Small Molecules as Inhibitors of PCSK9: Current Status and Future Challenges

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Abbreviations:

AMPK, adenosine 5'-monophosphate activated protein kinase; BBR, berberine; BET, bromodomain and extra-terminal; BRDs, bromodomains; CES1, carboxylesterase 1; CETP, cholesteryl ester transfer protein; CRISPR, clustered regularly interspaced short palindromic repeats; CVD, cardiovascular disease; EGF(A), epidermal growth factor A; EKO, exploring key orientations; FoxO3a, forkhead box O3a; GOF, gain-of-function; HFNAPS, highly functionalized nucleic acid polymers; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; HNF-1α, hepatocyte nuclear factor 1α; HS, heparan sulfate; HSPGs, heparin sulfate proteoglycans; HTS, high-throughput screening; LDL-C, low-density lipoprotein-cholesterol; LDLRs, low-density lipoprotein receptors; LOF, loss-of-function; mAbs, monoclonal antibodies; MW, molecule weight; NARC1, neural apoptosis-regulated convertase 1; NUTs, nutritional compounds; PCSK9, proprotein convertase subtilisin/kexin type 9; PDB, protein data bank; PPI, protein-protein interaction; PROTAC, proteolysis-targeting chimera; Q3G, quercetin-3-glucoside; qPCR, quantitative polymerase chain reaction; SAR, structure

activity relationship; siRNA, small interfering RNA; SPR, surface plasmon resonance; SREBP-2, sterol regulatory element binding protein-2; TC, total cholesterol; TR-FRET, time-resolved fluorescence resonance energy transfer; TRIB1, tribbles homolog 1; XZK, xuezhikang.

Abstract

Proprotein convertase subtilisin/kexin type 9 (PCSK9) plays an important role in regulating lipoprotein metabolism by binding to low-density lipoprotein receptors (LDLRs), leading to their degradation. LDL cholesterol (LDL-C) lowering drugs that operate through the inhibition of PCSK9 are being pursued for the management of hypercholesterolemia and reducing its associated atherosclerotic cardiovascular disease (CVD) risk. Two PCSK9-blocking monoclonal antibodies (mAbs), alirocumab and evolocumab, were approved in 2015. However, the high costs of PCSK9 antibody drugs impede their prior authorization practices and reduce their long-term adherence. Given the potential of small-molecule drugs, the development of small-molecule PCSK9 inhibitors has attracted considerable attention. This article provides an overview of the recent development of small-molecule PCSK9 inhibitors disclosed in the literature and patent applications, and different approaches that have been pursued to modulate the functional activity of PCSK9 using small molecules are described. Challenges and potential strategies in developing small-molecule PCSK9 inhibitors are also discussed.

Keywords

PCSK9 protein, inhibitor, small-molecule, LDL-C, hypercholesterolemia, natural products, patent

Contents

1. Introduction

Cardiovascular disease (CVD) is the leading cause of mortality worldwide despite advances in lifestyle and the available of various cardiovascular drugs.[1] Hypercholesterolemia has long been considered as an independent risk factor for CVD, and lowering low-density lipoprotein cholesterol (LDL-C) has been shown to reduce cardiovascular risk in clinical trials.[2] Several agents have been widely applied for reducing LDL-C levels in the treatment of hypercholesterolemia, including statins, ezetimibe, niacin, and bile acid sequestrates.[3] To date, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) have been widely regarded as the first line of pharmacological treatment for lowering LDL-C, reducing risk of primary and secondary cardiovascular events.[4] Despite the successful application of statins alone or in combination with other lipid-lowering drugs, residual risk persists in a large portion of statin-treated patients (~25%) who fail to attain desirable LDL-C levels with the maximally tolerated statin therapy[5] or are intolerant to statins owing to various side effects such as muscle symptoms, headache, sleep disorder, rash, arthritis, etc.[6] Thus, safer and more effective nonstatin therapies for the treatment of dyslipidemia to reduce cardiovascular risk are needed.

PCSK9 is a recently identified but well-validated novel drug target that lowers LDL-C levels for hypercholesterolemia treatment.[7] This protein plays an important role in regulating the degradation of hepatic low-density lipoprotein receptors (LDLRs), thereby increasing circulating LDL-C levels.[8] Various therapies have been designed to inhibit PCSK9 and consequently inhibit LDLR degradation.[9] In 2015, two fully human monoclonal antibodies (mAbs), alirocumab[10] and evolocumab,[11] were approved as PCSK9 inhibitors. They are efficacious, well tolerated, and have no obvious side effects.[12, 13] Recent clinical data of evolocumab have shown a reduction in cardiovascular events by 15% after a median treatment of 2.2 years, confirming the clinical benefit of PCSK9 inhibition as a drug target.[11]

The most studied and clinically approved approach to inhibit PCSK9 is the use of mAbs, and comprehensive reviews have summarized the development process, safety and tolerability of anti-PCSK9 antibodies.[14-17] Given the intravenous administration

and high cost of mAbs (>$14,500 per year), the appeal of small-molecule inhibitors of PCSK9 with lower cost and ease of administration is clear.[18] However, targeting PCSK9 with small-molecule approaches has proven a great challenge due to the lack of druggable pockets on PCSK9. Nevertheless, there has been a rapid increase in reports about small-molecule PCSK9 inhibitors in recent years.[19] Here, we'd like to provide a comprehensive overview of studies on the small molecules targeting PCSK9 in scientific literature and patent applications. We will first give an overview of the biogenesis, structure and physiological role of PCSK9 in lipid metabolism. Then, current strategies reported in the literature based on reducing the synthesis of PCSK9 or disturbing the binding of PCSK9 to the LDLR will be introduced in detail; in addition, several natural products modulating the expression, activity or functionality of PCSK9 will also be described. All international patent applications (2010-2018) protecting novel small molecules targeting PCSK9 will be covered. Finally, novel strategies that have the potential to assist the further development of small-molecule PCSK9 inhibitors and future challenges will be discussed.

2. Background

2.1. Biogenesis and structure of PCSK9

The human *PCSK9* gene, initially called 'neural apoptosis-regulated convertase 1' (*NARC1*), was discovered by Seidah et al., who reported the identification of the ninth member of the mammalian proprotein convertase family located on chromosome 1p32.3.[20, 21] The *PCSK9* gene on the short arm of chromosome 1 is 25 kb long and contains 12 exons and 11 introns (Figure 1a). *PCSK9* encodes an inactive glycoprotein (pre-PCSK9) with 692 amino acids comprising four major components: a signal sequence (1-30) and *N*-terminal prodomain (31-152), followed by a subtilisin-like catalytic domain (153-425) and a *C*-terminal domain (426-692, also called the V domain).[22] Once the signal peptide is cleaved from pre-PCSK9 in the endoplasmic reticulum, pro-PCSK9 (31-692) is formed and then converted to mature secretory PCSK9 through autocatalytic cleavage of the prodomain between Gln152 and Ser153[23] in the Golgi apparatus (Figure 1b).[24] However, unlike the other

proprotein convertases, the cleaved prodomain of PCSK9 remains tightly associated with the catalytic domain, where it inhibits further catalytic activity.[25]

PCSK9 binds to LDLRs on the cell surface, leading to their degradation. The binding site of PCSK9 localizes to the EGF(A) (epidermal growth factor A) domain of the LDLR (Figure 1c). The binding surface of PCSK9 is formed primarily by residues 367-381, constructing an exposed, slightly convex ~500 $Å^2$ region. The key interactions of PCSK9 with EGF(A) are made by an antiparallel β-sheet formed between residues 377-379 of PCSK9 and 308-310 of EGF(A). In addition, Arg^{194} and Asp^{238} of PCSK9 are also important for the binding. Other residues of PCSK9 such as Phe^{379}, Ile^{369}, and Cys^{378} also contribute to this interface (Figure 1d).[26]

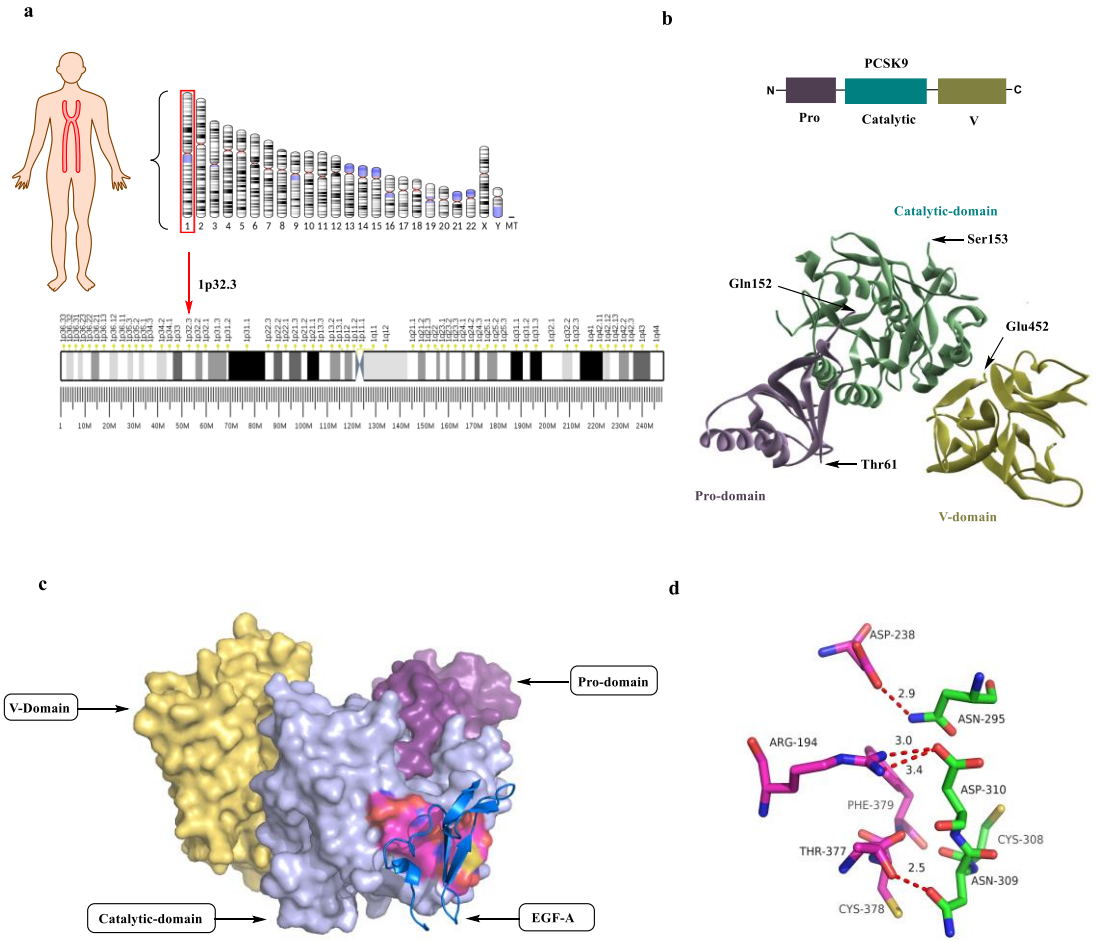


Figure 1. Gene location (human) and structure of PCSK9. (a) Gene location of PCSK9. (b) Structure of PCSK9, with the pro-domain (violet), the catalytic domain (forest), and the V-domain (pale yellow). (c) The binding surface of PCSK9 with EGF-A. The

surface of PCSK9 buried upon binding to EGF-A is colored according to element type: carbon, warmpink; nitrogen, blue; oxygen, red; sulfur, limon. EGF-A is represented as a marine cartoon model. (d) Key interactions between PCSK9 (magenta) and EGF-A (Green).

2.2. Physiological role of PCSK9

In 2003, novel gain-of-function (GOF) mutations (S127R, F216L) in the PCSK9 gene were identified by Abifadel *et al.* in two French families with autosomal dominant hypercholesterolemia.[27] By contrast, loss-of-function (LOF) mutations have the opposite effect and result in hypocholesterolemia. Since then, PCSK9 has emerged as a novel therapeutic target for the treatment of dyslipidemia. Several lines of evidence have demonstrate an LDL-C lowering mechanism in which PCSK9 lowers the amount of hepatic LDLR proteins and thus compromises the liver's ability to remove LDL-C from circulation.[28] As shown in Figure 2, under normal physiological conditions, circulating LDL is bonded to LDLRs at the hepatocyte surface and then internalized into hepatocytes through clathrin-mediated endocytosis as a receptor-ligand complex. The low pH in the endosome leads to dissociation of the LDL from its receptor, and the LDL is degraded in the lysosome, while the LDLR is recycled to the cell surface.[29, 30] However, in the presence of PCSK9 at the hepatocyte surface, the catalytic domain of secreted PCSK9 associates with the EGF(A) domain of the LDLR, and the PCSK9/LDLR complex is internalized into hepatocytes.[31] The low pH of the endosome enhances the affinity of PCSK9 for the LDLR, preventing the receptor from being recycled to the cell surface. Instead, the complex is directed to the lysosome, where LDLR is concomitantly degraded along with the complexed LDL particle.[32, 33]

2.3. Approaches to PCSK9 inhibition

After the identification of LOF and GOF mutations of PCSK9 in human, great efforts have been made to discover PCSK9 inhibitors that act as therapeutic agents to lower LDL-C.[34, 35] At least nine different modalities targeting PCSK9 have arisen,

including mAbs, peptidomimetics, antisense oligonucleotides, small interfering RNA (siRNA), vaccines, CRISPR (clustered regularly interspaced short palindromic repeats) therapeutics, antibody mimetics (including adnectin and anticalin), and small molecules.[36] The potential advantages and disadvantages of these modalities as well as the clinical studies of their effectiveness are summarized in Table 1.

In terms of mechanism, the success of these modalities is roughly attributed in this review to three strategies that target PCSK9 (Figure 2). Strategy 1 is to directly or indirectly prevent binding of PCSK9 to the LDLR on the cell surface with an antibody, a peptide, or a small molecule. Strategy 2 is to interfere with the maturation and secretion processing or the biological function of PCSK9. Strategy 3 is to develop inhibitors of PCSK9 synthesis and expression at the mRNA or protein levels. These three strategies have been successfully applied to develop small-molecule PCSK9 inhibitors in recent years. Although only one PCSK9 small molecule is currently active in clinical trials (see section 3.6.2), there has been a steady increase in available data on small-molecule PCSK9 inhibitors in recent years. This review will provide an updated view of small-molecule PCSK9 inhibitors reported in scientific journals and patent applications.

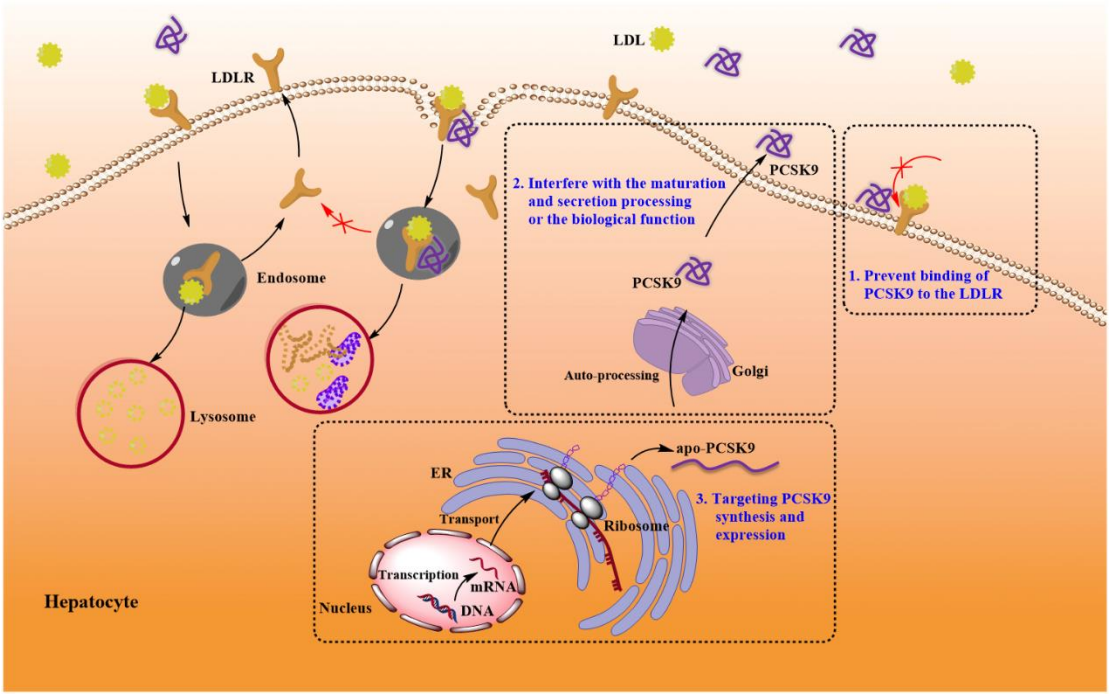


Figure 2. PCSK9 is produced mainly by the liver and is secreted into the bloodstream. Upon binding to liver LDLRs, the entire PCSK9/LDLR complex is endocytosed and subsequently directed to lysosomes for degradation. Strategies targeting the synthesis, processing, and binding of PCSK9 could be adopted to develop PCSK9 inhibitors.

Table 1

Pharmaceutical approaches targeting PCSK9

Approach	Mode of action	Advantages	Disadvantages	Agent[Ref]	Stage	Company
Monoclonal antibodies (mAbs)	Blocking interaction with LDLR or neutralizing PCSK9 activity.	Efficient and safe High specificity Low toxicity	Short shelf-life compared with siRNA High cost Parenteral administration	Evolocumab [10] Alirocumab [11] Bococizumab[37] LY-3015014[38, 39] RG-7652[40, 41]	Approved-2015 Approved-2015 Phrase III (Discontinued) Phase II Phase II	Amgen Sanofi Pfizer Lilly Genentech/Roche
Small Peptides	Blocking interaction with LDLR	Cheap	Instability in plasma Parenteral administration	Pep2-8[42]	Preclinical	Genentech
Antisense oligonucleotides	Silence mRNA Leading to mRNA degradation	High specificity	High production costs Parenteral administration	SPC5001[43] BMS-844421[44] Civi-007[45]	Phase I (Discontinued) Phase I (Discontinued) Phase I	Roche BMS Roche

siRNA	Target PCSK9 mRNA and arrest translation	Long-acting Safe Less-frequent dosing	Parenteral administration	Inclisiran (ALN-60212) [46, 47]	Phase III	Alnylam/The Medicines Company
Adnectin	Blocking interaction with LDL-R	High binding affinity Relative cheaper production cost	Short plasma half-life	BMS-962476[48]	Phase I	BMS
Anticalin	Blocking interaction with LDL-R	Antibody mimetic but smaller than mAbs, Mass production at low cost	Design and screening	DS-9001a[49]	Phase I (Discontinued)	Daiichi Sankyo/Pieris Pharmaceuticals
Vaccine	Immunization against the body's own PCSK9 to produce autoantibodies	High immune response against PCSK9 without side effects Long-term effects	Possible irreversibility Autoimmune response	AT04A& AT06A[50]	Phase I Phase I	AFFiRis AFFiRis
CRISPR	Genome editing disrupting *PCSK9* gene	Permanent reduction in LDL-C levels	Potential off-target mutagenesis	Academic project and AstraZeneca[51-55]	Preclinical	AstraZeneca
Small molecules	As detailed below	Cheap Oral administration	High likelihood of off-target	As detailed below	Preclinical	As detailed below

3. Small-molecule PCSK9 inhibitors reported in scientific journals

3.1. Blocking the ribosome: from R-IMPP to PF-06815345

In the search for small molecules that inhibit PCSK9 production, researchers from Pfizer have presented a fascinating work determining PCSK9 secretion through high-throughput screening (HTS).[56] R-IMPP (**1**, PF-00932239, Figure 3) was identified as a new anti-PCSK9 small lead through a phenotypic screen from a screening set of 2.55 million compounds. This process was challenging based on the following threshold values: for ProLabel tag chemiluminescent assays, $IC_{50} < 10$ μM; for secreted alkaline phosphatase nonspecific secretion assays, $IC_{50} > 10$ μM; and for ATP cytotoxicity assays, $IC_{50} > 10$ μM. Finally, R-IMPP was identified as a weak PCSK9

antisecretagogue with an IC_{50} value of 4.8 μM in a CHO-K1 cell line overexpressing recombinant ProLabel-tagged PCSK9 and an IC_{50} of 2 μM in native cell lines.[57] The pharmacokinetic properties of PF-00932239 were not satisfactory, and structural changes were made to two regions of hit compound (**2**) to improve its pharmacokinetic properties and potency, resulting compound PF-06446846 (**3**).[58] PF-06446846 inhibited the secretion of PCSK9 with an IC_{50} of 0.3 μM in Huh7 cells. The mechanism of action of this compound was shown to be new, as it demonstrated the ability to selectively bind to human (but not *Escherichia coli*) 80S ribosomal complex, causing the ribosome to stall on mRNA near the position predicted to generate a 35 amino acid peptide.[59] This work generated fascinating new insight into the basic biology of ribosomal processing.[60, 61]

Although PF-06446846 (**3**) exhibits a high degree of specificity for inhibiting the expression of PCSK9, the narrow margin between PCSK9-lowering and hematopoietic effects prevents the clinical development of PF-06446846. Thus, efforts to achieve better safety by narrowing the tissue distribution of these compounds was continued using a hepatic targeting approach.[62] An acidic/anionic functionality was introduced into benzamide (**4**, **5**) to facilitate liver-targeted delivery of these PCSK9 inhibitors according to previous structure-activity relationships (SARs).[63, 64] Unfortunately, these zwitterions lacked anti-PCSK9 pharmacology in cell-based assays due to their poor permeability. Thus, an exquisite ester, which is labile to carboxylesterase 1 (CES1) in the liver, was added to compounds **4** and **5** to make prodrugs.[65] Metabolism of these prodrugs in hepatocytes revealed the active zwitterion that was retained by its poor passive permeability. Finally, compound **6** (PF-06815345) was selected as a candidate, as it was relatively well tolerated in 28 day preclinical toxicity studies in rats and monkeys. Further studies are needed to understand the potential of this therapeutic modality and the liver targeting approach.

In parallel, efforts to transform compound **3** into molecules with high systemic bioavailability and an improved balance of safety and efficacy suitable for further development were also explored.[66] It was found that subtle structural changes around benzamide and *N*-triazolopyridine moieties of **3** yielded significant changes in

pharmacology and off-target toxicity. Synthetic SAR evaluations were performed abound these two regions to establish a correlation between substituent and toxicology to seek out the analogues of interest, and resulted in identification of compounds **7** and **8** with similar potencies and improved in vivo toxicology profiles. The improved safety profile may be related to diminish binding of these compounds to nontranslating ribosomes, but great efforts should be made to understand the translational selectivity.

The strategy adopted by Pfizer was extremely innovative, as translational inhibitors were generally confined to antibacterials;[67] however, Pfizer demonstrated that small molecules can specifically inhibit the translation of a single disease-associated protein by stalling the ribosome chain, truly opening new paradigms to overcoming 'undruggable' proteins. But whether this strategy could be applied to other target to find highly selective translational inhibitors remains unclear.

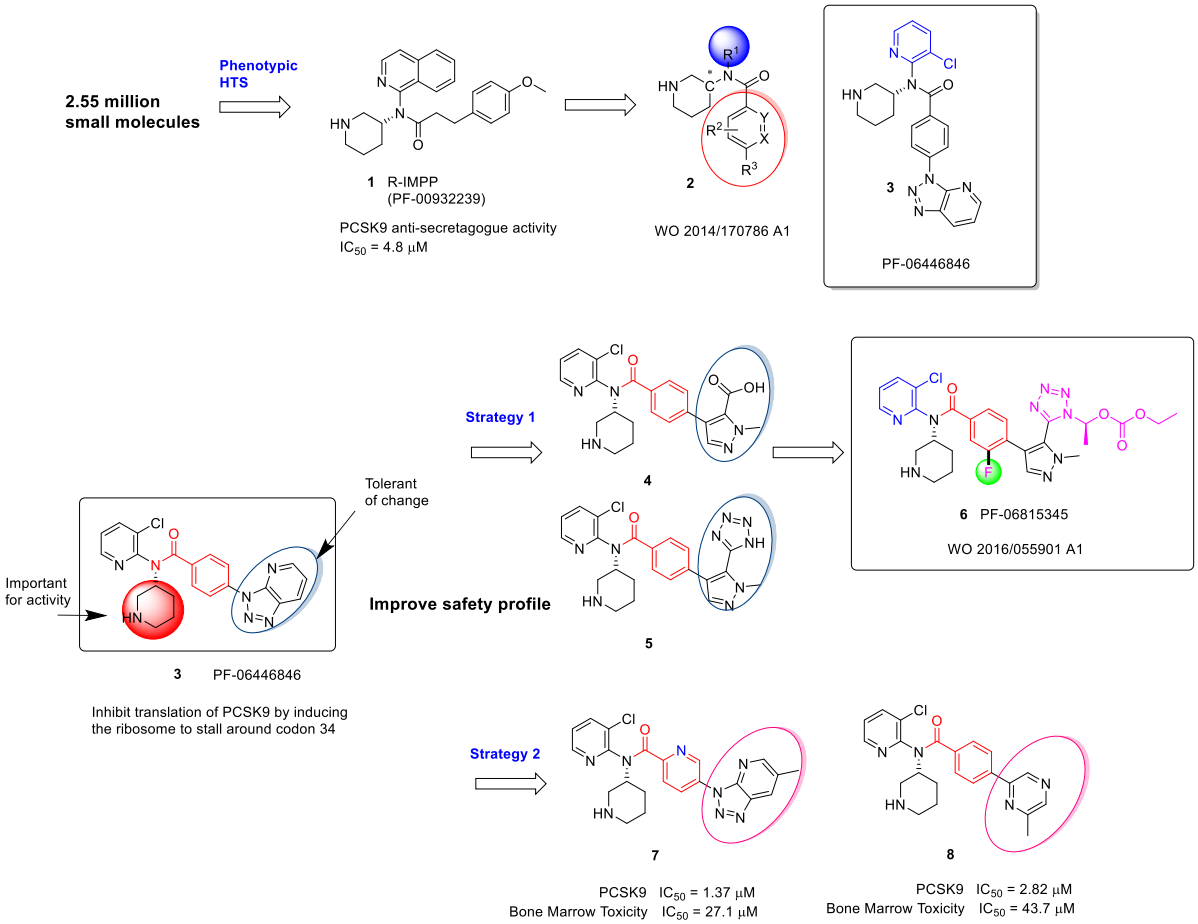


Figure 3. Strategies adopted by Pfizer for the development of PF-06815345 and compounds **7** and **8**.

3.2. Heparan sulfate mimetics

It is well known that PCKS9 interacts with the LDLR, causing the receptor to internalize, and consequently reduces the clearance of LDL. However, the PCSK9/LDLR binding constant is in the range of 170-628 nM, while the PCSK9 plasma concentration is approximately 6 nM.[68] Thus, as it is unlikely that the PCSK9 in the circulating system binds directly at normal physiological concentrations, some co-receptor may be involved in the interaction between PCSK9 and LDLR. Recently, Gustafsen and colleagues proposed the provocative concept that heparin sulfate proteoglycans (HSPGs) participate as a receptor for PCSK9 in the liver and facilitate the clustering of PCSK9 to LDLR.[69] The authors proved that HSPGs on the hepatocyte surface capture PCSK9 and present it to the LDLR, ensuring the optimal

conditions for PCSK9/LDLR complex formation. HSPGs participate in the regulation of a wide range of physiological and pathological phenomena, including developmental processes, angiogenesis, blood coagulation, and tumor metastasis.[70] Heparan sulfate (HS) is a linear polysaccharide chain composed of repeating linked pyrosulfuric acid and 2-amino-2-deoxy glucopyranose residues, and two or three HS chains are covalently attached to serine residues of the core proteins to form HSPGs. The HS-binding site on PCSK9 is located in the prodomain of PCSK9 and formed by surface-exposed basic residues interacting with trisulfated heparan sulfate disaccharide repeats. Heparin or heparan sulfate mimetics, interrupting the HS/PCSK9 interaction directly through binding to the HS-binding surface on PCSK9, would reduce PCSK9 activity and to be developed as PCSK9 inhibitors.[69]

Several molecules mimicking the structure of heparin, including dextran sulfate (**9**), pentosan sulfate (**10**), suramin (**11**) and the phosphorothioate oligonucleotide S-dC-36 (**12**), were screened for their abilities to inhibit PCSK9 and increase LDLR in HepG2 cells. S-dC-36 (**12**) was the most potent HS mimetic binding PCSK9 with a K_D of 4.8 μM. Interestingly, five clinical trials for anticoagulation with low-dose heparin at subtherapeutic amounts all reported marked reductions in cardiac events, although the mechanism for this has remained unclear.[71-75] The results from Gustafsen and colleagues may partly explain this phenomenon. In addition, chitosan oligosaccharides, linear polymers of *N*-acetyl-D-glucosamine and deacetylated glucosamine, were recently reported to enhance the level of lipid droplets via downregulation of *PCSK9* gene expression, suggesting saccharide might be a potential source of PCSK9 inhibitors.[76] It should be noted that the binding affinities of these heparan sulfate mimetics to PCSK9 were not high, further structural modifications with these molecules as leading compounds to find potent small-molecule PCSK9 inhibitors might be an effective method.

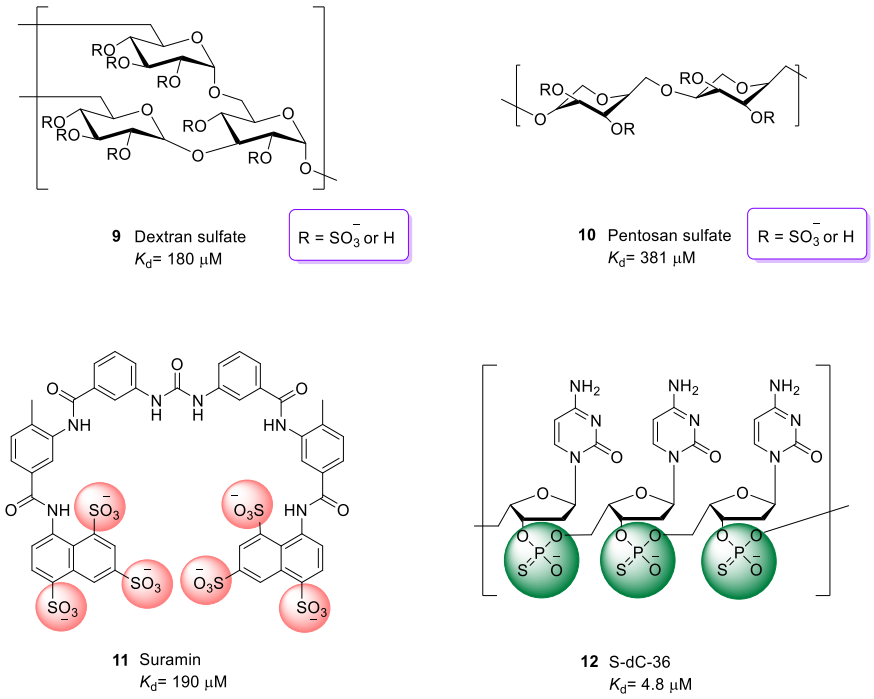
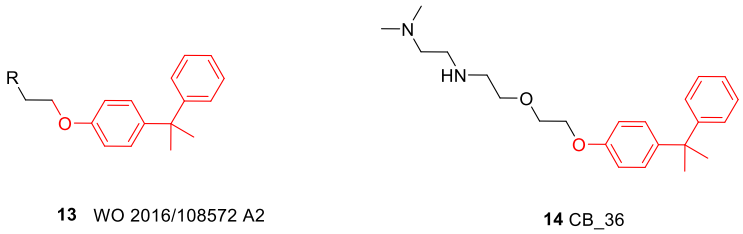


Figure 4. Heparan sulfate mimetics as PCSK9 inhibitors.

3.3. CB_36 from in silico screening

Although the motif of PCSK9 that binds to the EGF(A) domain of LDLR has been well characterized, the application of this interaction for the *in silico* virtual design of small molecules is a great challenge due to the lack of appropriate binding pocket. Min and co-workers tried to develop small-molecule inhibitors using a *in silico* virtual screening approach based on the PCSK9/LDLR interaction.[77] PCSK9 amino acids from 367 to 381, located where the EGF(A) domain of the LDLR binds, were extracted and assigned to be the target of the inhibitors. The PCSK9 protein structure from Protein Data Bank (PDB) was pretreated by removing the EGF(A) domain and water molecules, followed by the supplementation of hydrogen atoms. Subsequently, GOLD software version 4.0.1 was used to calculate the docking scores of ~450,000 chemicals from the ChemBridge Express database. The top 100 chemicals with the highest docking scores were selected to evaluate their *in vitro* activities using a PCSK9/LDLR binding assay, immunoblot analysis, and an LDL-C uptake assay. Most of the potent compounds (**13**) have fragments that structurally resemble the plasticizer bisphenol A and were protected in WO 2016/108572 A2.[78] The most potent chemical, CB_36 (**14**), was

validated as an inhibitor perturbing the linkage between PCSK9 and LDLR in a dose-dependent manner. However, whether this compound directly binds to PCSK9 and exerts its effects mediated by PCSK9 in a specific-manner remains to be proven.



13 WO 2016/108572 A2 **14** CB_36

Figure 5. CB_36 from in silico screening.

3.4. Imidazole-based minimalist peptidomimetic

Disrupting the protein-protein interaction (PPI) between PCSK9 and LDLR is an effective strategy, and several peptides have been developed accordingly.[42, 79] However, the search for alternatives to peptides, such as secondary-structure mimics of the main recognition motifs, that can be induced to fold into protein-like bioactive conformations that include α-helices, β-turns and β-strands, has received less attention.[80] The X-ray crystal structure of PCSK9/LDLR clearly showed a β-strand-mediated interaction formed by the residues C378-F379-V380-S381 of PCSK9 and V328-C329-N330-D331 of LDLR.[81] This evidence suggests that a small β-strand peptidomimetic resembling these native β-strand sequences (**15**), can function as a PCSK9 inhibitor through interfering with the PCSK9/LDLR reciprocal interaction.

Stucchi and co-workers reported a multicomponent synthesis of a novel imidazole-based peptidomimetic (**17**), that mimicked the conformation of a β-strand in terms of both the side chain orientation and the interstrand H-bond donating capability (Figure 6).[82] This peptidomimetic is more like a small molecule than a peptide as its molecule weight is 395 Da. Remarkably, the in vitro PCSK9-LDLR binding assay showed that this compound induces a concentration-dependent inhibition of the PPI and that the IC_{50} value is 11.2 ±0.2 μM. Reducing the number of imidazole rings from 4 to 3 caused a significant decrease in the IC_{50}, suggesting the importance of the tetraimidazole scaffold. However, a fluorescent-LDL uptake experiment showed that this compound

increased the LDL uptake with an EC_{50} of 6.04 μM, which was lower than the IC_{50} for the binding affinity of this compound to PCSK9.

Although β-strand peptidomimetics have been successfully applied as enzyme inhibitors,[83] this report is the first of an imidazole-based minimalist β-strand mimic as a PCSK9 inhibitor. This β-strand mimic paves the way for the future development of a new class of PCSK9 inhibitors. Future development of peptidomimetics might be focused on the number of imidazoles and the substitutions of imidazoles and *N*-Me groups to mimic the LDLR β-strand more strictly to improve the specificity.

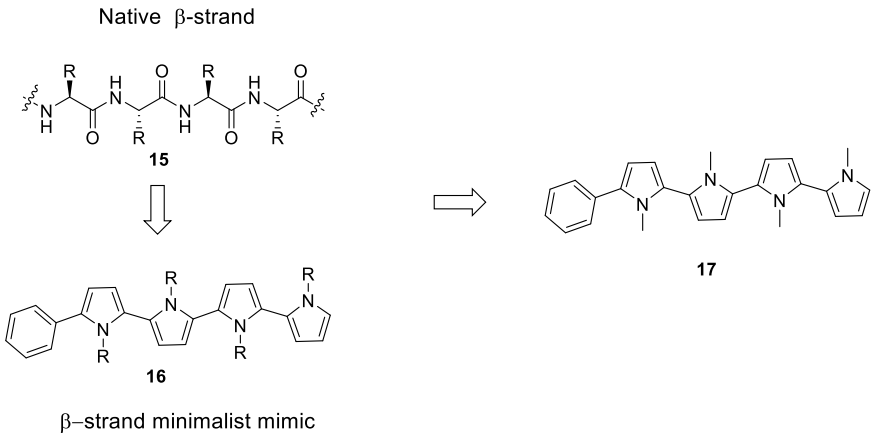


Figure 6. Small β-strand peptidomimetics as PCSK9 inhibitors.

3.5. Discovery of semirigid scaffold using Exploring Key Orientations

A recent story about peptidomimetics comes from Burgess and co-workers.[84] They hypothesized that a set of privileged conformational mimics of semirigid scaffolds bearing amino acid side-chains could be compared with PPI-interface regions to facilitate the development of peptidomimetics to perturb the PPI. They call this concept Exploring Key Orientations (EKOs), and developed a data mining algorithm to find such chemotypes that match PPI interfaces and, thereby perturb the interface.[85, 86] In 2018, several candidate chemotypes were conceived and screened as PCSK9 inhibitors using the EKO approach by the Burgess group. They found that molecule **18** was superimposed on LDLR in the PCSK9/LDLR complex in several ways. Specifically, five groups of interface side-chains of LDLR in the PCSK9/LDLR crystal

structure (Asp^{299}, Leu^{298}, and Asp^{301}; Cys^{297}, Asn^{301}, and Asp^{299}; Leu^{298}, Asp^{299}, and Asn^{301}; Val^{307}, Cys^{308}, and Leu^{318}; Asn^{309}, Cys^{308}, and Leu^{318}), might be overlaid by compound **18**. Interestingly, Asp^{299}, Leu^{318}, and Asn^{309} were implied as "hot spots" in LDLR side-chains implicated in the PCSK9/LDLR interface based on crystallographic studies.[28] Subsequently, the R^1-R^3 side chains in compound **18** were replaced with the natural side chains from the LDLR protein, and a series of compounds were prepared and tested using a time-resolved fluorescence resonant energy transfer (TR-FRET) assay. However, inconclusive results compelled the authors to improve the affinity of these compounds by appending additional pharmacophores using an iterative docking and energy minimization procedure. Consequently, structures **19** were conceived by including an Arg at the *C*-terminus of structure of **18** to fill the negative pocket in PCSK9. A solid-phase synthesis method was applied to install the R^1-R^4 side chains of chemotype **19**, and 15 compounds were finally prepared for screening. Three hit compounds exhibited promising dissociation constants for PCSK9 binding in surface plasmon resonance (SPR) analyses: **20** (LDLL-1dlnr, K_d 24.8±9.1 μM), **21** (DLLD-1nclk, K_d 41.2±17.5 μM), and **22** (LDLL-1dl(CN)r, K_d 35.8±11.4 μM). In addition, LDLL-1dlnr (**20**) was modified with a photoaffinity label and found to form a covalent conjugate with PCSK9 upon photolysis. It should be noted, as complex unnatural amino acids, further modifications on these amino acid side-chains to regulate the lipid-water partition coefficient and improve bioavailability are need.

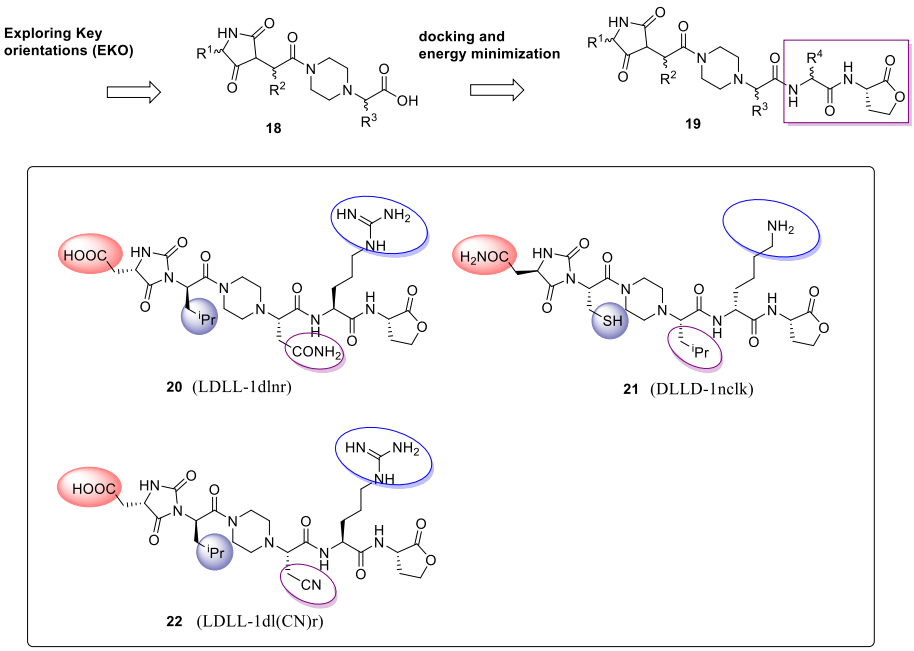


Figure 7. Discovery of semirigid scaffold using Exploring Key Orientations strategy.

3.6. Natural products

Despite of the high efficacy of approved PCSK9 antibody drugs, a few studies of developing natural products as lipid-lowering agents have been reported in recent years.[87, 88] Some of these natural products have been reported to hinder the function of PCSK9 directly or indirectly.[89, 90] Berberine, corydaline, curcumin, lignans, polydatin, flavones, etc. have been widely studied and identified to lower the expression of PCSK9. The mechanism studies of these natural products showed that most of these compounds alter PCSK9 status through modulating either *PCSK9* expression or mature protein secretion. However, more mechanistic investigations of these natural products are required, and these studies would facilitate reveling the signaling pathways associated with PCSK9. In the following section, we will review natural products with potential PCSK9 inhibitory effects.

3.6.1. Berberine

Berberine (**23**, BBR, Figure 8) is an isoquinoline plant alkaloid isolated from *Berberis*, such as *Rhizoma coptidis*, *Scutellaria baicalensis* and *Phellodendron amurense*.[91] In recent years, increasing evidence has suggested an ability of BBR to improve lipid

metabolism, and several clinical studies have also tested the efficacy of BBR in humans[92, 93] and implied that BBR administration might be a potential therapeutic approach for hypercholesterolemia. Studies have shown that BBR significantly increased LDLR expression through a posttranscriptional mechanism that stabilizes the LDLR mRNA[94] and that it reduced the expression and secretion of PCSK9.[95] However, the detailed mechanism by which BBR affects PCSK9 expression remains unclear. Cameron and co-workers suggested that BBR decreased PCSK9 mRNA and protein levels in a time- and dose-dependent manner and that this effect was not due to increased degradation of PCSK9 mRNA but most likely to decreased transcription of *PCSK9*.[95] Subsequently, sterol regulatory element binding protein-2 (SREBP-2) and hepatocyte nuclear factor 1α (HNF-1α) were identified to have roles in the BBR-mediated suppression of PCSK9 transcription.[96] A highly conserved HNF-1 binding site located between the SRE and Sp1 sites was identified as a critical *cis*-regulatory sequence of the *PCSK9* promoter.[96]

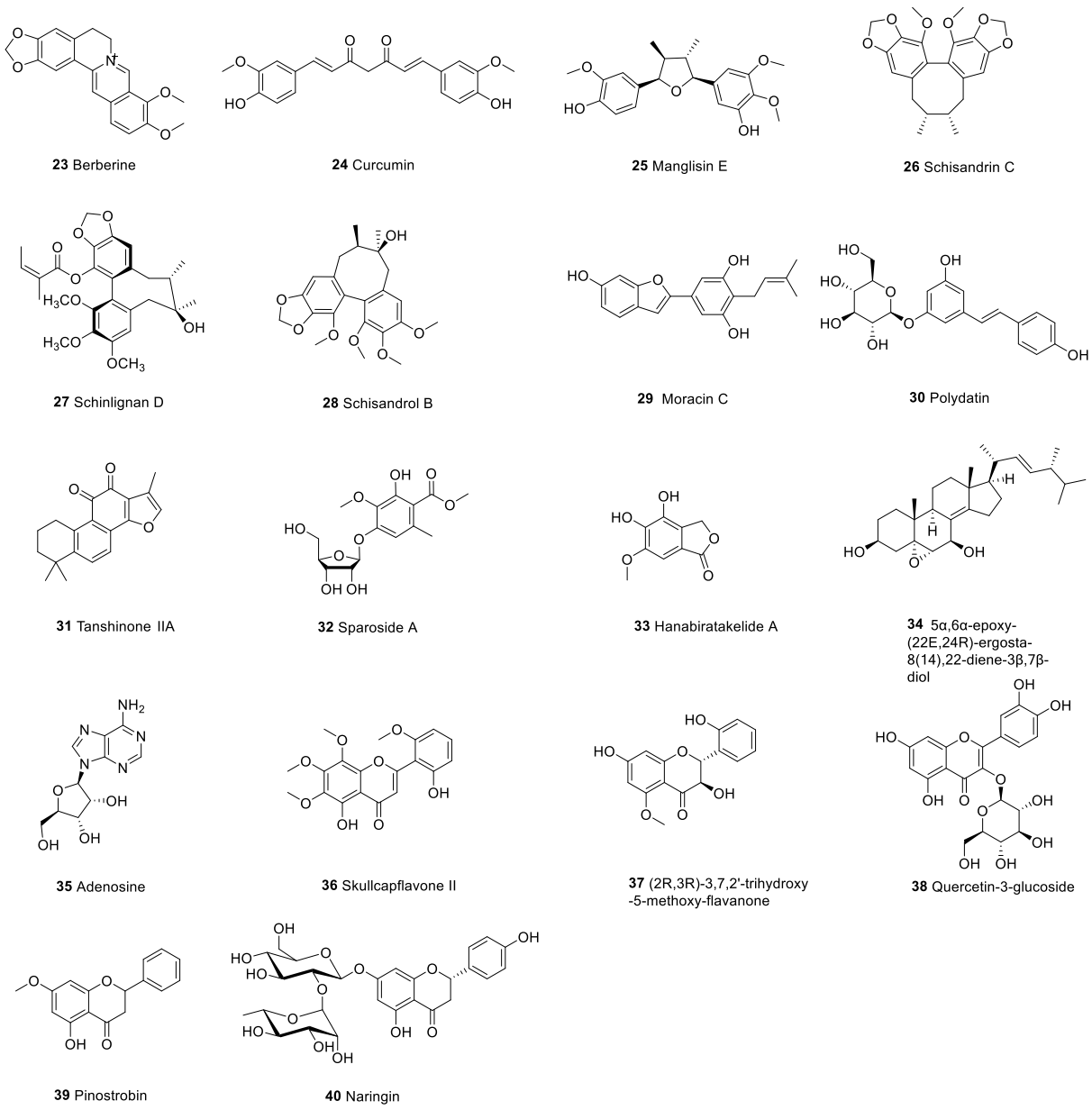


Figure 8. Structures of some natural products with potential PCSK9 inhibitory effects.

3.6.2. CVI-LM001 from corydaline

Corydaline (**41**, Figure 9) is an isoquinoline alkaloid with antiacetylcholinesterase, antiallergic and antinociceptive activities isolated from traditional Chinese herb *Corydalis yanhusuo*.[97, 98] Recent studies showed that corydaline also possesses several other pharmacological activities, including antienterovirus,[99] antiplatele,[100] and antifungal activities.[101] The lipid-lowering effect of corydaline or its derivatives have rarely been reported. Nevertheless, in 2008, CVI Pharmaceuticals (CVI) disclosed the hypocholesterolemic effects of compounds or derivatives from *Corydalis*. In their

disclosed patent, a series of isoquinolinyl-containing alkaloids, such as corydaline, corlumidin, corypalmine, bicuculline, tetrahydropalmatine, egenine and their derivatives were protected as hypocholesterolemic agents.[102, 103] The compounds disclosed there also lower total cholesterol (TC), LDL-C, and triglycerides. CVI-LM001 (**42**), the fluorobenzenesulfonate derivative of corydaline, was selected for preclinical studies, and the results confirmed the hypolipidemic effect of CVI-LM001 in hypercholesterolemic animal models, including a hamster model of hyperlipidemia and a diet-induced hyperlipidemia model in rabbits. The mechanism studies demonstrated that increases in hepatic LDLR expression, inhibition of PCSK9 expression and activation of AMP-activated protein kinase (AMPK) might explain the lipid-lowering efficacy of CVI-LM001. In February 2016, CVI initiated two small phase I clinical trials in China to estimate the preliminary pharmacokinetic and safety profiles of CVI-LM001.[104] It should be noted that although CVI-LM001 is a derivative of a natural product, it is the world's first orally administered small-molecule PCSK9 inhibitor in clinical studies. Recently, Liu and other inventors from the Shanghai Institute of Materia Medica described that phenyl [a]indole[2,3-g]quinolizine compounds can be used for treating diseases related to PCSK9 (WO 2017/167202 A1)[105]. The disclosed compounds possess a quinolizine skeleton, which is similar to natural product corydaline (**41**), typical compound C97 (**43**) is described in Figure 9.

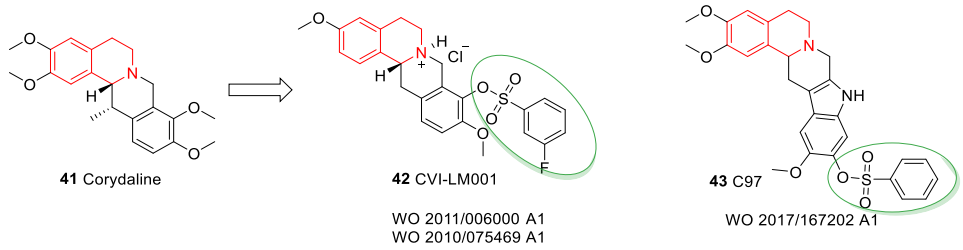


Figure 9. Development of CVI-LM001 from corydaline and related analogues reported in patent WO 2017/167202 A1.

3.6.3. Curcumin

Curcumin (**24**, Figure 8) is a polyphenolic diarylheptanoid found in turmeric, a spice derived from the rhizomes of the plant *Curcuma longa* Linn.[106] Mounting evidence

has demonstrated its positive effects in the treatment and prevention of cancer, Alzheimer's disease, multiple sclerosis, etc.[107] The lipid-lowering effect of curcumin has been witnessed in several experimental and clinical studies.[108, 109] In 2014, Tai and co-workers showed that curcumin increased LDLR expression through the suppression of PCSK9 expression and that HNF-1α is the curcumin-responsive element of the PCSK9 regulator.[110] Recently, the inhibitory effect of curcumin on PCSK9 in rat liver cirrhosis was also evaluated, and the results showed that curcumin protected against intestinal origin endotoxemia by inhibiting PCSK9 to promote LDLR expression.[110] However, the global pharmacological effects of curcumin may impend the further development of curcumin as PCSK9 expression inhibitor due to its multiple molecular targets. It should be noted that curcumin shows positive results in most drug screening assays and is regarded as a pan-assay interference compound, which may lead to misinterpretations of results. Some researchers even conclude that "curcumin is an unstable, reactive, nonbioavailable compound and, therefore, a highly improbable lead".[111]

3.6.4. Lignans

The lignans are a large group of polyphenols found in many vascular plants and have relevant health properties, including anticarcinogenic, anti-inflammatory and antioxidant activity.[112] Recently, a bioactivity-guided isolation of the fruits of *S. chinensis* using a PCSK9 mRNA expression screening assay identified the hexane-soluble extract as the active part. It total, 30 compounds were characterized, and four compounds potently inhibited PCSK9 mRNA expression: a tetrahydrofuran-type lignan (manglisin E (**25**), with an IC_{50} value of 3.15 μM), and three dibenzocyclooctadiene-type lignans (schisandrin C (**26**), schinlignan D (**27**), and schisandrol B (**28**), with IC_{50} values of 3.85, 0.36, and 1.10 μM, respectively) (Figure 8).[113]

3.6.5. Moracin C

Several nutritional compounds (NUTs) have been used against hypercholesterolemina as an alternative to pharmacological therapy. One innovative NUT combination

(LopiGLIK®), containing red yeast rice (monacolin K 3.3 mg), berberine (531.25 mg) and leaf extract of *Morus alba* (200 mg), has exhibited beneficial effects in reducing the plasma levels of the TC and LDL-C by 9.8% and 12.6%, respectively.[114] The hypolipidemic activity and mechanism of berberine have been studied extensively; however, the chemical constituents and mechanism responsible for the hypolipidemic activity of *Morus alba* extracts still need deep research.[115] Recently, a study from Pel and co-workers showed that moracin C (**29**) inhibited PCSK9 mRNA expression and thereby decreased the degradation of LDLR, which could be the active component responsible for the hypolipidemic effect of *Morus alba* extracts.[116] A PCSK9 mRNA expression monitoring assay in HepG2 cells first isolated and identified 15 compounds from a chloroform-soluble extract of dried *Morus alba* fruits, and subsequent assays showed that moracin C inhibited PCSK9 mRNA expression with an IC_{50} value of 16.8 μM in HepG2 cells.

3.6.6. Polydatin

Resveratrol is a biologically active stilbenoid present in various plant species and known to have numerous health-promoting effects. Since polydatin (**30**) is a glycoside precursor of resveratrol (resveratrol-3-O-β-mono-D-glucoside), its regulation on lipid metabolism has attracted considerable attention.[117] To explore the possible mechanism by which polydatin acts on lipid metabolism, Wang et al. evaluated the role of polydatin in insulin-resistant HepG2 cells and *db*/*db* mice. The results showed that polydatin not only upregulated the protein level of LDLR but also inhibited both the expression of PCSK9 and its interaction with LDLR in insulin-resistant HepG2 cells. In addition, polydatin significantly enhanced glucokinase and LDLR protein levels and inhibited PCSK9 expression in the liver.[118]

3.6.7. Tanshinone IIA

Tanshinone IIA (**31**) is a phenanthrene-quinone constituent of the Chinese medicinal herb Danshen (*Salvia miltiorrhiza*), which has been used clinically in Asia for the prevention and treatment of CVD.[119] A hypocholesterolemic effect of tanshinone

IIA was reported recently by Chen and co-workers.[120] They demonstrated that tanshinone IIA significantly increased the amount of LDLR and LDL uptake activity in HepG2 cells via posttranscriptional regulation. The expression of PCSK9 mRNA and mature protein was also inhibited by tanshinone IIA in HepG2 cells. Further mechanism studies showed that tanshinone IIA increased the nuclear forkhead box O3a (FoxO3a) level, enhanced FoxO3a/PCSK9 promoter complex formation and decreased the PCSK9 promoter-binding ability of HNF-1α, resulting in the suppression of PCSK9 gene expression. These results demonstrate that tanshinone IIA modulates cholesterol via downregulation of PCSK9 expression in hepatic cells. However, Jia et al. showed the conflicting result that tanshinone IIA promoted PCSK expression in the livers of hyperlipidemic rats, by upregulating the mRNA expression levels of *SREBP-2* and *PCSK9*.[121] Further studies might be required to investigate the underlying molecular mechanisms of tanshinone IIA and make a definitive conclusion.

3.6.8. Aromatic compounds from Sparassis crispa

Sparassis crispa (Wulf.) is a species of fungus in the genus *Sparassis*. It is a medicinal mushroom whose fruiting bodies possess various bioactive substances such as β-glucan, benzoate derivatives, maleic acids, and sesquiterpenoids.[122] Recently, fourteen compounds were isolated by Bang and co-workers from the EtOAc-soluble extracts of the fruiting bodies of *S. crispa*.[123] Among them, four compounds exhibited potent inhibition of PCSK9 mRNA expression: sparoside A (**32**, IC_{50} = 20.07 μM), hanabiratakelide A (**33**, IC_{50} = 7.18 μM), 5α,6α-epoxy-(22*E*,24*R*)-ergosta-8(14),22-diene-3β,7β-diol (**34**, IC_{50} = 8.23 μM), and adenosine (**35**, IC_{50} = 18.46 μM).

3.6.9. Flavonoids and flavanones

Flavonoids and flavanones have been reported to down-regulated the *PCSK9* gene expression. A methanolic extract of *S. baicalensis* was recently found to inhibit PCSK9 mRNA expression by Chin and co-workers. Nine compounds were isolated and compounds **36** (skullcapflavone II) and **37** (3,7,2'-trihydroxy-5-methoxy-flavanone) exhibited inhibitory activity at 20 μM (42.4% and 84.4%, respectively). Further studies

showed that the expression of *SREBP-1* mRNA was downregulated by compounds **36** and **37**, indicating that inhibition of PCSK9 mRNA was mediated by SREBP-1.[124] Quercetin is one of the most abundant dietary flavonoids with an average daily consumption of 25-50 mgs.[125] Quercetin is the aglycone form of many flavonoid glycosides, such as quercitrin, guaijaverin, hyperoside, and isoquercitin, and quercetin-3-glucoside (Q3G, **38**) is the quercetin 3-O-β-D-glucoside. This flavonoid glycoside has been shown to reduce diet-induced hyperlipidemia and atherosclerosis in mice.[126] Mbikay et al. examined the effects of micromolar concentrations of Q3G on LDLR and PCSK9 expression using human Huh7 hepatocytes, and the results showed that Q3G decreased the mRNA expression of PCSK9 by 20-30% while increasing the mRNA and protein expression of LDLR by 60 and 300-400%, respectively. Treatment with Q3G significantly reduced PCSK9 secretion, thereby causing its intracellular accumulation, and this PCSK9 retention might be partly due to induced sortilin deficiency.[127] Pinostrobin (**39**) was also found to inhibit the PCSK9 promoter activity and the suppression of PCSK9 mRNA expression in HepG2 cells through the up-regulation of the FoxO3a level.[128] Very recently, naringin (**40**), a flavanone-7-O-glycoside, was shown to activate AMPK, resulting in down-regulated expression of PCSK9 in obese C57BL/6J mice.[129]

3.6.10. Other extracts from natural products

Apart from the abovementioned compounds, some crude extracts from natural products also demonstrated lipid-lowering effects by regulating PCSK9 directly or indirectly. An aqueous extract *Phaleria macrocarpa* (Scheff.) Boerl fruit was reported by Chong et al. to reduce cholesterol levels *in vitro* and *in vivo*.[130] Further mechanism studies showed that *P. macrocarpa* extract significantly upregulated both LDLR and PCSK9 at the protein and mRNA levels, suggesting a mechanism that is similar to statin-induced effects. Xuezhikang (XZK) is an extract from red yeast rice that contains a mixture of lovastatin, plant sterols, isoflavones, and other components. XZK is believed in China to be a useful nutraceutical for patients with cardiovascular disorders.[131] Recent studies demonstrated that XZK increased PCSK9 levels through the SREBP-2

pathway,[132] which was similar to the mechanism behind the lipid-lowering effects of simvastatin.[133] In addition, some studies demonstrated that dietary *n*-3 polyunsaturated fatty acids reduced plasma PCSK9 concentration and hepatic PCSK9 mRNA expression, thereby exerting lipid-lowering effects.[134]

4. Small-molecule PCSK9 inhibitors reported in patents

The interface between PCSK9 and LDLR is extended, flat, and without deep binding pockets, which makes the interface a difficult target for small molecules. There is limited scientific literature reporting small molecules that directly inhibit the interaction between PCSK9 and LDLR; however, a few companies and institutions developed a series of small molecules targeting PCSK9/LDLR PPI, and some even disclosed the technical details.[135] In general, the strategies adopted by these companies targeting PCSK9/LDLR PPI are (a) altering the conformation of the protein PCSK9 through an allosteric mechanism and (b) targeting the "hot spots" of binding sites between PCSK9 and LDLR. In addition, a considerable portion of the pharmaceutical company's strategies focused on developing small molecules that inhibit PCSK9 expression at the genetic or protein levels. However, these small-molecule PCSK9 expression modulators might target other signal pathways and modulate PCSK9 expression indirectly, causing potential off-target effects. In addition, there are several patents that did not disclose the mechanism of their molecules. In this section, we will review the international patent publications reporting novel small molecules targeting PCSK9 for the treatment of CVDs. A brief summary of these patents is given in Table 2.

4.1. Small molecules targeting the PCSK9/LDLR PPI

Three patents, all from Portola Pharmaceuticals, disclosed that tetrahydroisoquinolines (**44-46**, WO 2017/034990 A1),[136] phenylalanines (**47-49**, WO 2017/034994 A1),[137] and phenylpiperazines (**50-52**, WO 2017/034997 A1) [138] increased LDL-uptake into liver cells, and LDLR cell surface populations (Figure 10). They declared that these compounds worked as ligands that directly bind the PCSK9 protein and differentially modify PCSK9 biological activity, changing the interactions between

PCSK9 and LDLR. However, researchers of Portola have not reported any direct evidence in these patents that these small organic molecules bind PCSK9.

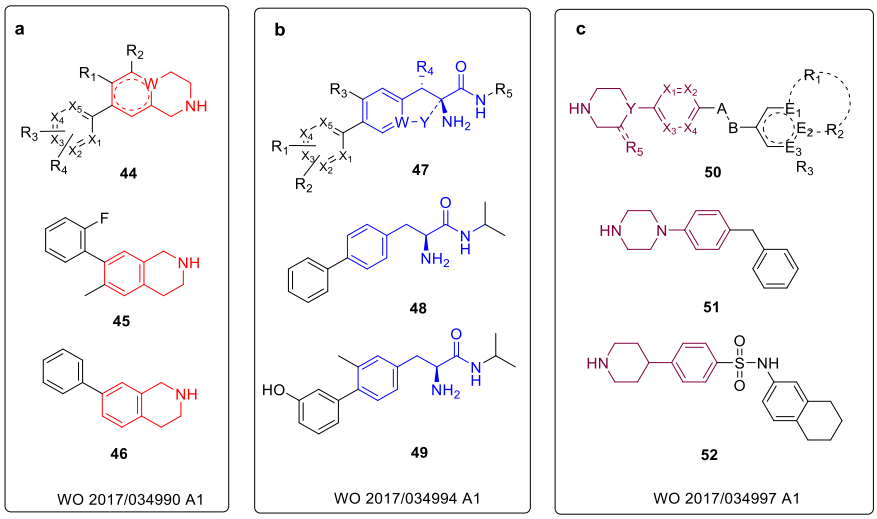


Figure 10. Markush structures and representative compounds from Portola. (a): tetrahydroisoquinolines; (b) phenylalanines; (c) phenylpiperazine.

Workers from the Shifa Biomedical Corporation have disclosed a series of compounds that modulate the physiological action of PCSK9, including its interaction with the LDLR. Various in vitro and in vivo assays have supported the biological activities of compounds claimed in three patents (WO 2014/150326 A1, WO 2014/150395 A1, and WO 2017/222953 A1).[139-141] Some compounds inhibited the PCSK9/LDLR interaction, and the detailed data were disclosed in WO2017222953. The most potent benzofuran compound against the PCSK9/LDLR interaction was SBC-115,337 (**56**), with an IC_{50} value of 0.5 μM (Figure 11). SBC-115,337 (**56**), SBC-110,686 (**57**), SBC-110,733 (**58**), SBC-110,736 (**59**), SBC-110,034 (**60**), and SBC-115,076 (**61**) were tested for their efficacy in male mice (C57BL/6 mice), and the data demonstrated that these compounds significantly lowered cholesterol levels (20%-38%).

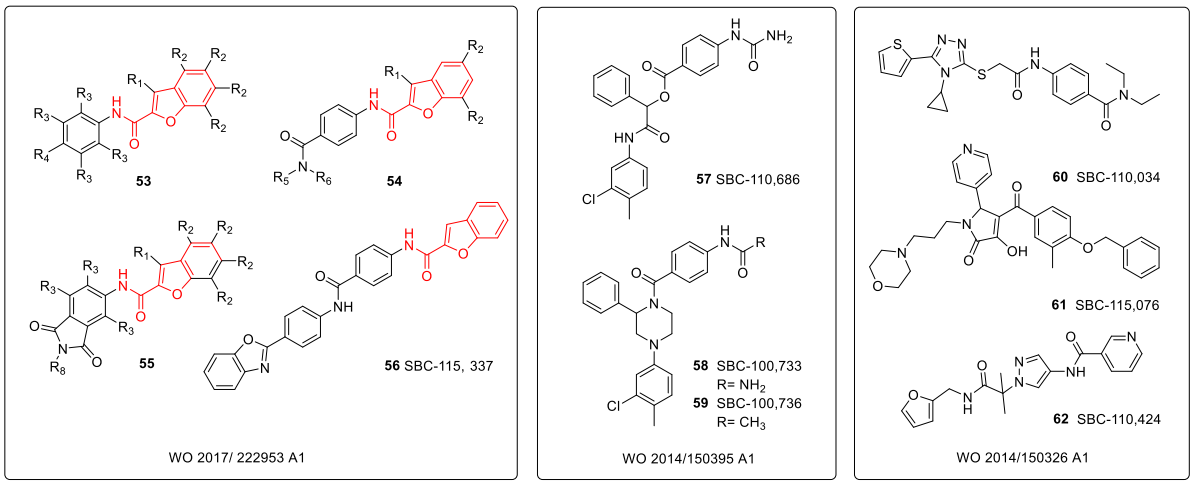


Figure 11. Markush structures and representative compounds from Shifa.

Many of the surfaces involved in the PPI are rather flat, making the development of small organic compounds to disrupt is difficult. However, most of the binding energy for the association of the two proteins is contributed by a few "hot spots" that can potentially be disrupted with small molecules. A group from Temple University disclosed a robust virtual screening method for the discovery of small molecules that bind PCSK9 and block its binding to the LDLR (Figure 12) (WO 2016/040305 A1).[142] Approximately 1 million drug-like compounds and over 400k large compounds (MW>500) from ZINC databases were processed with screening campaigns, and eleven compounds were finally identified as lead hits. Coincidentally, some of these compounds have already been disclosed by the Shifa Biomedical Corporation. Further SAR studies and lead optimization based on the pyrazoles, represented by SBC-110,424 (**62**, Figure 12), and the 4-hydroxy-5-oxopyrroles, represented by SBC-115,076 (**61**), have been facilitated and accelerated by docking poses, but the results were not reported in detail in this patent. The detailed virtual screening process disclosed by Temple University for the development of small molecules blocking the PPI between PCSK9 and LDLR provided a reference to future research.

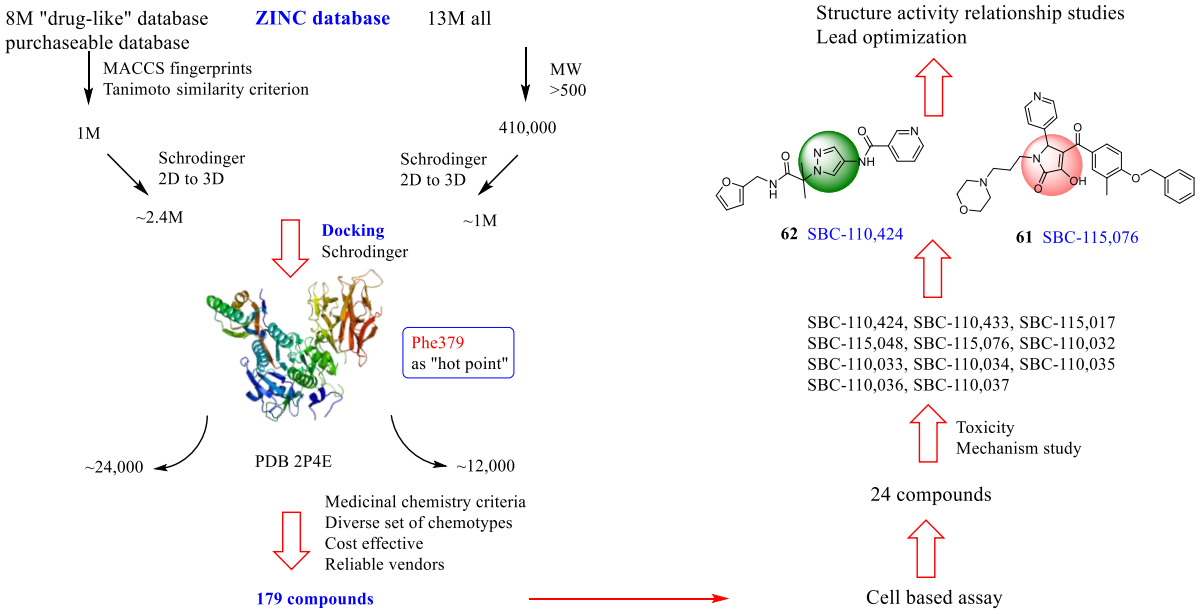


Figure 12. Virtual screening method used to identify small molecules blocking the binding of PCSK9 to LDLR.

In 2014, the SRX Cardio LLC described a set of synthetic peptides of 3-8 amino acids as allosteric inhibitors of PCSK9 (WO 2014/127316 A2).[143] These compounds were reported to bind and alter the conformation of the PCSK9 protein, changing the kinetics of the interaction between PCSK9 and LDLR. The most efficacious peptide is SRX55 (**63**, Figure 13a). Improved peptides were then designed by incorporating a negatively charged polar group to obtain better drug-like properties, and SRX60 (**64**), which contains a phosphate group, is a typical example. Two year later, the chemists from the SRX Cardio LLC simplified these peptides into small organic compounds, and negatively charged polar groups such as phosphate and tetrazole were retained (**65-68**, Figure 13b, WO 2016/029037 A1).[144] Considerable biological data were presented in this patent, including direct binding assays measured using backscatter interferometry. The most potent compound presented was SRX200 (**66**), which bound to recombinant human PCSK9 with a measured K_d of 24±8 nM. Allosteric modulation of PPI by small molecules binding to an allosteric site provides potential way forward for the difficult targets without apparent deep pockets in the interface.[145] Developing allosteric inhibitors of PCSK9 which bind to the allosteric site of PCSK9 and alter the

kinetics of the interaction between PCSK9 and LDLR seems to be a promising strategy identified by SRX Cardio LLC in this patent, which is worth following up.

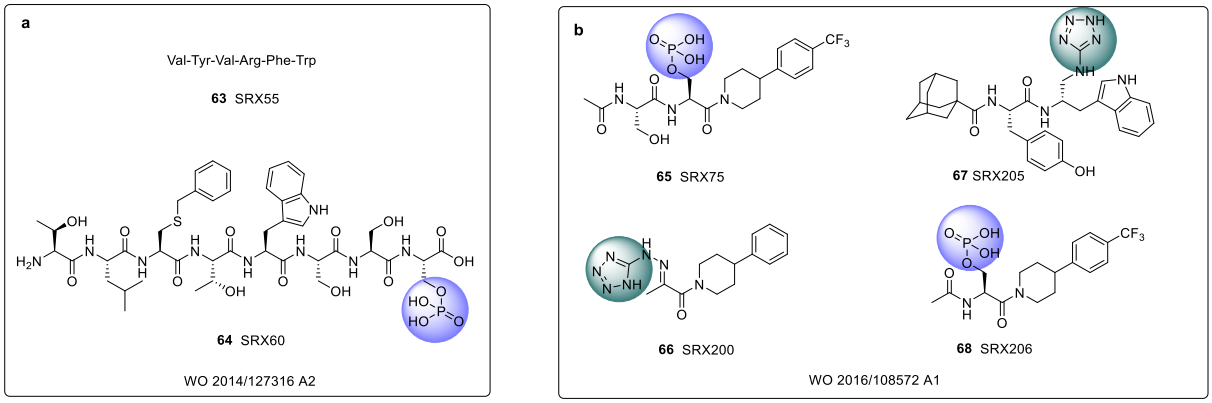


Figure 13. Representative compounds from SRX Cardio LLC. (a) Synthetic peptides; (b) Small organic compounds simplified from peptides.

4.2. Small molecules inhibiting PCSK9 expression

Recently, inventors from Paris have observed that steady-state PCSK9 mRNA levels in mouse primary hepatocytes surprisingly decreased by more than 90% after 24 h of treatment with anticancer agents IBET-151 (**69**, 2.5 μM) and JQ1 (**70**, 2.0 μM) (Figure 14a).[146] Interestingly, JQ1 and IBET-151 are potent, highly specific, competitive inhibitors of acetyl-lysine for the bromodomain and extraterminal (BET) family of bromodomains (BRDs). These results show that BRD inhibitors may represent a novel way to inhibit PCSK9 expression and hence reduce circulating LDL levels; however, the detailed mechanism of the invention was not disclosed.

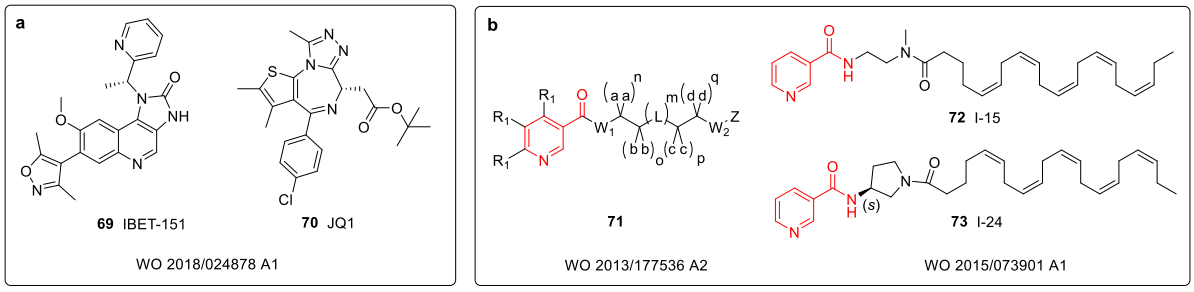


Figure 14. Small molecules inhibiting PCSK9 expression. (a) Structure of JQ-1 and IBET-151; (b) Markush structure and representative compounds of fatty acid niacin conjugates.

A set of novel fatty acid niacin conjugates (**71**) was disclosed by Catabasis Pharmaceuticals in 2013 for treating or preventing metabolic diseases by inhibiting the production or lowering the serum levels of PCSK9 protein (WO 2013/177536 A2).[147] Two years later, a more extensive patent presenting significant activities for two selected compounds (**72** and **73**, Figure 14b) was disclosed by the same company (WO 2015/073901 A1).[148] The surprising benefit of these *N*-alkylated fatty acid niacin conjugates is their ability to preferentially accumulate in the liver rather than other tissues. In 2016, the biological data supporting this result appeared in the scientific literature.[149] The fatty acid niacin conjugate **72** has been advanced into clinical trials to evaluate its effect on various lipid parameters.

Chengdu Bestchiralbio Pharmaceuticals has also patented a set of substituted nitrogen-containing heterocyclic derivatives as inhibitors of PCSK9 that down regulate PCSK9 expression at the mRNA level. At least 166 analogs are exemplified in WO 2016/107603 A1, and at least 87 analogs are exemplified in WO 2016/107602 A1.[150, 151] Most of the compounds in these series have a substituted piperazine or piperidine ring. Figure 15a shows the in vivo assays of typical potent compounds in these two patents (**74**, **75**). In addition, these compounds activated AMPK and can be used to treat type 2 diabetes.

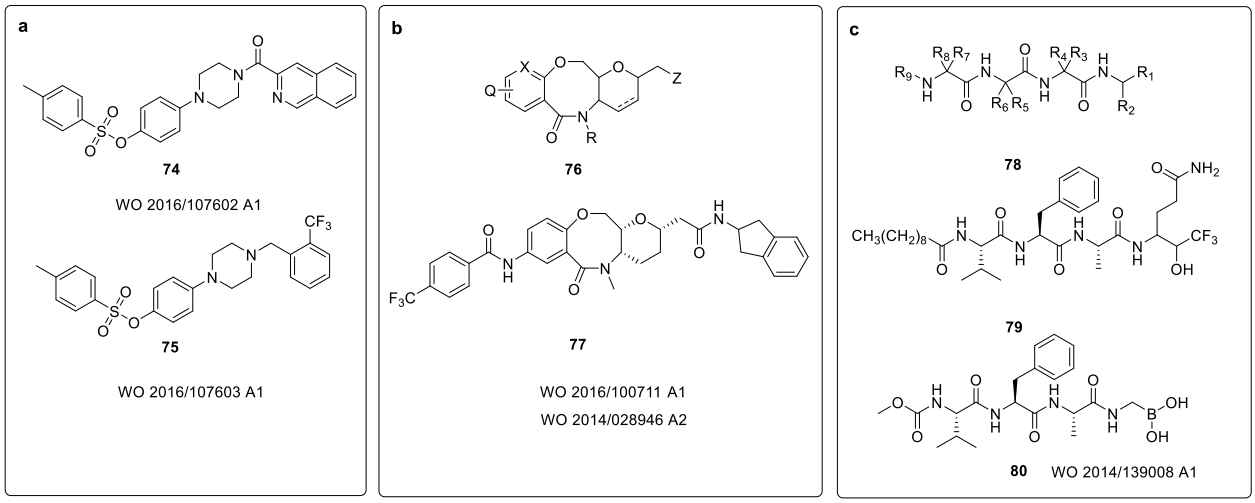


Figure 15. Representative compounds from (a) Chengdu Bestchiralbio; (b) Broad Institute; (c) Amorchem Holdings.

The Broad Institute described small molecules that modulate hepatic cholesterol by downregulating the expression levels of *PCSK9* (inhibit the expression of *PCSK9* mRNA and secretion of PCSK9 protein) and upregulating the expression level of Tribbles homolog 1 (*TRIB1*) (WO 2014/028946 A2, and WO 2016/100711 A1).[152, 153] The general formula (**76**, Figure 15b) is exemplified in 148 explicit examples. Compound **77** is the reference compound representing the series and was found to downregulate *PCSK9* with an EC_{50} of 0.36 μM, as monitored by quantitative polymerase chain reaction (qPCR) analysis.

At least 91 aminoacid-based compounds have been disclosed by a worker from AmorChem Holdings Inc., for preventing or treating LDL-C-related disease (WO 2014/139008 A1).[154] Compounds of the invention were screened using biological PCSK9-based assays, and at least 24 compounds were identified to inhibit PCSK9 secretion in a dose-dependent manner. Compounds **78-80** were typical examples (Figure 15c). However, the toxicity of some examples containing boric acid demands attention due to the potential carcinogenicity.[155]

4.3. Additional patents

Since 2011, Cadila Healthcare Pharmaceuticals have continuously published five patents (WO 2011/051961 A1, WO 2012/090220 A1, WO 2013/132509 A1, WO 2014/002105 A1, and WO 2014/002106 A1) claiming compounds for the treatment of dyslipidemia and related diseases. The initial Markush structure in these patents is extremely broad, but all of the compounds contain a substituted tetrahydropyran moiety. Although a PCSK9/LDLR in vitro binding assay was performed to support the claimed formulas, the mechanism that is responsible for the hypocholesterolemic effect of these compounds has not yet been fully clarified in all these patents.[156-160]

In 2018, researchers from Merck disclosed PCSK9 allosteric binding compounds bearing tetrahydroisoquinoline moiety (**81**, Figure 16).[161] These PCSK9 allosteric binders capable of stabilizing PCSK9 may stabilize PCSK9 in an inactive conformation, a conformation that inhibits PCSK9's ability to bind to the LDLR, increasing the cell

surface function of LDLR. In addition, some compounds in this invention exhibit the ability to lower steady state concentrations of both the pro and mature forms of PCSK9. For example, compound **82** was found to degrade mature PCSK9 level with DC_{50} value of 1.05 μM. Interestingly, some PCSK9 allosteric binders specifically bind PCSK9 at a unique site that does not interfere with the binding of PCSK9 with the LDLR, can be used, *inter alia*, in the detection, quantitation and imaging of PCSK9 in a sample when coupled with a detectable marker, e.g. a fluorescence probe or biotin (**83**, **84**). These PCSK9 allosteric binding compounds disclosed by Merck have different functions when binding to PCSK9 at different allosteric sites, extending the capability of allosteric modulators. However, no further information about these allosteric sites was disclosed in this patent.

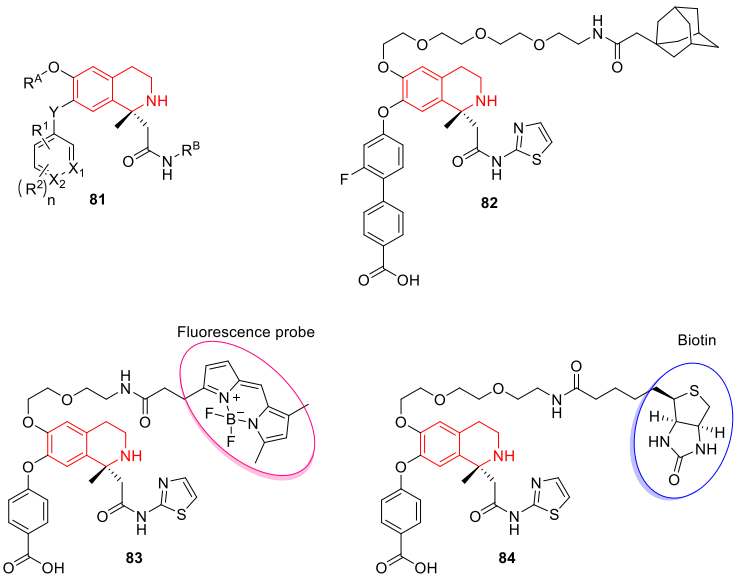


Figure 16. Markush structure of PCSK9 allosteric binders and representative labeling probes from Merck.

It is noteworthy that some compounds protected in patents have been reported in scientific journals and have been discussed in the first section of this review (WO2018/054959 A1, WO2018/053517 A1, WO2016/108572 A2, WO2014/170786 A1, WO2016/055901 A1, and WO2014/005224 A1). Finally, it is difficult to obtain a comprehensive view of all small-molecule PCSK9 inhibitors reported in non-English patents due to language barriers (WO 2016/021706 A1, WO 2014/017569 A1, and WO

2013/137371 A1); thus, other patents related to molecules that target PCSK9 might exist.

Table 2

Selected international patent applications related to small-molecule inhibitors of PCSK9.

NO.	Title	Mechanism	Publication number	Publication date	Applicant(s)
1	Methods of inhibiting PCSK9	Not provided	WO2018/129205 A1	12-07-2018	The Trustees of Dartmouth College
2	Compounds for treatment of lipoprotein metabolism disorders	Heparin analogues prevent PCSK9/LDLR complex formation	WO2018/054959 A1	29-03-2018	AARHUS Universitet
3	Substituted 1-methyl-1,2,3,4-tetrahydroisoquiniline molecules as PCSK9 allosteric binders	PCSK9 allosteric binding compounds	WO2018/057409 A1	29-03-2018	Merck Sharp & Dohme Corp.
4	Inhibitors of LDLR-PCSK9 protein-protein interaction and methods of their use	Disrupt the PPI between PCSK9 and LDLR	WO2018/053517 A1	22-03-2018	The Texas A&M University
5	Methods and compositions for reducing PCSK9 expression	Inhibit PCSK9 expression	WO2018/024878 A1	08-02-2018	Centre National de la Recherche Scientifique (CNRS); Institut National de la Santé et de la Recherche Médicale (INSERM); Université Paris Descartes
6	Antiproprotein convertase subtilisin kexin type 9 (anti-PCSK9) compounds and methods of using the same in the treatment and/or prevention of cardiovascular diseases	Modulate the physiological action of PCSK9, including its interaction with the LDLR	WO2017/222953 A1	28-12-2017	Shifa Biomedical Corporation
7	Phenyl [a]indole[2,3-g]quinolizine compounds, preparation method therefor, pharmaceutical composition, and applications thereof	Not provided	WO2017/167202 A1	29-03-2017	Shanghai Institute of Materia Medica, Chinese Academy of Sciences
8	Phenylpiperazine proprotein convertase subtilisin/kexin type 9 (PCSK9) modulators and their use	Bind to PCSK9 and alter the conformation of the protein	WO2017/034997 A1	02-03-2017	Portola Pharmaceuticals, Inc.

9	Composition and methods of use of tetrahydroisoquinoline small molecules to bind and modulate PCSK9 protein activity	Bind to PCSK9 and alter the conformation of the protein	WO2017/034990 A1	02-03-2017	Portola Pharmaceuticals, Inc.
10	Composition and methods of use of novel phenylalanine small organic compounds to directly modulate PCSK9 protein activity	Bind to PCSK9 and alter the conformation of the protein	WO2017/034994 A1	02-03-2017	Portola Pharmaceuticals, Inc.
11	Composition for preventing and treating cholesterol-related diseases	Inhibit the linkage between PCSK9 and LDLR	WO2016/108572 A2	07-07-2016	Industry-ACA-Demic Cooperation Foundation Yonsei University
12	Substituted nitrogen-containing heterocyclic derivatives and applications thereof	Inhibit PCSK9 expression	WO2016/107603 A1	07-07-2016	Chengdu Bestchiralbio LLC.
13	Substituted nitrogen heterocyclic derivatives and use thereof	Inhibit PCSK9 expression	WO2016/107602 A1	07-07-2016	Chengdu Bestchiralbio LLC.
14	Modulators of hepatic lipoprotein metabolism	Inhibit the expression of PCSK9 mRNA and secretion of PCSK9 protein	WO2016/100711 A1	23-06-2016	The Broad Institute, Inc.
15	Substituted amide compounds	Inhibit PCSK9 translation	WO2016/055901 A1	14-04-2016	Pfizer Inc.
16	PCSK9 inhibitors and methods of use thereof	Disrupt the PPI between PCSK9 and LDLR	WO2016/040305 A1	17-03-2016	Temple University-of the Commonwealth System of Higher Education
17	Composition and methods of use of small molecules as binding ligands for the modulation of proprotein convertase subtilisin/kexin type 9 (PCSK9) protein activity	Alter the conformation of the protein PCSK9	WO2016/029037 A1	25-02-2016	SRX Cardio, LLC
18	Fatty acid niacin conjugates	Inhibit PCSK9 expression and/or production	WO2015/073901 A1	21-05-2015	Catabasis Pharmaceuticals, Inc.
19	N-piperidin-3-ylbenzamide derivatives for treating cardiovascular diseases	Inhibit the translation of PCSK9 mRNA to protein	WO2014/170786 A1	23-10-2014	Pfizer Inc.
20	Small molecule modulators of PCSK9 and methods of use thereof	Inhibit PCSK9 secretion	WO2014/139008 A1	18-09-2014	Adaerata Limited Partnership; AmorChem Holdings Inc.
21	Antiproprotein convertase subtilisin kexin type 9 (anti-PCSK9)	Modulate the physiological action of	WO2014/150326 A1	25-09-2014	Shifa Biomedical Corporation

	compounds and methods of using the same in the treatment and/or prevention of cardiovascular diseases	PCSK9, including its interaction with the LDLR			
22	Anti-PCSK9 compounds and methods for the treatment and/or prevention of cardiovascular diseases	Modulate the physiological action of PCSK9, including its interaction with the LDLR	WO2014/150395 A1	25-09-2014	Shifa Biomedical Corporation
23	Proprotein convertase subtilisin/kexin type 9 (PCSK9) allosteric binding ligands to modulate serum low density lipoprotein (LDL) levels	Alter the conformation of the protein PCSK9	WO2014/127316 A2	21-08-2014	SRX Cardio, LLC
24	Modulators of hepatic lipoprotein metabolism	Inhibit the expression of PCSK9 mRNA and secretion of PCSK9 protein	WO2014/028946 A2	20-02-2014	The Broad Institute, INC.
25	Quercetin-3-glucoside and uses thereof	Increase the amount of PCSK9 secreted by hepatocyte cells	WO2014/005224 A1	09-01-2014	Mbikay Majambu Sirois Francine Chretien Michel Mayne Janice
26	Novel compounds for the treatment of dyslipidemia and related diseases	Not provided	WO2014/002106 A1	03-01-2014	Cadila Healthcare Limited
27	Compounds for the treatment of dyslipidemia and other diseases	Not provided	WO2014/002105 A1	03-01-2014	Cadila Healthcare Limited
28	Novel compounds for the treatment of dyslipidemia and related diseases	Not provided	WO2013/132509 A1	12-03-2013	Cadila Healthcare Limited
29	Methods of lowering proprotein convertase subtilisin/kexin types (PCSK9)	Inhibit the production of PCSK9	WO2013/177536 A2	28-11-2013	Catabasis Pharmaceuticals, INC.
30	Heterocyclic compounds suitable for the treatment of dyslipidemia	Not provided	WO2012/090220 A1	05-07-2012	Cadila Healthcare Limited
31	Compounds for the treatment of dyslipidemia and related diseases	Not provided	WO2011/051961 A1	05-05-2011	Cadila Healthcare Limited
32	Berberine derivatives useful for modulating lipid levels and their methods of synthesis	Inhibit PCSK9 expression	WO2011/006000 A1	13-01-2011	Liu Haiyan Li Gaoping Wang Junbo Liu Jingwen
33	Corydaline derivatives useful for reducing lipid levels	Inhibit PCSK9 expression	WO2010/075469 A1	01-07-2010	CVI Pharmaceuticals Limited

5. Potential strategies for the design of small molecules

The search is on to develop small-molecule inhibitors of PCSK9, and some potential strategies or techniques that might be applied to the design of novel small-molecule PCSK9 inhibitors will be introduced in this section.

5.1. Another binding site on PCSK9 for the design of small-molecule inhibitors

The abovementioned characteristics of PCSK9 implied that developing small-molecule inhibitors based on the EGF(A)-binding site is difficult. Altering the conformation of PCSK9 by allosteric inhibitors has been successfully displayed by SRX Cardio LLC, but the relevant patents did not uncover the allosteric site. A recent study from the Kirchhofer group discovered a cryptic peptide-binding site next to the LDLR-binding site on PCSK9, which is possible for small molecules to target this site and inhibit LDLR binding by a steric mechanism.[162] The findings suggested that the P'-helix (Ser^{153}-Thr^{162} of the catalytic domain) is intrinsically labile and adopts different conformational states; in the 'out' state, the groove becomes accessible for peptides to bind, which could antagonize LDLR binding by a steric mechanism. Grooved-binding antagonistic peptides that share sequence homology with the native P'-helix are potent PCSK9 inhibitors, affording a new strategy for the development of small molecules from these peptides to antagonize PCSK9.[163]

5.2. Aptamers

Aptamers are synthetic oligonucleotides or peptides that bind to a specific target molecule with high affinity. Aptamers are now used ubiquitously as binding agents for a broad range of applications.[164] They function similarly to mAbs, but have more advantages over antibodies. The fact that unmodified DNA or RNA aptamers have less chemical diversity than antibodies limits their wide application. Recently, a group from SomaLogic, Inc. reported the selection and identification of DNA aptamers in which one or both pyrimidines were replaced by side-chain-functionalized variants that bind and inhibit PCSK9 with dissociation constants similar to those of approved antibodies (evolocumab, K_D = 8.0 pM, and alirocumab, K_D = 0.58 nM).[165] Recently, Liu and co-workers developed a ligase-mediated DNA-templated polymerization and in vitro

selection system, and diverse highly functionalized nucleic acid polymers (HFNAPS) were prepared. HFNAPs that bind PCSK9 were selected for further diversification and reselection, resulting in an improved PCSK9-binding HFNAP with a K_D of 3 nM, which potently inhibited the binding between PCSK9 and the LDLR. These studies shed light on designing extensively chemically functionalized aptamers as potential PCSK9 inhibitors.[166]

5.3. Proteolysis-targeting chimera technology

It seems from the current data that low PCSK9 function and LDL-C levels caused by PCSK9 inhibitors or LOF mutations did not trigger possible deleterious effects.[167] An extreme example comes from a patient who was a compound heterozygote for the Y142X and ΔR97 LOF mutations, which disrupt the synthesis and processing/secretion of PCSK9, respectively. No additional adverse events were observed in this patient, although the levels of PCSK9 were below the limit of detection and the LDL-C level was as low as 14 mg/dL.[167] RNA interference, antisense RNA and CRISPR-Cas9 techniques are often used to knock down the target protein, but proteolysis-targeting chimera (PROTAC) technology has recently been successfully applied to selective degradation of multiple protein targets other than PCSK9.[168] PROTAC methods utilize endogenous cellular quality control machinery by recruiting it to the target protein to induce its degradation. Thus, the targeted degradation of PCSK9 protein using PROTAC technology might be possible, although there have been no related reports in the literature.

5.4. Targeting the signal transduction cascade

Relatively little is known about the PCSK9 promoter. PCSK9 and LDLR are coregulated by SREBP-2.[169] Reducing the amount of PCSK9 mRNA by disrupting the SREBP pathway would be expected to down-regulate transcription of the LDLR gene, which would counteract a beneficial effect of lowering PCSK9 levels. The Kowa Company reported a new cholesteryl ester transfer protein (CETP) inhibitor, K-312, that suppressed PCSK9 expression through modulation of its transcription by

decreasing SREBP levels, representing a novel strategy to reduce LDL-C.[170] Thus, with the development of the molecular biology of PCSK9, more signaling pathways and interconnected factors of PCSK9 will be unveiled and facilitate the search for new inhibitors of PCSK9 activity.[171] Several natural products have been widely reported to modulated PCSK9 levels without detailed mechanisms, and mechanism studies about these natural products may facilitate reveling the signaling pathways associated with PCSK9.

6. Summary and future directions

The cost of current PCSK9-blocking antibodies ($US14,500 and $US14,100 per year for evolocumab and alirocumab, respectively) has aroused a fierce debate, as only 15% relative risk reduction resulted from these expensive antibodies.[172] The cost-effectiveness ratio may be acceptable for patients currently with familial hypercholesterolemia, but for patients with conventional vascular disease, these antibodies are not so cost effective.[173, 174] The appeal of small-molecule inhibitors of PCSK9 over antibodies is rather clear, because you can achieve the same goal with only hundreds of dollars, let alone the potential for delivering the inhibitors via oral pills instead of injectable antibodies. In addition, despite antibodies perturbing the binding of PCSK9 to LDLR, by their nature, anti-PCSK9 mAbs are unable to modulate PCSK9 and LDL-C levels in an intermediate fashion, while the inhibition of PCSK9 synthesis by a small molecule could provide additional options for patients and physicians by offering different levels of inhibition to balance safety and efficacy. Thus, orally administrated small molecule is a preferred approach to inhibit PCSK9.

Base on the above descriptions, three strategies can be concluded that have been applied to inhibit PCSK9 with small molecules, including 1) preventing the binding of PCSK9 to LDLR; 2) interfering with the maturation and secretion processing or the biological function of PCSK9; and 3) inhibiting the synthesis and expression of PCSK9. Designing small molecules disrupting the PPI between PCSK9 and the LDLR is the most immediate strategy but also the toughest way to discover small-molecule PCSK9 inhibitors. The K_D for PCSK9/LDLR is in submicromolar level,[175] which makes

small-molecule inhibitors targeting the relatively flat EGF(A)-binding site on PCSK9 quite challenging. The discovery of “hot spots” makes it possible to prevent the binding of PCSK9 to the LDLR directly; in addition, altering the conformation of PCSK9 (allosteric modulators) can also change the interactions between PCSK9 and LDLR, leading to reduced levels of circulating LDL-C.[176] However, considering the potent binding affinity between PCSK9 and LDLR, most of the currently reported inhibitors targeting the PPI between PCSK9 and LDLR directly may not be potent enough to disrupt the binding. Altering the conformation of PCSK9 by allosteric inhibitors or targeting the newly found adjacent P’-helix site may offer more hope for disrupting the PCSK9/LDLR interaction with small molecules in the future. Maturation and secretion processing are required for PCSK9 function, and a small-molecule inhibitor that interferes with these processes would block, suppress, and reduce PCSK9 biological activity or decrease the amount of mature, secreted PCSK9. However, few small-molecule inhibitors have been reported that reduce LDL-C levels through this pathway. Transcription and translation of PCSK9 are controlled by many factors, and a small molecule targeting these factors would also reduce the synthesis and expression of PCSK9 protein. Many small-molecule inhibitors, like natural products, have been reported to reduce PCSK9 at the gene or protein levels, but their detailed mechanisms need further study. Cell-based HTS afforded opportunities to overtake PPI-based strategies. In addition, mechanism of action studies of compounds from HTS or natural products might disclose novel mechanisms and facilitate the discovery of small-molecule PCSK9 inhibitors.

Up to date, antibody targeting mature PCSK9 is the most advanced modality, other points of intervention and modalities have also been widely reported. In all cases, small molecule progress has lagged behind. Although researchers have dabbled with the discovery of PCSK9 inhibitors, no significant progress toward small-molecule PCSK9 inhibitors has been made up to date. In recent five years, great efforts have been made to develop small-molecular PCSK9 inhibitors. This review, which highlights a few of the recent advances in small-molecule PCSK9 inhibitors, is a witness to the growing interest for and importance of this field. A pill that inhibits PCSK9 is "a Holy Grail" in

the world of lowering LDL-C, and many companies and institutions are struggling to commercialize the first PCSK9 small-molecular inhibitors and win the chance to build a franchise in what is expected to be a blockbuster. Nevertheless, "a Holy Grail" is still at the top of the mountain, waiting for anyone who is keen and ambitious enough to pluck it.

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