Development of chemicals which reduce the expression of PCSK9 as therapeutics for treatment of hypercholesterolemia

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Development of chemicals which reduce the expression of PCSK9 as therapeutics for treatment of hypercholesterolemia

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실험에 대해 아주 작은 질문들도 귀찮은 내색 없이 항상 친절하게 대답해주고 자신의 일처럼 신경 써주었던 부남이형, 앞으로의 일들을 친동생처럼 신경 써주면서 방향을 제시해주던 현석이형, 저의 논문 공개발표를 저보다 더 긴장하면서 지켜봐주고 진심으로 축하해주던 영주형에게도 감사의 인사를 전합니다. 더불어 실험실에서 만난 많은 선후배, 동료들에게 고마움을 전합니다. 처음 실험실에서 익숙하지 않아 힘들어 할 때 많은 조언을 해주신 김경아 선생님, 많은 관심과 애정 어린 충고를 아끼지 않으셨던 성균관대 선배님 고은진 선생님, 항상 옆 팀에 계시면서 우리 팀처럼 챙겨 주시는 김미영 선생님, 커피 한 잔 하면서 많은 애기를 나누었던 효경누나, 수연누나 감사합니다. 그리고 이사할 때마다 도와줬던 윤희, 현우, 혁구, 정환이, 항상 반갑게 인사하는 후배 재성이에게도 진심으로 고마움을 전합니다.

마지막으로 실험을 하며 보낸 기나긴 나날 동안 나의 사랑하는 가족들이 베풀어준 정성과 지원에 감사를 드립니다. 한번도 힘들고 아픈 것 내색하지 않으시고 저를 믿으며 6년을 한결같이 힘이 되어주신 아버지와 어머니께 감사 드리며 사랑의 마음을 전합니다. 몸이 멀리 있어 항상 맘뿐인 미안한 형 대신 든든한 동생 동건이 덕분에 맘 편히 학위를 마무리 할 수 있었습니다. 그리고 저를 배려해주신 장인어른, 장모님과 귀엽고 재치 넘치는 처제 영옥이와 영은이게도 감사의 마음을 전합니다. 2007년 시작된 우연이 아름다운 인연으로 결실을 맺고 더 큰 행복을 알게 해준 나의 예쁜 아내에게도 사랑의 마음을 가득 전합니다.

2014년 12월, 논문을 마무리하며 모든 분들께 감사의 마음을 전합니다.

<div align="right">민 동 국</div>

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Abstract

**Development of chemicals which reduce the expression of
PCSK9 as therapeutics for treatment of hypercholesterolemia**

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(Directed by Professor Sahng Wook Park)

Proprotein Convertase Subtilisin/kexin type 9 (PC1SK9), the ninth member of subtilisin serine protease, promotes the degradation of the low density lipoprotein receptor (LDLR), thereby increasing the plasma concentration of LDL-cholesterol. Several studies have strongly suggested that inhibition of PCSK9 action is a promising therapeutic modality to treat hypercholesterolemia. As a strategy for development of PCSK9 inhibitors,

the chemical library that consists of 3,000 randomly selected compounds was primarily screened by western blot analyses for the chemicals that reduce the amount of protein levels of PCSK9 with reciprocal increase in the LDLR expression in HepG2 cells. A set of chemicals (C935 and related chemicals) with the the common scaffold structure of 1,4-naphthoquinone reduced the amounts of the protein and mRNA for PCSK9, and transcriptional activity of the PCSK9 promoter, while they increased the amount of the LDLR protein. Functional relevance of the increased amount of in the LDLR was confirmed by the increased uptake of fluorescence-labeled LDL as well as the increase in the LDLR protein level. These results suggest that these chemicals increase the uptake of LDL into the cells by the increased LDLR expression which may be driven by reduction of PCSK9 expression in HepG2 cells. To elucidate the mechanism by which selected chemicals to reduce the transcriptional activity of PCSK9, microarray analysis was performed in HepG2 cells after treatment of chemicals. Among the genes of which amounts of mRNA was changed by C935, Nuclear factor (erythroid-derived 2)-like 2 (NRF2) was deduced to play an important role on regulation of the PCSK9 expression, although the reduction of PCSK9 by C935 does not involve NRF2 directly. These findings suggest that decrease in the PCSK9

expression by NRF2 is a novel mechanism of cells to unburden to synthesize cholesterol de novo synthesis under oxidative and/or electrophilic stress conditions. In addition, it is suggested that modulation of NRF2 activation along with PCSK9 might serves as a new target of lowering the plasma concentration of LDL Cholesterol.

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Development of chemicals which reduce the expression of

PCSK9 as therapeutics for treatment of hypercholesterolemia

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I. INTRODUCTION

Hypercholesterolemia increases the risk of atherosclerosis, coronary artery disease and cholesterol-related disease[1,2]. Therefore much effort was taken to low the level of plasma LDL cholesterol and the access method to overcome hypercholesterolemia is to increase the amount of LDLR expressed on the hepatocyte surface[3]. LDLR is the essential protein

in regulating the level of LDL-cholesterol by intracellular uptake of LDL-cholesterol in bloodstream. The elevated expression of LDLR leads to the reduced amount of LDL cholesterol; on the other hand, the decreased LDLR expression results in growing level of plasma LDL-cholesterol[4].

Statin is the most universal therapeutic agent applied toward treating hypercholesterolemia[5]. Statin is pharmaceutical inhibitor of hydroxyl methyl glutaryl coenzyme A (HMG-CoA) reductase, an enzyme involved in controlling the rate of cholesterol synthesis[6]. In addition, statin induces sterol regulatory element binding protein 2 (SREBP2) expression, which increases the expression of LDLR. Use of statins increases LDL cholesterol clearance by inhibiting HMG-CoA and increases the expression of LDLR in the liver[7]. SREBP2 also up-regulates PCSK9 through stimulation of *PCSK9* gene transcription[8].

Variety of proteins is produced as precursor forms that undergo limited proteolytic cleavage at specific sites, yielding matured bioactive proteins[9,10,11]. The nomenclature of PCSK, one of the most limited proteolytic enzymes is as Proprotein Convertase Subtilisin/kexin due to its structural similarity with bacterial 'subtilisin' and yeast 'kexin'[12].

Proprotein Convertase Subtilisin/Kexin type9 (PCSK9) is the ninth known member of the Proprotein Convertase family[13], and its function is

to enhance low density lipoprotein receptor (LDLR) degradation expressed on the cell surface[14]. Catalytic domain of PCSK9 binds to EGF-A region that is present in the extracellular domain of LDL receptor, and the complex is internalized to endosomes/lysosomes for degradation[15]. Thereby LDL cholesterol level is increased due to reduced expression of LDL receptor.

Gain-of-function mutations of *PCSK9* reduced the expression of the LDL receptor and resulted in elevated plasma LDL cholesterol[16,17]. Those with gain-of-function mutations of *PCSK9* acquire autosomal dominant familial hypercholesterolemia[16,17,18]. Loss-of-function mutations of *PCSK9* increase the density of the LDLR on the hepatocyte cell membrane and lower LDL cholesterol level in plasma[15]. African-Americans with Y142X or C679X nonsense mutation of *PCSK9* were observed to have28% reduction of LDL-cholesterol level and nearly 90% low incidence of coronary artery disease[19]. Studies in *PCSK9* knockout mice model revealed that the increased number of LDLR leads to reduced amount of plasma LDL cholesterol[20]. Thus, it was expected that there are two strategies to decrease plasma cholesterol level, either through the inhibition of PCSK9 synthesis or inhibition of the binding of PCSK9 to LDLR.

Paradoxically, the expression of PCSK9 is increased by statins[7],

indicating that statin therapy along with PCSK9 inhibition could create a synergistic effect of lowering LDL Cholesterol. Up-regulated PCSK9 by statin leads to lower the expression of LDLR[20]. Accordingly, the effect of statin is offset by dual regulation of the LDLR and PCSK9 expression through activation of SREBP2.

Recently, the strategies of inhibiting the action of PCSK9 have been demonstrated to be successful to some extent. A Locked Nucleic Acid Antisense Oligonucleotide (LNA) efficiently silenced *PCSK9* mRNA and elevated the expression of LDLR in high-fat diet mice, which subsequently reduced LDL cholesterol level[21]. Silencing *PCSK9* with formulated in lipidoid nanoparticle siRNAs reduced *PCSK9* mRNA level by 50-70% and decreased plasma LDL cholesterol level in nonhuman primates for three weeks[22]. A natural plant extract, berberine reduced the expression of PCSK9 in HepG2 cells[23,24]. It has been reported that ongoing clinical studies involving monoclonal antibodies to PCSK9 showed that therapies against PCSK9 effectively decreased LDL-cholesterol level[25]. These advanced studies strongly suggest that inhibiting PCSK9 is a therapeutic treatment, targeting hypercholesterolemia.

To discover the PCSK9 inhibitors, this study focused on screening the chemical library for selecting a set of chemicals which reduce the

expression of PCSK9 while increasing that of the LDL receptor. The selected chemicals share the same structure, 3-anilino-2-chloro-1,4-naphthoquinone(CAS No. 1090-16-0). These chemicals decreased the expression of PCSK9, while up-regulated LDLR in HepG2 cells. Also, they increased the uptake of fluorescence-labeled LDL particles (Dil-LDL); these results suggest that these chemicals increased the amount of the LDL receptor on the cell surface of HepG2 cells, resulting in increased uptake of LDL into the cells. In addition, the use of these effective chemicals, known as Rosuvastatin supplements, along with Rosuvastatin led to higher expression of LDLR than when Rosuvastatin was applied alone, suggesting that the implication of these PCSK9 inhibitors for therapeutic application of hyper-cholesterol treatment.

To elucidate the mechanism of chemicals to reduce the transcriptional activity of PCSK9, microarray experiment was performed. From the data, Nuclear factor (erythroid-derived 2)-like 2 (NRF2) was focused the relation with PCSK9. NRF2 is transcription factor that is known to regulate many antioxidant enzymes[26]. NRF2 encodes basic leucine zipper transcription factor[27]. Under normal conditions, NRF2 is kept in the cytoplasm by a cluster of proteins that undergoes rapid ubiquitination by CUL3 and KEAP1[28]. CUL3 is an ubiquitin ligase and

KEAP1 is a substrate adaptor, which helps the Cul3 ubiquitination of the NRF2 protein[28]. Under stress conditions, the KEAP1-NRF2 complex is disrupted and NRF2 is trans-located to the nucleus[29]. In nucleus, NRF2 binds with a small Maf protein and binds to Antioxidant Response Element (ARE) in the promoter region of many anti-oxidative genes, and initiates their transcription[30]. So, NRF2 is a critical mediator comprehensively regulates the expression of numerous stress responsive enzymes and detoxification enzymes[31].

Recently, the studies of NRF2 pathway in the body benefits manage the metabolic syndrome have been reported. These studies proposed that the role of NRF2 in the development of obesity[32] and in the highly regulated process of adipocyte differentiation[33] through its interaction with other transcription factors and receptors implicated in metabolic regulation.

To evaluate the regulation of NRF2 on PCSK9 expression, NRF2 was knocked downed using siRNA transfection. Knockdown of NRF2 gene enhanced the expression of *PCSK9* mRNA as well as PCSK9 Protein significantly. However, the microarray data showed that C935 decreased the of *NRF2* mRNA level. Therefore, it needs to find out the mechanism that C935 regulates the PCSK9 expression and clarify the relationships between C935, PCSK9 and NRF2 gene.

II. MATERIALS AND METHODS

1. Chemicals and reagents

About 4,000 chemicals were purchased from Chemdiv Inc.(San Diego, CA). Dulbecco's modified Eagle's medium (DMEM) and 100 units/ml penicillin and 100 µg/ml streptomycin sulfate were purchased from invitrogen (Carlsbad, CA, USA). Delipidated serum (DLPS) was prepared from Fetal Bovine Serum (FBS) as described previously[8]. Rosuvastatin was kindly provided by AstraZeneca (Mölndal, Sweden). Sodium mevalonate was prepared from mevalonic acid lactone (Sigma-Aldrich, Co. St. Louis, MO, USA) as follows. To prepare 1.0 M sodium mevalonate, 5 g of mevalonic acid lactone was dissolved in distilled water, and then 4 ml of 10 N NaOH was added drop-wise and stirred for 40 min at room temperature. The pH of the solution was adjusted by 0.5 N HCL to pH 7.5, then the final volume was brought to 38.4 ml with distilled water. The stock solution was filter-sterilized, divided into aliquots, and stored at -20°C until use. Dil-LDL was purchased from Biomedical Technologies Inc. (MA, USA).

2. Antibodies

The polyclonal antibodies against human PCSK9, SREBP2 were prepared as previously described by Jeong et al[8]. The polyclonal antibody against the human LDLR was raised in rabbits using synthetic peptide spanning amino acids 832-841 of the bovine LDLR according to the standard technique. NRF2 polyclonal antibody was purchased from Santa Cruz Biotechnology (CA, USA). GAPDH polyclonal antibody was purchased from Cell Signaling Technology, Inc. (Beverly, MA). Horseradish peroxidase-conjugated secondary antibodies were obtained from Pierce (Rockford, IL).

3. Cell culture

HepG2 (American Type Culture Collection number HB-8065)cells were maintained in DMEM containing 100 U/ml penicillin and 100 mg/ml streptomycin sulfate supplemented with 10% (v/v) FBS at 37°C in a humidified atmosphere of 5% CO_2 in air. For the treatment of cells with chemicals, HepG2 cells were set up at 1.5 x 10^5cells/well in 12 well plate in DMEM medium containing 100 units/ml penicillin and 100 µg/ml streptomycin sulfate supplemented with 10% FBS on day 0. On day 1, cells were washed with phosphate-buffered saline

(PBS) twice and change to the DMEM medium containing 100 units/ml penicillin and 100 µg/ml streptomycin sulfate supplemented with 10% DLPS. On day 2, the medium was replaced with fresh medium supplemented with each concentration of chemicals with or without 0.1 µM Rosuvastatin and 50 µM sodium mevalonate for 18 hrs. On day 3, cells were washed twice with PBS, harvested for further analyses.

4. Cell fractionation

HepG2 cells were set up at 1.5 x 10^6 cells per 10cm plate with DMEM supplemented with 10% FBS. On day1, cells were washed twice with PBS, then were switched to fresh DMEM supplemented with 10% DLPS. After treating t-BHQ or H_2O_2 or Sulforaphane for 18hr, HepG2 cells were harvested by scraping in cold PBS and collected by 1,000 x g for 5 min at 4°C. Discard the supernatant and then freeze the cells in liquid nitrogen. Cells were resuspended pellet in buffer A containing 10 mM HEPES, 1.5 mM MgCl$_2$, 10 mM KCl, 5 mM EDTA, 5 mM EGTA, 250 mM Sucrose, and protein inhibitors (1 mM DTT, 10 µg/ml leupeptin, 1 mM PMST, 2 µg/ml aprotinin, and 50 µg/ml N-acetyl-leucine-leucine-norleucinal) and stand on ice. After

15 min, cells were disrupted by passing 30 times through a 23-G needle on a 1cc syringe. Spin nuclei down at 1,000 x g in swing bucket rotor for 7 min at 4°C. Supernatant was fractionated to cytosol and membrane, and nuclear extract was fractionated from the pellet. Cytosol fraction was collected by centrifugation at 55,000 rpm for 30 min at 4°C. After spin down, membrane fraction pellet was resuspended with NUN buffer containing 0.33 M NaCl, 1.1 M urea, 1% Nonidet P-40, 25 mM HEPES (pH 7.6). Nuclear pellet was resuspended in Buffer C containing 20 mM HEPES, 1.5 mM MgCl$_2$, 0.42 M NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 % Glycerol with protease inhibitors by pipetting. Resuspended pellet was incubated at 4°C for 1hr with rotation, and then centrifuged at 55,000 rpm for 30 min at 4°C. Save the supernatant to extract the nuclear fraction.

5. **Immunoblot analysis**

After treating chemicals as described in each figure legends, cells were washed with PBS twice and lysed with 150 μl NUN buffer and protein inhibitors by adding directly onto the plate, then shaking for 10 min at room temperature. The cells were transferred in pre-chilled 1.5ml tube on ice and vortex for 10 min at 4°C. Lysates were cleared

by centrifugation at 16,000 g for 10 min at 4°C, and the supernatants were collected as whole cell lysate. Protein quantitative analysis was performed with BCA kit (Pierce, Rockford, IL). After quantitation of protein, 20 µg of proteins were subjected to 10% SDS-polyacrylamide gels and separated by electrophoresis. Proteins were transferred onto nitrocellulose ECL membranes (GE Healthcare Bio-Science, Piscataway, NJ). The membranes were blocked in PBS containing 0.05% (v/v) tween 20 and 5% (w/v) non-fat dried milk for 1hr at room temperature. Primary antibodies were probed in fresh blocking solution for 1hr at room temperature. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies were used to detect the protein using the Super Signal West Pico Chemiluminescent Substrate System (Pierce).

6. Quantitative real time PCR

Total RNA was prepared from HepG2 cells using an RNeasy Mini kit (Qiagen, Inc., Valencia, CA), according to the manufacturer's instruction. Removal of DNA from RNA was achieved with RNase-free DNase (Qiagen). cDNA was synthesized from 2 µg of DNase-treated total RNA using a High-Capacity cDNA Archive kit (Applied

Biosystems, Foster City, CA). Quantitative real-time PCR was performed using the PowerSYBR® Green PCR master (Applied Biosystems) and all reactions were analyzed using the StepOne™ Real-time PCR systems (Applied Biosystems). All reactions were done in triplicate, and the relative amounts of all mRNAs were quantified by the comparative cycle-time method as described previously[8]. RPLP0 or GAPDH mRNA was used as the invariant control. The primers used for RT-PCR are shown in Table 1.

Table 1. Primers used for real-time PCR

Gene symbol	Genbank Accession No.	Sequence (5´ to 3´)
PCSK9	NM_174936.3	5´-GGCAGGTTGGCAGCTGTTT -3´ 5´-CGTGTAGGCCCCGAGTGT -3´
LDLR	NM_000527.4	5´-AGGAGACGTGCTTGTCTGTC -3´ 5´-CTGAGCCGTTGTCGCAGT -3´
SREBP-1	NM_001005291.2	5´-AAACTCAAGCAGGAGAACCTAAGTCT -3´ 5´-GTCAGTGTGTCCTCCACCTCAGT -3´
SREBP-2	NM_004599.3	5´-CGGTAATGATCACGCCAACAT -3´ 5´-TGGTATATCAAAGGCTGCTGGAT -3´
FASN	NM_004104.4	5´-TCGTGGGCTACAGCATGGT -3´ 5´-GCCCTCTGAAGTCGAAGAAGAA -3´
HMGCR	NM_000859.2	5´-CAAGGAGCATGCAAAGATAATCC -3´ 5´-GCCATTACGGTCCCACACA -3´

NFE2L2	NM_006164.4	5´-TGGCTTCTGGACTTGGAAC -3´
		5´- GACGGTATGCAACAGGACAT-3´
GAPDH	NM_002046.5	5´-GCCCCAGCGTCAAAGGT -3´
		5´-GGCATCCTGGGCTACACTGA -3´
RPLP0	NM_001002.3	5´-TGCATCAGTACCCCATTCTATCA -3´
		5´-AAGGTGTAATCCGTCTCCACAGA -3´

7. Transient transfection and reporter gene assay

The pGL3-PCSK9 promoter-reporter construct, (-D4)was prepared as previously described by Jeong et al.[8] HepG2 cells were maintained in medium A (DMEM containing 100 U/ml penicillin and 100 μg/ml streptomycin sulfate) supplemented with 10% (v/v) FBS. Transfection of DNA into HepG2 cells in suspension was carried out using Lipofectamine™2000 (Invitrogen) according to the method described by Notarangelo et al.[34] with minor modifications. Briefly, plasmids were complexed in Opti-MEM (Invitrogen) using Lipofectamine™2000 according to the manufacturer's instruction. While the DNA-Lipofectamine complex was prepared, HepG2 cells were trypsinized and suspended in medium A supplemented with 10% FBS. The complex was mixed with 2×10^5 cells per well/12well plate in 0.8 ml of medium A supplemented with 10% FBS and rocked gently for 30 min at 37°C in the tube. Aliquots of mixtures were plated on 12-well plates and cultured overnight at 37°C under a humidified atmosphere of 5% CO_2. For the treatment of chemicals, on day 1, cells were washed twice with PBS and changed to medium A supplemented with 10% DLPS. On day 2, cells were washed twice with PBS, harvested, and analyzed for luciferase activity using the

dual-luciferase assay system with passive lysis buffer (Promega, Madison, WI) according to the manufacturer's instructions. The firefly luciferase activity was normalized to the renilla luciferase activity and the amounts of protein in the lysate.

8. Fluorescence microscopy and Dil-LDL uptake assay

HepG2 cells were set up at 1.0×10^5 cells per well in 12-well plate with DMEM supplemented with 10% FBS. On day1, cells were washed twice with PBS, then were switched to fresh DMEM supplemented with 10% DLPS. After treating chemicals at 10 µM for 18hr, HepG2 cells were washed with PBS and treated with Dil-LDL (Biomedical Technologies, Inc., Stoughton, MA) at the 2 µg/ml concentrations for 3hr at 37 ℃. Cells were washed twice with PBS and fluorescence images were gained by using fluorescence microscope (Olympus, Tokyo, Japan) with rhodamine filter. For quantification of LDL uptake, cells were trypsinized to obtain a single-cell suspension. The mean fluorescence intensities of 10,000 cells were analyzed by fluorescence-activated cell sorting on the FACScan (BD Bioscience, San Jose, CA, USA).

9. Microarray

Each total RNA sample (200ng) was labeled and amplified using Low Input Quick Amp labeling kit (Agilent technologies, CA). The Cy3-labeled aRNAs were resuspended in 50µl of hybridization solution (Agilent technologies, CA). After labeled aRNAs were placed on Agilent SurePrint G3 Human GE 8x60K array (Agilent technologies, CA) and covered by a Gasket 8-plex slide (Agilent technologies, CA). The slides were hybridized for 17hr at 65 °C oven. The hybridized slides were washed in 2 X SSC, 0.1 % SDS for 2 min, 1 X SSC for 3 min, and then 0.2 X SSC for 2 min at room temperature. The slides were centrifuged at 3000 rpm for 20 sec to dry.

10. Microarray data analysis

The arrays were analyzed using an Agilent scanner with associated software. Gene expression levels were calculated with Feature Extraction v10.7.3.1 (Agilent technologies, CA) Relative signal intensities for each gene were generated using the Robust Multi-Array Average algorithm. The data were processed based on median polish normalization method using the Gene Spring GX 7.3.1 (Agilent technologies, CA). This normalization method aims to make the

distribution of intensities for each array in a set of arrays the same. The normalized, and log transformed intensity values were then analyzed using Gene Spring GX 7.3.1 (Agilent technologies, CA). Fold change filters included the requirement that the genes be present in at least 200% of controls for up-regulated genes and lower than 50% of controls for down-regulated genes. Hierarchical clustering data were clustered groups that behave similarly across experiments using Gene Spring GX 7.3.1 (Agilent technologies, CA). Clustering algorithm was Euclidean distance, average linkage.

11. Statistical analysis

Three experiments were performed for all in vitro studies. The results are presented as means ± standard error of the mena (SEM). The data were subjected to a two-tailed Student's t-test. Statistical analyses were carried out using SPSS version 18.0 for Windows (Statistical Package for the Social Science, SPSS, Ins., Chicago, USA). All p values less than 0.05 were considered statistically significant.

III. RESULTS

1. Chemical Library Screening and Selection of Effective Chemicals

Chemical library screening was performed to discover any effective chemicals that could inhibit the expression of PCSK9. HepG2 cells were treated with random chemicals for eighteen hours, and PCSK9 expression was confirmed by western blotting using PCSK9 antibodies. In all screening experiment, the medium was switched from DMEM supplemented with 10% FBS to DMEM supplemented with 10% DLPS (Delipidated serum) to overexpress PCSK9 by activating SREBP2.

Selected five chemicals share specific structures of naphthoquinone are found to reduce the expression of PCSK9. Chemical C935 is the basic structure among the five chemicals, and the others are derived from C935. Table 2 and Table 3 list the respective chemicals structures and names. Residues of Fluorine, Hydrocarbon, and Nitrogen Dioxide were additional composition from basic structure C935.

5 μM of each chemical more effectively reduced the expression
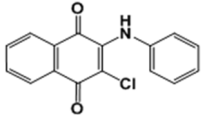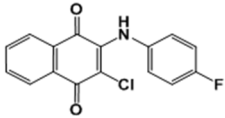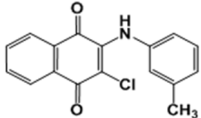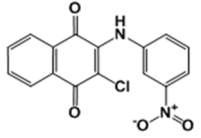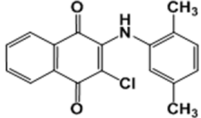
of PCSK9 and increased the expression of LDLR when compared to treating with DMSO, which is a negative control purified from HepG2 cells (Fig. 1A). PCSK9 and LDLR expressions were reduced and raised respectively as the concentrations of C935 and C1168, C1182, C1184 and C1186 were increased: C935 from 0.6 µM to 10 µM (Fig. 1B) and C1168, C1182, C1184 and C1186 from 1.2 µM to 20 µM (Fig. 1C). With this PCSK9 expression is reduced by chemicals which share the specific structure.

Table 2. List of effective chemicals to reduce the PCSK9 expression

Chemical ID[1]	Molecular Formula	Chemical Name
C935	$C_{16}H_{10}ClNO_2$	2-chloro-3-(phenylamino)naphthalene-1,4-dione
C1168	$C_{16}H_9ClFNO_2$	2-chloro-3-[(4-fluorophenyl)amino]naphthalene-1,4-dione
C1182	$C_{17}H_{12}ClNO_2$	2-chloro-3-(3-toluidino)-1,4-dihydronaphthalene-1,4-dione
C1184	$C_{16}H_9ClN_2O_4$	2-chloro-3-[(3-nitrophenyl)amino]naphthalene-1,4-dione
C1186	$C_{18}H_{14}ClNO_2$	2-chloro-3-[(2,5-dimethylphenyl)amino]naphthalene-1,4-dione

Arbitrarily numbers for the screening of chemical library

Table 3. Structure of the effective chemicals

Chemical ID	Chemical Structure
C935	
C1168	
C1182	
C1184	
C1186	
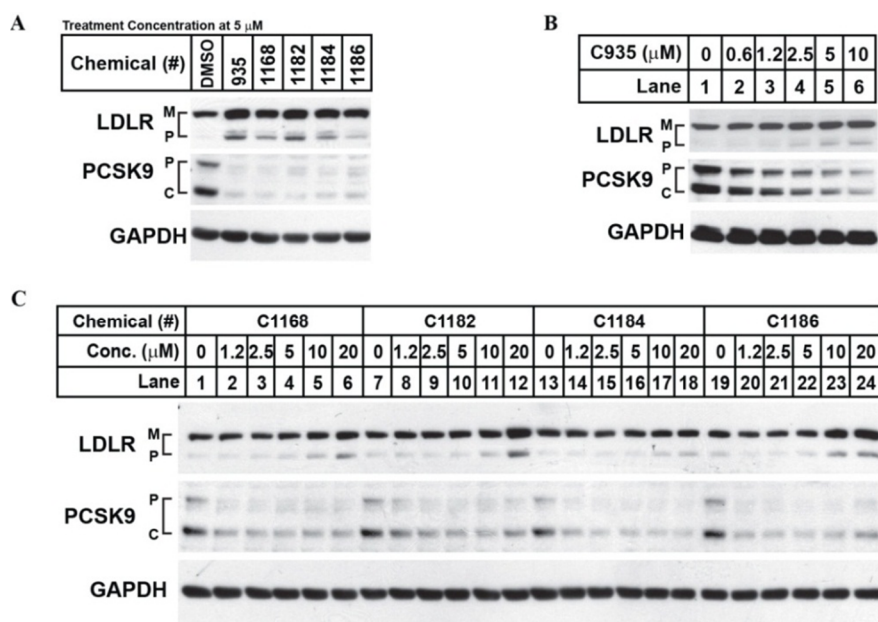
Figure 1. Effects of chemicals on PCSK9 and the LDLR expression in HepG2 cells

(A) On day 0, HepG2 cells were set up at 1.5×10^5 cells per well in 12-well plate with DMEM supplemented with 10% FBS. On day1, cells were washed twice with PBS, and then were switched to fresh DMEM supplemented with 10% de-lipidated serum (DLPS) at the 5 µM concentration of each chemical. After 18hr incubation, cells were harvested, and whole cell lysates were subjected to immunoblot analyses with antibodies against PCSK9, the LDL receptor. GAPDH was used as an invariant control. (B and C) HepG2 cells were treated with each chemical

26

in dose dependent manner and incubated for 18 hr. M and P for LDLR represent the mature and precursor forms of LDLR, respectively. P and C for PCSK9 represent the proprotein and cleaved forms of PCSK9, respectively.

2. The Chemicals Inhibit the Induction of PCSK9 in Response to Rosuvastatin

In the presence or absence of each chemical, HepG2 cells were treated with 50 µM mevalonate and different concentrations of Rosuvastatin in order to verify the possibility of statin being used as an adjuvant. According to the Rosuvastatin concentrations, LDLR and PCSK9 expressions are increased by Rosuvastatin effect (Fig. 2A, B, C lane1-5). However, treatment with 5 µM of each chemical more successfully reduces and elevates the expression of PCSK9 and that of LDLR respectively than when in absence of chemicals (Fig. 2A, B, lane6-10, Fig. 2C, lane 6-20).

In two different experimental conditions, one with Rosuvastatin and another without, the HepG2 cells were treated with 1.25 µM and 5 µM of each chemical to confirm the elevation of LDLR expression. As expected, each chemical decreases the expression of PCSK9 and increases the expression of LDLR in a dose-dependent manner (Fig. 2D lane 1-11). Through the effect of Rosuvastatin, PCSK9 and LDLR expressions are increased (Fig. 2D lane1, 12). When Rosuvastatin alone was applied to HepG2 cells, LDLR expression was elevated. In addition, when the cells were treated the chemicals

in addition to Rosuvastatin, LDLR was further increased due to the synergistic effect of blocking PCSK9 expression by the chemicals. Among the five chemicals, C935 showed the greatest effect on the expression of PCSK9 and LDLR. These findings suggest that five chemicals could remedy statin's shortcomings and maximize the LDLR expression which plays an important role of plasma LDL cholesterol adjustment.
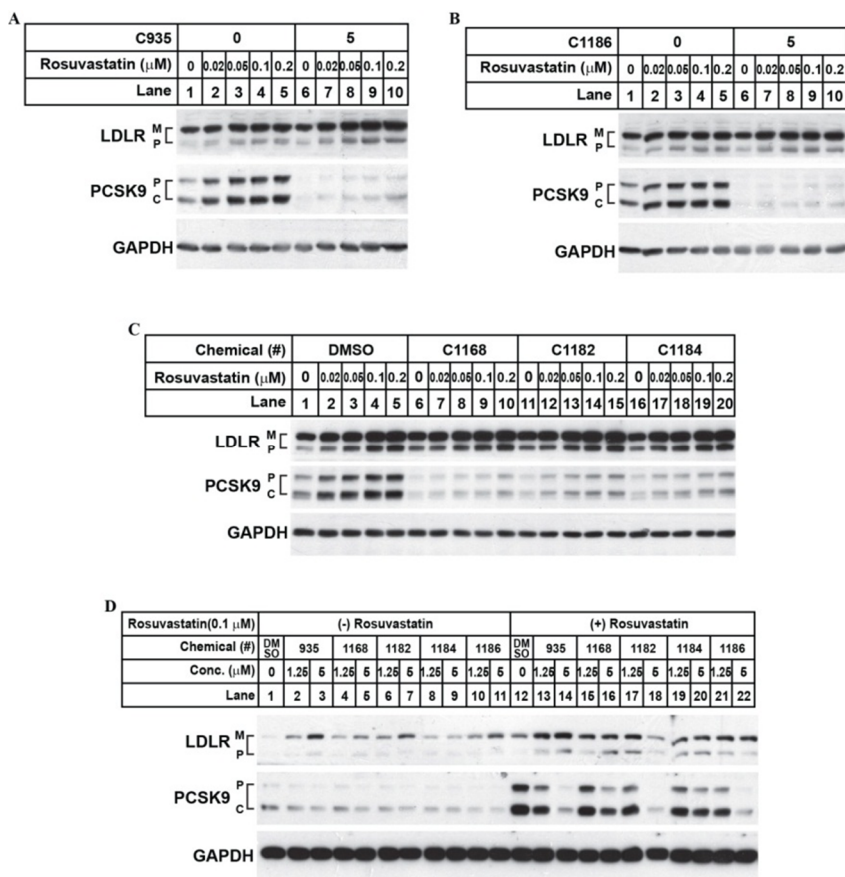
Figure 2. The greater effect of chemicals applied with Rosuvastatin on the expression of PCSK9 and LDLR

(A) The effect of C935 on the expression of LDLR and PCSK9 with the elevated concentration of Rosuvastatin. (B) The effect of C1186 and (C) the effects of C1168, C1182 and C1184 on PCSK9 and LDLR expression were verified with western blot analysis. (D) HepG2 cells were treated

with 1.25 µM and 5 µM of each chemical with the absence (lane 1-11) or presence (lane 12-22) of 0.1 µM Rosuvastatin and 50 µM mevalonate. On day 0, HepG2 cells were set up at 2.0 x 10^5 cells per well in 12-well plate with DMEM supplemented with 10% FBS. On day1, cells were washed twice with PBS, then were switched to fresh DMEM supplemented with 10% de-lipidated serum (DLPS) with the indicated concentration of Rosuvastatin, 50 µM mevalonate and 5 µM each chemical. After 18hr incubation, cells were harvested with nun buffer, and whole cell lysates were subjected to immunoblot analyses with antibodies against PCSK9 and the LDL receptor.

3. **The chemicals repress the *PCSK9* Promoter Activity and *PCSK9* mRNA**

To elucidate the mechanism of the chemical effect on PCSK9, total RNA was isolated from each chemical-treated HepG2 cells for 18hrs and was analyzed with real-time PCR (Fig. 3A). Each chemical not only decreased the *PCSK9* mRNA effectively, but also Pcsk9 protein level. There was a slight increase in *LDLR* mRNA level by C935, C1168 and C1182, and minor decrease by C1184 and C1186. Nevertheless, the range of deviation of *LDLR* mRNA level can be considered narrow, and therefore the main effect five chemicals produce is contributed to *PCSK9* regulation.

Following test was performed to determine the transcriptional changes of lipogenic genes (Fig. 3B). The amount of *PCSK9* mRNA was decreased while that of *LDLR* mRNA was increased slightly by the gradual increment in C935 concentration. The transcriptions of *SREBP-2* and *HMG-CoA reductase*, which are involved in cholesterol biosynthesis, were not influenced by C935. The *SREBP-1* mRNA showed a moderate decrease in a dose-dependent manner, but *FASN*, which is the target gene of *SREBP-1*, was changed slightly in mRNA level (Fig. 3B). These findings reaffirm that

PCSK9 is the main target of C935 and show the powerful effect, decreasing the transcriptional regulation of PCSK9.

To determine whether the *PCSK9* promoter is activated by each chemical or not, the *PCSK9* wild-type construct D4 was transfected into HepG2 cells and the luciferase activity was measured[8]. The *PCSK9* promoter activity was decreased by each chemical in two different conditions: 1) transient transfection of D4-construct into HepG2 (Fig. 4A); 2) stable transfection of D4-construct into HepG2 cells (Fig. 4B). When HepG2 cells stably expressing D4 construct were treated dose-dependently by C935, which is the basic structure among other chemicals, *PCSK9* promoter activity was attenuated effectively (Fig. 4C). Together, the mechanism of the chemicals can be summarized as the repression of promoter activity, followed by the reduction of both the mRNA level and protein expression of PCSK9.
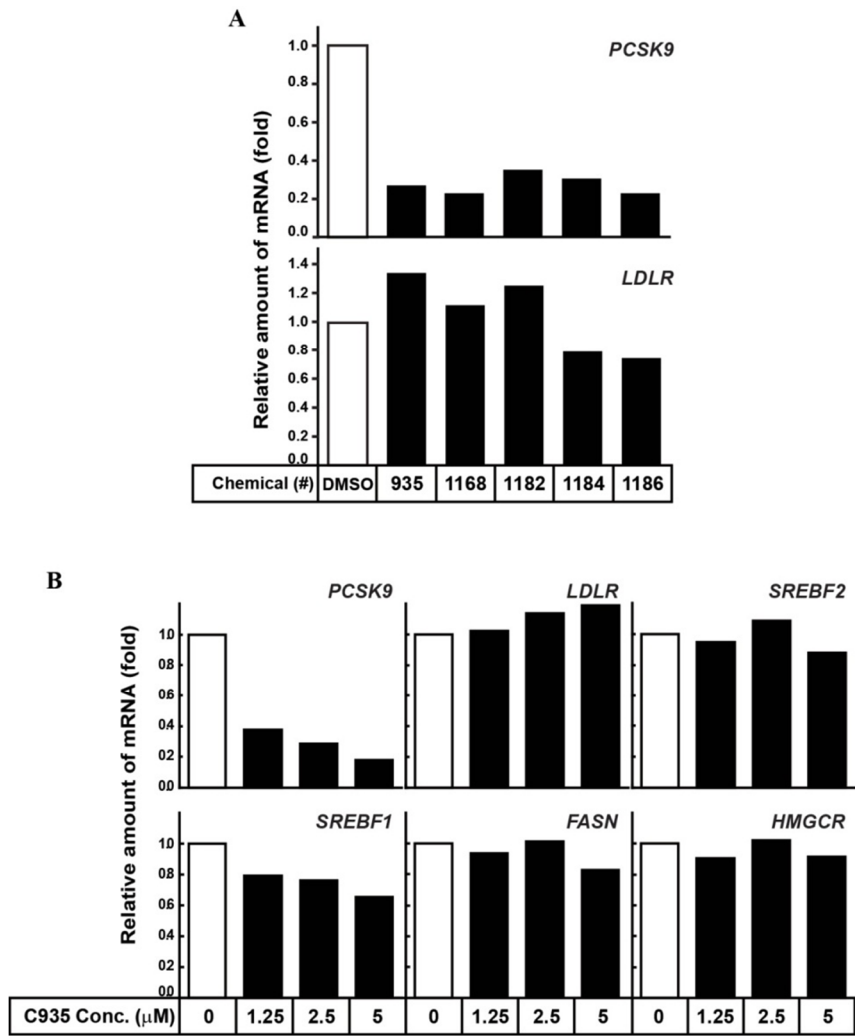
Figure 3. The effective chemicals suppressed the *PCSK9* mRNA in HepG2 cells

(A) The effect of reducing the *PCSK9* and *LDLR* mRNA level were

analyzed with quantitative real-time PCR. Total RNAs were prepared from HepG2 cells treated with indicated chemicals at 5 μM for 18 hr. (B) C935 effect on the *PCSK9*, *LDLR* and other lipogenic enzymes mRNA level were analyzed with quantitative real-time PCR. Total RNAs were prepared from HepG2 cells treated with C935 at the indicated concentration for 18 hr. cDNA was synthesized by reverse-transcription, and subjected to the analyses by quantitative real-time PCR. Each value represents the amount of mRNA relative to that in the cells grown with vehicle (DMSO), which is arbitrarily defined as 1. The values represent means from duplicate reactions. GAPDH was used as an invariant control (data not shown). Similar results were obtained in three independent experiments.
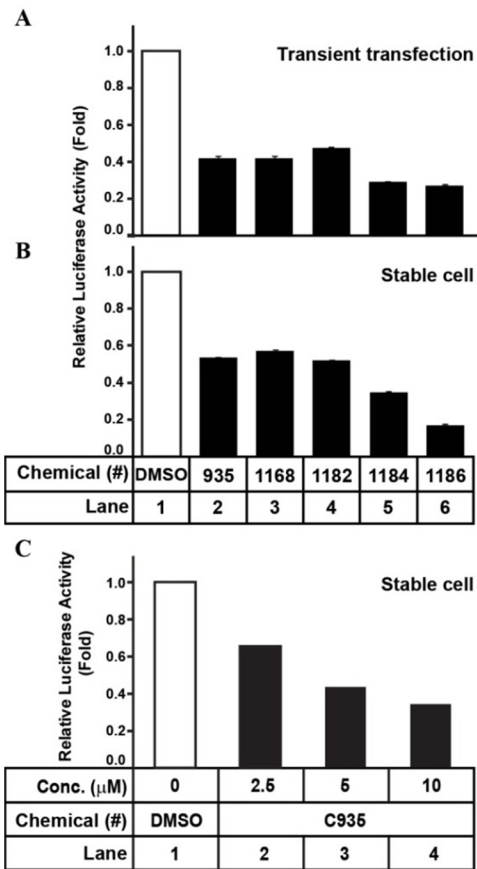
Figure 4. Reduce the human *PCSK*9 promoter activity by the effective chemicals in HepG2 cells

(A) HepG2 cells were transiently transfected with pGL3-*PCSK9* promoter-reporter construct (D4) on day 0. On day 1, cells were changed to DMEM supplemented with 10% DLPS, and treated with the indicated chemicals at 5 μM for 18hr. On day 2, cells were washed twice with ice-cold PBS, and

cell lysates were prepared with passive lysis buffer. The luciferase activities were measured with the relative to that of vehicle (DMSO) treated cells, which is arbitrarily defined as 1. The luciferase activities were normalized with the amounts of proteins used for the assays. (B and C) pGL3-*PCSK9* promoter-reporter construct (-D4) was stably transfected in HepG2 cells were set up on day 0. On day 1, each chemical were treated in DMEM supplemented with 10% DLPS. After 18hr, cell lysates were prepared and luciferase activities were measured using the dual-luciferase assay system. The values represent means +SD from triplicate reactions. Similar resultswere obtained in three independent experiments.

4. The Chemicals Enhanced the Dil-LDL Uptake in HepG2 Cells

As a functional test to LDLR, human LDL which is tagged with fluorescence probe (Dil-LDL) was applied to HepG2 cells after incubation with DMSO and each chemical for 18hrs. With theelevated expression of LDLR, the more binding between Dil-LDL and LDLR is induced; thus, fluorescence intensity is subsequentlystrengthened. Due to the effect of the chemicals, PCSK9 minimizes the degradation of LDLR and consequently increases LDLR expression. As expected, treating cells with each chemical yielded more intensefluorescence than treated with DMSO alone (Fig. 5). Dil-LDL uptake was measured by flow cytometry and quantified with the relative mean fluorescence intensity (MFI) by flow-jo program. These results suggest that these chemicals increased the amount of the LDL receptor on the cell surface of HepG2 cells, resulting in increased uptake of LDL into the cells.
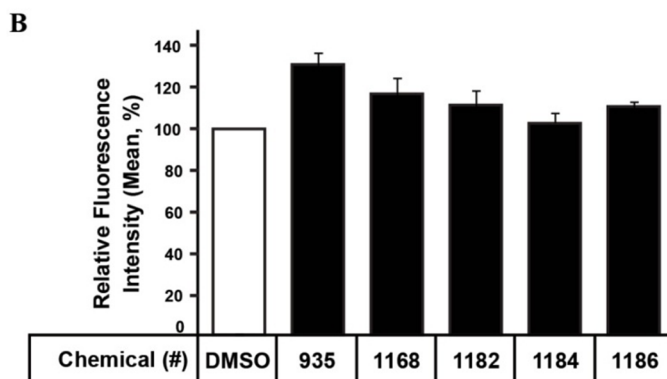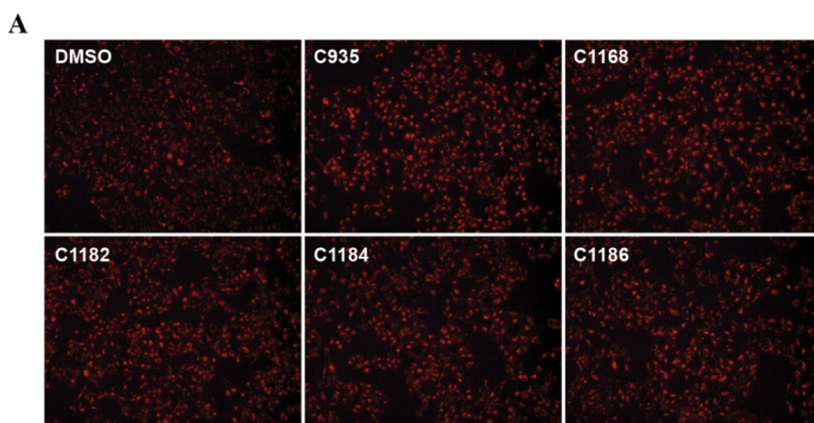
Figure 5. Enhanced the uptake of Dil-LDL in HepG2 cells by the chemicals

(A) HepG2 cells were grown in DMEM supplemented with 10% DLPS in the presence of each chemical for 18h at the concentration of 10µM. After washing with PBS, cells were treated with Dil-LDLat the concentration of 2 µg/ml for 3hr at 37 °C. Fluorescence images were gained by using

fluorescence microscopy. (B) After gain the fluorescence image, cells were trypsinized and fixed with 3% formaldehyde for 20min. Cells were washed twice with PBS and relative mean fluorescence intensity (MFI) was analyzed with flow cytometry. The MFI was calculated as the ratio of fluorescence intensity of vehicel (DMSO) treated cells. Each value represents the mean + SD of three independent experiments.

5. Gene Expression Changes in HepG2 cells applied with C935

To elucidate the mechanism of C935 to reduce the expression of PCSK9, microarray experiment was requested to GenoCheck Ltd,. Based on the microarray data, following experiments were focused on discover the target transcription factors due to the result of decreasing the transcriptional activity of PCSK9 by C935. Compared to HepG2 cells applied to DMSO, negative control, there was a considerable decrease of PCSK9 expression by C935 (Table 4). Microarray data showed that more than 2,000 genes expression was changed two fold or more as a result of high reactivity of C935 (data not shown). These microarray results suggested that *PCSK9* might be a critical target of C935.

Table 4. Genes regulated by C935 in HepG2 cells

Gene Symbol	Description	Fold-change
Lipid metabolism related genes		
ELOVL7	ELOVL family member 7, elongation of long chain fatty acids	7.30
DEGS2	Degenerative spermatocyte homolog 2, lipid desaturase	7.20
CYP3A5	Cytochrome P450, family 3, subfamily A, polypeptide 5	5.60
AGPAT9	1-acylglycerol-3-phosphate O-acyltransferase 9	4.70
TPI1	Triosephosphateisomerase 1	4.18
HSD17B2	Hydroxysteroid (17-beta) dehydrogenase 2	4.14
PLAUR	Plasminogen activator, urokinase receptor	4.14
AKR1C4	Aldo-ketoreductase family 1, member C4	3.91
CPT1C	Carnitinepalmitoyltransferase 1C	3.90
ADM	Adrenomedullin	3.82
PLA2G3	phospholipase A2, group III	0.18
NR1H4	nuclear receptor subfamily 1, group H, member 4	0.21
PHCA	phytoceramidase, alkaline	0.22
DGKK	diacylglycerol kinase, kappa	0.23
PCSK9	proproteinconvertasesubtilisin/kexin type 9	0.27
ABCA2	ATP-binding cassette, sub-family A (ABC1), member 2	0.29
ALDH3B1	aldehyde dehydrogenase 3 family, member B1	0.31
DHRS2	dehydrogenase/reductase (SDR family) member 2	0.32
DGAT1	diacylglycerol O-acyltransferase homolog 1 (mouse)	0.36
ACSM2B	acyl-CoA synthetase medium-chain family member 2B	0.36

Transcription Factor related genes

HAXA10	homeobox A10	11.3
RAB13	RAB13, member RAS oncogene family	9.44
CSDC2	cold shock domain containing C2, RNA binding	7.80
EGR3	early growth response 3	6.59
PTMA	prothymosin, alpha	6.20
FOSL1	FOS-like antigen 1	5.15
ZNF763	zinc finger protein 763	5.02
HMX1	H6 family homeobox 1	4.98
LHX1	LIM homeobox 1	4.95
POLR1C	polymerase (RNA) I polypeptide C, 30kDa	4.91
OSM	oncostatin M	4.88
ZNF38	zinc finger protein 738	0.15
TCEA3	transcription elongation factor A (SII), 3	0.18
ZIK1	zinc finger protein interacting with K protein 1 homolog	0.20
CBX7	chromobox homolog 7	0.21
NR1H4	nuclear receptor subfamily 1, group H, member 4	0.21
E2F2	E2F transcription factor 2	0.24
PHF10	PHD finger protein 10	0.28
MIXL1	Mix1 homeobox-like 1 (Xenopuslaevis)	0.28
NFE2L2	nuclear factor (erythroid-derived 2)-like 2	0.28

6. *NRF2* Knockdown induced the Expression of PCSK9 in HepG2 cells

To assess the regulation of NRF2 on PCSK9 expression, *NRF2* was knocked down using a RNAi technique. The efficacy of small interfering RNA (siRNA) against human NRF2 was evaluated; the amount of *NRF2* mRNA was detected by real-time qPCR 48hr after transfection of si-NRF2 or NC1 (Negative control) in HepG2 cells. The *NRF2* mRNA level was decreased by 18% compared with the mRNA level in NC1 transfected control cells (Fig. 6B). These results suggest that the si-NRF2 used in this study successfully knocked down the *NRF2* mRNA in HepG2 cells. *NRF2* knockdown significantly enhanced the expression of PCSK9 protein as well as mRNA (Fig. 6). However, microarray data showed that C935 decreased the expression of *NRF2* mRNA (Table 4). These results were contrary to expected. Though the microarray data and the result of *NRF2* knock down test, it was necessary to precede additional experiments for finding the regulation of PCSK9 expression by C935 and NRF2.
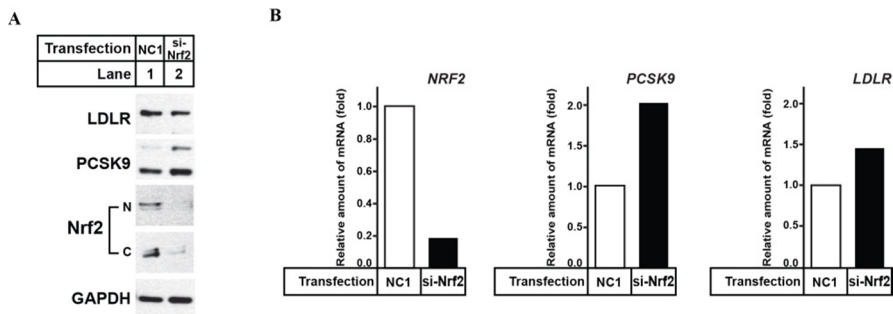
Figure 6. Effect of *NRF2* knock down on the expression of PCSK9

(A) HepG2 cells were transiently transfected with NC1 and si-NRF2 on day 0. On day 1, cells were changed to DMEM supplemented with 10% DLPS. On day 2, cells were fractionated as described in materials and methods. N and C for NRF2 represent the nuclear and cytosol fraction, respectively. (B) The *PCSK9* and *LDLR* mRNA level were analyzed with quantitative real-time PCR. Total RNAs were prepared from HepG2 cells 48 hr after transfected with NC1 and siNRF2 and cDNA was synthesized by reverse-transcription. Each value represents the amount of mRNA relative to that in the cells transfected with NC1, which is arbitrarily defined as 1. The values represent means from duplicate reactions. GAPDH was used as an invariant control (data not shown). Similar resultswere obtained in three independent experiments.

7. Activated NRF2 decreased the expression of PCSK9 in HepG2 cells

tert-butylhydroquinone (t-BHQ), Resveratrol and sulforaphane (SFN) were used as activators of NRF2. These materials are known to lead to translocate and accumulatethe NRF2 proteins in nucleus[35]. t-BHQ and Resveratrol effectively increased the expression of NRF2 level in nucleus and slightly decreased the amount of NRF2 levels in cytosol (Fig. 7A, E). While Sulforaphane increased the NRF2 expression in both fractions (Fig. 7I).

t-BHQ, Resveratrol and SFN were effectively reduced the expression of PCSK9 when compared to treating with DMSO, which is a negative control purified from HepG2 cells (Fig. 7A, E and I): t-BHQ from 125μM to 250μM (Fig. 7A), Resveratrol from 50μM to 100μM (Fig. 7E) and SFN from 25μM to 100μM (Fig. 7I).

Total RNA was isolated from t-BHQ or Resveratrol or SFN-treated HepG2 cells for 18hrs and was analyzed with real-time PCR (Fig.7B, F and J). t-BHQ, Resveratrol and SFN decreased the *PCSK9* mRNA effectively, and the mRNA level of *HMOX1* which is the target gene of NRF2 was enormously increased. There was a slight increase by t-BHQ (Fig. 7B) and great increase by SFN (Fig.

7J) in *LDLR* mRNA level. From this, Sulforaphane could raise the expression of LDLR by the effect of decrease the PCSK9 expression as well as by increase the *LDLR* mRNA levels. Figure 7B, F and J showed that t-BHQ, Resveratrol and SFN induced the NRF2 activity and identically reduced the mRNA level of *PCSK9* effectively.

Activation of NRF2 by effect of the t-BHQ, Resveratrol and SFN led to decrease the expression of PCSK9. This effect minimizes the degradation of LDLR and consequently increases the amount of LDLR on the cell sulface. Treating cells with t-BHQ, Resveratrol and SFN respectively yielded more intense fluorescence than treated with DMSO alone (Fig. 7C, G and K). These results suggest that t-BHQ, Resveratrol and SFN increased the expression of the LDL receptor on the cell surface of HepG2 cells, resulting in increased uptake of LDL particels into the cells.

These findings suggest that NRF2 is a new target for regulating the LDL cholesterol level in plasma and the specific NRF2 activators are expected to be therapeutic agents of hypercholesterolemia.
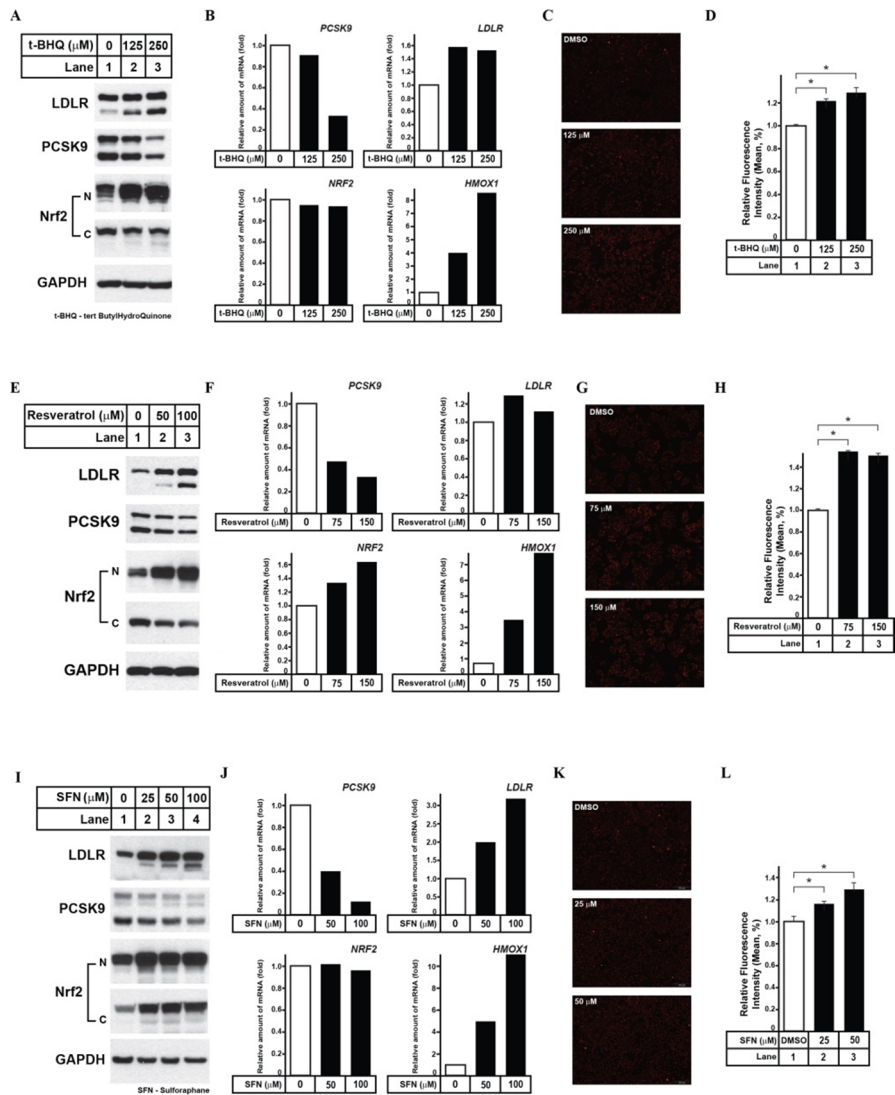
Figure 7. Activated NRF2 by t-BHQ, Resveratrol and Sulforaphane decreased the expression of PCSK9 in HepG2 cells

HepG2 cells were set up at 10 cm plate with DMEM supplemented with 10% FBS. On day1, cells were washed with PBS, then pre-incubated with fresh DMEM supplemented with 10% DLPS. On day 2, cells were treated with (A) t-BHQ, (E) Resveratrol and (I) Sulforaphane at the indicated concentration. After 18hr incubation, cells were fractionated as described in materials and methods. N and C for NRF2 represent the nuclear and cytosol fraction, respectively. The effect of the *PCSK9* and *LDLR* mRNA level by (B) t-BHQ, (F) Resveratrol and (J) Sulforaphane were analyzed with quantitative real-time PCR. Total RNAs were prepared from HepG2 cells treated with t-BHQ, Resveratrol and Sulforaphane respectively at the indicated concentration for 18 hr. cDNA was synthesized by reverse-transcription, and subjected to the analyses by quantitative real-time PCR. Each value represents the amount of mRNA relative to that in the cells grown with vehicle (DMSO), which is arbitrarily defined as 1. The values represent means from duplicate reactions. GAPDH was used as an invariant control (data not shown). Similar results were obtained in three independent experiments. (C, G and K) HepG2 cells were grown in DMEM supplemented with 10% DLPS in the presence of t-BHQ, Resveratrol and Sulforaphane respectively for 18h at the indicated concentrations. After washing with PBS, cells were treated with Dil-LDL

at the concentration of 2 µg/ml for 3hr at 37 °C. Fluorescence images were gained by using fluorescence microscopy. (D, H and L) After gain the fluorescence image, cells were trypsinized and fixed with 3% formaldehyde for 20min. Cells were washed twice with PBS and relative mean fluorescence intensity (MFI) was analyzed with flow cytometry. The MFI was calculated as the ratio of fluorescence intensity of vehicel (DMSO) treated cells. Each value represents the mean + SD of three independent experiments.

8. Knockdown of *PCSK9* leads to decrease the expression of NRF2 in nucleus

To elucidate the effect of C935 on the expressions of PCSK9 and NRF2, HepG2 cells were transfected with siPCSK9 or siNrf2. As showed above, knock downed *NRF2* led to increase the PCSK9 expression and decrease the LDLR expression (Fig. 8A lane 3). Meanwhile, the expression of NRF2 in nucleus was reduced when the amount of PCSK9 was decreased (Fig. 8A lane 2). This result means that C935 decreases the expression of PCSK9, resulting in reducing the NRF2 expression in nucleus to recover the expression of PCSK9 again and maintain the cholesterol homeostasis. Figure 7B shows the quantifications of LDLR, PCSK9 (65KD), nucleus-NRF2 and cytosol-NRF2 expression respectively.
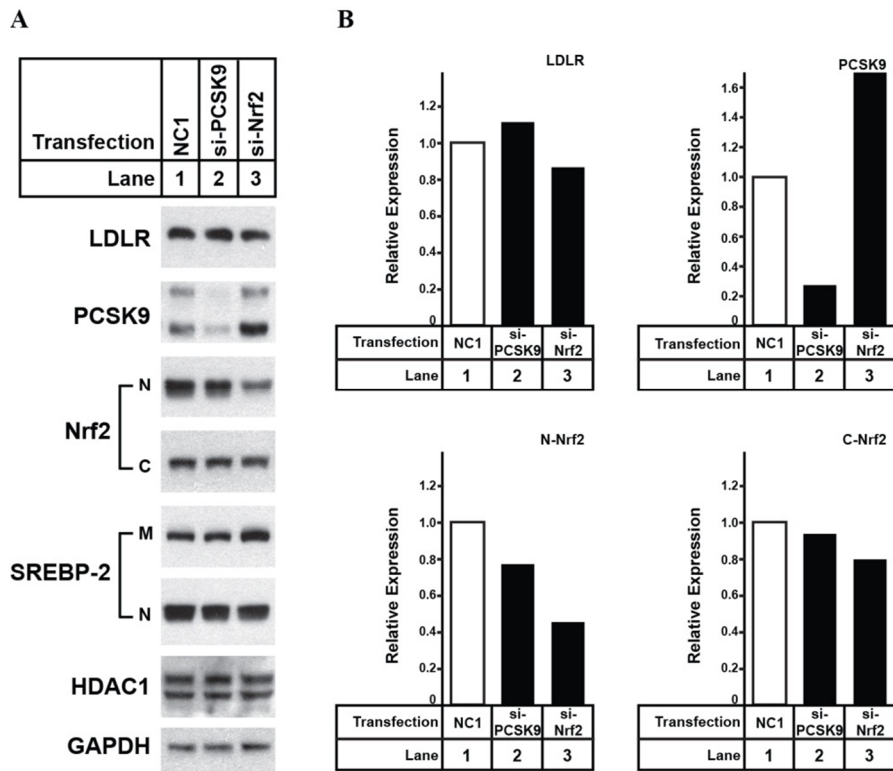
Figure 8. Effect of *PCSK9* knock down on the expression of NRF2

(A) HepG2 cells were transiently transfected with NC1, siPCSK9 and siNRF2 on day 0. On day 1, cells were changed to DMEM supplemented with 10% DLPS and the following day cells were fractionated and cell lysates were subjected to western blot analyses with antibodies against PCSK9, the LDLR, NRF2 and SREBP-2. GAPDH and HDAC1 were used as an invariant control. N and C for NRF2 represent the nuclear and

cytosol fraction, respectively. M and N for SREBP-2 represent the membrane and nuclear fraction, respectively. (B) The band intensities of LDLR, PCSK9, nucleus-NRF2 and cytosol-NRF2 in figure 9A were quantified using Image J software.
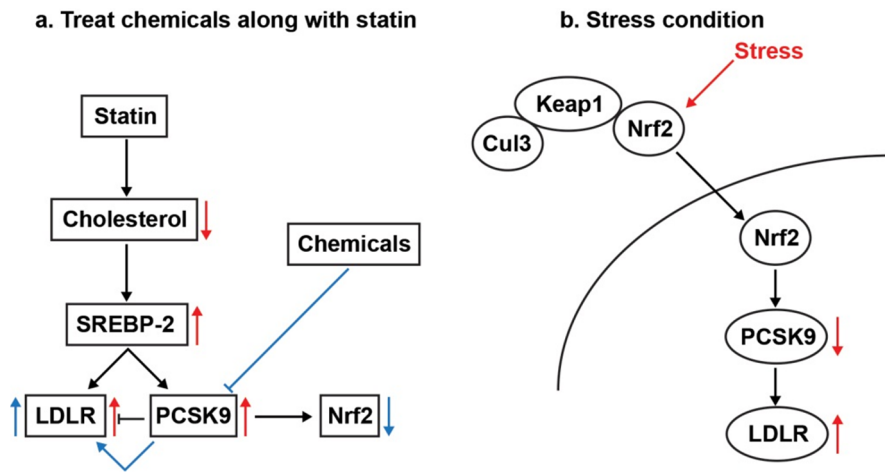
Figure 9. Diagram about the regulation of PCSK9 by the chemicals with statin and NRF2 action

(A) The chemicals inhibit the induction of PCSK9 in response to statin resulting in further increased of LDLR expression. (B) In stress condition, NRF2 is activated, and then decrease the amount of PCSK9.

IV. DISCUSSION

The level of plasma LDL cholesterol is regulated by coordinated functions of proteins participating in uptake of cholesterol from diets and in synthesis of cellular cholesterol *de novo*. In particular, overall regulation of these proteins are well known to be regulated by the transcription factor SREBP-2 which binds to the SRE motif in promoter regions of genes[8]. In a condition deprived from sterol, cells increase the amount of the LDLR on a cell surface to increase the cellular concentration of cholesterol by activation of SREBP-2, which is an important consequence of statin action[5, 7, 8]. Unfortunately, the expression of PCSK9 which antagonizes the role of the LDLR is up-regulated simultaneously by activation of SREBP-2 under the same condition[36]. Accordingly, the development of PCSK9 inhibitors is considered invaluable as a new modality to treat hypercholesterolemia as well as in a way that it helps supplement statins. In this study, a chemical library was screened to search for chemicals as PCSK9 inhibitors which have the ability to reduce the expression of PCSK9.

By screening the commercially available chemical library, a set of chemicals which decreased the expression of PCSK9 effectively with

concurrent increase in that of the LDLR was selected. These chemicals shared 3-anilino-2-chloro-1,4-naphthoquinone, as a scaffold structure The range of decrease in PCSK9 by chemicals seemed to be related to chemical nature and positions of their side chains. It is necessary to discover the correlation between the effect on the expression of PCSK9 and kinds of side chains attached of chemicals in the future studies. The five chemicals finally selected showed the significant decrease in the amount of both protein and mRNA for *PCSK9* while that of *LDLR* and other lipogenic enzymes remained relatively unaffected. These results suggest that the effects of selected chemicals are relatively confined to the expression of PCSK9. In addition, C935 and related chemicals effectively blocked the Rosuvastatin-induced PCSK9 expression while they increased the amount of the LDLR in a larger amount than that in cells grown without Rosuvastatin. These results correspond to the hypothesis that blocking PCSK9 induction has beneficial augmentative effect to increase the LDLR by statins. The increase in the amount of the LDLR was confirmed to be relevant to functional increase in LDL-cholesterol uptake assay by fluorescence-labeled Dil-LDL uptake in HepG2 cells. However, unfortunately, this study could not provide the evidence that C935 can lower the plasma concentration of cholesterol in mice. The *in vivo*

application of C935 or the related chemicals should be carried out in further studies.   The decrease in PCSK9 expression by chemicals was caused by transcriptional suppression of the *PCSK9* promoter assessed by promoter-reporter assay. According to Jeong H.J. et al.[8], five sp1 sites and one SRE region are found in the *PCSK9* promoter-reporter construct D4, which contains the 5' flanking region of the *PCSK9* promoter (-94 ~ -440). Selected five chemicals showed the decrease in *PCSK9* promoter activity at 5 µM concentration regardless of the presence of sterol. To determine transcription factors that mediate the regulation of the *PCSK9* promoter activity by chemicals, we used the microarray analysis of mRNAs of which amounts were altered by C935 in HepG2 cells, and the computational analysis of the *PCSK9* promoter region for prediction of transcription factor binding sites. Several transcription factors were revealed as potentially functional for regulation of the PCSK9 promoter by C935. However, no significant transcription factor except NRF2 was revealed to be functional in regulation of PCSK9 expression by C935 as far as experiments applied in this study: site-directed mutagenesis of the transcription binding sites, over-expression of transcription factors and knock-down of the transcription factors by target specific siRNA. NRF2 is a transcription factor that is known to up-regulate many antioxidant

enzymes by binding to antioxidant response element sites in their promoter region[30]. NRF2 is a critical mediator that regulates comprehensively the expression of several stress responsive enzymes and detoxification enzymes. Recently, there are a few studies that show the NRF2 activation may prevent or treat obesity and associated metabolic syndrome[37-39]. These studies suggested that a novel mechanistic linkage between metabolic syndrome and oxidative stresses[32, 33, 40]. Interestingly, knock-down of NRF2 using siRNA enhanced the expression of *PCSK9* mRNA as well as PCSK9 Protein significantly. This induction of PCSK9 by NRF2 knock-down was contrary to the result in microarray analysis that deduced causative relationship of *NRF2* mRNA reduction by C935 with decrease in PCSK9 expression. However, it was evident that the PCSK9 expression could be regulated by transcription factor NRF2, this relationship between NRF2 and PCSK9 was further elucidated in this study.

Knocking-down NRF2 increased the PCSK9 expression and decreased the LDLR expression, which suggests that NRF2 is a suppressor of the PCSK9 expression. Interestingly, when the expression of PCSK9 was knocked-down, the expression of NRF2 in nucleus was reduced. This may support the result that C935 simultaneously reduced the expression of PCSK9 and NRF2, and suggests that this simultaneous reduction may be

resulted from a mechanism secondary to changes in PCSK9 the LDLR expression. Treatment of HepG2 cells with specific NRF2 activators like t-BHQ, resveratrol, and sulforaphane [41], effectively reduced the expression of PCSK9 and increased amounts of expression of LDLR and the nuclear form of NRF2 in a dose-dependent manner. These results suggest that NRF2 is activated under the stress condition, and then activated NRF2 causes to decrease the amount of PCSK9 while to increase the amount of the LDLR to saving cellular metabolism to reduce de novo cholesterol biosynthesis. These results also suggest that NRF2 is a novel regulator of the LDL-cholesterol metabolism in plasma through modulating the expression of PCSK9. In addition, the specific NRF2 activators could be proposed as a new therapeutic agent for hypercholesterolemia.

In summary, this study provided the scientific basis for development of small molecular chemicals as PCSK9 inhibitors, and elucidated a novel mechanism of regulation of PCSK9 expression by NRF2. These results will be invaluable evidence for development of therapeutic agents for atherosclerosis and cardiovascular diseases.

V. CONCLUSION

Through the screening of 3,000 chemicals, five effective chemicals to reduce the expression of PCSK9 were selected and they share the common scaffold structure of 1,4-naphthoquinone. These chemicals reduce the expression of PCSK9 by transcriptionally and enhance the LDLR expression and LDL uptake into HepG2 cells. And the chemicals overcome the statin drug shortcoming which up regulates the PCSK9 expression. Although the effect of these chemicals may not be involved in NRF2 action or control by feedback mechanism, NRF2 can mediate the regulation of the expression of PCSK9. Together, invaluable cholesterol-lowering chemicals applied with statin or NRF2 activators are expected to be therapeutic agents targeting hypercholesterolemia.

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Abstract (in Korean)

화합물 리이브러리 선별과정을 통해 고콜레스테롤혈증 치료

표적으로서 PCSK9 발현 억제 물질 선정 및 규명

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민 동 국

혈중 low density lipoprotein(LDL) cholesterol 농도 증가는 동맥경화 및 고혈압, 협심증 등의 심혈관계 질환의 가장 주된 유발 요인이다. 따라서 이러한 질환을 치료 및 예방하기 위하여 혈중 LDL cholesterol의 농도를 감소시키기 위한 다양한 시도가 이루어지고 있다.

Proprotein convertase subtilisin/kexin type 9 (PCSK9)은 혈액으로 분비된 후 세포막에 존재하는 LDL receptor와

결합하여 세포 내로 LDL receptor를 유입시킨 후 LDL receptor의 분해를 촉진시키는 역할을 하는 단백질로서 고콜레스텔롤혈증의 유발 원인 중의 하나이다. 즉 PCSK9의 과발현은 LDL receptor의 양적인 감소를 초래하여 콜레스테롤이 대사되지 못하여 고콜레스테롤혈증이 발병하며, 반대로 PCSK9의 발현을 억제시킴으로써 세포막에 존재하는 LDL receptor를 증가시켜 혈중 콜레스테롤 농도를 감소시키는 것으로 보고되고 있다. 본 연구에서는 PCSK9의 발현을 감소시키고 LDL receptor의 발현을 증가시키는 물질을 선정하기 위하여 3,000여 개의 chemicals로 이루어진 chemical library를 screening 하였다. 이 과정을 통해 HepG2 세포에서 1,4-naphthoquinone의 특정 구조를 가진 chemicals이 PCSK9의 발현을 감소시키데 효과적임을 단백질과 mRNA의 level을 통해 확인하였고, 증가된 LDL receptor를 통해 세포 내로 LDL cholesterol의 유입이 증가됨을 확인하였다. 또한 chemicals는 Statin 약물이 SREBP-2를 활성화 시킴으로써 PCSK9의 발현을 증가시키는 단점을 극복하고 LDL receptor의 발현을 더욱 증가시키는 효과를 보여주었다. Chemicals과 PCSK9

사이의 메커니즘을 밝히고자 microarray 실험이 진행되었고, data 분석을 통해 여러 transcription factors 중 C935에 의해서 감소되는 Nuclear factor (erythroid-derived 2)-like 2 (NRF2)가 PCSK9의 발현 조절과 관련성이 있음을 확인하였다. NRF2는 스트레스 상화에서 antioxidant response pathway에 관련된 여러 유전자의 발현을 증가시키는 전사인자로 알려져 있다. siRNA를 이용하여 NRF2를 저 발현 시켰을 때 PCSK9의 발현이 증가되었고, NRF2를 활성화 시킬 수 있는 t-BHQ, Resveratrol 그리고 Sulforaphane을 처리하였을 때 NRF2가 활성화 되고, PCSK9의 발현이 감소됨을 확인하였다. 하지만 이 결과는 C935에 의해 감소된 NRF2를 통해 PCSK9의 발현이 감소되는 가설에 반대되었고, 이런 현상을 뒷받침 하기 위해 PCSK9의 저 발현을 통한 피드백 검정 실험을 진행하였다. PCSK9의 발현이 감소되었을 때 nuclear form의 NRF2의 발현이 감소됨을 확인하였고, 이 결과를 통해 C935에 의해 NRF2의 발현이 낮아지는 현상은 감소된 PCSK9의 발현을 다시 증가시키기 위한 피드백 작용임이 증명되었다.

이러한 결과를 통해 Chemicals과 함께 NRF2를 활성화 시키는 물질을 동시에 처리하였을 때 PCSK9의 발현을 더욱 억제할 수 있는 가능성을 제시하였다. 또한 chemicals과 독립적으로 PCSK9의 발현을 감소시키는 전사인자인 NRF2 와 PCSK9과의 상관관계에 대해 밝힐 수 있는 연구가 필요하다.

이러한 연구를 토대로 chemicals은 PCSK9 억제를 통하여 statin약물의 보조약제로써 고콜레스테롤혈증 치료제로의 이용 가능성을 보여주었고, NRF2는 PCSK9과 피드백 메커니즘으로 조절되면서 스트레스 상황에서 NRF2가 활성화 되었을 때 스트레스를 극복하기 위한 하나의 방법으로 PCSK9의 발현을 감소시키고 LDL receptor의 양을 증가시켜 세포 내 cholesterol level을 높게 유지시키는 현상으로 판단된다.