New CETP Inhibitor K-312 Reduces PCSK9 Expression
A Potential Effect on LDL Cholesterol Metabolism

Katsutoshi Miyosawa1,2, Yuichiro Watanabe1,2, Kentaro Murakami1,2, Takeshi Murakami1,2, Haruki Shibata2, Masaya Iwashita1,2, Hiroyuki Yamazaki2, Koichi Yamazaki2, Tadaaki Ohgiya2, Kimiyuki Shibuya2, Ken Mizuno1,2, Sohei Tanabe2, Sasha A. Singh1, Masanori Aikawa1

1Center for Interdisciplinary Cardiovascular Sciences, Brigham and Women's Hospital, Harvard Medical School, Boston, MA
2Tokyo New Drug Research Laboratories, Kowa Company, Ltd., Tokyo, Japan

Running title: New CETP Inhibitor K-312 Reduces PCSK9 Expression

Address for Correspondence:
Masanori Aikawa, MD, PhD
Center for Interdisciplinary Cardiovascular Sciences, Brigham and Women's Hospital, Harvard Medical School, 3 Blackfan Circle, CLSB, Floor 17, Boston, MA 02115
Tel: 617-730-7777
Fax: 617-730-7791
Email: maikawa@rics.bwh.harvard.edu

Copyright © 2015 by the American Physiological Society.
Abstract

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 (IC_{50}: 0.06 μM). Administration of K-312 to cholesterol-fed New Zealand White rabbits for 18 weeks 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 active forms of sterol regulatory element binding proteins 1 and 2 (SREBP-1 and SREBP-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-C and PCSK9 levels, serving as a new therapy for dyslipidemia and cardiovascular disease.
Key words: atherosclerosis, SREBP, lipoproteins, mass spectrometry, parallel reaction monitoring
Introduction

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 dalcetrapib, was terminated due to the increased mortality presumably caused by off-target effects of increasing blood pressure, unrelated to CETP inhibition itself, or due 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). HMG-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
PCSK9 inhibitors represent one of the most promising LDL lowering therapeutics (46). PCSK9, which is mainly produced 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 as compared to 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 CHD (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, 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.
LDL-lowering agents (e.g., statins), which are administered orally, small molecule PCSK9 inhibitors would have substantial benefits compared to biotherapeutics. We synthesized the novel small molecule CETP inhibitor K-312, which inhibits CETP as potently as do other CETP inhibitors, anacetrapib and evacetrapib, but does not indicate the adverse side effect of elevated blood pressure, when compared to 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 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): beta actin (4326315E), HINFP (Hs00210105_m1), HNF1α (Hs00167041_m1), HNF1β (Hs01001602_m1), PCSK9 (Hs00545399_m1), SREBP-1 (Hs001088691_m1), SREBP-2 (Hs01081784_m1). Other primer sequences are available upon request. CETP inhibitors (K-312, anacetrapib, evacetrapib, torcetrapib, dalcetrapib) were synthesized in Tokyo New Drug Research Laboratories of Kowa Company, Ltd. (Tokyo, Japan).

**Animal experiments**

Animal studies were carried out at Kowa Tokyo New Drug Research Laboratories and all animals used and experimental procedures were approved by in-house committee of the laboratory. Male Syrian golden hamsters (7 weeks old, Japan SLC, Shizuoka, Japan) fed
chow diet were administered with K-312 or anacetrapib suspended in 0.5% methylcellulose by gavage for 2 weeks. Male New Zealand White rabbits (2.0 – 2.5 kg, Kitayama Labes, Nagano, Japan) were fed a rabbit chow containing 0.25% of cholesterol. K-312 suspended in 0.5% methylcellulose was administered once a day by gavage. For the analysis of atherosclerosis, after 18 weeks of administration of K-312, the aorta was dissected and stained with Oil red-O to measure the atheroma area. For the analysis of PCSK9, animals were treated with K-312 for 2 weeks then plasma and livers were collected. Male Sprague Dawley rats (Japan SLC) were used for blood pressure monitoring and primary hepatocyte isolation.

Biochemical analysis
Plasma lipid levels were measured with automatic analyzer (Labospect 003, HITACHI, Tokyo, Japan). Plasma lipoprotein cholesterol levels were determined using HPLC (Shimadzu LC-20A system) equipped with Superose 6 column (GE Healthcare, Fairfield, CT)(48).

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 (EMEM, ATCC) supplemented with 10% fetal bovine serum (FBS), 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 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 RAW 264.7 cells (ATCC) were cultured for 24 hours in DMEM containing 50 μg/mL acetyl-LDL (BT-906, Biomedical Technologies, Stoughton, MA) and 2 μCi/ml of [3H] cholesterol (PerkinElmer, Waltham, MA). After stimulation with LXR agonist (T0901317 1 μM) for 24 hours, the rabbit HDL fraction (1.125 < d < 1.210 g/ml), isolated by ultracentrifugation (19), was added to the medium. The media was collected 4 hours 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 subcloned into the pGL3-basic vector (Promega, Madison, WI). Mutation constructs for the various transcription factor binding sites were generated with the method previously reported (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 by On-Target plus SMART pool siRNA (Dharmacon, Lafayette, CO). On-Target plus non-targeting 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, St. Louis, MO) to halt transcription. K-312 was added to the culture media and incubated for the indicated time. After RNA isolation, gene expression was measured by real-time qPCR to determine the decay of mRNA.

Detection of SREBP-1 and SREBP-2 by Western blot

To improve the detection of SREBP, immunoprecipitation (IP) of SREBP was carried out prior to the Western blot. Cell lysate was pre-cleared with Dynabeads (Life Technologies, Thermo Fisher) and normal rabbit IgG (R&D systems) for 1 hour. The lysate was then incubated for 4 hours with either anti-SREBP-1 antibody (sc-8984, Santa Cruz) or anti-SREBP-2 antibody (10007663, Cayman Chemical) followed by the incubation with Dynabeads Protein G for 1 hour. After washing the beads, 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 nuclear extract kit (Active Motif), then 20 to 30 μg of the lysate was analyzed by the Western blot technique with the same
antibodies used above.

**Chromatin Immunoprecipitation (ChIP) 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 average size of 200 – 500 bp. ChIP was carried out with anti-SREBP-1 antibody (sc-8984, Santa Cruz) or anti-SREBP-2 antibody (10007663, Cayman Chemical). The immune complex was collected with Dynabeads Protein G (Life Technologies) then purified with 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 specific primer set. Reactions were performed in triplicate and the fold enrichment was calculated against non-target region IGX1A (QIAGEN).

**CETP activity assay**

In vitro CETP inhibitory activity of compounds was measured using methods previously reported (8). CETP inhibitors were incubated with human plasma for 4 hours at 37˚C prior to measure the CETP activity. Ex vivo CETP activity was measured with hamster or rabbit plasma treated with K-312 or anacetrapib.

**Immunoprecipitation and sample preparation for mass spectrometry-based quantification of PCSK9**

Rabbit plasma 500 μl was incubated for 18 hours 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 then boiled to elute proteins from beads. Immunoprecipitate was resolved by SDS-PAGE and the area between 50 - 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 C-terminus were unsuccessful. However, an amplicon was obtained with a reverse primer corresponding to 45 base pairs from the predicted C-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 mass spectrometry**

The PCSK9H tryptic 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) non-overlapping 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 (more below).

**PRM for quantification of rabbit plasma PCSK9**

In order to generate a standard curve, several dilutions of PCSK9<sup>H</sup> were mixed with 500 μl of plasma to achieve the same matrix effect as endogenous/light (PCSK9<sup>L</sup>); 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 mono-isotopic peaks of two to three fragments per peptide were quantified and then plotted to establish the lower limit of detection and quantification for the various peptides. For absolute quantification of the PCSK9<sup>H</sup> standard, 1.0 fmol of FLEX-peptide<sup>L</sup> (TENLYFQGDISR, m/z = 721.86) were spiked into the samples. The XIC corresponding to the area under the curve (AUC) of each FLEX<sup>H</sup> (PCSK9<sup>H</sup>) fragment and the corresponding FLEX<sup>L</sup> fragment was measured. The absolute quantity of the PCSK9<sup>H</sup> standard was calculated by the FLEX<sup>L</sup>:FLEX<sup>H</sup> ratio, which was then correlated to the AUCs of the five PCSK9<sup>H</sup> fragment ions (43).

The AUCs for the five endogenous/PCSK9<sup>L</sup> peptide fragments were then measured and quantified using the PCSK9<sup>H</sup> standard curves. The absolute quantity of each individual peptide was then calculated based on the median value for its fragment ions. The final
quantity for PCSK9L was then calculated by taking the median value for all peptides for each rabbit (43).

**Mass spectrometry**

Peptide samples were analyzed with the high resolution/accuracy Q Exactive mass spectrometer fronted with a Nanospray FLEX ion source, and coupled to an Easy-nLC1000 HPLC pump (Thermo Scientific, Waltham, MA). The peptides were subjected to a dual column set-up: an Acclaim PepMap RSLC C18 trap column, 75 µm X 20 mm; and an Acclaim PepMap RSLC C18 analytical column 50 µm X 150 mm (Thermo Scientific). The analytical gradient was run at 250 nl/min from 5 to 28% Solvent B (acetonitrile/0.1% formic acid) for 30 min, followed by 5 min of 95% Solvent B. Solvent A was 0.1% formic acid. All reagents were HPLC-grade. For standard data dependent MS, the instrument was set to 140 K resolution, and the top 10 precursor ions (within a scan range of 380-1500 m/z) were subjected to higher energy collision induced dissociation (HCD, collision energy 25 +/- 10%, isolation width 1.6 m/z, and MS/MS resolution set to 17.5 K). The dynamic exclusion feature was disabled for spectral counting experiments. An inclusion list with light or heavy PCSK9 peptide m/z values and their corresponding retention times was used for PRM (isolation window, +/- 1.5 m/z; resolution, 140 K) using the DIA module on the Q Exactive software.

**Data analysis**

The MS/MS data were queried against the Rabbit Uniprot database (downloaded on March 27, 2013) using the SEQUEST search algorithm, via the Proteome Discoverer (PD) Package.
(version 1.3, Thermo Scientific) (14) using a 10 ppm tolerance window in the MS1 search space, and a 0.02 Da fragment tolerance window for HCD. Methionine oxidation was set as a variable modification, and carbamidomethylation of cysteine residues was set as a fixed modification. The peptide false discovery rate (FDR) was calculated using Percolator provided by PD: the FDR was determined based on the number of MS/MS spectral hits when searched against the reverse, decoy Rabbit database (13, 22). Skyline (30) was used for XIC based quantification and for candidate MS/MS ion filtering.

**Statistical Analysis**

Statistical differences were tested by unpaired t-test, or by one-way ANOVA followed by post-hoc analysis with Dunnett’s test or Tukey’s test. Differences with $p<0.05$ were considered statistically significant. All experiments were performed in triplicate or quadruplicate unless otherwise mentioned and the data are presented as mean ± standard error of mean (SEM).

**Results**

**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 inhibited plasma CETP activity at comparable levels (Figure 1A). When
administered for 2 weeks, K-312 and anacetrapib significantly increased HDL-C levels and
decreased LDL-C levels (Figure 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
weeks of treatment period (Figure 2A). Furthermore, K-312 tended to lower LDL-C, VLDL-C
and triglyceride levels (Figure 2A). Plasma CETP activity was suppressed more than 90% at
week 18 (Figure 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, Figure 3A, 3B). K-312
administration (10 and 30 mg/kg) reduced plaque area by up to 54.8%. Further analyses
linked atherosclerotic plaque area with changes of plasma lipid levels. Lesion area correlated
with area under the curve (AUC) of LDL-C levels whereas no significant relation was
observed with AUC of HDL-C (Figure 3C), indicating that 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 to that
isolated from vehicle control (Figure 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 and primary hepatocytes**

To further explore pharmacological features of K-312 other than the CETP inhibitory activity,
we conducted gene expression profiling using DNA micro-array analysis of the human
hepatoma cell line HepG2. We identified PCSK9 as one of the genes whose expression
decreased with K-312 treatment. PCSK9, mainly secreted 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 up to 69.2% in HepG2 cells (real-time qPCR, Figure 4A). Other CETP inhibitors
anacetrapib and torcetrapib also decreased PCSK9 mRNA but at lower efficacies than that of
K-312 (Figure 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 hours after its addition,
reaching the maximum reduction at 24 hours (Figure 4B). Treatment with K-312 decreased
protein levels of PCSK9 in the culture media of HepG2 cells in a dose-dependent manner,
which is in accordance with PCSK9 mRNA levels (Figure 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 (Figure 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%, Figure 4E) and cultured the cells in serum-free media 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 (Figure 4F). Primary rat hepatocytes, which do not express CETP, also demonstrated a decrease in PCSK9 mRNA with K-312 treatment (Figure 4G). Collectively, these data indicate that the suppression of PCSK9 by K-312 occurs in human and rat hepatocytes, seemingly via the 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 K-312 decreases 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 (Figure 5A). We then studied the effect of K-312 on the transcriptional activity of PCSK9 using the promoter-luciferase assay (Figure 5B). Luciferase activity with PCSK9 promoter -1 bp to -3150 bp, relative to translation start site, was suppressed by 60% when treated with 3 μM K-312 (Figure 5C). Deletion analysis showed that inhibition of PCSK9 promoter activity by K-312 decreased when the promoter was shortened to less than -450 bp, suggesting the existence of key responsive elements around -450 bp (Figure 5C). Previous reports identified the binding sites for HNF1 (-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 accordance 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 (Figure 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 HNF1α, HNF1β and HINFP by examining the expression of these transcription factors in HepG2 cells. K-312 treatment for 24 hours (0.3 to 3 μM) did not change the mRNA or protein levels of HNF1α, HNF1β and HINFP (Figure 6A, B). K-312 treatment produced no significant changes in active HNF1 levels in HepG2 nuclei (Figure 6C). We then analyzed the expression of SREBP-1 and SREBP-2. SREBP-1 mRNA substantially decreased with K-312 treatment at 1 and 3 μM, whereas SREBP-2 mRNA slightly but significantly decreased at 3 μM (Figure 6D). SREBP functions as a transcription factor when activated through the cleavage of the full-length form into the nuclear or active form then translocates to nuclei. K-312 treatment decreased nuclear forms of SREBP-1 and SREBP-2 at 4 and 24 hours in HepG2 whole cell lysate (Figure 6E). Furthermore, the full length SREBP-1, but not SREBP-2, decreased with K-312 for 24 hours, which reflects the gene expression results. We also examined nuclear levels of SREBP-1 and SREBP-2. K-312 decreased both SREBP nuclear forms by at 4 and 24 hours (Figure 6F).

K-312 reduces the binding activity of SREBP-1 and SREBP-2 to the PCSK9 promoter

We performed chromatin immunoprecipitation (ChIP)-qPCR to evaluate the occupancy of PCSK9 promoter with SREBPs. After the immunoprecipitation of SREBP-DNA complex,
real-time qPCR was performed using primers to amplify the PCSK9 promoter region including the sterol regulatory element (SRE). Both SREBP-1 and SREBP-2 PCSK9 promoter occupancies decreased at 4 and 24 hours of treatment with K-312 (Figure 6G). Of note, 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 hours (Figure 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 possibly due to the compensatory increase of SREBP-2 levels (Figure 7A, B). However, when both SREBP-1 and SREBP-2 were suppressed at the same time, PCSK9 levels decreased further compared to when SREBP-2 was silenced alone (Figure 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% (Figure 8A), whereas LDLR mRNA levels were not decreased by K-312 (Figure 8B). In order to determine whether this decrease in PCSK9 mRNA produces a significant decrease in PCSK9 in plasma, we turned to mass spectrometry-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, PCSK9\textsuperscript{H}, as a standard for optimization of
immunochemistry- and mass spectrometry-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 (Figure 8C). Using the
PCSK9\textsuperscript{H} 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
PCSK9 signal sufficient for spectral counting based comparison between the two groups.
Spectral counting revealed significantly lower levels of PCSK9 in the K-312 treated versus
control group (reduction of 76%) where one rabbit in the K-312 group had no observed
PCSK9 signal (Figure 8D).

In parallel, we capitalized on a recently introduced mass spectrometry method,
parallel reaction monitoring (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 PCSK9\textsuperscript{H} peptides for high resolution MS/MS based quantification
included non-overlapping retention times (Figure 9A, B); five peptides met all criteria (see
Materials and Methods for more details of this approach). Serial dilution spiking experiments
of PCSK9\textsuperscript{H} 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 endogenous (Light) or PCSK9\textsuperscript{H} standard
(Heavy). The lower limit of detection was calculated to be 10 – 100 amol on column for each peptide (Figure 9D). PRM also demonstrated that PCSK9 was 57% lower in K-312 treatment group (Figure 9E). Moreover, this method quantified PCSK9 signal from the rabbit whose spectral counts were zero (Figure 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 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, however, with higher potency for K-312 (Figure 4A). A recent study showed that anacetrapib, torcetrapib and evacetrapib decreased PCSK9 levels via SREBP-2 in vitro and in vivo (11). Our more in-depth study into the PCSK9 promoter occupancies by SREBP-1 and -2, however, 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 (Figure 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 (Figure 4F, 4G). These lines of evidence demonstrated that suppression of PCSK9 by K-312 involves 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 two weeks of treatment with K-312 in cholesterol-fed rabbits, however, liver LDLR mRNA levels did not decrease by K-312 treatment (Figure 8B), indicating the possibility that reduction of PCSK9 with K-312 treatment was mainly due 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. reported that PCSK9 levels in plasma is affected by insulin likely via SREBP-1c in mice (9). 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 do HDL-C levels (24). In the present study, HDL isolated from K-312 treated rabbits exhibits enhanced cholesterol efflux capacity from cholesterol-loaded macrophages (Figure 3D), suggesting a potential anti-atherogenic effect of this compound.

Finally, in order 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 (MRM), that is performed on the highly selective but low resolving triple quadrupole (31, 35). Our workflow, employing a stable isotopically 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 for understanding not only 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 do other CETP inhibitors. Small molecule inhibitors are generally better tolerated than biotherapeutics, which require injection. K-312, the small molecule dual inhibitor of CETP and PCSK9, may therefore 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 originally designed 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 (Figure 2B) and the lack of correlation between LDL-cholesterol and PCSK9 levels ($R^2=0.078$, $p=0.151$; data not shown) at the 18th week also support this speculation and also denies the possibility that the loss of K-312’s effects on either CETP inhibition or PCSK9 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 weeks; yet, the effect was
diminished at 11 to 16 weeks (32), although in humans, torcetrapib treatment showed consistent LDL-C reduction for at least 12 months (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 anti-atherogenic 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 residual global burden of cardiovascular diseases.

Acknowledgments:
The authors would like to thank Alexander Mojcher, Brett Pieper and Tan Pham for their excellent technical assistance.

Disclosures: This study was supported by a research grant from Kowa Company, Ltd., Tokyo, Japan (to M.A.). K.Miyosawa, Y.W., K.Murakami, T.M., H.S., M.I., H.Y., K.Y., T.O., K.S., K.Mizuno and S.T. are employees of Kowa Company, Ltd.
References:


45. **Stein EA, Ose L, Retterstol K, Tonstad S, Schleman M, Harris S, and Sager P.**


Table 1. In vitro CETP inhibitory activity

<table>
<thead>
<tr>
<th></th>
<th>Human plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-312</td>
<td>0.06</td>
</tr>
<tr>
<td>Anacetrapib</td>
<td>0.20</td>
</tr>
<tr>
<td>Evacetrapib</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Figure 1. Effects of K-312 on lipoprotein cholesterol levels and CETP activity in hamsters. A, Plasma CETP activity after single administration of K-312 or anacetrapib 10 mg/kg in Syrian golden hamsters. Plasma was collected at indicated time points then plasma CETP activity was measured (n=3). †; p<0.01 (4 hour), ‡; p<0.05 (6 hour) v.s. 0 hour in K-312 treated group, §; p<0.05 (4 hour) v.s. 0 hour in anacetrapib treated group (Dunnett’s test). B, Vehicle (C), K-312 or anacetrapib (Ana) was administered in chow-fed Syrian golden hamsters. Plasma lipoprotein cholesterol levels were measured after 2 weeks of administration (n=8-9). *p<0.05, **p<0.01, ***p<0.001 versus control group (Dunnett’s test).

Figure 2. Effects of K-312 on lipids and CETP activity in cholesterol-fed rabbits. A, K-312 was administered in cholesterol-fed New Zealand White rabbits. Plasma lipid levels were measured at the indicated time points (n=14). B, CETP activity was measured in plasma collected at four hours after the final administration at 18th week. *p<0.05, ***p<0.001 versus control group (Dunnett’s test).

Figure 3. K-312 suppresses atherosclerotic plaque formation. A and B, En face analysis of rabbit aorta fed 0.25% cholesterol diet administered with K-312. Representative images of oil red O staining (A) and quantitative analysis of atherosclerotic lesion in the aorta (B) (n=14). Scale bars represent 1 cm. C, Regression analysis between percent lesion area and (area under the curve) AUC of HDL-C or LDL-C. D, Cholesterol efflux from RAW264.7 cells to HDL isolated from rabbits treated with K-312 for 18 weeks (n=6). Cells were labeled with [3H]cholesterol then incubated with LXR agonist (T0901317 1 μM) for 24 hours, followed by the addition of rabbit HDL (100 μg protein/ml). *p<0.05, ***p<0.001 versus control group (Dunnett’s test).

Figure 4. K-312 decreases PCSK9 levels in HepG2 and primary hepatocytes. A, PCSK9 mRNA levels in HepG2 cells treated with various CETP inhibitors for 24 hours (Ana: anacetrapib, Tor: torcetrapib, Dal: dalcetrapib, n=4). B, Time course of PCSK9 mRNA
expression after the addition of K-312 (3 μM) in HepG2 cells (n=3). Data shows relative mRNA levels in K-312 treated cells compared to DMSO treated cells at each time point. C, PCSK9 protein concentration in HepG2 culture media 24 hours after K-312 or anacetrapib treatment (n=3). D, PCSK9 mRNA levels in human primary hepatocytes treated with K-312 or anacetrapib for 24 hours (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 non-target control or CETP then cultured for 24 hours. Cells were then incubated with serum-free media containing DMSO, K-312 or anacetrapib 3 μM (n=3). G, PCSK9 mRNA levels in primary rat hepatocytes treated with either K-312 or anacetrapib 10 μM for 24 hours (n=3). Each mRNA data show mRNA levels relative to DMSO control (Cont) normalized by β-actin (A-D, G) or Rplp0 (E, F). *p<0.05, **p<0.01, ***p<0.001 versus control (Dunnett’s test (A, C, D, G), unpaired t-test (E), Tukey’s test (F)).

Figure 5. K-312 suppresses PCSK9 promoter activity. A, The decay of PCSK9 mRNA levels in HepG2 cells treated with 5 μg/ml actinomycin D with DMSO or 3 μM of 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 of K-312 for 24 hours (n=3). D, Diagram of mutation constructs for HNF1, HINFP and 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 shows % inhibition of promoter activity by K-312 compared with promoter activity in DMSO treatment.

Figure 6. K-312 decreases SREBP levels thereby suppressing PCSK9. A and B, mRNA (A) and protein (B) levels of HNF1α, HNF1β and HINFP in HepG2 cells after treatment with K-312 or anacetrapib for 24 hr (n=4 for A). C, Active HNF1 levels in HepG2 cells after K-312 or anacetapib treatment. Activity of HNF1 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 anacetrapib (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, Nuclear protein levels of SREBP-1 and SREBP-2. LXR agonist T0901317 1 μM (T1317) and cholesterol depletion with pitavastatin 0.3 μM 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, ChIP assay to examine the occupancy of PCSK9 promoter by SREBPs shown as fold enrichment of PCSK9 promoter region relative to non-target sequence, IGX1A (n=3). The mRNA data are relative to vehicle
control normalized by β-actin. *p<0.05, **p<0.01, ***p<0.001 versus control (Dunnett’s test).

C: control, Ana: anacetrapib.

Figure 7. Both SREBP-1 and SREBP-2 contribute to PCSK9 expression. A, mRNA and nuclear form protein levels of SREBP-1 and SREBP-2 after silencing of either SREBP-1 or SREBP-2 alone or both SREBP-1 and SREBP-2. B, PCSK9 mRNA and protein levels in culture medium after silencing of either SREBP-1 or SREBP-2 alone or both SREBP-1 and SREBP-2. *p<0.05, **p<0.01, ***p<0.001 versus control (Dunnett’s test (A), Tukey’s test (B)).

Figure 8. K-312 decreases PCSK9 protein levels in cholesterol-fed rabbits. A and B, New Zealand White rabbits fed a high cholesterol diet were treated with either vehicle (Control, n=7) or K-312 (30 mg/kg, n=7) for 2 weeks. Liver PCSK9 (A) or LDLR (B) mRNA levels were measured at the end of the study period. C, Screening for an anti-PCSK9 antibody to detect rabbit PCSK9. In vitro synthesized rabbit PCSK9 (6xHis-tag) was detected by Western blotting using various anti-PCSK9 antibodies. Arrowheads indicate the bands corresponding to rabbit PCSK9. D, Measurement of plasma PCSK9 levels by a combined immunoprecipitation and mass spectrometry approach. Relative peptide-spectrum matches (PSMs) show that plasma PCSK9 levels decrease in K-312 treated rabbits. N.S.: not significant, **p<0.01, versus control group (unpaired t-test).

Figure 9. A parallel reaction monitoring (PRM) approach for improved detection and quantification of plasma PCSK9. A, Amino acid sequence of rabbit PCSK9 (G1U461: www.uniprot.org) with five tryptic peptides (black bold) 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 [13C, 15N-Arg/Lys] PCSK9 peptide. D, Standard curves for the fragment ions used for PRM. The area under the curve of the extracted ion chromatogram was plotted for the corresponding volume of PCSK9 standard. E, K-312 treatment for 2 weeks significantly decreased PCSK9 protein levels in plasma of cholesterol-fed rabbits as shown by PRM. **p<0.01, versus control group (unpaired t-test). A carbamidomethyl cysteine is indicated as lower case “c” in the peptide sequences.
Figure 2

(A) HDL-C and LDL-C levels over time in control and treated groups. * indicates significant differences compared to control.

(B) CETP activity as a percentage. *** indicates significant differences.
Figure 3

(A) Control, K-312 10 mg/kg, K-312 30 mg/kg

(B) Atherosclerotic plaque area

(C) Lesion, % vs. LDL AUC_{0-18 week} (week-mg/dl) and HDL AUC_{0-18 week} (week-mg/dl)

(D) Cholesterol efflux
Figure 5

A

PCSK9 mRNA

Relative mRNA expression

- Act.D
- Act.D+K-312

Time (hour)

0 2 4 6 8 10

B

-3150 PCSK9 promoter
-2535
-2112
-1572
-1057
-450
-372
-335
pGL3 (Control)

Luc

C

Length of PCSK9 promoter

3150
2535
2112
1572
1057
450
372
335
0

Inhibition of promoter activity (%)

D

Wild type
HNF1 mut
HINFP mut
SRE mut

-450
-1

Luc

E

Wild type
HNF1 mut
HINFP mut
SRE mut

-10 0 10 20 30 40

Inhibition of promoter activity (%)

- HNF1 binding site
- HINFP binding site
- SRE
Figure 7

A

SREBP-1 mRNA

IB: SREBP-1

SREBP-2 mRNA

IB: SREBP-2

B

PCSK9 mRNA

PCSK9 in culture medium

IB: SREBP-1

IB: SREBP-2
Figure 8

A) PCSK9 mRNA

Relative mRNA expression

Control K-312

Exposure: 10 min

B) LDLR mRNA

Relative mRNA expression

Control K-312

Exposure: 1 min

C) Immunoblot analysis

Anti-6x His

Rabbit anti-human PCSK9

Mouse anti-human PCSK9

Sheep anti-human PCSK9

Goat anti-mouse/rat PCSK9

kDa

Exposure: 1 min

Exposure: 10 min

D) Plasma PCSK9

PSMs

Control K-312

**
Figure 9

A

RLGQDFWRLQQTVVVLKEECSREQTERTAR
RLQARAARCGYFTKVLHLFHDLFPGFLVKMSS
DLLALMLSMVKYIEDDSVFQOFWNLERIF
PVGQYDHEIRPSNGSGLVGEYVLLOOTSI5RSGRH
EKGYTVTDIFPVEEDIGDFRQESK
CDSH
GTHLAGVVSGRDAVAKGTSLSRLVNRNCGR
RGAVSSLMQELIQKHNLAQTVPLVVLPLL
GGYRSYVLNAACQR
V1RTGV/LVAAAGNFRERD
ACLYSPASEVTVYGATNAQDOPLTELTG
YGR
CDHLFAPEIKGTVGAASDCPCMFLQSGT
SQAASAVIAAMALSTPGSPEAILHRLH
SDKVINETCFEPDQRVLTPNLVVALPPSTGTAA
GGQLFCRTVWSAHSGPMCMATATARCAPDE
ELLCSFSSRSGKRGERIKYRGGRV
VCLAHI
NAFGGEGVYAIRSCCLPQANCVHTAPPAPAR
AGGELCHQPQGVHTGCSHWEASLHTHR
OPAPRPGHAQCVHREASIHASCCHAPG
LVCK
VKEKHGIPGPAEQVSVACEEDGWTLG
cNAPGASLVGAYVDNTCVKSRDAGTAGGT
TSEEPTGAVAICRRPS

B

Relative Abundance

Time (min)

VL N A AcQR
(b7) cDSHGHTRLAAGVVSGR
(y6) EASIHASCCHAPG
VcLHNAFGGE[GV][AIAR

C

Relative Abundance

m/z

VL NA AcQR
VcLAHNAFGGE[GV][AIAR

D

Log10 fmol on column

Log10 fmol on column

E

Plasma PCSK9

Control

K-312

*