports identified the binding sites for HNF-1 (−386 to −374 bp), HINFP (−362 to −356 bp), and SREBP (−345 to −336 bp) on the PCSK9 promoter (12, 26). Mutation of each transcription factor binding site decreased the basal luciferase activity, which is in accord with the results from previous studies (21, 26). Suppression of luciferase activity by K-312 treatment was almost completely abolished in each of the mutation constructs (Fig. 5E), indicating that these transcription factors or binding sites may mediate the suppressive effect of K-312 on PCSK9 expression.

**K-312 decreases the expression of full-length SREBP-1 and active forms of SREBP-1 and SREBP-2.** We examined the effect of K-312 on HNF-1α, HNF-1β, and HINFP by examining the expression of these transcription factors in HepG2 cells. K-312 treatment for 24 h (0.3 to 3 μM) did not change the mRNA or protein levels of HNF-1α, HNF-1β, or HINFP (Fig. 6, A and B). K-312 treatment produced no significant changes in active HNF-1 levels in HepG2 nuclei (Fig. 6C). We then analyzed the expression of SREBP-1 and SREBP-2. SREBP-1 mRNA decreased substantially with K-312 treatment at 1 and 3 μM, whereas SREBP-2 mRNA decreased slightly but significantly at 3 μM (Fig. 6D). SREBP functions as a transcription factor when activated through the cleavage of the full-length form into the nuclear or active form and then translocates to nuclei. K-312 treatment decreased nuclear forms of SREBP-1 and SREBP-2 at 4 and 24 h in HepG2 whole cell lysate (Fig. 6E). Furthermore, the full-length SREBP-1, but not SREBP-2, decreased with K-312 for 24 h, which reflects the gene expression results. We also examined nuclear levels of SREBP-1 and SREBP-2. K-312 decreased both SREBP nuclear forms at 4 and 24 h (Fig. 6F).

**K-312 reduces the binding activity of SREBP-1 and SREBP-2 to the PCSK9 promoter.** We performed ChIP-qPCR to evaluate the occupancy of the PCSK9 promoter with SREBP(s). After the immunoprecipitation of the SREBP-DNA complex, real-time qPCR was performed using primers to amplify the PCSK9 promoter region, including the sterol regulatory element. Both SREBP-1 and SREBP-2 PCSK9 promoter occupancies decreased at 4 and 24 h of treatment with K-312 (Fig. 6G). Of note is that this decrease in SREBP-1 and SREBP-2 occupancy may account for the K-312-mediated suppression of PCSK9 mRNA observed as early as at 4 h (Fig. 4B).

Loss-of-function analysis in HepG2 cells examined the relative contribution of SREBP-1 and SREBP-2 on the PCSK9 transcriptional regulation. Silencing of SREBP-2 decreased PCSK9 mRNA and protein levels, whereas silencing of SREBP-1 increased PCSK9 levels, which was due possibly to the compensatory increase of SREBP-2 levels (Fig. 7, A and B). However, when both SREBP-1 and SREBP-2 were suppressed at the same time, PCSK9 levels decreased further compared with when SREBP-2 alone was silenced (Fig. 7B). These results suggest that K-312 decreases both SREBP-1 and SREBP-2 nuclear form levels, which decrease the binding of these transcription factors on the PCSK9 promoter, thereby suppressing PCSK9 transcription.

Quantitative proteomics demonstrates that K-312 decreases plasma levels of PCSK9 in cholesterol-fed rabbits. To seek the in vivo evidence for the effects of K-312 on PCSK9 production, we aimed to measure its protein levels in cholesterol-fed rabbits. K-312 treatment showed a tendency to lower liver PCSK9 mRNA levels by 63% (Fig. 8A), whereas LDLR mRNA levels were not decreased by K-312 (Fig. 8B). To determine whether this decrease in PCSK9 mRNA produces a significant decrease in PCSK9 in plasma, we turned to MS-based approaches since available anti-rabbit PCSK9 ELISA assays did not reliably indicate PCSK9 signal from rabbit plasma. We synthesized rabbit PCSK9 in vitro in a [13C,15N]Arg/Lys-labeled (heavy) form, PCSK9H, as a standard for optimization of immunochemistry- and MS-based detection and quantification methods (42). Without the availability of an anti-rabbit PCSK9 antibody, we tested the ability of several anti-PCSK9 antibodies to recognize rabbit PCSK9 by Western blot (Fig. 8C). Using the PCSK9H as a control, the sheep anti-human antibody proved to be the most promising candidate for immunoprecipitation of PCSK9 from plasma. Indeed, immunoprecipitation of PCSK9 from plasma derived from the seven control and seven K-312-treated rabbits enriched the PCSK9 signal sufficiently for spectral counting-based comparison between the two groups. Spectral counting revealed significantly lower levels of PCSK9 in the K-312-treated vs. control group (reduction of 76%), where one rabbit in the K-312 group had no observed PCSK9 signal (Fig. 8D).

In parallel, we capitalized on a recently introduced MS method, PRM, that relies on high resolution and accurate mass measurements acquired on the bench top quadrupole Orbitrap platform (31, 35). Pilot studies into the candidacy of PCSK9H peptides for high-resolution MS/MS-based quantification included nonoverlapping retention times (Fig. 9, A and B); five peptides met all criteria (see MATERIALS and METHODS for more details of this approach). Serial dilution spiking experiments of PCSK9H into rabbit plasma were performed to account for plasma-specific matrix effects on the ionization properties and to establish the lower limits of quantification of the PCSK9 peptides. In addition, as the standard is isotopically labeled, there is insignificant interference by the light/endogenous PCSK9 peptides during data acquisition. Figure 9C is an example MS/MS spectrum for a peptide derived from either the endogenous (light) or PCSK9H standard (heavy). The lower limit of detection was calculated to be 10–100 amol on column for each peptide (Fig. 9D). PRM also demonstrated that PCSK9 was 57% lower in the K-312 treatment group (Fig. 9E). Moreover, this method quantified the PCSK9 signal from the rabbit whose spectral counts were zero (Fig. 9B), demonstrating the sensitivity of the method. Taken together, our results demonstrate that K-312 lowers PCSK9 levels in vivo.

**DISCUSSION**

LDL-C lowering is an established therapy for cardiovascular disease. Human genetic studies positively correlated CETP and LDL-C levels (20). In fact, CETP inhibition is now recognized as an LDL-C lowering therapy, although its clinical impact on cardiovascular outcomes needs to be clarified (23). The new CETP inhibitor K-312 decreased LDL-C in animal models. Furthermore, our study revealed that K-312 suppresses PCSK9 levels in vitro and in vivo. Through its dual inhibitory actions on CETP and PCSK9, K-312 may serve as a novel LDL-lowering drug. We demonstrated that K-312 suppresses PCSK9 expression in the human hepatoma cell line HepG2 and
Fig. 6. K-312 decreases SREBP levels, thereby suppressing PCSK9. A and B: mRNA (A) and protein (B) levels of HNF-1α, HNF-1β, and HINFP in HepG2 cells after treatment with K-312 or Ana for 24 h (n = 4 for A). C: active HNF-1 levels in HepG2 cells after K-312 or Ana treatment. Activity of HNF-1 in HepG2 nuclear extract was measured using TransAM transcription factor ELISA (active motif). D: mRNA levels of SREBP-1 and SREBP-2 in HepG2 cells after treatment with K-312 or Ana (n = 4). E: protein levels of precursor (P) and nuclear forms (N) of SREBP-1 and SREBP-2. SREBPs were immunoprecipitated from HepG2 whole cell lysate followed by Western blotting. F: mRNA levels of SREBP-1 and SREBP-2. LXR agonist T0901317 (1 μM; T1317) and cholesterol depletion with 0.3 μM pitavastatin in 5% lipoprotein-deficient serum (Chol) serve as positive control to increase nuclear forms of SREBP-1 and SREBP-2, respectively. TATA-binding protein (TBP) serves as loading control for nuclear protein. G: chromatin immunoprecipitation (ChIP) assay to examine the occupancy of PCSK9 promoter by SREBPs shown as fold enrichment of PCSK9 promoter region relative to nontarget sequence IGX1A (n = 3). The mRNA data are relative to vehicle control normalized by β-actin. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. control (C; Dunnett’s test). IB, immunoblot.
that its mechanism of action involves SREBP-1/2. In addition, K-312 decreased PCSK9 expression in human primary hepatocytes, which express SREBP-1c as a dominant isoform of SREBP-1 similar to the liver tissue (41). K-312 treatment also attenuates the progression of atherosclerotic lesions in hyperlipidemic rabbits. Moreover, these K-312-treated rabbits possess decreased protein levels of PCSK9 in plasma, providing the in vivo evidence for the impact of this compound on PCSK9. Collectively, our study indicates that K-312 may serve as a potent lipid modulator and lower cardiovascular risk in patients.

In this study, we demonstrate suppression of PCSK9 mRNA levels in HepG2 cells not only by K-312 but also anacetrapib and torcetrapib, albeit with higher potency for K-312 (Fig. 4A). A recent study showed that anacetrapib, torcetrapib, and evacetrapib decreased PCSK9 levels via SREBP-2 in vitro and in vivo (11). However, our more in-depth study into the PCSK9 promoter occupancies by SREBP-1 and -2 demonstrated decreased binding of both transcription regulators in response to K-312 treatment, a candidate mechanism for the increased potency of K-312 treatment over the other CETP/PCSK9 inhibitors (Fig. 4). We then had to determine whether a CETP inhibitory action of K-312 contributes to PCSK9 suppression as a class effect. Silencing of CETP expression by siRNA in HepG2 cells did not compromise the suppression of PCSK9 mRNA levels by K-312, nor did lacking CETP expression itself, as determined by rat primary hepatocytes studies (Fig. 4, F and G). These lines of evidence demonstrated that suppression of PCSK9 by K-312 involves a CETP-independent mechanism.
K-312 decreases active forms of both SREBP-1 and SREBP-2, which raises the concern if K-312 treatment compromises LDLR expression since transcription of LDLR gene is induced (or activated) mainly by SREBP-2. After 2 wk of treatment with K-312 in cholesterol-fed rabbits, however, liver LDLR mRNA levels did not decrease by K-312 treatment (Fig. 8B), indicating the possibility that reduction of PCSK9 with K-312 treatment was due mainly to SREBP-1 in our rabbit model. Although SREBP-2 has been recognized as a transcriptional regulator of PCSK9, some studies support the involvement of SREBP-1 as another key transcriptional regulator of PCSK9. Costet et al. (9) reported that PCSK9 levels in plasma are affected by insulin likely via SREBP-1c in mice. Furthermore, in a human study, a high-fructose diet increased PCSK9 levels without affecting cholesterol synthesis, which is regulated by SREBP-2, suggesting the regulation of PCSK9 expression by SREBP-1 (4). Further analysis will be required to address the relative contribution of SREBP-1 and SREBP-2 on transcription of PCSK9.

Growing evidence indicates that cholesterol levels in HDL may not represent cardioprotective properties, suggesting that the functionality of HDL plays a role (16). Considering that the ultimate goal of HDL therapy is enhancing reverse cholesterol transport from peripheral tissues to the liver to prevent or cure atherosclerosis, more comprehensive analyses on HDL functionality are necessary to further refine HDL therapeutics (15, 51). As a previous study suggested, cholesterol efflux from cholesterol-enriched macrophages to apolipoprotein B-depleted serum, a surrogate for the HDL fraction, better predicts atherosclerotic burden than HDL-C levels (24). In the present study, HDL isolated from K-312-treated rabbits exhibits enhanced cholesterol efflux capacity from cholesterol-loaded macrophages (Fig. 3D), suggesting a potential antiatherogenic effect of this compound.

Finally, to verify whether PCSK9 levels were in fact lower in rabbit plasma, we took advantage of a relatively new high-resolution MS/MS platform, also referred to as PRM (31), to quantify circulating PCSK9. This technology serves as a powerful tool when an ELISA-based system is not available. Moreover, high-resolution MS/MS is emerging as a promising alternative to the traditional targeted mass spectrometry method, known as multiple reaction monitoring, that is performed on the highly selective but low resolving triple quadrupole (31, 35). Our workflow, employing a stable isotope-labeled rabbit PCSK9 standard in combination with high-resolution MS/MS, is the first of its kind to be presented. We believe that this and similar high-resolution MS/MS technologies will be critical not only for understanding the underlying mechanisms of cardiovascular diseases but also facilitating the development of new therapies.

In the clinic, statins are one of the most prescribed drugs for hypercholesterolemia; however, the treatment is known to increase PCSK9 levels, which compromises the LDL-C-lowering effect of statins (12). For this reason, therapies to inhibit expression or action of PCSK9 would strengthen the LDL-C-lowering effect of statins. Thus, when used with statins, K-312 may enhance their LDL-C-lowering action more potently than other CETP inhibitors. Small-molecule inhibitors are generally better tolerated than biotherapeutics, which require injection. Therefore, K-312, the small-molecule dual inhibitor of CETP and PCSK9, may be a promising add-on therapy to existing drugs such as statins.

Our preclinical study has a few limitations, including a high-cholesterol-fed rabbit model. Although we provided clear
evidence for the beneficial effects of K-312 on PCSK9 expression, its LDL-lowering effects were not sustained at the later time points. The present study was designed originally to examine whether K-312 suppresses the development of atherosclerosis in rabbits. High-cholesterol feeding was thus necessary to accomplish this primary goal. As clearly documented in other studies (25, 49), however, excessive cholesterol stored in rabbits due to cholesterol feeding may have offset the effects of K-312. For instance, excess cholesterol suppresses SREBP activity in the liver (3), which could suppress downstream gene expression such as LDLR and PCSK9, crucial molecules for LDL metabolism. Sustained effects on CETP inhibition (Fig. 2B) and the lack of correlation between LDL-C and PCSK9 levels ($r^2 = 0.078$, $P = 0.151$; data not shown) at the 18th wk also support this speculation and also deny the possibility that the loss of K-312’s effects on either CETP inhibition or PCSK9

Fig. 9. A parallel reaction-monitoring (PRM) approach for improved detection and quantification of plasma PCSK9. A: amino acid sequence of rabbit PCSK9 (Uniprot ID: G1U461; www.uniprot.org) with 5 tryptic peptides (boldface letters) chosen for PRM. B: extracted ion chromatogram from example fragment ions used in PRM. C: MS/MS ion spectra of representative light (endogenous) or heavy [$^{13}$C, $^{15}$N-Arg/Lys] PCSK9 peptide. D: standard curves for the fragment ions used for PRM. AUC of the extracted ion chromatogram was plotted for the corresponding volume of PCSK9 standard. E: K-312 treatment for 2 wk significantly decreased PCSK9 protein levels in plasma of cholesterol-fed rabbits, as shown by PRM. *$P < 0.01$, vs. control group (unpaired t-test). A carbamidomethyl cysteine is indicated as lowercase c in the peptide sequences.
suppression led to the reduced effects on LDL lowering at the later time points. In fact, in a similar cholesterol-fed rabbit model, the CETP inhibitor torcetrapib reduced non-HDL cholesterol until 5.5 wk, and yet the effect was diminished at 11 to 16 wk (32), although in humans, torcetrapib treatment showed consistent LDL-C reduction for at least 12 mo (2). Despite the loss of LDL-lowering effects in these rabbits, however, we were able to demonstrate statistically significant effects of K-312 on atherogenesis. Furthermore, this may indicate that the antiatherogenic effects of HDL raising by K-312 outweighed increased LDL levels in our rabbit model. Our study provides molecular and preclinical bases for future clinical studies that can address whether K-312 suppresses PCSK9 and LDL cholesterol levels and retards the progression of atherosclerotic plaques in humans, all of which may contribute to the reduction of cardiovascular risk.

In conclusion, the new CETP inhibitor K-312 suppresses PCSK9 expression through the modulation of its transcription by decreasing SREBP levels. Through this unique mechanism, K-312 may raise HDL-C and lower LDL-C in patients, two major goals in the development of new lipid modulators beyond statins. K-312 may thus represent promising novel strategies to reduce the residual global burden of cardiovascular diseases.

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DISCLOSURES


AUTHOR CONTRIBUTIONS


REFERENCES


